RESEARCH ARTICLE

Studies on the physicochemical properties, antioxidants, and hypoglycemic activities of lotus (*Nelumbo nucifera*) leaf polysaccharides before and after fermentation

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Lotus (*Nelumbo nucifera*) leaf has the effects of lowering lipids, weight, and blood glucose with its polysaccharide as the most important functional component. This study used *Eurotum cristatum* to ferment the lotus leaf and investigated the changes in the structure and activity of lotus leaf polysaccharides before and after fermentation. Six components including WFJ-1, WFJ-2, WFJ-3 before fermentation and FJH-1, FJH-2, and FJH-3 after fermentation were isolated from Lotus leaf by ethanol precipitation method and DEAE-52 column. The monosaccharide composition and average molecular weight of the six components were characterized. The results showed that the extraction rate of polysaccharides from lotus leaves was 4.31 ± 0.45% before fermentation and 6.01 ± 0.52% after fermentation. The main constituent monosaccharides and dominant monosaccharides were the same before and after fermentation, but fermentation decreased the proportion of monosaccharides and glucosamine and significantly increased the proportion of glucuronic acid. Compared with WFJ-3, the average molecular weight and dispersion index of FJH-3 were significantly lower. The antioxidants as well as gluconeogenic enzyme inhibitory activities of FJH-3 were significantly enhanced compared to the pre-fermentation period. The improvement results of IR-HepG-2, an insulin-resistant human liver cancer cell model generated by inducing HepG-2 cells with high insulin or fatty acids, showed that WJH-1 and WJH-3 significantly induced the expression of hexokinase and improved the glucose metabolism in HepG-2 cells, whereas FJH-3 significantly increased the transcription of pyruvate kinase, increased glycogen synthesis, and promoted IR-HepG-2 cell glucose consumption, thus improving glucose metabolism in HepG-2 cells. The glucose-lowering mechanism of both WFJ-3 and FJH-3 was through significant up-regulation of the mRNA transcript levels of *IRS-1*, *Akt*, *PI3K*, and *GLUT4* in the PI3K/Akt insulin signaling pathway.

Keywords: lotus (*Nelumbo nucifera*) leaf; *Eurotum cristatum*; fermentation; functional activity; *IRS-1*; *Akt*; *PI3K*; *GLUT4*.

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Introduction

Lotus (*Nelumbo nucifera*) is an aquatic plant of the Water Lily family. Its dried leaf has the efficacy of relieving summer heat and clearing heat, generating and eliminating “Yang Qi”, dispersing fetish, and stopping bleeding. The functional activity of lotus leaf is closely related

to its chemical composition, which includes alkaloids, flavonoids, terpenoids, lignans, polysaccharides, *etc*. [1]. Among these, lotus polysaccharides have high content in the chemical composition of lotus leaf, and it is found that lotus polysaccharides have the roles of antioxidant, bacteriostatic, hypoglycemic, and enhanced immunomodulation, *etc*. [2]. In food nutrition and food processing, lotus leaf is mainly used in the form of dried leaves to produce bagged tea, which is used for the development of functional foods such as hypoglycemia, hypolipidemia, and weight loss. However, this single processing method makes it difficult to utilize the multifaceted efficacy of lotus leaves fully.

In recent years, with the development of the fermentation industry, research and development of fermented foods have become more extensive. Fermentation is a traditional means of Chinese medicine concoction and food production, where microbial fermentation of herbal materials can enhance efficacy, reduce toxicity or side effects, and even produce new active substances. Some studies have used *Enterococcus faecium* to ferment lotus leaves, resulting in extracts that significantly slow weight gain in obese SD rats, exhibit good lipid-lowering effects, and show improved antibacterial ability [3]. Other studies indicated that fermentation by microbes like *Eurotium cristatum* could significantly improve the antioxidant and anti-aging effects of materials such as mogro-fruit pomace and alleviate conditions like experimental colitis by improving intestinal flora imbalance [4, 5]. Therefore, according to the principle of tea fermentation, it is of great significance to conduct research on the fermentation of lotus leaf for developing new lotus leaf foods. *Eurotium cristatum* is a fungus derived from *Poria cocos* tea. Many studies have shown that *Eurotium cristatum* has significant health benefits including lipid-lowering and weight-loss, lowering blood glucose and blood pressure, inhibiting inflammation, protecting the liver, and regulating intestinal flora [6, 7].

This study used *Eurotium cristatum* to ferment lotus leaf to investigate the structural and functional effects of this fermentation on the polysaccharide components in lotus leaves. The research extracted and purified polysaccharides from lotus leaves before and after *Eurotium cristatum* fermentation to evaluate and compare the physicochemical properties, *in vitro* antioxidant activities, and regulation of glucose metabolism. The results of this study provided theoretical support for the development of novel lotus leaf products, potentially offering enhanced or new functional benefits through biotransformation.

Materials and methods

Preparation of fermented Lotus leaf polysaccharide

The raw materials of dried lotus leaves were purchased from Traditional Chinese Medicine market in Bozhou, Anhui, China and were identified by China Jiliang Uiversity, Hangzhou, Zhejiang, China. This research adopted the fermentation method developed by Xu *et al*. with slightly modification [8]. Briefly, the dried lotus leaves were crushed by a pulverizer and passed through a 60-mesh sieve. 10 g of lotus leaf powder was mixed with 5 mL of distilled water in a conical flask and sterilized at 121°C for 15 min. After cooling down on the ultra-clean bench, 1 mL of *Eurotum cristatum* (CICC 2099) (China General Microbiological Culture Collection Center, Beijing, China) spore suspension was inoculated with the concentration of 1.62 ± 0.56 × 10^5 spores/mL and incubated at 28°C for 2 days. The samples were taken once every 2 days and stored at 4°C. The total sugar content was measured by using the phenol and concentrated sulfuric acid method based on the formation of indophenols or their quinoid derivatives. Concentrated sulfuric acid was used as both a sulfonating agent and a powerful dehydrating/condensing agent, facilitating the reaction between the sample and phenol to create highly conjugated colored compounds. The alkaline medium stabilized and intensified

this color, and the fermented lotus leaves with higher polysaccharide content were selected for the next step of isolation and purification.

Extraction and purification of polysaccharides from lotus leaves

The dried lotus leaf powder was mixed with 80% ethanol (v/v) in a ratio of 1:20 and then sonicated for 1.0 h at room temperature. The extracts were combined, concentrated, precipitated with ethyl alcohol, and the crude polysaccharide was obtained by vacuum freeze-drying. The extraction rate of crude polysaccharide was calculated as follows [9].

$$\text{Polysaccharide extraction rate } (\%) = \frac{m_1}{m_0} \times 100\% \quad (1)$$

where m_1 was the dry weight of extracted polysaccharide of lotus leaf before and after extraction and fermentation (g). m_0 was the dry weight of crude lotus leaf powder (g). The crude polysaccharides were deproteinized by trichloroacetic acid (TCA) according to the method of Chen *et al*. [10]. D101 macroporous resin (Shanghai Yuanye Biotechnology Co., LTD, Shanghai, China) was used for decolorization [11], while DEAE-52 cellulose column (Shanghai Yuanye Biotechnology Co., LTD, Shanghai, China) was used to eluate with distilled water, 0.2 mol/L NaCl solution, and 0.4 mol/L NaCl solution in turn. The sugar content of each tube was detected by phenol-sulfuric acid method, and the elution curve was drawn [12]. The eluates were dialyzed at 4°C for 48 h and stored lyophilized. The polysaccharide eluted before fermentation with distilled water, 0.2 mol/L NaCl, and 0.4 mol/L NaCl were labeled as WJH-1, WJH-2, and WJH-3, while the polysaccharides eluted from Lotus leaves after fermentation with distilled water, 0.2 mol/L NaCl, and 0.4 mol/L NaCl were labeled as FJH-1, FJH-2, and FJH-3.

