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Effect on hypoglycemic activity and UPLC–MS/MS profiling of *Rosa roxburghii* fruit fermented with Chinese traditional distiller's yeast

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Abstract

To illuminate changes in the secondary metabolic spectrum and hypoglycemic activity between *Rose roxburghii* fruit fermented (RRFF) by Chinese traditional distiller's yeast and *R. roxburghii* fruit (RRF) using UPLC–MS/MS and streptozotocin-induced diabetic mice. Secondary metabolomics in the RRFF apparently differs from those in RRF; 32 pentacyclic triterpenoids were identified first, and ursolic acid-substituted roxburix acids were found to be the marker compounds. Testing of HepG2 cells indicated that RRFF had good glucose consumption capacity. Additionally, fasting blood glucose levels, glucose tolerance tests, plasma insulin levels and insulin resistance in diabetic mice indicated that RRFF was better than RRF. More importantly, RRFF exhibited good structure in pancreatic tissue. These results indicate that the fermentation process is beneficial for increasing the content of triterpenoids and enhancing hypoglycemic activity. Traditional fermentation with Chinese traditional distiller's yeast can provide a way to expand the application of nutritious fruits with short shelf life.

Keywords: *Rosa roxburghii* tratt; hypoglycemic effect; UPLC–MS/MS profiling; fermentation by Chinese traditional distiller's yeast, streptozotocin-induced diabetic mice.

Practical Application: We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome

1 Introduction

Diabetes mellitus is a complex chronic illness associated with a state of high blood glucose levels or hyperglycemia, occurring from deficiencies in insulin secretion, action, or both (Lin et al., 2022; Chaudhury et al., 2017). There were approximately 451 million people (aged 18–99) with diabetes globally in 2017, which is estimated to increase to 693 million by 2045, according to a report of the International Diabetes Federation (IDF) (Cho et al., 2018). Poorly controlled diabetes can lead to numerous diseases. In addition, the novel coronavirus disease 2019 (COVID-19) pandemic is associated with markedly elevated blood glucose levels and a remarkable degree of insulin resistance (Hayden, 2020). There are numerous hypoglycemic medications available in the clinic, but these medications are associated with many side effects with long-term medication. For example: hypoglycemia, insulin resistance, nausea, vomiting and congestive heart failure. In addition, insulin is inconvenient for daily carrying (Maruthur et al., 2016). Considering these drawbacks, it is necessary to investigate new effective medicines with few side effects. Plant medicines have been applied for the treatment of diabetes in some ethnic minority groups (Mahomoodally et al., 2016). In addition, the multicomponent and synergistic multitarget action of herbal medicine contributed to blood glucose homeostasis with few side effects during the clinical treatment of diabetes. Thus, it is important to investigate natural hypoglycemic products (Zhumabayev et al., 2022; Wu et al., 2019).

Rosa roxburghii Tratt (*R. roxburghii*), belonging to the Rosaceae family, has high edible and medicinal value as a source of homologous medicine and food (Wang et al., 2018). According to the record of the "Guizhou Tong Zhi", the fruit of *R. roxburghii* has been historically used for 380 years as an ancient Chinese medicine for the treatment of different diseases (Wang et al., 2020). Its raw juice is the most consumed as an astringency flavor beverage in folk medicine and claimed to have functions of tonifying the spleen, cuing diarrhea, and clearing summer heat. *R. roxburghii* fruit contains unusually high levels of vitamin C (Vit C, L-ascorbic acid) (AsA; similar to 1300 mg 100 g(-1) FW) (Huang et al., 2014; Lu et al., 2016). Furthermore, there are numerous functional ingredients in *R. roxburghii* juice and its pomace, such as polysaccharides, flavonoids, phenolic acids and triterpenoids. (Xu et al., 2014).These components have been reported to exert antioxidant, anti-inflammatory, antitumor, antimutagenic, antiatherogenic, radioprotective activities, etc. (Wang et al., 2018; Chen & Kan, 2018; Dai et al., 2007). Although *R. roxburghii* fruits have a long history of safe usage as traditionally healthy food, the shelf life and marketability of *R. roxburghii* fruits are significantly reduced due to being not

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stored for more than forty days under uncontrolled conditions. Thus, the fermentation processing of fresh *R. roxburghii* fruits to produce is one of the worth options for long-term storage and utilization of fruit residues (Guan et al., 2020). Fermentation is not only known as an outstanding and effective processing method to preserve foods (Mustafa & Chua, 2019) but is also reported to transform edible raw matter into novel bioactive compounds with unique immune, glycemic, and anti-inflammatory properties (Shimoga & Kim, 2022; Minamiyama et al., 2003). The literature is very rich in reports that fruit fermentation should increase the health benefits of natural food sources, especially probiotic dairy foods are beneficial to postprandial glycemia (Lee et al., 2016; Grom et al., 2020). Currently, the use of a well-defined strain starter culture is a core modern fermentation technology ensuring the consistency and quality of fermented products (Zhao et al., 2022; Mustafa & Chua, 2019). The people of the minority areas in China have developed rich ethnobotanical knowledge on producing diverse Chinese traditional distiller's yeast by fermentation, resulting in diverse microbial resources for making their traditional wine and fermented foods (Li et al., 2020). Our preliminary work shows that there are many active ingredients in *R. roxburghii* fruit pomace. To the best of our knowledge, there are no reports on *Rose roxburghi*i fresh fruit fermented by Chinese traditional distiller's yeast and hypoglycemic activities in vivo.

Therefore, the purpose of the present study was to illuminate changes in the common and specific chemical constituents between RRFFs by Chinese traditional distiller's yeast and RRF. Furthermore, the hypoglycemic activities were evaluated in vitro by testing HepG2 cells and in vivo in streptozotocin-induced diabetic mice by oral glucose tolerance, fasting blood glucose level, body weight and histopathological analysis assays. These results indicate that fermentation enhances hypoglycemic activity. This study improves our knowledge of the fermentation process of fruit-fermented beverages from the perspective of substrates and effects.

