

# Kombucha fermentation of mulberry leaves and its effect on the content and $\alpha$ -glucosidase inhibitory activity of flavonoid extracts

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**Abstract.** As the main drug for the treatment of type II diabetes, presently used  $\alpha$ -glucosidase inhibitors generally have serious side effects. Developing new, effective, safe, and economical  $\alpha$ -glucosidase inhibitors from medicinal and edible plant resources have become a research hotspot. In this paper, Kombucha was used for the fermentation of dried mulberry leaves (ML-DS) and fresh mulberry leaves (ML-FS), with the aim of increasing the content and  $\alpha$ -glucosidase inhibitory efficiency of active substances in ML. The research results indicated that both dried and fresh mulberry could be used as fermentation substrates for Kombucha, especially ML-FS. After 12 d of fermentation, pH value and reducing sugar concentration of the ML-FS was rapidly decreased from 6.46 to 2.78 and from 108.43  $\pm$  5.33 mg/ml to 19.40  $\pm$  1.67 mg/ml, respectively. As for the total flavonoid content in the extract of ML (MLF), the MLF content in the ML-FS that fermented for 12 d (ML-D12-FS) was increased by 49.46% compared to that in the unfermented ML (ML-D0-FS). Meanwhile, Kombucha fermentation also can significantly improve the  $\alpha$ -glucosidase inhibitory activity of MLF. At the concentration of 45.6 mg/L, the inhibition rate of ML-D12-FS on  $\alpha$ -glucosidase reached to 85.88%, and the IC50 value decreased to 5.5 mg/L which was 16.7 mg/L for ML-D0-FS. This paper would provide an economical and green method for the production of  $\alpha$ -glucosidase inhibitors from mulberry leaves.

## 1 Introduction

Diabetes mellitus is a chronic disease that leads to disorder of glucose and lipid metabolism due to islet function recession, which seriously affects human health [1]. According to the 10th edition of the Global Diabetes Map (GDM) released by the International Diabetes Federation in December 2021, there were currently 537 million diabetic patients in the world [2]. It was predicted that it will reach 783 million by 2045. However, diabetic patients are usually accompanied by serious complications, such as impaired renal function, eye diseases and neuropathy [3]. Therefore, the research on diabetes has become a hot spot in the world [4].

Among all types of diabetes, type II diabetes accounts for more than 80%, and its prevalence rate has increased in recent years with the changes in people's diet and living habits. Alpha-glucosidase inhibitor is one of the main drugs in the treatment of type II diabetes, which can effectively inhibit the increase of postprandial blood sugar in patients. It is mainly because the ingested carbohydrates can be decomposed into small-molecule oligosaccharides under the action of  $\alpha$ -amylase, and the latter continue to be hydrolyzed into glucose under the action of  $\alpha$ -glucosidase. The addition of  $\alpha$ -glucosidase inhibitor can effectively inhibit this hydrolysis process or slow down the conversion rate of polysaccharide and disaccharide to monosaccharide, thus reducing the blood sugar concentration [5]. At present, the main  $\alpha$ -glucosidase inhibitors used in clinic are acarbose, voglibose, miglitol.

However, taking drugs for a long time will cause a variety of adverse reactions, including bloating, stomachache, obesity and so on [6]. On the other hand, the treatment cost of diabetes is extremely high. As mentioned in the GDM the cost caused by diabetes will reach 1 trillion US dollars by 2030 [3]. Therefore, develop economical drugs with strong therapeutic effects and minimal side effects from natural medicinal and edible plants has attracted worldwide attention [7].

At present, the research on extracting  $\alpha$ -glucosidase inhibitors from plants to treat diabetes mostly focuses on the following aspects: one is the mechanism of lowering blood sugar [1], the other is the inhibition of  $\alpha$ -glucosidase by natural active ingredients [8], and the third is the improvement and enrichment of  $\alpha$ -glucosidase inhibitors [9]. Mulberry leaves (ML) as a new edible plant resource have been widely used to treat diabetes since ancient times. The Compendium of Materia Medica written by Li Shizhen in Ming Dynasty first recorded the efficacy of ML in treating diabetes [10]. Modern research shows that its hypoglycemic activity is related to alkaloids, flavonoids and polysaccharides [11]. Therefore, ML has become an excellent source for developing new  $\alpha$ -glucosidase inhibitors.