Determination of chemical composition

The total sugar content before and after fermentation was determined by the phenol-sulfuric acid method [13], and the glyoxylate content was determined by the carbazole sulfate method with D-glucuronic acid as the standard [14]. Protein content was determined by using bovine serum albumin (BSA) as a standard, and residual protein content of Lotus leaf polysaccharide before and after fermentation was detected according to the Caulophylline blue method. Total polyphenol content was determined by the ferrous tartrate method using gallic acid as a standard reagent. Total flavonoid content was determined by the modified Al $(NO_3)_3$ colorimetric method using rutin as standard [15].

Monosaccharide composition analysis

10 - 20 mg of purified polysaccharide samples were mixed with 5 mL of 2 mol/L trifluoroacetic acid (TFA) (for liquid samples, an equal volume of 4 mol/L TFA was mixed with the sample solution at 1:1 ratio) in 10 mL hydrolysis tube. After filling N_2 sealing tube at 10 L/min for 1 min, the sample was hydrolyzed at 110°C for 2 h. After cooling, 1 mL of methanol was added in the ratio of 1:1 before blowing dry with N_2 in a 70°C water bath. The addition of methanol and blow dry with N_2 was repeated 2 times to remove the TFA. 1 mL of 0.3 mol/L NaOH solution was then added to fully dissolve the residue. 400 µL of mixed monosaccharide standard solution or polysaccharide hydrolysis solution was placed in a 5 mL stoppered test tube followed by adding 400 µL of 1-phenyl-3-methyl-5-pyrazolone (PMP) methanol solution, mixing well, and then reacting at 70°C in a water bath for 2 h. After cooling down to room temperature, 400 µL of 0.3 mol/L HCl was added to neutralize the solution to pH 6 to 7 followed by chloroform extraction two times. The aqueous phase was filtered through a 0.45 µm microporous membrane and then analyzed by using Agilent 1100 High-Performance Liquid Chromatography (Agilent, Santa Clara, California, USA) with DAD detector and GraceSmart RP18 C18 column (250 mm × 4.6 mm, 5 µm) under the mobile phase A of 100 mM sodium phosphate buffer (pH 6.7), mobile phase B of acetonitrile with gradient elution, the detection wavelength of 250 nm, the column temperature of 30°C, the flow rate of 1 mL/min, and the injection volume of 5 µL.

Determination of molecular weight

The molecular weight (Mw) and dispersion coefficient (Mw/Mn) of lotus leaf polysaccharides before and after fermentation were determined by using high-performance size-exclusion chromatography (HPSEC) (Agilent Technologies, Palo Alto, CA, USA) with multi-angle laser scattering combined with a MALLS Oscillometric Detector (Wyatt Technology Co., Santa Barbara, CA, USA). The chromatographic column employed a Shodex OHpak SB-806M HQ (300 mm × 8.0 mm, i.d.) gel column tandem with a Shodex OHpak SB-G 6B (50 mm × 6 mm) gel column at a temperature of 35°C. 0.9% NaCl aqueous solution at a flow rate of 1 mL/min was used as the mobile phase, and the sample concentration was 1 mg/mL with a 40 µL injection volume.

DPPH radical scavenging

According to the method of Marecek *et al*. with slightly modification [16], 400 µL of 0.35 mM DPPH (1,1-diphenyl-2-picryl-hydrazyl radical) solution prepared from 50% ethanol was mixed with 50 µL of different concentrations of Lotus leaf polysaccharide and incubated in the dark at 37°C for 30 min before measuring optical density at 519 nm. Vitamin C was used as positive control, while distilled water was used as blank control. The DPPH-cleavage rate of the polysaccharides was calculated before and after the fermentation as follows.

$$\text{DPPH free radical scavenging rate }/\% = \left(1 - \frac{A_2 - A_3}{A_1}\right) \times 100\% \quad (2)$$

where A_1 was the absorbance of the blank. A_2 was the absorbance of the experimental sample. A_3 was the absorbance of the positive control.

ABTS$^+$ free radical scavenging activity

ABTS$^+$ working solution was prepared by mixing 7 mM 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS$^+$) with 2.6 mM potassium persulfate solution in equal proportion and diluted with PBS buffer (pH 6.6) until the absorbance reaching 0.75 ± 0.02 [17]. Vc was used as positive control. 20 µL of different concentrations of lotus leaf polysaccharides were mixed with 200 µL of ABTS$^+$ working solution, and the reaction was shaken and protected from light for 6 min before obtaining the absorbance at 734 nm. The ABTS$^+$ clearance was calculated below.

$$ABTS^+ \text{ free radical scavenging rate } (\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (3)$$

where A_1 was the sample absorbance. A_0 was the blank group absorbance. A_2 was the absorbance of control group.

Determination of total reducing power

0.2 mL of lotus leaf polysaccharide before and after fermentation with different concentration gradients diluted by distilled water was mixed with 0.2 mL of each 0.1 mol/L PBS and 0.1% potassium ferricyanide. The reaction was terminated by adding 0.2 mL of 10% TCA and centrifuged at 50°C for 5 mins. 0.2 mL of supernatant was removed and mixed with 0.2 mL of distilled water and 0.2 mL of 0.1% $FeCl_3$. The reaction mixture was placed at room temperature for 10 min before measuring the absorbance at 700 nm to determine the total reducing power as follows [18].

$$\text{Total reducing power} = A_1 - A_0 \quad (4)$$

where A_0 was the absorbance of the blank group. A_1 was the absorbance of the experimental sample.

Hydroxyl radical scavenging activity

1 mL of polysaccharide solution with different concentrations was mixed with 1 mL of 9 mmol/L ferrous sulfate and 1 mL of hydrogen peroxide. After incubation at 37°C for 10 min, 1 mL of 9 mmol/L salicylic acid was added. The reaction continued at 37°C for 30 min in dark before the absorbance was measured at 510 nm. The hydroxyl radical scavenging rate was calculated below [19].

$$\text{Hydroxyl radical scavenging rate } (\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (5)$$

where A_0 was the absorbance of blank group. A_2 was the absorbance of control group using

ethanol. A_1 was the absorbance of the experimental sample.