2 Materials and methods

2.1 Chemicals and reagents

Chromatographic grade methanol and acetonitrile were purchased from Chaoyan Biotechnology Co., Ltd. (Merck, Germany). Other reagents used were of analytical grade and were all supplied by Tianjin Kemiou Chemical Reagent Co., Ltd. Standard samples: Vitamin C, catechin and oleanolic acid were purchased from Guizhou Dida Biotechnology Co., Ltd. (Guizhou, China). DMEM cell culture medium was purchased from Gibco (USA), 96-well plates, culture flasks were purchased from Cornig (USA), penicillin/streptomycin double-resistant fluid and fetal bovine serum were purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Phosphate-buffered saline (PBS) was supplied by BI Co., Ltd. (Suzhou, China). Thiazolyl blue tetrazolium blue was supplied by Bomeibio Co., Ltd. (Hefei, China). Dimethyl sulfoxide (DMSO) was supplied by Sigma–Aldrich Corporation (St Louis, USA). Glycogen kits and the insulin ELISA kit were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Metformin was obtained from Shanghai Medicinal Co., Ltd. (Shanghai, China). Streptozotocin (STZ) was purchased from Solarbio Co., Ltd. (Beijing, China). A high-fat diet was purchased from Trophic Animal Feed High-tech Co., Ltd. (Nantong, China). Chinese traditional distiller's yeast were purchased from pectinase and were purchased from Guizhou Brewing Engineering Technology Development Center, Guizhou Keli Industrial Co., Ltd. (Guiyang, China), batch number: q-qk05-2003.

2.2 Samples preparation

Plant material: *R. roxburghii* Tratt was collected from Guizhou Province, and fresh fruit was collected in September 2019. All sapples were precisely identified by the authors and stored at -80 °C in the Key Laboratory of Chemistry for Natural Products of Guizhou Province and Chinese Academy of Sciences, China.

Extraction of ethanol fraction (**EE**): One hundred grams of fresh *R. roxburghii* Tratt fruits were washed with pure water, cut into small pieces (thickness approximately 0.1 cm), and refluxed with 70% ethanol 3 times (3.0 h each time). The 70% ethanol extraction of the investigated paste was subjected to an assessment of their metabolic profiles and hypoglycemic activities.

Extraction of the fermentation fraction (FE): One hundred grams of fresh *R. roxburghii* Tratt fruits were washed and cut into small pieces (thickness approximately 0.1 cm). Twice the amount of sterilized distilled water was added to beat for one minute by a homogenizer. *R. roxburghii* Tratt fruit pulp was treated with 0.5% Chinese traditional distiller's yeast and 0.2% pectinase along with adding 10% white sugar according to the slurry quality into a sterilized triangular flask and incubated at a temperature of 35 °C in a shaker incubator at 120 rpm for 20 days. A fermented dryness sample was achieved by vapourizing under reduced pressure (at 40 °C).

2.3 UPLC–MS/MS data acquisition

Preparation of the EE and FE samples

The two dried extracts were added to 1 mL of 70% methanol internal standard extract, vortexed for 5 minutes to mix, and then centrifuged (12000 r/min, 4 °C) for 10 minutes. The supernatant was filtered with a microporous membrane (0.22 μm) and stored in a sample bottle for UPLC–MS testing.

UPLC-Q/TRAP-MS Conditions

The sample extracts were analysed by using UPLC-Q/TRAP-MS on a SHIMADZU Nexera X2 UPLC system (SHIMADZU, Jappen) equipped with an AB4500 Q TRAP mass spectrometer (AB SCIEX, America) equipped with an electrospray interface (ESI). The analytical conditions were set as follows: the UPLC on an Agilent SB-C18 (1.8 μm, 2.1 × 100 mm) column with a linear gradient solvent system 5% B - 95% B (9 min) - 95% B (10 min) - 5% B (11.1 min) - 5% B (14 min) at a flow rate of 0.35 mL/min, [A: 100% H2O + 0.1% formic acid; B: 100% acetonitrile + 0.1% formic acid]. The oven temperature was set at 40 °C, and the injection volume was 4 μL. The extracts were acquired in both positive and negative ion modes with ion spray voltages of 5500 V and -4500 V, respectively. The source

temperature was 550 °C, gas flow 10 L/min, nebulizer pressure 50 psi and collision-activated dissociation (CAD) conducted in the high model. A specific set of MRM transitions was monitored for each period according to the metabolites eluted. The full scan range was from m/z 50 to 3000.

PCA and HCA

Unsupervised principal component analysis (PCA) was carried out by the statistics function prcomp within R (www.r-project.org), and the data were unit variance scaled before unsupervised PCA.

Hierarchical cluster analysis (HCA) was applied to determine the optimal number of clusters and to classify the heatmaps of metabolites.

2.4 Determination of total phenolic content (TPC), total flavonoid content (TFC) and total triterpenoid content (TTC)

The total phenolic contents of the EE and FE were determined using Folin-Ciocalteu's method. Generally, the extracts of two samples (12.5 mg) were mixed with H_2O (6.5 mL), Na_2CO_3 solution (2.0 mL, 7.5% m/v), and Folin-Ciocalteu phenol reagent (0.5 mL). The mixture was vortexed (1 min) and incubated at 70 °C for 30 min. Then, the filtrate was analysed using a spectrophotometer (750 nm, UV-180; Shimadzu, Kyoto, Japan). The total phenolic content was presented in gallic acid equivalents based on the standard curve of gallic acid.