Microbial fermentation is an effective means to increase the content of effective components in plants and produce new effective components [12]. And a large number of studies have shown that microbial fermentation can improve the content of active substances in mulberry leaves, and then improve the  $\alpha$ -glucosidase inhibitory

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activity of mulberry leaf extracts [13]. For example, Luo reported that the crude extracts from mulberry tea fermented by *Inonotus hispidus* could significantly improve its anti-tumor efficiency. Liu and Qiu found that fresh mulberry leaves fermented by *Eurotium cristatum* CY-1 could enhance the content of flavonoids in mulberry leaves (MLF) obviously. Moreover, the IC<sub>50</sub> values of MLF that extracted from the 10 d-fermented ML and unfermented ML were 4.925 µg/mL and 25.995 µg/mL, respectively [14]. However, at present, the existing research is mainly based on dried mulberry leaves. There is a lack of research on the fermentation of fresh mulberry, as well as its effects on the content and on α -glucosidase inhibition efficiency of active substances. Kombucha is rich in many beneficial microorganisms, such as acetic acid bacteria, yeast and lactic acid bacteria, which form a strong symbiotic relationship and complex metabolic pathways [15]. Therefore, the fermentation of Kombucha can produce a large number of active ingredients [16]. In this paper, taking dried and fresh mulberry leaves as substrate, respectively, the effects of Kombucha fermentation on the content and inhibitory activity on α -glucosidase of main active substances were investigated.

## 2. Material and methods

### 2.1. Materials and reagents

Fresh mulberry leaves were commercially available. Alpha-glucosidase (source of yeast), rutin standard (≥98%) and 4-nitrophenyl-α-D-glucopyranoside (pNPG, ≥99%) were purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China). Dinitrosalicylic acid, sodium nitrite, aluminum nitrate, sodium hydroxide and anhydrous ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Reinecke salt were purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). 4-hydroxypiperidine (≥98%) were purchased from Macklin Biochemical Co., Ltd (Shanghai, China).

### 2.2. Preparation of ML substrate

Added three times the wet weight of fresh mulberry leaves to sterile water, crushed them in a beater, and stored them at 4 °C for later use. Recorded them as Fresh ML substrate (ML-FS). Then crushed the dried mulberry leaves after natural air drying to 60 mesh and recorded them as the dried mulberry substrate (ML-DS).

### 2.3 Preparation of Kombucha inoculum

Socked 2 g of black tea in 200 ml of 90 °C hot water for 20 min. Collected the tea infusions and added with 10% (w/v) glucose. Then, prepared the Kombucha inoculum (KI) by incubating the Kombucha starter into the tea infusions and cultivating at 28°C for 2-3 d.

At ratio of 3 times of dry weight and 10 times of wet weight sterile water was added into FML and DML, respectively. After stirring evenly, ultrasound extraction

of flavonoids from ML was performed at 40 °C and 70kHz for 30 minutes (KQ5200DE, Kun Shan Ultrasonic Instruments Co., Ltd, Jiangsu, China). Added 3.5% glucose (w/v) into fresh mulberry extract filtrate (MLFE) or dried mulberry extract filtrate (MLDE), respectively. After stirring evenly, 10 ml pre-cultured KI was inoculated and statically cultivated at 28-30 °C for 3 d to obtain the Kombucha inoculum in ML-FS extract (KI-MLFE) and ML-DS extract (KI-MLDE), respectively.

### 2.4 Fermentation of ML with Kombucha

Took about 20 g of FML added with 80 ml sterile water and 3.5% glucose (w/w) to prepare a fresh mulberry fermentation substrate. Took 2 g of DML added with 100 ml of sterile water and 3.5% glucose (w/v) to prepare a dry mulberry fermentation substrate. Then, 20 ml KI-MLFE or KI-MLDE was inoculated into the above fermentation substrate, respectively, and statically incubated at 28 °C. Took 1 ml of the supernatant at intervals and stored the samples at 4 °C for use.

Monitored the the pH value of the supernatant. Measured the reducing sugar content in the supernatant by DNS method with a UV-Vis spectrophotometer (754 PC, Jinghua Technology Co., Ltd., Shanghai, China). The undegraded ML residues was dried to constant weight and calculated the weight loss rate (%) using the following formula (1).

$$\text{Weight loss rate (\%)} = \frac{DW_0 - DW_F}{DW_0} \times 100\% \quad (1)$$

where,  $DW_0$  and  $DW_F$  are the dried weight of mulberry leaves before and after fermentation, respectively.