***In vitro* hypoglycemic activity**

(1) Inhibitory activity of α-amylase

100 μL of polysaccharides in different concentrations were reacted with an equal volume of 5 U/mL α-amylase at 37°C for 30 mins with acarbose as the positive control followed by the addition of 200 μL of 0.5% soluble starch solution. After 10 min reaction, 100 μL of the reaction mixture was added to 400 μL of 3,5-dinitrosalicylic acid (DNS). The OD value was determined at 540 nm. The α-amylase was calculated as follows [20].

$$\text{Alpha} - \text{amylase}\ (\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (6)$$

where A_1 was the absorbance of the sample. A_2 was the background absorption of PBS buffer. A_0 was the absorbance of the blank control group.

(2) Inhibition of α-glucosidase

200 μL of different concentrations of lotus leaf polysaccharides was mixed with 40 μL of 0.5 U/mL α-glucosidase at 37°C for 30 mins with acarbose as the positive control followed by adding 100 μL of 4 mM of p-nitrophenyl-D-α-pyranoside (pNPG) to continue the reaction for 10 mins before measuring the absorbance of the polysaccharides at 405 nm. The α-glucosidase was calculated below [20].

$$\text{Alpha} - \text{glucosidase}\ (\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (7)$$

where A_1 was the absorbance of the sample. A_2 was the background absorption of PBS buffer. A_0 was the absorbance of the blank control.

Hypoglycemic effect of polysaccharides on HepG2 cells before and after fermentation

(1) Screening of polysaccharide concentration

2 mg each of WFJ-3 and FJH-3 were mixed with 2 mL PBS to formulate a 1 mg/mL master batch followed by series dilution to 0.05, 0.1, 0.5, and 1.0 mg/mL for the cell viability assay. HepG2 cells (SCSP-510) were obtained from the Typical Culture Collection Center of the Chinese Academy of Sciences (Wuhan, Hubei, China) and divided into blank, control, and experimental groups, where the blank group was cell-free with an equal volume of medium and the control group having 1×10^5/mL cells. The experimental group was consisted with 1×10^5/mL cells and 0.05, 0.1, 0.5, and 1.0 mg/mL of Hepatitis C polysaccharide medium (Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China), respectively. 6 replicates were set up for each group. After 24 h incubation, CCK-8 kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) was applied following manufacturer's instructions. The absorbance was measured at 450 nm, and the viability of the cells was calculated as follows.

$$\text{Cell viability}\ (\%) = \frac{OD_1 - OD_0}{OD_2 - OD_0} \times 100\% \quad (8)$$

where OD_1 was the absorbance of experimental group. OD_2 was the absorbance of control group. OD_0 was the absorbance of blank group.

(2) Establishment of HepG-2 insulin resistance model

Cells were grouped into blank group, control group, and model group with 6 replicates in each group. 100 μL of Dulbecco's modified eagle medium (DMEM) was used as blank group. The control and model groups were HepG-2 cells in logarithmic growth phase, where Malt extract medium was used in the control group, and the same medium containing glucosamine at concentrations of 5, 10, 14, 18, and 22 mmol/L was used in the model group. Three wells were set up for each concentration. After 24 h induction, the cell viability was determined by using CCK-8 kit and was calculated according to the formula (8). The glucose consumption ratio was determined by using glucose assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following manufacturer's instruction. The absorbance was measured at 505 nm to determine the optimal induction concentration for modeling as follows.

$$\text{Glucose consumption (mmol/L)} = A_0 - \left(\frac{A_1 - A_2}{A_3 - A_2} \times C_3 \times \text{Sample dilution factor}\right) \quad (9)$$

$$\text{Glucose consumption ratio} = \frac{\text{Glucose consumption in experimental group cells}}{\text{Glucose consumption of cells in the normal group}} \quad (10)$$

where A_1 was the absorbance of the model group at different glucosamine concentrations. A_2 was the absorbance of the blank group. A_3 was the absorbance of the standard in the kit. C_3 was the concentration of the standard (5.55 mM). A_0 was the absorbance of the different concentrations of glucosamine only.

(3) Effects of lotus leaf polysaccharides on glucose uptake and glycogen content in IR-HepG2 cells

After successfully constructed insulin resistance HepG2 cell model, the cells were divided into blank group (Control), positive group (PC), model group (Model), and drug-delivered group (WJH-1, WJH-3, FJH-1, and FJH-3). 100 µL of cell culture medium was added into the blank and model control groups, while 100 µL of polysaccharide dilution solution was added to the drug-delivered group, and 100 µL of 100 µmol/L metformin hydrochloride was added to the positive group. After incubation at 37°C, 5% CO_2 for 24 h, the culture solution was extracted and analyzed by using CCK-8 kit, glucose assay kit (GOD-POD method), and glycogen content assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The cell viability of each group was calculated using equation (8). The glucose uptake ratio was calculated using equations (9) and (10). The glycogen content was determined as follows.

$$\text{Glycogen (mg/mgprot)} = \frac{(A_1 - A_0)}{(A_3 - A_0)} \times C_1 \times 10 \div 1.11 \div C_{pr} \quad (11)$$

where 1.11 was the glucose content measured by anthraquinone method converted to glycogen content constant. C_1 was the concentration of 0.01 mg/mL. Cpr was the sample protein concentration (mg/mL). A_2 was the OD_{620} of the model group, the positive group, and the drug-delivered group. A_0 was the OD_{620} of the blank group. A_3 was the OD_{620} of the standard in the kit.

(4) Determination of the effects of lotus leaf polysaccharide on the antioxidant, hexokinase, and pyruvate kinase activities of IR-HepG2 cells

the protein content was determined by using protein quantification kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following manufacturer's instruction. The antioxidant activities were then determined using the superoxide dismutase (SOD) activity assay kit, cellular malondialdehyde (MDA) activity assay kit, and trace reduced glutathione (GSH) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following manufacturer's instructions. Further, the activities of pyruvate kinase and hashish kinase were determined by using pyruvate kinase (PK) kit and hashish kinase (HK) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following manufacturer's instructions.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was employed to examine the expression levels of the specific genes related to the insulin signaling pathway and glucose metabolism. After 24 h treatment, the cells were collected, and total RNA was extracted using TRIzol reagent. Primer Premier 6.0 and Beacon Designer 7.8 (https://www.premierbiosoft.com) were used for the design of the qRT-PCR primers (Table 1), which were synthesized by Sangon Biotech (Shanghai) Co., Ltd (Shanghai, China). The reaction consisted of 10.0 µL of Power SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.5 µL of each 10 µM forward and reverse primers, 1.0 µL of cDNA template synthesized by using SuperScript™ III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Waltham, MA, USA), and 8.0 µL of sterile nuclease-free water to 20 µL. The reaction was carried out in a Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the program as 95°C for

Table1. qRT-PCR primers information.