The total flavonoid contents of the EE and FE were measured using a colorimetric method with slight modifications. In short, the extracts were mixed with ethanol (9 mL, 80% v/v) and 5% $NaNO_3$ solution (300 mL). The mixture was shaken well and left for 6 min at room temperature. Then, 10% $Al(NO3)_3$ solution (300 µL) and 1 N NaOH (0.5 mL) were added, shaken well and left for 10~15 min. The mixture was filtered with a 0.45 µm Millipore membrane filter and analysed using a spectrophotometer (517 nm, Shimadzu). The total flavonoid content of the samples was quantified based on a calibration curve of catechin, which was used as the standard.

The total triterpenoid content (TTC) was determined by the method of Fan and He with slight modification. A total of 0.5 mL MeOH was added to the test tube. After methanol was evaporated at 100 °C in a water bath, 0.40 mL (5%) vanillin glacial acetic acid solution and 1 mL perchloric acid were added and heated at 60 °C in a water bath for 15 min. Then, the mixture was transferred into an ice water bath, and 5.00 mL glacial acetic acid was added, shaken well and left for 10~15 min. The mixture was filtered with a 0.45 µm Millipore membrane filter and analysed using a spectrophotometer (545 nm, Shimadzu). The total triterpenoid content of the samples was quantified based on a calibration curve of oleanolic acid, which was used as the standard.

2.5 Animals and animal treatment

For all experiments, adult male C57BL/6J mice weighing 18-22 g (purchased from SPF (Beijing) Biotechnology Co., Ltd, SCXK (Beijing) 2019-0010) were used, according to the international rules for animal experiments (a proved by animal Ethics Committee of Guizhou Medical University, clearance No. 2000876) and according to guidelines for the appropriate use of experimental animals (NIH publication No. 80-23; revised 1978). The mice were acclimatized at a temperature of 23 ± 2 °C with free access to water and standard food pellets. The mice were housed in our animal facility for at least 1 week prior to the experiment. Adult male C57BL/6J mice (6 weeks) fed a high-fat diet for 30 days were intraperitoneally injected with 70 mg/kg body weight STZ (Beijing Solarbio Science & Technology Co., Ltd.). Seventy-two hours after the injection, fasting blood glucose (FBG) levels were measured from the tail vein of overnight fasting mice by a glucometer (Omron Healthcare Co., Ltd., China). The mice that exhibited FBG levels higher than 16 mmol/L were considered hyperglycemic in the study. The hyperglycemic mice were randomly divided into seven groups: normal group control (NC), diabetic group control (DC), metformin group (MF), EE group, FE-L, FE-M, and FE-H.

2.6 Evaluation of hypoglycemic activity

Hypoglycemic activity of RRFF on HepG2 cells

The HepG2 cell line was supplied by the Cell Bank of the Institute of Cell Biology (Shanghai, China). Then, the cells were cultured in DMEM containing 10% FBS with penicillin (100 U/mL)/streptomycin (100 µg/mL) in a humidified incubator (5% CO_2) at 37 °C. The medium was renewed every day.

HepG2 cells were seeded into 96-well plates in DMEM supplemented with 10% FBS and penicillin (100 U/mL)/ streptomycin (100 µg/mL) and cultured in a humidified incubator (5% CO_2) at 37°C for 24 h. HepG2 cells were incubated with fresh medium containing 1% FBS and 10^{-6} mol/L bovine insulin for 24 h. Cells were treated with 10^{-6} mol/L metformin, EE (50 µg/mL, 10 µg/mL, 2 µg/mL) and FE (50 µg/mL, 10 µg/mL, 2 µg/mL) containing 1% FBS. After incubation for 24 h, the glucose concentrations in the cell supernatant were determined at a wavelength of 505 nm by the glucose oxidase method.

Experimental design for the hypoglycemic effects of EE and FE in SD mice

The SD male C57BL/6J mice were divided into 7 groups, with 6 animals in each group. The animals were fasted with free access to water for 12 h before the experiment. Using an intragastric tube, Group 1 mice were administered EE (442 mg/kg bw) (0.5% carboxymethylcellulose). The animals in Group 2 were administered metformin (110 mg/kg) as a standard hypoglycemic drug (0.5% carboxymethyl-cellulose), and Groups 3-5 were treated with FE at 3 dose levels (225, 450 and 675 mg/kg bw). The remaining groups were treated with 0.9% solution in saline. The dosing volume was kept constant (20 ml/kg) for all the orally treated groups. The C57 mice were weighed weekly, and blood glucose concentrations were measured in the fasting state once every 10 days.

Measurement of fasting blood glucose level (FBG), body weight (BW) and food intake

The FBG levels of all mice were measured after overnight fasting and ad libitum access to water on the 0, 10th, 20th and 30th days by using a glucometer (Omron Healthcare Co., Ltd., China). During the course of treatment, the BW and food intake of the mice were measured every week.

Oral glucose tolerance test (OGTT)

On day $^{30\,of}$ treatment, the OGTT was conducted as follows: the SD mice were orally loaded with glucose (2 g/kg bw) after overnight fasting and ad libitum access to water. The levels of blood glucose in the mice at 0, 30 and 120 min were measured. Glucose tolerance was assessed by computing the total area under the curve (AUC) of blood glucose.

Blood and tissue sample collection

Overnight fasting mice in this study were anesthetized and sacrificed after 30 days. Subsequent blood samples were obtained from the femoral artery, and the blood samples were centrifuged at 4500 rpm at 4°C for 15 min to obtain the serum. The pancreatic tissue samples for histopathological investigation were stored in 10% neutral buffered formalin. The contents of insulin, triglycerides (TGs), total cholesterol (TC) and free fatty acids (FFAs) in serum were quantified using assay kits.