### 2.5 The extraction and assay of flavonoids in ML

Took an appropriate amount of sample added with 70% ethanol solution at a ratio of 1:10 (w/v). Ultrasounded the solution at 70 kHz for 30 min at 40 °C, and filtered the supernatant to obtain the crude extract of mulberry leaf flavonoids (MLF). NaNO<sub>2</sub>-Al(NO<sub>3</sub>)<sub>3</sub>-NaOH chromogenic method were used to determin the total flavonoid content of MLF. Using rutin as the standard, the absorbance was measured at 510 nm using spectrophotometer (754 PC, Jinghua Technology Co., Ltd., Shanghai, China). The standard curve was obtained with rutin content as the abscissa axis and absorbance value as the ordinate, then the total flavonoid content of MLF was calculated according to the standard curve [17].

### 2.6 The inhabitation activity of ML extract on α-glucosidase

The measurement of α-glucosidase inhibition rate was conducted in the 96- microplate reader (MB-96B, Suzhou Pinhuai Technology Co., Ltd. Jiang Su, China). Four experimental groups were setted, blank group (B), control group (C), sample blank group (SB), and sample group (S). The reagent contained in each group was added according to the following description. Microplate of Group B contained 80 µL phosphate buffer solution (PBS). Microplate of group C contained 70 µL PBS and 10 µL α-

glucosidase solution. Microplate of group SB contained 60  $\mu$ L PBS and 20  $\mu$ L tested inhibitor solution. Microplate of group S contained 50  $\mu$ L PBS, 20  $\mu$ L tested inhibitor solution and 10  $\mu$ L  $\alpha$ -glucosidase solution. After mixed evenly, each group were reacted at 37  $^{\circ}$ C for 15 min. Then, 20  $\mu$ L 10 mmol/L pNPG substrate was added and reacted at 37  $^{\circ}$ C for 30 min. Finally, the reaction was terminated by adding 100  $\mu$ L 1 mol/L sodium carbonate solution. Afterwards, measured the absorbance at 405 nm and calculated the inhibition rate of different inhibitors on  $\alpha$ -glucosidase according to formula (2).

Originpro 8.5 software was used to fit the inhibitory rate of different MLF on  $\alpha$ -glucosidase, and the half inhibitory concentration (IC<sub>50</sub>) was calculated according to the fitting equation.

$$\text{Inhibitory rate of } \alpha\text{-glucosidase (\%)} = \left(1 - \frac{A_S - A_{SB}}{A_C - A_B}\right) \times 100\% \quad (2)$$

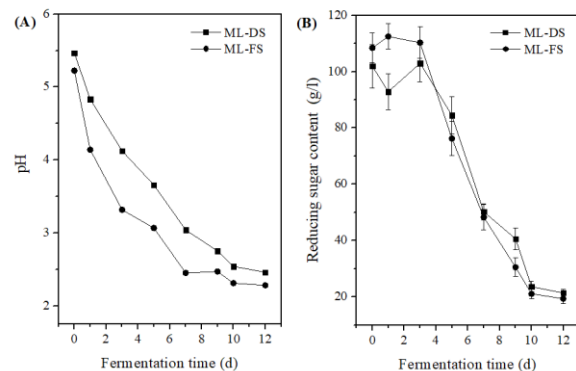
Where, AS and ASB are the OD<sub>405</sub> of sample group and sample blank group, respectively. AC and AB are the OD<sub>405</sub> of blank group and control group, respectively.