Gene	Genbank accession No.	Primer sequences (5' to 3')	Size (bp)	Annealing (℃)
Human GAPDH	NM_002046.5	CCATGACAACTTTGGTATCGTGGAA GGCCATCACGCCACAGTTTC	107	60
Human Akt	NM_005163.2	GCCCCACTTCCCCCAGTTCT CCGCCTCTCCATCCCTCCAA	94	60
Human GLUT-4	M20747	TCGACCAGCATCTTCGAGACAG CCACCAACAACACCGAGACCAA	106	60
Human IRS-1	NM_005544	AGAACTCACTCGGCAGGCACATC TGGTGGGTAGGCAGGCATCATCT	111	60
Human PI3K	NM_001242466.1	GAGCGGTACAGCAAAGAATACATAGA GCCTGCTTCTTCAAGTCTTCTTCCA	155	60
Human GAPDH	NM_002046.5	CCATGACAACTTTGGTATCGTGGAA GGCCATCACGCCACAGTTTC	107	60
Human G6PC1	NM_000151.4	CCCAAGTCGAGCTGGTCTT GCAGTAGGGGATGACACTGA	86	60
Human PEPCK	NM_004563.4	CCCTGGGAGATGGTGACTTT GCTGCCGAAGGAGATGATC	152	60

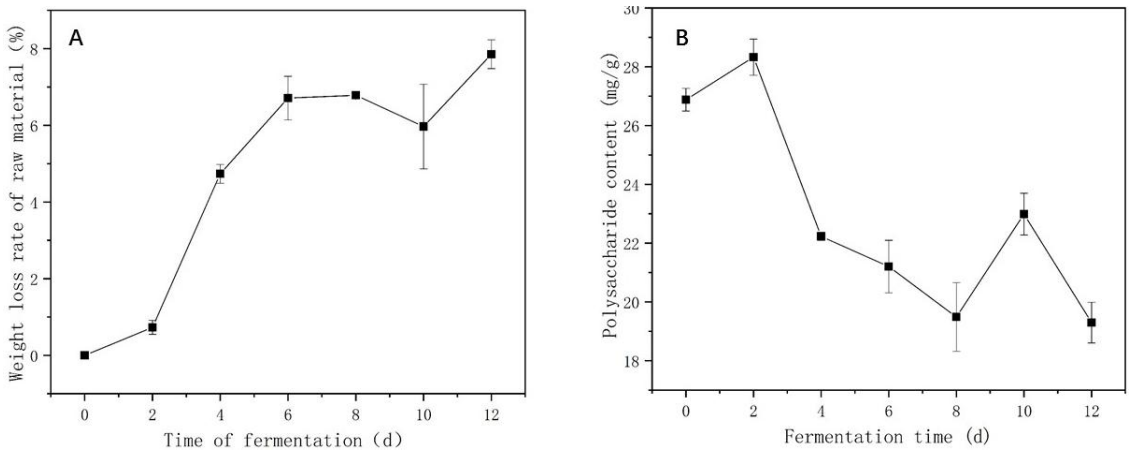

Figure 1. Changes in weight loss and total sugar of lotus leaves after 12 days of fermentation. **A.** the weight loss rate of lotus leaf. **B.** the total sugar of lotus leaf.

1 min followed by 40 cycles of 95°C for 15 s, 63°C for 25 s to collect fluorescence signal. The melt curve analysis was performed from 55°C to 95°C to confirm PCR product specificity. Each sample was repeated three times, and the relative expression level of each gene was statistically analyzed by $2^{(\text{Ct internal reference gene - Ct target gene})}$.

Statistical analysis

SPSS 18.0 (IBM, Armonk, NY, USA) was employed for statistical analysis. Data was presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used to analyze the inter-group significance level with *P* value less than 0.05 as statistically significant difference.

Results and discussion

Weight loss rate and total polysaccharide content of lotus leaves after fermentation

During the initial growth phase of 0 - 2 days, *Eurotum cristatum* consumed relatively few substances, and the polysaccharide content

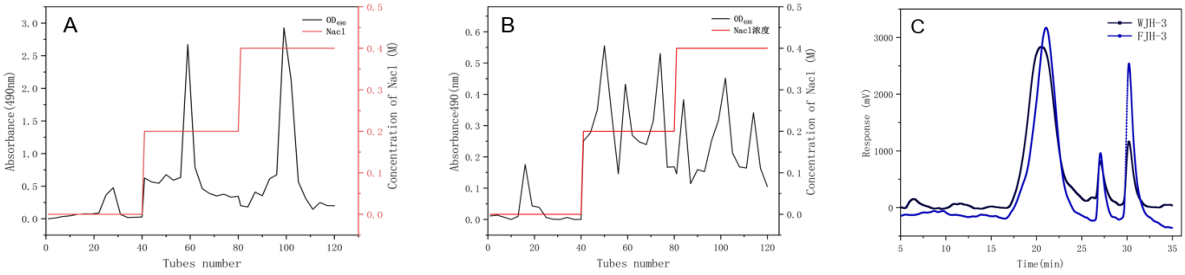

Figure 2. Elution curves of lotus leaf polysaccharide before (**A**) and after fermentation (**B**), and average molecular weight of WJH-3 and FJH-3 (**C**).

Table 2. Chemical composition of polysaccharides before and after fermentation.

Chemical composition (%)	WJH-1	WJH-3	FJH-1	FJH-3
Polyphenol	7.45	2.31	5.83	1.74
Flavones	1.88	1.79	2.82	0.34
Uronic acid	39.97	79.97	28.15	92.70
Protein	4.12	3.72	4.62	5.71
Polysaccharide content	82.01	93.27	89.38	91.29

increased during this period, which might be due to *Eurotum cristatum* consuming lotus leaves during the pre-fermentation stage to produce new polysaccharides, providing nutrients for subsequent growth or might be due to *Eurotum cristatum* producing cellulase and pectinase, which led to an increase in polysaccharide content. With further subsequent growth of 2 -6 days of *Eurotum cristatum*, substrate consumption increased substantially until it stabilized (Figure 1). Therefore, lotus leaves from the second day of fermentation were selected in this study.

Polysaccharide extraction rate of lotus leaves before and after fermentation

The lotus leaf polysaccharide extraction rate before and after fermentation were 4.31 ± 0.45% and 6.01 ± 0.52%, respectively. The extraction rate of crude polysaccharides was greatly increased by the fermentation of *Eurotum cristatum* (Figures 2A and 2B). The purification of polysaccharides from lotus leaves before fermentation yielded three polysaccharides of WFJ-1, WFJ-2, WFJ-3 with yields of 17.50%, 5.55%, 10.86%, respectively. The purification of fermented lotus leaf polysaccharides also yielded three polysaccharides of FJH-1, FJH-2, FJH-3 with yields of 21.25%, 8.53%, 24.78%, respectively.