Histopathological analysis

On the last day of the study, all mice were sacrificed, and pancreatic tissue was collected. All tissues were kept in 10% neutral formalin solution for H&E staining analysis. Fixed samples were dehydrated with ethyl alcohol and then embedded in paraffin. Pathological observation with a microscope (Olympus-BX51, Tokyo, Japan) was conducted.

2.7 Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM) by using the Statistical Package for Social Sciences 18.0 program (SPSS Inc., Chicago, USA). Statistical differences were assessed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test. $p < 0.05$ was considered statistically significant.

3 Results and discussions

3.1 Determination of bioactive secondary metabolite content

Bioactive secondary metabolites from *Rose roxburghii* fruit are the material basis of diverse functions. Triterpenoids, flavonoids and phenolics are the major active compounds. However, there are only a few reports of qualitative and quantitative analyses of *R. roxburghii* in the literature. In this study, the change in the secondary metabolic spectrum between Rose roxburghii fruit fermented (RRFF) by Chinese traditional distiller's yeast and *R. roxburghii* fruit (RRF) using UPLC–MS/MS was investigated.

The total phenolic content, total flavonoid content and total triterpenoid content of samples from EE and FE were detected by spectrophotometric assays. The results for total phenolic content are expressed as gallic acid equivalents, total flavonoid content is expressed as rutin equivalents, and total triterpenoid content is expressed as oleanolic acid equivalents. Fermentation effectively enriched the phenolic and triterpenoid compounds and led to a total phenolic content of 13.8013 ± 0.8923 mg/g extract and a total triterpenoid content of 13.4426 ± 0.7302 mg/g extract ($p < 0.05$).

unfermented (TPC and TTC of 10.6112 ± 0.8822 mg/g extract and 10.4214 ± 0.8233 mg/g extract, respectively; $p < 0.05$). However, the fermented *R. roxburghii* fruit samples had the lowest TFC of 5.6507 ± 0.9302 mg/g extract compared with that of prefermentation. Table S1 of the supporting information is shown in the supporting information. The total phenolic content and total triterpenoid content postfermentation were approximately higher than those prefermentation, indicating that phenolic and triterpenoid compounds in *R. roxburghii* fruit possessed relatively lower polarity, consistent with a moderate dielectric constant of ethanol ($\varepsilon = 25.7$).

Although the main chemical constituents of *R. roxburghii* fruit are phenolic acids, flavonoids and triterpenoids, the secondary metabolic spectrum of the extract and fractions is due to the presence of some possible interfering compounds, such as lipids, aromatic amines, and organic acids. Thus, a detailed analysis of the secondary metabolic spectrum of pro- and postfermentation must be validated using the UPLC–MS/MS method.

3.2 Secondary metabolomics analysis

UPLC–MS/MS analysis of *R. roxburghii* fruit fermented by Chinese traditional distiller's yeast and *R. roxburghii* fruit is a powerful tool to identify and locate changes in chemical composition compounds. In negative ionization mode of the total ion current chromatogram, it was known that because chemical compositions compounds were presence of hydroxyl, glycoside, and/or carboxylic acid groups. With respect to obtained m/z accuracy values, MS/MS fragments with standard compounds, or reported data in references or mass bank, the comparison study was performed to identify positively or tentatively chemical compounds. According to the structural characteristics, 871 compounds were identified, including 176 flavanols, 133 phenolic acids, 114 lipids, 93 amino acids and derivatives, 91 organic acids, 62 terpenoids, 49 nucleotides and derivatives, 50 tannins, 26 alkaloids, 16 lignans and coumarins, 52 quinones and 9 other compounds. Detailed information on the secondary metabolic spectrum obtained from the UPLC–MS analysis is summarized in Table S2 of the supporting information.

To comprehensively understand the change in the secondary metabolic spectrum between *Rose roxburghii* fruit fermented by Chinese traditional distiller's yeast and *R. roxburghii* fruit. The raw data were processed by MS-DIAL and then analysed by SIMCA-P 14.1 multivariate statistical software. Samples were ordered and classified clearly by hierarchical clustering. The processed raw data and the LC–MS/MS methods used are reported in the Supplemental material (Table S2).

The PCA and OPLS-DA score plots showed a clear classification of the different samples. In brief, HCA classified the seven samples into three types, with PCA and OPLS-DA showing similar clustering. The fresh fruit and the samples after fixation, rolling, and first drying clustered together, and the samples after the second drying and yellowing clustered together, while the roasted sample was a third type. These results suggested that, of the whole manufacturing process, yellowing and roasting are the two critical steps that result in changes in the chemical constituents. To identify the chemical responsible for the classification of the various samples and which can serve as process markers, the S-plot was generated, and some critical compounds are listed in Figure 1.

The results presented in this report and the data in the literature show that typical secondary metabolites are an effective discrimination for unfermented *R. roxburghii* fruit (Table S2). For example, some triterpenoids are used as markers to discriminate fermented samples, such as camaldulenic acid, corosolic acid, maslinic acid, 2-hydroxyoleanolic acid and rosamultic acid. Moreover, we also found that some terpenoids, tentatively identified by referring to our previous results, increased slightly after fermentation. To explore the cause of the formation, a targeted metabolomics study provided a novel and sensitive approach to study the markers.

Although the nontargeted metabolomics results suggested a tentative classification of the *R. roxburghii* fruit fermentation process, many biologically active compounds have yet to be identified as marker compounds similar to those found in other metabolomics studies on *R. roxburghii*. Unique metabolomic studies are needed to further elucidate the metabolic profile before and after fermentation, focusing on a few specific compounds correlated with fermentation. On the basis of the expected fragmentation patterns, a series of roburic acid derivatives were identified in the RRFF product. Roburic acid and/or its derivatives are worth investigating and identified with reference to mass fragment data (Figure S1). In a previous study, eight oleanolic acid and ursolic acid-substituted euscaphic acid derivatives were isolated and identified in ripe *R. roxburghii* fruit. Roburic acid is the chemical precursor for biotransformation and can be used for the synthesis of Kajiichigoside F1. Some ursolic acid derivatives showed some degree of changes before and after fermentation, which identified them as critical compounds (Figure 2).

