Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2018

www.elsevier.com/locate/jbiosc

Antioxidant and hypoglycemic effects of *Diospyros lotus* fruit fermented with *Microbacterium flavum* and *Lactobacillus plantarum*

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Received 16 November 2017; accepted 9 January 2018
Available online xxx

Diospyros lotus, a member of the Ebenaceae family, has long been used as a traditional sedative in China. In this study, the antioxidant and hypoglycemic effects of non-fermented and microorganism-fermented _D. lotus_ were explored. The total phenolic and vitamin C contents of microorganism-fermented _D. lotus_ for 24–72 h were less than those of non-fermented. High-performance liquid chromatography showed that the tannic, catechinic, and ellagic acid contents increased significantly upon fermentation for 24 h. _D. lotus_ fermented with _Microbacterium flavum_ for 24 h exhibited the highest DPPH radical scavenging activity (IC_{50} = 4.18 μg mL^{-1}), and the highest ABTS radical scavenging activity was exhibited at 72 h of fermentation (IC_{50} = 29.18 μg mL^{-1}). The anti-α-glucosidase activity of fermented _D. lotus_ was higher (2.06–4.73-fold) than that of non-fermented one. Thus, fermented _D. lotus_ is a useful source of natural antioxidants, and a valuable food, exhibiting antioxidant and hypoglycemic properties.

[**Key words:** *Diospyros lotus*; Fermentation; Antioxidant; Hypoglycemic; *Microbacterium flavum*; *Lactobacillus plantarum*]

Natural products derived from Chinese herbs have been used to treat a variety of diseases, especially in Asian countries, and continue to be useful even as Western medicine advances (1). To date, over 7000 medicinal plants or herbs have been utilized by 80% of the world's population (2). Many studies have revealed the medicinal activities, especially the antioxidant properties, of natural products used to treat various diseases including hypertension, cancer, Alzheimer's disease, HIV infection, diabetes, and Parkinson's disease (3). Recently, natural antioxidants have become the focus of attention, because artificial antioxidants may actually trigger certain chronic diseases (4,5). Natural products from various Chinese herbs exert remarkable effects on diseases caused by oxidative damage, and may play valuable roles in disease prevention and treatment (6,7).

The date plum persimmon (*Diospyros lotus* L.) of the genus *Diospyros* in the family Ebenaceae is native to China, the Balkans, the Caucasus, and Japan (8). The seed (a traditional Chinese medicine) is used both as a sedative and a febrifuge, and the fruits are commonly employed to promote secretion. *D. lotus* extracts exhibit antioxidant activities, eliminating free radicals and protecting against oxidative stress (9). Extracts exhibited dose-dependent DPPH radical scavenging activity, and served as reductants and chelators, attributable principally to the contained phenolics and flavonoids (10). However, limited information is available on the development of antioxidant and hypoglycemic activities in the microorganism-fermented *D. lotus*.

Traditional fermentation changes the biochemical profile and the ratio of nutritive to non-nutritive components. In recent decades, there has been an increasing interest in the bioconversion of polyphenols by probiotics. Particularly, *Lactobacillus* species have received tremendous attention due to their potential biological activity. Fermentation by selected lactic acid bacteria enhanced the antimicrobial, antioxidant, and immunomodulatory features of several varieties of mulberry and blueberry, and of medicinal plants including *Echinacea* spp. (11). Increasingly, wild plant-associated microbes are used to ferment various plant materials to improve biological activities (12). *Microbacterium lacticum* fermentation yields L-lysine, which may be added to human foods and animal feeds to improve protein quality (13). Therefore, fermentation is an effective approach to simultaneously increase both nutritional variety and biological activity.

In this study, the *D. lotus* was treated with the traditional fermenters *Lactobacillus plantarum* B7 and the wild plant-associated microbes *Microbacterium flavum* YM18-098, and changes in the levels of phenolic compounds and vitamin C were evaluated. The anti-α-glucosidase and anti-trypsin activities, DPPH and ABTS radical scavenging activities were also examined. The correlations among variables were determined.