The chemical composition and monosaccharide composition of lotus leaf polysaccharides

The total sugar contents of WFJ-1 and WFJ-3 before fermentation were 82.01% and 93.27%, and the glucuronic acid content was 39.97% and 79.97%, respectively. After fermentation, the total sugar contents of FJH-1 and FJH-3 were 89.38% and 91.29%, and the glucuronic acid contents were 28.15% and 92.70%, respectively. In addition, the protein contents of WFJ-1, WFJ-3, FJH-1, and FJH-3 were low (Table 2). Chemical composition analysis showed that the four polysaccharides before and after fermentation were heteroacidic polysaccharides with different monosaccharide contents. WJH-3 was mainly composed of galactose, rhamnose, arabinose, glucose, mannose. and xylose with a molar ratio of 14.71: 11.56: 9.86: 3.92: 1.76: 1.71. FJH-3 was also composed of galactose, rhamnose, arabinose, glucose, mannose, and xylose with a molar ratio of 9.98: 9.36: 6.61: 2.35: 1.71 (Table 3). The results showed that WJH-3 and FJH-3 had the same major monosaccharides, and the dominant monosaccharide was galactose. Further, *Saccharomyces cerevisiae* fermentation

decreased the content of monosaccharides and glucosamine. FJH-3 did not even contain galactosamine and greatly increased the content of glucuronic acid, especially D-galacturonic acid, which could be attributed to the production of pectinase by *Saccharomyces cerevisiae* during the fermentation process and the destruction of the cell wall. It was consistent with the results of the increase in the extraction rate after fermentation.

Table 3. Main monosaccharide composition (%) of polysaccharide before and after fermentation.

Monosaccharide	WJH-3	FJH-3
L- Guluronic acid	0.302	0.236
D- Mannuronic acid	0.120	0.206
D- Mannuronic acid	1.759	1.160
D- Glucosamine	0.986	0.034
D- Ribose	0.545	0.049
L- Rhamnose	11.563	9.358
D- Glucuronic acid	1.779	1.380
D- Galacturonic acid	51.947	66.740
D- Galactosamine	0.189	0.000
D- Glucose	3.915	2.352
D- Galactose	14.708	9.975
D- Xylose	1.713	1.230
L- Arabinose	9.863	6.610
L- Fucose	0.612	0.670

Average molecular weight after fermentation

The results of Mw (weight average molecular weight) and Mw/Mn (number average molecular weight) before and after fermentation showed that the Mw of WFJ-3 was 33.14 KDa and the MW/Mn was 2.68, while the Mw of FJH-3 was 28.17 KDa and the MW/Mn was 2.26 (Figure 2C), which indicated that the moderate molecular weight was favorable to cross the cell membrane barrier and thus showed good biological activity. The average molecular weight and dispersibility of FJH-3 were lower than those of WFJ-3, which might show better biological activity. Meanwhile, compared with high molecular weight polysaccharides, low molecular weight polysaccharides had more exposed reducing ends to accept and eliminate free radicals and therefore had better antioxidant properties [21].

***In vitro* antioxidant activity of lotus leaf polysaccharide fermentation**

Excessive reactive oxygen species break the homeostasis of the antioxidant defense system, leading to aging of the organism, multiple metabolic diseases, inflammation, cardiovascular diseases, and cancer [22]. The antioxidant properties of lotus leaf polysaccharides were evaluated before and after fermentation by determining DPPH radical scavenging, ABTS+ radical scavenging, hydroxyl radical scavenging, and total reducing power. The results showed that antioxidant capacity was enhanced with increasing concentration. The DPPH radical scavenging rate, ABTS+ radical scavenging rate, hydroxyl scavenging rate, and total reducing power level of the post-fermentation lotus leaf polysaccharide were higher than that of pre-fermentation one when the concentration of lotus leaf polysaccharide was 8 mg/mL. The post fermentation power levels were higher than those before fermentation. The ABTS+ radical scavenging ability, total reducing power, and hydroxyl radical scavenging rate (IC_{50}) of FJH-1 were higher than those of the other five fractions, while the DPPH radical scavenging ability and the highest hydroxyl radical scavenging rate of FJH-3 were higher than the others. In comparison of the three lotus leaf polysaccharide fractions before fermentation, the activities of the four antioxidant indexes of WJH-1 were higher than those of WJH-2 and WJH-3 (Figure 3).

***In vitro* hypoglycemic activity of fermented lotus leaf polysaccharides significantly increased**

α-glucosidase is a glycoside hydrolase, which can regulate the level of glucose metabolism in the body. α-amylase plays a key role in the decomposition of starch in the body. Inhibition of α-amylase activity can effectively reduce the decomposition of starch and glycogen in the body, delay the absorption rate of starch and high molecular carbohydrates, and improve the rapid rise of blood glucose in diabetic patients after meals [23]. The results showed that, in α-glucosidase inhibition, the inhibition rate first increased and then decreased with increasing

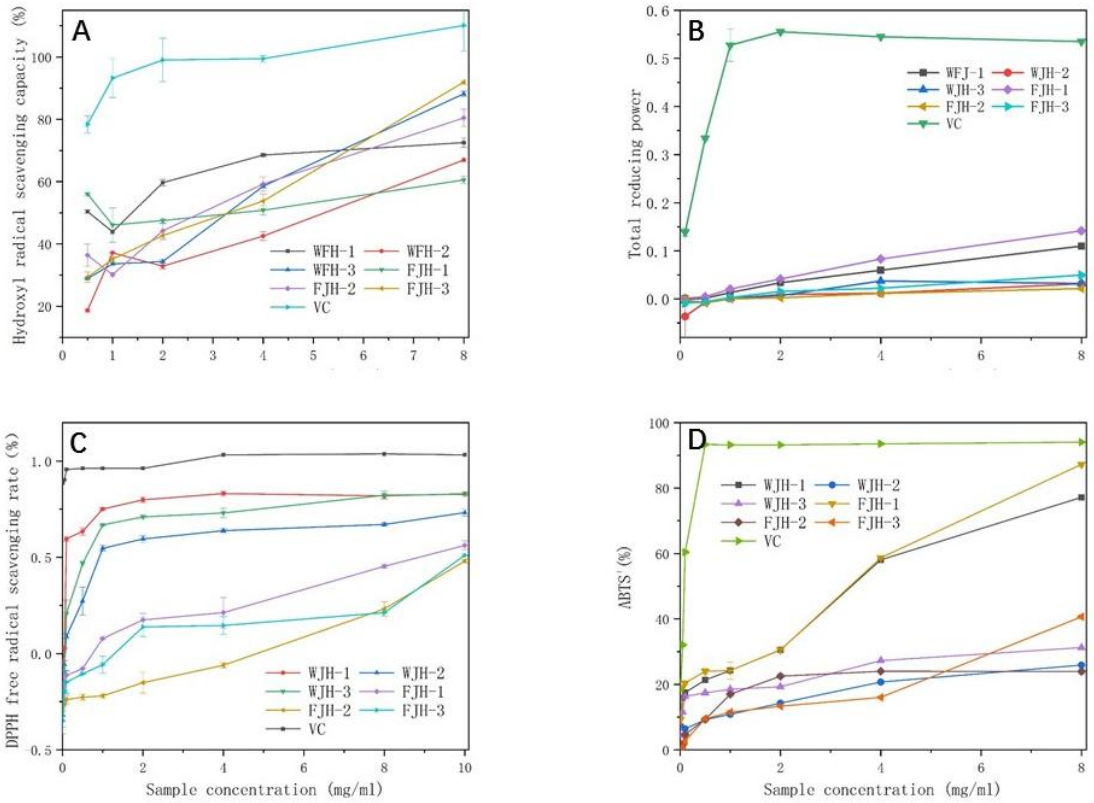

Figure 3. *In vitro* antioxidant activity of lotus leaf polysaccharides before and after fermentation. **A.** the DPPH radical scavenging rate. **B.** the ABTS+ radical scavenging rate. **C.** the hydroxyl radical scavenging rate. **D.** the total reducing power level of the polysaccharide.