To illuminate the effect on the hypoglycemic activity of *R. roxburghii* fruit fermented with Chinese traditional distiller's yeast, it is necessary to perform hypoglycemic activity studies on secondary metabolites.

3.3 Evaluation of hypoglycemic activity

Determination of glucose consumption on insulin resistance HepG2 cells in vitro

To observe the hypoglycemic activity of *R. roxburghii* in vitro, glucose consumption testing of *R. roxburghii* in HepG2 cells was performed. Metformin (MF) was used as a case–control. As shown in Table 1, the glucose rate (%) of fermentation of R. *roxburghii* was 10.91 ± 0.91, 14.50 ± 2.85 and 14.90 ± 1.67 and showed a dose-dependent manner. A slightly higher value of glucose consumption of R. *roxburghii* fermented extraction was shown, but no significant difference was observed between the EE group and FE group. Furthermore, the cell inhibition rate of EE ranged from 2.01% to 3.12%, while the cell inhibition rate of FE was below the lower limit of quantitation.

Table 1. glucose consumption testing of *R. roxburghii* in HepG2 cells.

Analytes	Concentration (μg/mL)	Cell inhibiting rate (%)	Glucose consumption rate (%)
EE	50	2.01 ± 3.51	14.00 ± 1.74
	10	2.76 ± 5.91	13.29 ± 2.69
	2	3.12 ± 2.74	10.55 ± 0.34
FE	50	0	14.90 ± 1.67
	10	0	14.50 ± 2.85
	2	0	10.91 ± 0.91
MF	1×10^{-6} mol/L	8.67 ± 1.78	12.38 ± 4.35

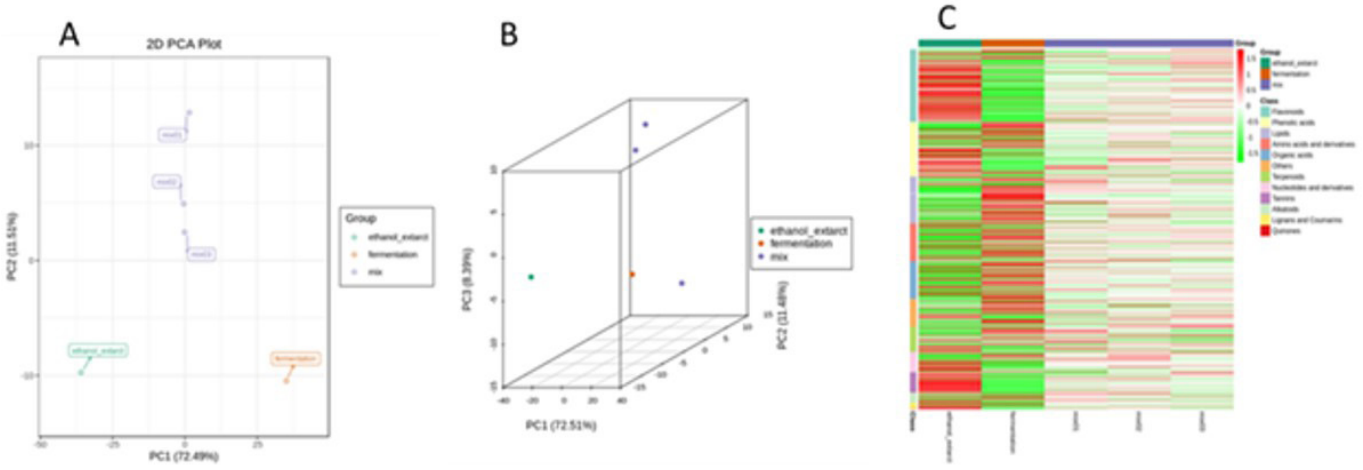

Figure 1. (A) 2D score plot of the principal component; (B) 3D score plot of the principal component; (C) Heatmap of compound classification.

Effect of EE and FE on food intake and BW in diabetic mice

As shown in Figure S2A, compared with the NC group, the food intake increased in the DC group ($p < 0.05$). Compared with the MF group and EE group, the food intake of the FE group decreased in the first week and showed an upwards trend in the 2nd, 3rd and 4th weeks. In Figure S2B, a slow upwards trend of the average body weight of the NC group was shown, whereas the average body weight of the DC group was rapidly increased. The results showed that the weight gain of SD mice could be reduced after FE and EE oral treatment, which indicated that RRFF was helpful for decreasing fat accumulation in C57 mice.

Effect of EE and FE on fasting blood glucose (FBG) and oral glucose tolerance test (OGTT)

The FBG levels increased significantly after injection with STZ at a dose of 70 mg/kg in SD mice. As shown in Figure 3A, SD mice demonstrated a significant increase in serum glucose levels compared to normal mice ($p < 0.01$). After treatment with EE and