### 3. Results and Discussion

#### 3.1 The variation of Kombucha fermented ML broth

Mulberry leaves contains rich nutrients, such as polysaccharides, proteins, minerals, and vitamins. It was reported that the crude protein content oin ML was approximately 13.61-24.97% [18]. The carbohydrates in ML are mainly glucose, galactose, mannose, fructose and so forth, which are belong to monosaccharides, disaccharides, and various polysaccharides. Among them, the soluble polysaccharide content is about 4-18% and the fiber content is about 52.9% [19,20]. Therefore, mulberry leaves was able to meet the carbon and nitrogen sources required for microbial growth to a certain degree. As shown in Fig. 1, it could be seen that when dried and fresh mulberry leaves were used as a substrate for Kombucha fermentation, the pH and total sugar content of the fermentation broth decreased rapidly, indicating that Kombucha achieved rapid growth. For pH value, the initial pH of fresh mulberry substrate was slightly higher than the dried one (Fig.1A), with values of 6.46 and 6.65 for fresh and dry mulberry, respectively. Because of the large amount of acetic acid bacteria and lactic acid bacteria in Kombucha, the pH of the fermentation broth rapidly decreased along with the fermentation. On the 12th day of fermentation, pH of the ML-FS and ML-DS was decreased to 2.78 and 3.26, respectively. Moreover, the pH value of ML-FS decreased faster than ML-DS. It was indicated that the fermentation rate of Kombucha in ML-FS was faster than that in ML-DS, which could also be proved by the change in total reducing sugar concentration(TRS) (Fig.1B). The TRS concentration in the ML-DS and ML-FS showed significant differences at the beginning of fermentation. On the first day of fermentation with ML-FS, the TRS concentration in the fermentation broth increased first and then decreased rapidly. For ML-DS, TRS content decreased rapidly on

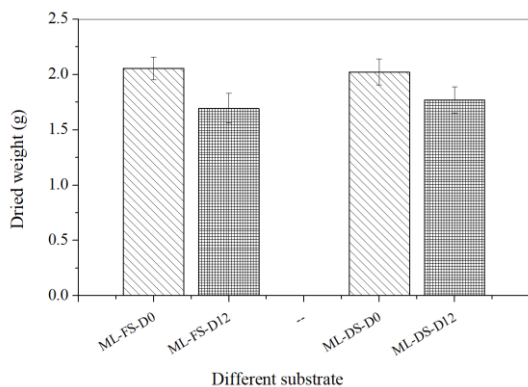
the first day of fermentation. But from the second day, it was increased first and then rapidly decreased. The brief increase in TRS concentration might be due to the microorganisms existed in Kombucha and their secreted enzymes. After fermentation, the TRS concentration in the ML-DS fermentation broth decreased from the initial 101.90  $\pm$  7.71 mg/ml to 21.45  $\pm$  1.34 mg/ml at the end of fermentation. As for ML-FS, it was decreased from 108.43  $\pm$  5.33 mg/ml to 19.40  $\pm$  1.67 mg/ml. The degradation rates of TRS concentration in ML-DS and ML-FS were 78.95% and 82.11%, respectively.



**Fig.1** The variation of pH (A) and total sugar concentration (B) during the Kombucha fermentation process of mulberry leaves (ML). ML-DS and ML-FS are the dried and fresh mulberry leaves substrate for Kombucha fermentation, respectively.

#### 3.2 Analysis of weight loss rate of mulberry leaves fermented by Kombucha

As shown in Fig.2 Kombucha fermentation would cause significant weight loss of ML-FS and ML-FS. The wet weight of the used ML-FS in this paper was 20.056  $\pm$  0.048 g and the dry weight of ML-FS was 10.25  $\pm$  0.49% determined by weight loss method. It could be calculated that the dry weight of ML-FS at the beginning of fermentation was 2.056  $\pm$  0.048 g. At the end of fermentation, the dry weight of the ML-FS decreased to 1.694  $\pm$  0.134 g. After 10 d of Kombucha fermentation, the the initial dry weight of ML-DS (2.023  $\pm$  0.021 g) dropped to 1.771  $\pm$  0.121 g. The weight loss rate for ML-FS and ML-DS after been fermented by Kombucha was 17.6% and 12.3%, respectively. It suggested that the rich microbial community of Kombucha or some enzymes secreted by Kombucha have the ability to grow and metabolize easily degradable components in ML.