polysaccharide concentration, eventually stabilizing. Comparison of the maximum inhibition rates revealed the order as Vc > FJH-3 > FJH-2 > FJH-1 > WJH-3 > WJH-2 > WJH-1. The post-fermentation polysaccharide inhibition rate was greater than the pre-fermentation. When the concentration of FJH-3 was 0.49 mg/mL, the maximum inhibition rate was 43.23%. However, it was still lower than the α-glucosidase inhibition ability of Vc. In terms of α-amylase inhibition, the α-amylase inhibition ability of FJH-3 was higher than that of Vc to a certain extent, and the *in vitro* hypoglycemic activity of FJH-3 was significantly better than that of Vc after fermentation. The α-amylase and α-amylase inhibition activity of FJH-3 was the best. The antioxidant activity of WJH-3 was the best among the three fractions before fermentation (Figure 4).

Correlation analysis of the chemical composition of lotus leaf polysaccharides with *in vitro* antioxidant and hypoglycemic activities before and after fermentation

The *in vitro* antioxidant activity and hypoglycemic activity of polysaccharides are affected by chemical composition factors. Pearson correlation analysis showed that a rational mechanism of fermentation affected the antioxidant and hypoglycemic activities of polysaccharides. The amount of free radical reduction appears to be related to the amount of electron- or hydrogen-supplying capacity in the antioxidant molecule. Ketone or aldehyde groups in polysaccharides are electrophilic groups that promote hydrogen release from the O-H bond under acidic media [24]. The results demonstrated that polyphenols, flavonoids, and the four indexes of antioxidants were positively correlated, in which the flavonoid content and the ABTS+ radical scavenging ability were significantly different. The flavonoid content of FJH-1 was the highest of 2.82%, which was

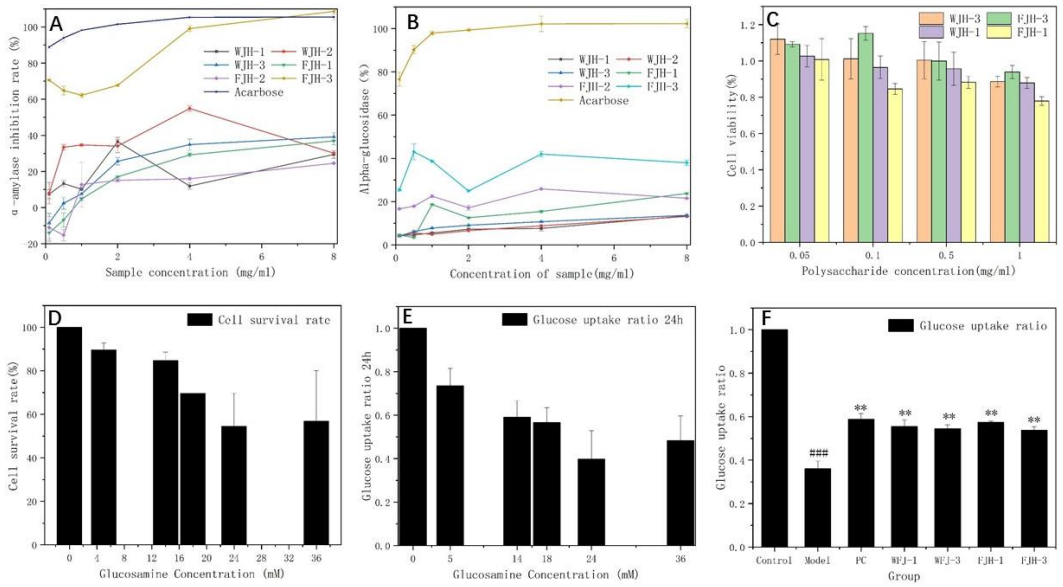

Figure 4. The glucose-lowering activity of polysaccharide before and after fermentation and the cell viability and glucose consumption of HepG2 induced by glucosamine and polysaccharide at different concentrations. **A.** inhibition rate of polysaccharide α-glucosidase. **B.** inhibition rate of polysaccharide α-amylase. **C.** effect of polysaccharide concentration on cell proliferation. **D.** cell viability of HepG2 induced by glucosamine at different concentrations. **E.** glucose consumption of HepG2 induced by glucosamine at different concentrations. **F.** glucose uptake of IR-HepG2 cells treated with polysaccharide. $^{\#}$: $P < 0.05$, $^{\#\#}$: $P < 0.01$, $^{\#\#\#}$: $P < 0.001$ compared to normal group. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ compared to model group.

Table 4. Correlation between chemical composition of lotus leaf polysaccharide before and after fermentation and *in vitro* antioxidant and hypoglycemic effects.

Coefficient of correlation	ABTS^{+}	Hydroxyl group clearance rate	Total reducing power	DPPH	α-glucosidase	α-amylase
Polyphenol	0.84	0.91	0.87	0.35	-0.59	-0.54
Flavones	0.96*	0.77	0.77	0.25	-0.79	-0.95*
Uronic acid	-0.95*	0.97*	-0.97*	-0.14	0.60	0.71

Note: *: $P < 0.05$.

identical to that of the previous research, and showed that the ABTS+ radical scavenging ability of FJH-1 was the highest. The aldehyde group in polysaccharides reacts with the hydrogen atom on the telomeric carbon, so polysaccharides with high content of glyoxalate usually have strong antioxidant properties. The results showed that the glyoxylate content of polysaccharides before and after fermentation was negatively correlated with their ABTS+ scavenging, total reducing power, and DPPH radical scavenging with the correlation coefficients (r) of -0.95, -0.97, and -0.14, respectively. The antioxidant properties of lotus leaf polysaccharides were influenced by their glyoxalate content, polyphenol content, and flavonoid content with higher flavonoid polyphenol content and lower glyoxalate content resulting in stronger antioxidant activity. Among the six polysaccharides, the highest antioxidant capacity of FJH-1 might be related to its lowest content of glyoxalate and highest content of flavonoids and polyphenols. The polyphenol and flavonoid contents were negatively correlated with the *in vitro* hypoglycemic indexes (α-glucosidase and α-amylase) with the correlation coefficients of polyphenols as -0.59, -0.54 and flavonoid correlation coefficients as -0.79, -0.95, respectively, whereas glucuronic acid content was positively correlated with the *in vitro* hypoglycemic indexes as 0.60, 0.71, respectively