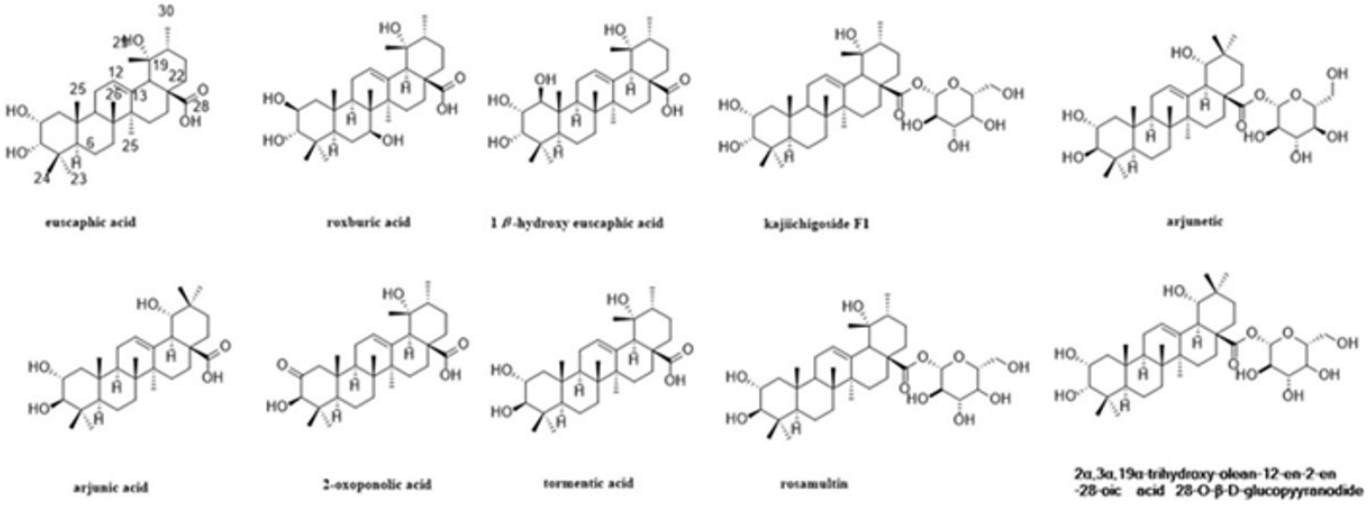

Figure 2. Representative oleanolic acid and ursolic acid-substituted euscaphic acid derivatives.

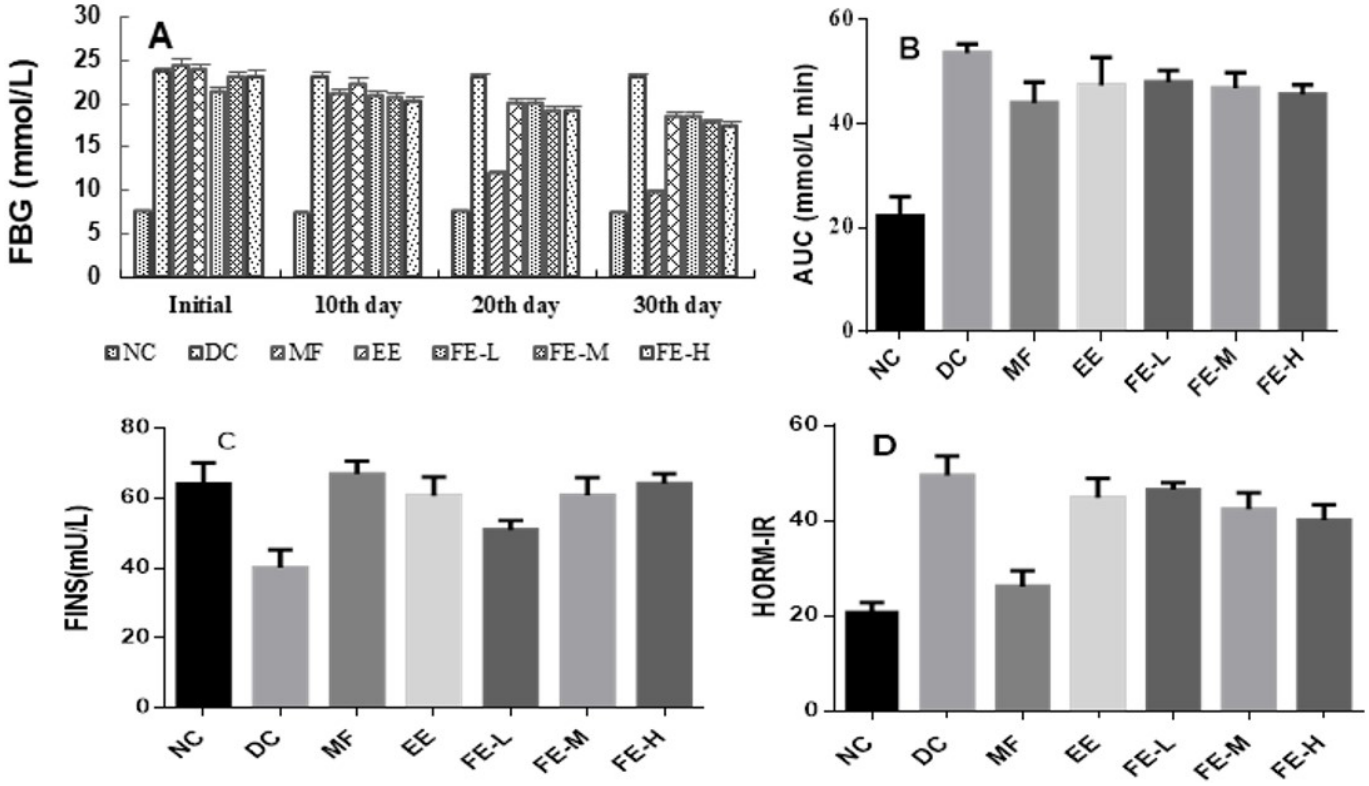

Figure 3. Effects of EE and FE treatment on blood glucose levels and insulin levels in SD mice. (A) Fasting blood glucose levels of each group of mice; (B) Oral glucose tolerance test of each group of mice; (C) Insulin level of each group of mice; (D) Insulin resistance of each group of mice.

FE, the FBG levels decreased significantly at the 20th day compared with the DC group ($P < 0.05$). FBG levels in the EE group ranged from 23.78 ± 1.54 to 18.51 ± 2.6, a reduction of approximately 22.2%. The decrease in glucose levels in the FE-M group ranged from 22.95 ± 2.41 to 17.67 ± 1.67, a reduction of approximately 23.0%. The decrease in glucose levels in the FE-H group ranged from 23.11 ± 2.26 to 17.50 ± 3.51, a reduction of approximately 24.3%.