MATERIALS AND METHODS

Materials *D. lotus* fruit was collected in Shexian county, Hebei province, China. *L. plantarum* B7 was isolated from fermented sour dough and stored in the China Center of Industrial Culture Collection (CICC 23121). *M. flavum* YM18-098 was a endophyte which isolated from crude *D. lotus* fruit. After cleaning and disinfecting,

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.01.005

D. lotus fruit was ground into a homogenizing solution with sterile saline. Diluents (200 uL) were added to LB medium and cultured for 72 h at 28°C. Single colony was selected for purification and identification. Both strains were characterized by 16S rDNA sequencing. MRS broth (g/L): peptone 10 g, beef extract 5 g, yeast extract 4 g, glucose 20 g, tween 80 1 mL, sodium acetate anhydrous 5 g, ammonium citrate dibasic 2 g, K_2HPO_4 2 g, Mg_2SO_4 0.2 g, $MnSO_4$ 0.05 g. LB broth (g/L): tryptone 10 g, yeast extract 5 g, NaCl 10 g. *L. plantarum* B7 and *M. flavum* YM18-098 were grown for 24 h in 100 mL amounts of MRS and LB broth, respectively, at 30°C with shaking at 120 rpm (incubator model SCGZ-1102C, Yongcheng Industrial Development Co. Ltd., Shanghai, China). All chemicals and reagents were of analytical grade.

Fermentation *Diospyros lotus* fruit *D. lotus* fruit (10 g) was suspended in 150 mL of distilled water and autoclaved. Single or mixed bacterial cultures (*L. plantarum* B7: *M. flavum* YM18-098 to 1: 1) were then added to the suspension, followed by incubation at 30°C for up to 72 h. The optimal fermentation temperature and time were determined in a pilot study. Changes in colony-forming units (CFU) and pH during fermentation were measured by plate count and pH meter, respectively. After fermentation, the suspensions were centrifuged at 12,000 rpm at 4°C for 30 min, and the supernatants were stored at −80°C prior to analysis.

Determination of total phenolic and vitamin C contents Total phenolic contents were determined using the classical Folin-Ciocalteu assay (14). Briefly, dilutions (1 mL) of the solutions received 1 mL amounts of 0.2 M Folin-Ciocalteau reagent, were neutralized with sodium carbonate (7% [w/v]), and incubated in the dark for 120 min at room temperature. The absorbance at 750 nm was then measured by spectrophotometer. Gallic acid served as the standard; the calibration curve was derived using gallic acid solutions of 0–20 mg/mL. The calibration equation was: $Y = 9.9429x + 0.0044$, $R^2 = 0.9982$, where Y was the absorbance at 750 nm, and x the phenolic concentration (mg/mL).

The molybdenum blue colorimetric test was used to measure total vitamin C contents (15). Extract (10 mL) was reacted with 10 mL oxalate-EDTA (0.05 mol/mL oxalate and 0.2 mol/mL EDTA) and the volume was then adjusted to 50 mL. The solution was centrifuged at 5000 rpm at 4°C for 10 min and the supernatant collected and mixed (10 mL) with 1 mL metaphosphoric acid-glacial acetic acid and 2 mL 5% (v/v) sulfuric acid. After shaking, 4 mL of an ammonium molybdate solution was added and the volume adjusted to 50 mL. After standing for 15 min, the absorbance was measured at 750 nm.

Determination of tannic, catechinic, and ellagic acid contents High-performance liquid chromatography-diode array detection (HPLC-DAD) analysis was performed with the aid of an LC-20A HPLC instrument (Shimadzu, Kyoto, Japan) fitted with a reverse-phase hypersil BDS C18 column (250 mm × 4.6 mm; 5 μm particle size) (Thermo, Waltham, MA, USA). Gradient elution used mobile phase A (100% acetonitrile) and mobile phase B (1% [w/v] phosphoric acid in deionized water, pH 2.15) as follows: 5% A for 0–5 min, 5–15% A for 5–6 min, 15% A for 6–15 min, and 15–23% A for 15–25 min. The flow rate was 1 mL/min at a temperature of 25°C, and the injection volume was 10 μL Ultraviolet/visible spectra were recorded from 200 to 600 nm at an acquisition rate of 1.25 scan/s. Eluted tannic, catechinic, and ellagic acid were quantified by absorbance at 270 nm. Authentic commercial standards were dissolved in acetonitrile or DMSO to 1 mg/mL, and then diluted in acetonitrile or DMSO to yield a series of standard solutions (1, 0.5, 0.1, 0.02, and 0.01 mg/mL).

Trypsin inhibition assay The ninhydrin method was used to measure inhibition of the activity of trypsin on casein (16). The sample solution (2 mL) and a trypsin solution (1 mL) were incubated for 10 min at 37°C. Casein was then added and incubation continued for 10 min at 40°C. The reaction was stopped by addition of 1 mL of 1 M trichloroacetic acid. After centrifugation (3000 rpm, 15 min), 0.5 mL of the supernatant was mixed with 1 mL of acetic acid buffer (2 mol/L, pH 5.0) and 1 mL of a ninhydrin solution, the volume was adjusted to 5 mL with distilled water, and boiled for 15 min. After cooling, the absorbance at 568 nm was measured. The content of released amino acids was determined with L-tyrosine as standard. One unit of trypsin was defined as the quantity of enzyme liberating 1.0 μmol of tyrosine per minute under the standard conditions.