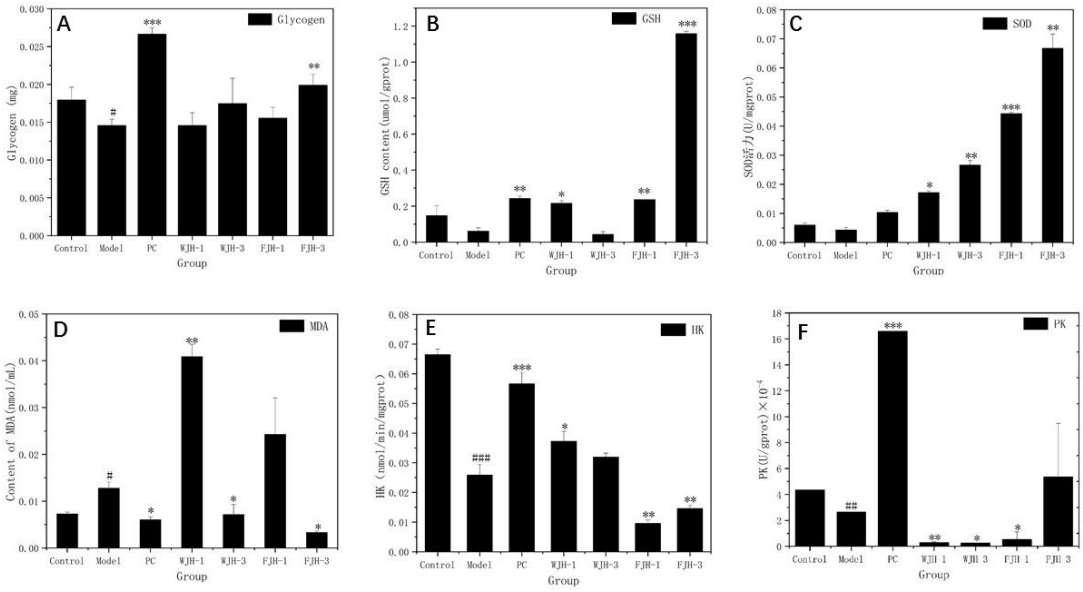

Figure 5. The glycogen content, antioxidant activity, PK activity, and HK activity of IR-HepG2 cells treated with lotus leaf polysaccharide before and after fermentation. **A.** glycogen content. **B.** GSH content. **C.** SOD content. **D.** MDA content. **E.** HK content. **F.** PK content.

(Table 4). The *in vitro* hypoglycemic performance was influenced by its flavonoid and polyphenol as well as glucuronic acid contents. The lower the flavonoid and polyphenol content and the higher the glucuronic acid content, the stronger the *in vitro* antioxidant activity. Among the six polysaccharides, the highest hypoglycemic ability of FJH-3 might be related to its lowest flavonoid and polyphenol content and highest glucuronic acid content.

Ameliorative effects of polysaccharides on insulin resistant HepG2 before and after fermentation

(1) Effect of polysaccharides on cell proliferation

The results showed that WFJ-1, WJH-3, FJH-1, and FJH-3 polysaccharides had little effect on the cell viability of HepG2 cells. All four polysaccharides basically did not affect the proliferation of the cells at a concentration of 0.5 mg/mL (Figure 4D). Therefore, the polysaccharide with a 0.5 mg/mL concentration was chosen in this study.

(2) Optimal modeling concentration of glucosamine

The results showed that glucose consumption increased rapidly at different concentrations of glucosamine and then remained stable as 54.53% at 24 mM with the HepG2 cells becoming insulin resistant (Figure 4E).

Fermented lotus leaf polysaccharide significantly enhanced glucose uptake as well as glycogen content of IR-HepG2 cells

The results demonstrated that glucose consumption was significantly lower in the model group than in the normal group, and it was increased by WFJ-1, WFJ-3, FJH-1, and FJH-3 interventions compared to the model group with FJH-3 having the highest glucose consumption but still lower than the glucose content in the PC group (Figure 4F). Compared with the normal control group, the intracellular glycogen content in the IR model group was significantly reduced, while, after polysaccharide treatments, the glycogen content of IR-HepG2 cells was elevated with the order of PC > FJH-3 > WJH-3 > FJH-1 > WJH-1 (Figure 5A), which indicated that the glycogen content was larger than that before fermentation. The results found that the glucose consumption and glycogen content of IR-HepG2 cells treated with FJH-3 were the highest, while

the best effect before fermentation was found in the IR-HepG2 cells treated with WFJ-1 and WFJ-3, indicating that HepG2 cells had glucose uptake and glycogen synthesis.

Fermented polysaccharides significantly increase the antioxidant activity of IR-HepG2 cells

Studies have shown that oxidative stress is closely associated with diabetes. The results showed that, at a glucosamine concentration of 24 mM, MDA content increased, while SOD enzyme activity decreased, and reduced GSH content decreased in IR-HepG2 cells, indicating that oxidative stress was involved in the pathological process of IR. The polysaccharide FJH-3 significantly decreased the content of MDA ($P < 0.05$), increased the activity of the SOD enzyme, and increased the content of reducing GSH in the cells (Figures 5B - 5D). The polysaccharides of lotus leaf after fermentation were enhanced in alleviating oxidative stress compared with those before fermentation.

Effects of polysaccharides on HK and PK activities before and after fermentation

There are many key rate-limiting enzymes in the glucose metabolism pathway, and hexokinase (HK) and pyruvate kinase (PK) are two of the core rate-limiting enzymes. HK increases insulin release and promotes glucose phosphorylation. PK changes phosphoenolpyruvate and ADP to ATP and pyruvate, which play an extremely important role in hepatic glucose metabolism. The results showed that, compared with the normal group, PK and HK activities were significantly reduced in the model group ($P < 0.01$). After administration of four polysaccharides of WJH-1, WJH-3, FJH-1, and FJH-3 to IR-HepG2 cells, WJH-1 and WJH-3 improved glucose metabolism in HepG2 cells by activating HK activity, while FJH-3 improved glucose metabolism in HepG2 cells by increasing PK activity, glycogen synthesis, and glucose consumption in the IR-HepG2 cell (Figures 5E and 5F).

Real-time fluorescence quantitative PCR (qRT-PCR) results

The effects of WJH-3 and FJH-3 on the expression of PI3K, PEPCK, IRS-1, AKT, G-6-P, and GLU-4 genes in IR-HepG2 cells showed that, compared with the normal group, the cells in the model group showed a significant decrease in the gene expressions of PI3K, IRS-1, AKT, GLUT-4 and a significant increase in PEPCK and G-6-P. Compared with the model group, the gene expressions of PI3K, IRS-1, AKT, GLUT-4 were increased after WJH-3 treatment in PC group. The gene expressions of PI3K, AKT, GLUT-4 were significantly increased ($P < 0.05$), indicating that WJH-3 could up-regulate the expression of IRS-1 gene in the cells, and meanwhile increase the expression of the IRS-1 downstream proteins PI3K and AKT, which also verified that the preceding WJH-3 increased the uptake of glucose in the cells and increased glycogen synthesis. After FJH-3 treatment, the gene expressions of PI3K, IRS-1, AKT, GLUT-4 were also increased, indicating that FJH-3 activated the PI3K/AKT signaling pathway and up-regulated the expression level of the GLUT-4 gene to promote the uptake of glucose in the cells by increasing the expression of the IRS-1 gene and the expression of PI3K and AKT proteins in the cells (Figure 6).