The regulatory effect of EE and FE on blood glucose in SD mice was evaluated using the OGTT. As shown in Figure 3B, the OGTT of diabetic mice decreased significantly after RRFF treatment. Compared with the DC group ($p < 0.01$), the AUCs of FE-L, FE-M and FE-H were significantly reduced, which indicated that RRFF improved the glucose tolerance of SD mice ($p < 0.01$).

Effect on insulin and insulin resistance (IR) in SD mice

Since insulin levels may be closely related to high blood glucagon levels in SD mice (Kim & Kim, 2006; Li et al., 2019) Accordingly, high-fat diet-fed and STZ-induced diabetic mice were developed insulin deficiency (Figure 3). As shown in Figure 3C, insulin secretion in the DC group mice was markedly lower than that in the NC group, MF group, EE group, and FE group ($p < 0.01$). The IR (Figure 3D) in the DC group mice was significantly higher than that in the EE group and FE group ($p < 0.05$). IR in SD mice was reduced after oral administration of *R. roxburghii* fermentation. The results indicated that insulin sensitivity could effectively be improved by FE.

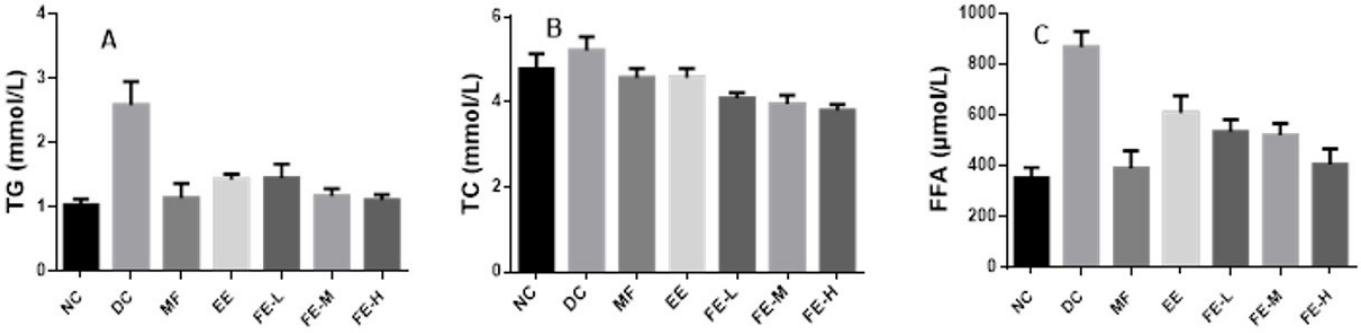

Figure 4. Effect of EE and FE treatment on lipid metabolism in SD mice. (A) Effect of EE and FE treatment on TG; (B) Effect of EE and FE treatment on TC; (C) Effect of EE and FE treatment on FFA.

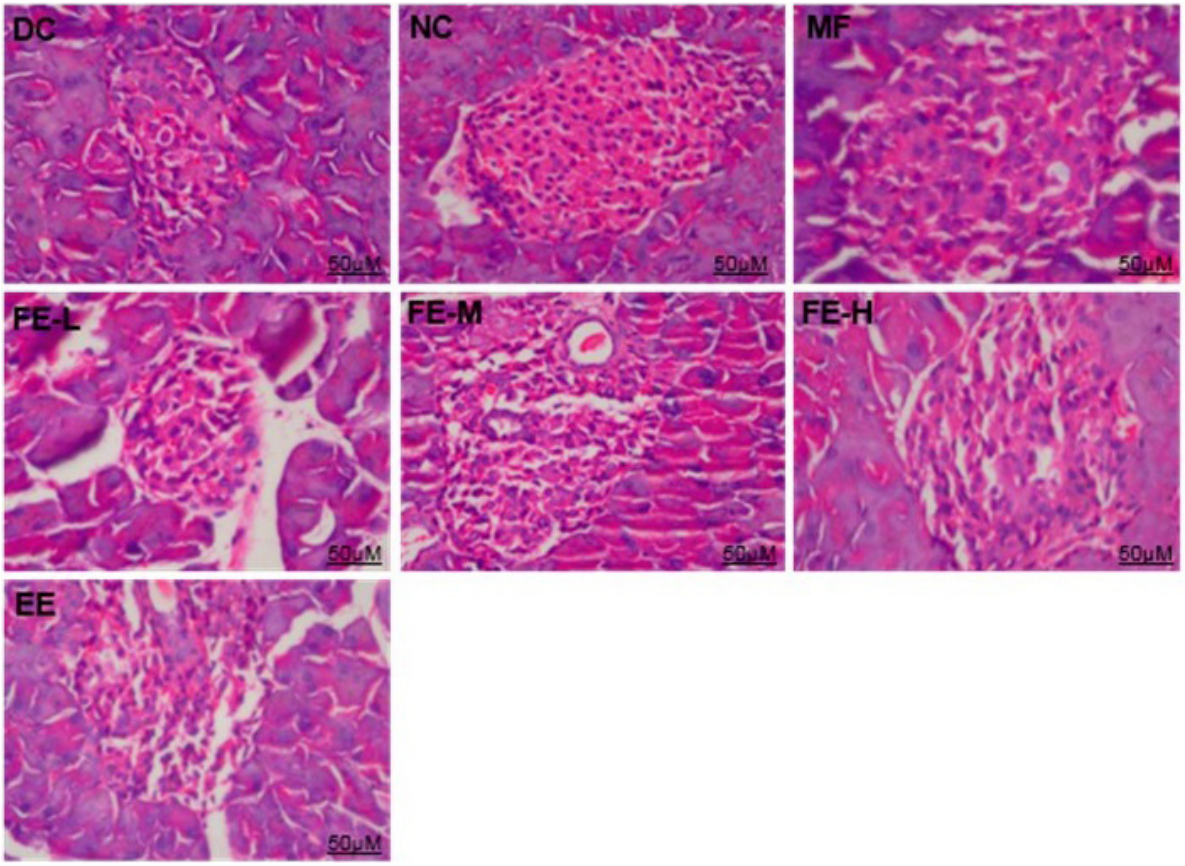

Figure 5. Hematoxylin and eosin (H&E) staining of the pancreas in streptozotocin in SD mice.