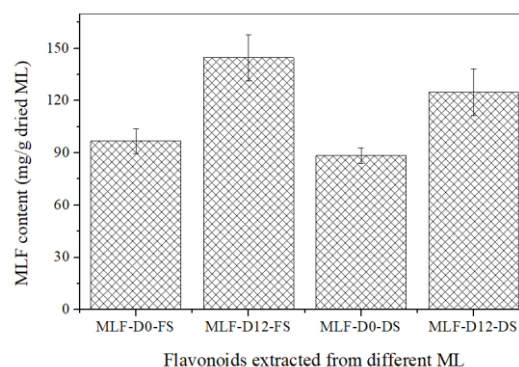


**Fig.2** Changes in dry weight of dried and fresh mulberry substrate before and after Kombucha fermentation. ML-FS-D<sub>0</sub> and ML-DS-D<sub>0</sub> are the initial dry weight (g) obtained from fresh and dry mulberry substrates, respectively; ML-FS-D<sub>12</sub> and ML-DS-D<sub>12</sub> are the dry weights (g) of the remaining fresh and dry mulberry substrates after 12 days of fermentation, respectively.

### 3.3 Variation of the content of active substances in mulberry leaves

Active compounds contained in ML which have alpha-glucosidase inhibitors are mainly polysaccharides, flavonoids, alkaloids and so on. This section investigated the effect of Kombucha fermentation on the total flavonoid content in the ML-DS and ML-FS (Fig. 3). Firstly, the flavonoid content in the unfermented ML-FS (MLF-D<sub>0</sub>-FS) was 96.80 ± 6.95 mg/g dried ML, which was 15.66% higher than that in unfermented ML-DS (MLF-D<sub>0</sub>-DS). After 12 d of Kombucha fermentation, the total flavonoid content extracted from the fermented residue of ML-FS (MLF-D<sub>12</sub>-FS) and ML-DS (MLF-D<sub>12</sub>-DS) was 144.67 ± 13.19 mg/g dried ML and 124.81 ± 13.33 mg/g dried ML, respectively. Compared to MLF-D<sub>0</sub>-FS, the content in MLF-D<sub>12</sub>-FS increased by 49.46%, while for ML-DS, the content increase was 41.16%. Thus it can be seen that Kombucha fermentation could obviously increase the content of flavonoids in the dried and fresh ML substrate, but the effect of drying on ML fermentation was not significant.

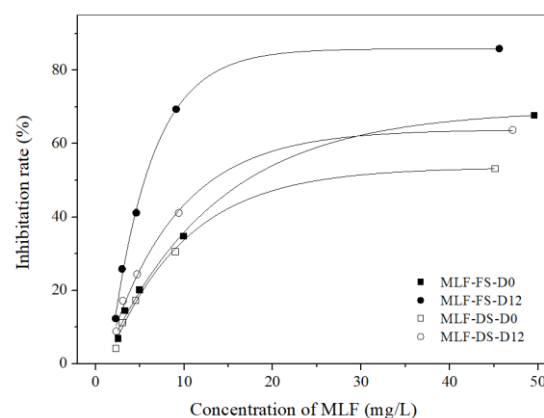
The research on microbial fermentation of ML has received widespread attention in recent years. Many different microbial strains have been applied for the ML fermentation, such as bacteria [21], yeast [21], macro-fungi [22], medicinal fungi [23], probiotics [24], and so on. But, there were no studies have been conducted on the Kombucha fermentation of ML. Kombucha is a probiotic that exists various microorganisms such as lactic acid bacteria, acetic acid bacteria, and yeast. The previous studies have shown that lactic acid bacteria fermentation could increase the content and efficacy of active substances in substrate. For example, the lactic acid fermentation could enhance the flavonoids content of African *Nightshade* leaves [25]. Chuah et al. [26] also found that the total phenolic content and total flavonoids content in mulberry leaves could be significantly increased after the fermentation of Lactic acid bacteria. Therefore, the increase of flavonoid content in ML that fermented by Kombucha might be closely related to lactic acid bacteria.



**Fig. 3** Effect of Kombucha fermentation on the content of flavonoids extracts in dry and fresh mulberry substrates. MLF-D<sub>0</sub>-FS and MLF-D<sub>0</sub>-DS are the initial flavonoid content in fresh and dried mulberry substrates, respectively; MLF-D<sub>12</sub>-FS and MLF-D<sub>12</sub>-DS are flavonoid content in fresh and dried mulberry substrate that fermented by Kombucha for 12 d, respectively.