With the improvement of living standards in recent years, people's dietary patterns have undergone significant changes, marked by a substantial increase in high-fat and high-sugar food consumptions. Obesity has been recognized as one of the world's most pressing public health challenges, characterized by fat accumulation, metabolic disorders, and gut microbiota imbalance leading to overweight [25]. Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder that remains incurable. The core of treatment lies in controlling blood glucose levels and reducing the occurrence of complications. Strictly controlling blood glucose and lipid levels in patients with T2DM is fundamental to reducing cardiovascular complications and lowering mortality rates. Current pharmacological

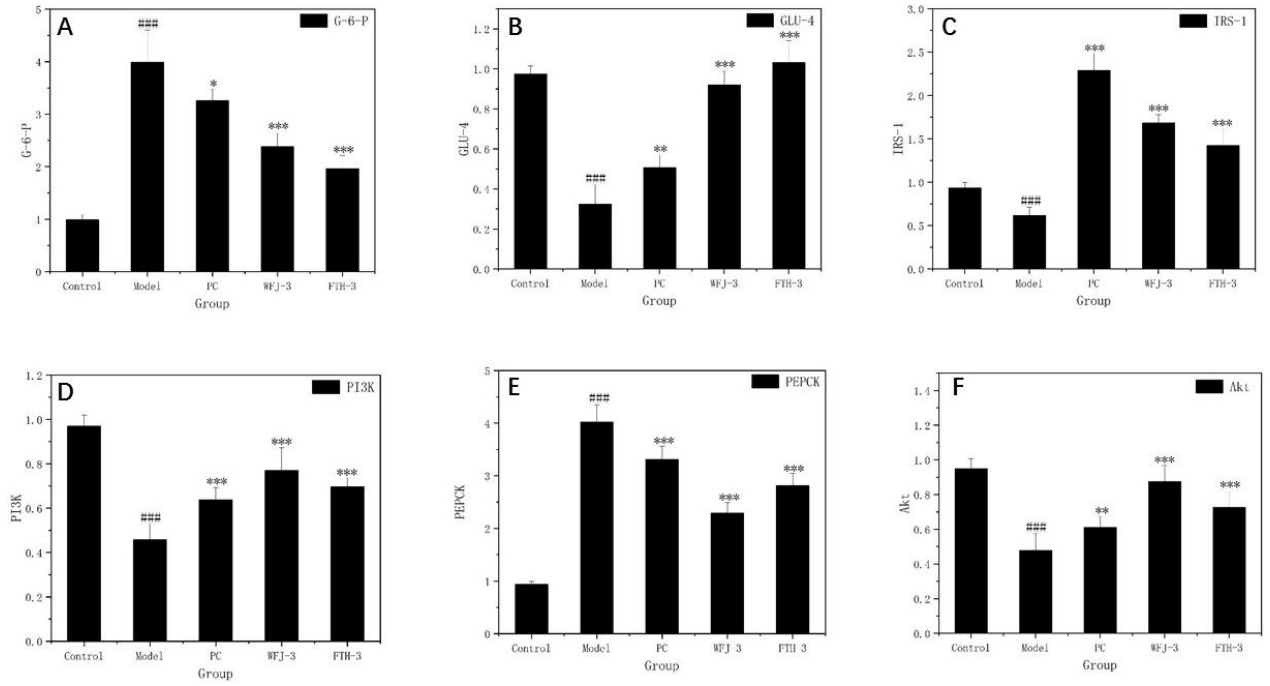

Figure 6. The expression levels of 6 genes in IR-HepG2 cells treated by WJH-3 and FJH-3. **A.** G-6-P gene. **B.** GLU-4 gene. **C.** IRS-1 gene. **D.** PI3K gene. **E.** PEPCK gene. **F.** AKT gene.

treatments rely on hypoglycemic agents such as metformin, pioglitazone, and sulfonylureas, which lower blood sugar through different mechanisms. While these medications effectively reduce patients' blood glucose levels, prolonged use of those medicines may cause adverse reactions, limiting their long-term clinical application. Metformin serves as the first-line treatment for T2DM, primarily working by decreasing hepatic glucose output and improving peripheral tissue insulin resistance (IR). However, it carries risks of lactic acidosis and gastrointestinal side effects [26, 27]. Therefore, continuous exploration and improvement the treatments for T2DM are still required in diabetes drug research. In recent years, plant polysaccharides as natural plant components have demonstrated significant potential in diabetes management. Their hypoglycemic effects feature diverse mechanisms, high safety, and minimal side effects. Compared to traditional hypoglycemic drugs, they are more readily accepted by patients and may offer broader benefits like immune enhancement and liver protection. Previous studies showed that cordyceps polysaccharide could alleviate weight gain in diabetic rats, reduce oxidative stress, and exert hypoglycemic effects [28]. Soybean polysaccharide improved gut microbiota imbalance in diabetes by activating intestinal flora-mediated tryptophan-indolepyrine metabolism and promoting indole metabolite production [29]. Atractylodes polysaccharides could exert hypoglycemic effects by increasing the abundance of *Bifidobacterium*, *Clostridium tenderum*, and *Pseudomonas aeruginosa*, while reducing the levels of *Escherichia coli-Shigella* spp. and *Escherichia coli* [30]. *Schisandra chinensis* polysaccharides could enhance fasting blood insulin levels and superoxide dismutase activity, lower fasting blood glucose and malondialdehyde (MDA) levels, improve STZ-induced pancreatic islet lesions, and inhibit JNK and related apoptosis proteins to prevent β cell apoptosis-mediated mechanisms, thereby achieving hypoglycemic effects [31]. Plant-derived polysaccharides provide new directions and possibilities for the treatment of T2DM.

Lotus leaf has been known for its weight loss and lipid-lowering effects since ancient times, and modern studies have found that lotus leaf polysaccharide as one of the effective components of lotus leaf has certain hypoglycemic effects. This study found that fermentation not only improved the yield of lotus leaf polysaccharide but also improved the *in vitro* antioxidant as well as hypoglycemic activity of lotus leaf polysaccharide. The mechanism of the hypoglycemic effect of fermented lotus leaf polysaccharide needs to be further investigated to provide a new direction and idea for the further development and utilization of lotus leaves and increase the possibility of finding natural and efficient hypoglycemic drugs. To study the changes in the structure and function of polysaccharides from lotus leaf before and after fermentation, three kinds of polysaccharides were obtained from the purification of lotus leaves polysaccharides before fermentation in this study including WFJ-1, WFJ-2, and WFJ-3, while the purification of fermented lotus leaf polysaccharides yielded FJH-1, FJH-2, and FJH-3 three polysaccharides. The results of *in vitro* hypoglycemic activity demonstrated that WJH-1 and WJH-3 improved glucose metabolism in HepG2 cells by activating HK activity, while FJH-3 improved glucose metabolism by increasing PK activity, increasing glycogen synthesis, and increasing glucose consumption. Thereby, FJH-3 had the most significant effect. Among them, the glucose-lowering mechanisms of both WFJ-3 and FJH-3 were through significant up-regulation of the mRNA expression levels of IRS-1, Akt, PI3K, and GLUT4 in the PI3K/Akt insulin signaling pathway.

Acknowledgements

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