Regulation of EE and FE on lipid metabolism

Diabetes, obesity and dyslipidemia are the most prevalent associated comorbidities (Parafati et al., 2018). Dyslipidemia is similar to IR and tissue injury in T2DM (Deng et al., 2012). In addition, T2DM is characterized clinically by elevated plasma triglycerides and cholesterol (Sas et al., 2018), and excessive energy is transferred to TG in peripheral adipose tissue. Thus, the regulation of lipid metabolism by FE was coducted. As shown in Figure 4, the results of the present study suggest that FE can reduce TG (Figure 4A), TC (Figure 4B) and FFA (Figure 4C) levels in SD mice in a dose-dependent manner. The results corresponded well with our previous work showing that FE could effectively reduce body weight gain in SD mice. The effects of RRFF on lipid metabolism and mechamnism associated with diabetes in the tissue of SD mice will be investigated in our future work.

Regulation of EE and FE on pancreas tissue

Hematoxylin and eosin (H&E) staining was conducted to observe the regulatory effect of FE on pancreatic tissue. The pancreatic tissue of the male C57 diabetic mice showed diffuse vacuolation of pancreatic acini, and islet cells were severely damaged, accompanied by mononuclear inflammatory cell infiltration. Smaller size and distorted islets were also observed in SD mice (DC group) (Figure 5). The situation was improved after EE and FE treatment. Pancreatic tissue of the NC group was complete, and the edge was clear and smooth.

4 Conclusion

The present study aimed to investigate the hypoglycemic activity and secondary metabolic spectrum of *R. roxburghii* fruit fermented by Chinese traditional distiller's yeast and R. roxburghii fruit using UPLC–MS/MS and streptozotocin-induced diabetic (SD) mice. The results reveal that RRFF has good glucose consumption properties on HepG2 cells. RRFF exhibited favorable inhibitory activities against IR, dyslipidemia and hyperglycemia. Moreover, the histopathological results showed that RRFF ameliorated the histopathologic changes in C57 pancreatic tissue. Overall, these results indicate that the fermentation process is beneficial for increasing the content of triterpenoids and enhancing hypoglycemic activity. Traditional fermentation with Chinese traditional distiller's yeast can provide a way to expand the application of nutritious fruits with short shelf life.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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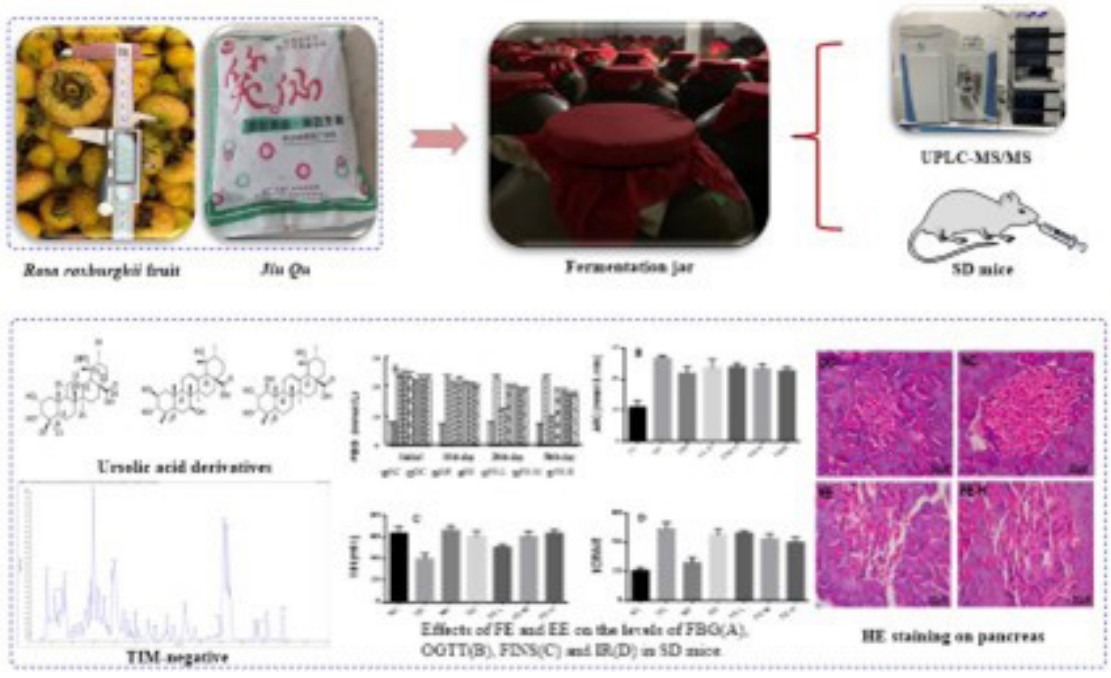
Graphical Abstract



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Supplementary material

Supplementary material accompanies this paper.

Table S**1**. Results of various substances in R. roxburghil samples (x±s, n=3)

Table S**2**. Significant change of substance before and after fermentation

Figure S1: MS peak of roxburic acid

Figure S2. Effects of FE and FE treatment on the food intake (A) and body weight (B) in the diabetic mice.

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