α-Glucosidase inhibition assay α-Glucosidase inhibition was measured using the method of Xiao (17). α-Glucosidase and pNP-G (1 mM) were dissolved in 0.1 M phosphate buffer (pH 6.9). α-Glucosidase solution (1 mL; 0.2 U/mL) and the sample solution (0.3 mL) were incubated for 10 min at 37°C followed by addition of 1 mL pNP-G solution and further incubation for 20 min at 37°C. Hydrolysis was stopped by addition of 1 mL anhydrous methanol and the absorbance at 405 nm was measured. The extent of inhibition was calculated as follows:

$$\text{Inhibition } \% = \frac{\Delta\text{Acontrol} - \Delta\text{Asample}}{\Delta\text{Acontrol}} \times 100\ \% \quad (1)$$

Determination of antioxidant activity DPPH radical scavenging activity was determined as described by Singh and Rajini (18). Briefly, a sample and freshly prepared DPPH solution were mixed (1:1 v/v) and held for 30 min at 25°C in the dark. Distilled water served as a control. The absorbance at 517 nm was measured. Measurement of ABTS radical scavenging activity was based on the fact that antioxidants destroy the blue/green (stable) ABTS radical produced by reaction of ABTS with potassium persulfate (19); the level of this radical was spectrophotometrically assessed after 30 min incubation by measuring the absorbance at 734 nm.

Statistical analysis All the experiments were performed at least three times. SPSS software was used for all data analysis. The data are reported as means ± standard deviations. The differences between groups were analyzed using one-way analysis of variance (ANOVA), and correction for multiple comparisons was made through a Dunnett's multiple comparison test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The changes of CFU and pH The growth curve of *L. plantarum* B7, *M. flavum* YM18-098 and mixed bacterial during fermentation is shown in Fig. 1, while the change of pH during fermentation is shown in Fig. 2. The growth curve indicates that the total viable count increased slowly in the first 6 h, increased rapidly from 6 h to 24 h, and remained in a stable state after 24 h. The pH value of the three kinds of fermentation broth

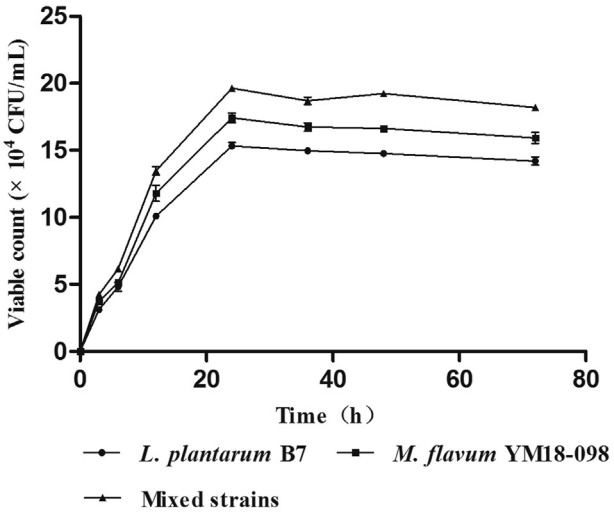

FIG. 1. Growth curve of *Microbacterium flavum* YM18-098, *Lactobacillus plantarum* B7 and mixed strains during fermentation.

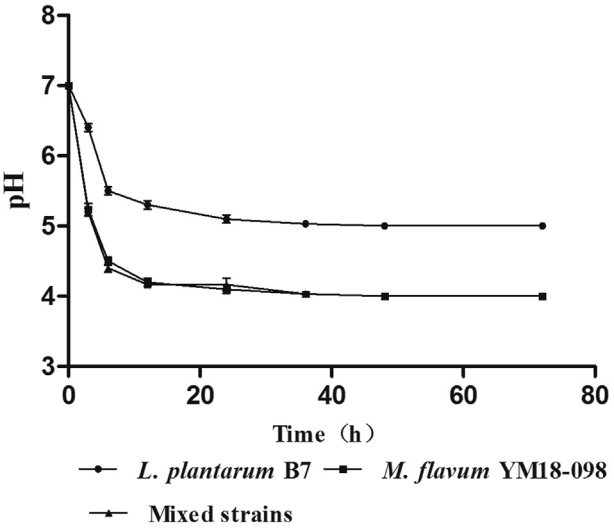

FIG. 2. Change in pH of *Microbacterium flavum* YM18-098, *Lactobacillus plantarum* B7 and mixed strains during fermentation.

Please cite this article in press as: Zhang, Z.-P., et al., Antioxidant and hypoglycemic effects of *Diospyros lotus* fruit fermented with *Microbacterium flavum* and *Lactobacillus plantarum*, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.01.005