### 3.4 Effect of Kombucha fermentation on the Alpha-glucosidase inhibition rate of active substances in ML

Effect of Kombucha fermentation on α-glucosidase inhibitory activity was studied using different concentrations of flavonoids extracted from ML fermented for 12 d and unfermented ML (Fig.4). The α-glucosidase inhibitory activity of flavonoid extracts from unfermented ML-FS and ML-DS showed little difference. The inhibition rate of 49.5 mg/L MLF-FS-D<sub>0</sub> and 45.1 mg/L MLF-DS-D<sub>0</sub> was 67.77% and 53.25%, respectively. The fermentation of Kombucha could significantly increase the α-glucosidase inhibitory activity of MLF. At the concentration of 45.6 mg/ml MLF-FS-D<sub>12</sub>, the α-glucosidase inhibitory activity was 85.88%. However, there was no significant difference in the inhibitory rate of flavonoids extracted from the dried mulberry substrate before and after Kombucha fermentation on α-glucosidase. The inhibitory rate of 47.1 mg/ml MLF-DS-D<sub>12</sub> on α-glucosidase was only 63.74%.



**Fig.4** Inhibition rate of different mulberry leaf flavonoid extracts on α-glucosidase. MLF-D<sub>0</sub>-FS and MLF-D<sub>0</sub>-DS are the initial flavonoid extracts obtained from fresh and dry mulberry substrates, respectively (mg/g dried ML); MLF-D<sub>12</sub>-FS and MLF-D<sub>12</sub>-DS are flavonoid extracts extracted from fresh and dry mulberry substrate residues after 12 d of fermentation, respectively (mg/g dried ML).

The corresponding kinetic equation was obtained by fitting the inhibitory activity of different mulberry leaf flavonoid extracts on glucosidase (Fig. 4), which indicated that the fitting degree of the kinetic equations was high, and the correlation coefficients reached above 0.99 (Table 1). According to the kinetic equation, the IC<sub>50</sub> values of different mulberry leaf flavonoid extracts on α-glucosidase were calculated (Table 1). The IC<sub>50</sub> value of MLF-FS-D<sub>12</sub> decreased from 16.7 mg/L of MLF-FS-D<sub>0</sub> to 5.5mg/L, while the IC<sub>50</sub> value of MLF-DS-D<sub>12</sub> was 12 mg/L, which was 52.3% lower than the IC<sub>50</sub> value of MLF-DS-D<sub>0</sub>. Therefore, it could be seen that Kombucha fermentation not only enriched the content of flavonoids in the dry and fresh mulberry substrate, but also enhanced its inhibitory activity of α-glucosidase. This also indicates that the complex metabolic pathways of microorganisms in Kombucha might have the ability to produce flavonoids with new structures, which have better α-glucosidase inhibitory activity.

**Table 1.** The Inhibition Kinetics of Different Flavonoids Extracts from Mulberry Leaves on α-glucosidase

Type of inhibitor	Dynamics equations	R <sup>2</sup>	IC <sub>50</sub> (mg/L)
MLF-FS-D0	$Y = -73.4 * \exp(-x/12.7) + 69.2$	0.992	16.7
MLF-FS-D12	$Y = -118.7 * \exp(-x/4.6) + 85.8$	0.997	5.5
MLF-DS-D0	$Y = -61.6 * \exp(-x/8.8) + 53.5$	0.992	25.2
MLF-DS-D12	$Y = -71.5 * \exp(-x/8.0) + 63.8$	0.993	12.0

#### 4. Conclusion

Dried and fresh mulberry leaves could be used as the fermentation substrate for Kombucha, causing a rapid decrease in the pH and total reducing sugar content of the fermentation broth. Fresh mulberry leaves substrate was more conducive to Kombucha fermentation than dried mulberry leaves substrate, which suggested that the complex microbial community or the enzymes secreted during Kombucha fermentation could utilize the easily degradable components in mulberry leaves as a substrate. After Kombucha fermentation, the content of flavonoids in the dried and fresh mulberry substrate significantly increased. On the other hand, the flavonoid extracts from the Kombucha fermented fresh mulberry leaves substrate have a higher α-glucosidase inhibitory activity than that extracted from the fermented dried mulberry leaves. More than 85% of α-glucosidase could be inhibited by 45.6 mg/ml of MLF-FS-D<sub>12</sub>. It might be owing to the organisms exist in Kombucha, which have the complex metabolic paths to degrade ML and generate new flavonoids with new structures and high α-glucosidase inhibitory activity.

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