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Research Article

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Phenolic profile of jujube fruit subjected to gut microbiota fermentation and its antioxidant potential against ethyl carbamate-induced oxidative damage

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Abstract: Objective: To evaluate the composition of bioactive substances and the antioxidant effects of jujube fruit under gut microbiota fermentation (GMF), and the inhibitory effect on cytotoxicity caused by ethyl carbamate (EC). Methods: Changes in the contents of flavonoids, polyphenols, total sugars, and reducing sugars of jujube fruit after GMF (0, 2, 6, 12, 24, and 48 h) were determined. The oxidation resistance of fermented jujube fruits (from 0 to 48 h fermentation) was evaluated using in vitro 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and ferric reducing antioxidant power (FRAP) assays. Inhibitory effects of 48 h-fermented jujube fruit at various concentrations (0.25, 0.50, 1.00, and 2.00 mg/mL) on EC-treated toxicity and DNA damage of Caco-2 cells were estimated using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and nuclear staining assays, respectively. Effects of different concentrations of jujube fruit on EC-treated Caco-2 cells' intracellular reactive oxygen species (ROS), glutathione (GSH) levels, and mitochondrial membrane potential (MMP) were also evaluated. Results: Jujube fruit has rich bioactive components after GMF and shows strong antioxidant capacity. Fermented jujube fruit can inhibit the cytotoxicity and DNA damage of Caco-2 cells caused by EC and reduce intracellular ROS generation, as well as restoring GSH and MMP. Conclusions: Fermented jujube fruit extracts produced by GMF still contain biologically active substances which retain biological activity and antioxidation capabilities.

Key words: Jujube; Gut microbiota fermentation; Polyphenols; Ethyl carbamate; Antioxidant activity

1 Introduction

Chinese jujube (*Ziziphus jujuba* Mill.) belongs to the Rhamnaceae family, and is native to China. It has been domesticated in China for more than 7000 years, with current production rates at approximately 6.25 million tons of dry fruit per year (Huang et al., 2016). The jujube is a famous traditional resource with numerous biological activity (Pahuja et al., 2011; Plastina et al., 2012) and is highly respected for its unique flavor and nutritional content, such as flavonoids, amino acids, polysaccharides, triterpene acids, and phenolic acids (Chen JP et al., 2013; Du et al., 2013). Jujube possesses various health benefits such as antioxidant activity (Zhang et al., 2018), anticancer properties (Ji et al., 2020), hepatoprotective properties (Li Y et al., 2018), and anti-inflammatory properties (Periasamy et al., 2020).

Polyphenols are important secondary metabolites available in plants with potential antioxidant properties (Ravisankar et al., 2019; Zhang et al., 2019; Zhu et al., 2019; Luca et al., 2020). High dietary intake of polyphenol-rich fruits and vegetables is negatively correlated with the onset of diabetes (Dinda et al., 2020) and cardiometabolic disorders (Mozaffarian and Wu, 2018). The intake of polyphenols prompts complex metabolic processes in the body; they form biologically active metabolites and decomposition products, and then interact with gut microbiota and microbial enzymes (Rowland et al., 2018; Su et al., 2020). The bioactivity of dietary polyphenols is more dependent on bioavailability than on their parent compounds (Cardona et al., 2013; Kawabata et al., 2019).

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Oxidative stress (OS) plays an important role in the development of type 2 diabetes (T2D) (Rocha et al., 2020), non-alcoholic fatty liver disease (NAFLD) (Podszun et al., 2020), aging (Luo et al., 2020), and cancer (Kou et al., 2020). Ethyl carbamate (EC) is recognized as a Group 2A carcinogen by the International Agency for Research on Cancer (IARC). It is commonly present in fermented foods and various alcoholic beverages. Cellular metabolites of EC are associated with oxidative stress and resulting DNA damage, and can form covalent bonds with DNA, RNA, or proteins (Zhao et al., 2013). Vinyl carbamate is generated from the adducts and C-hydroxylation of EC and then converted to epoxides which interact with DNA (Colombo et al., 2015). Previous study has shown that EC treatment leads to increased production of reactive oxygen species (ROS) in human intestinal epithelial cells (Chen et al., 2016b). Although there have been some studies on the biological activity of jujube fruit (Wang, 2011; Huang et al., 2017), the antioxidant activity of its metabolites after undergoing the gut microbiota fermentation (GMF) which is part of the digestive process has not been studied in detail. This knowledge is important to determine whether jujube fruit has the potential for significant antioxidant activity in the body when eaten.

Therefore, in this study we aimed to investigate the antioxidant activity and biologically active components of jujube fruit after GMF and to evaluate whether compounds from jujube fruit could scavenge EC-induced ROS overproduction after GMF. In addition, we evaluated the effect of jujube fruit after GMF on mitochondrial dysfunction caused by EC treatment in vitro and revealed its possible mechanism.

2 Materials and methods

2.1 Chemicals and materials

Rhodamine 123 (Rh123), 2', 7'-dichlorofluorescin diacetate (DCFH-DA), and naphthalene-2, 3-dicarboxaldehyde (NDA) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). In addition, 3-(4,5-dimethyl-2-thiazolyl) -2, 5-diphenyl-2H-tetrazolium bromide (MTT) and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chromatographic-grade (≥99.9%) acetonitrile and methanol were purchased from Aladdin, Inc. (Shanghai, China). The gallic acid, protocatechuic acid, catechol, catechin, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, epicatechin, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, cinnamic acid, and quercetin used were of high-performance liquid chromatography (HPLC) grade (≥99%), and were purchased from Shanghai Yuanye Biotechnology Company (Shanghai, China). All reagents used were of analytical grade (≥99%) unless otherwise mentioned.

We obtained fresh jujube fruit (*Zizyphus jujuba* cv. Junzao) from Xinjiang Uygur Autonomous Region, China in October 2018, and screened it for uniform shape, size, and color. The initial average moisture content was 65.82% (wet basis) and the fruit was oven-dried to a constant weight at 105 °C.

2.2 In vitro gut microbiota fermentation

The gastrointestinal digestion procedure was first carried out on the fresh jujube fruit according to a previously described method (Bao et al., 2018, 2019). In short, 10 g of fresh jujube fruit was homogenized and then diluted with distilled water (10 mL). The sample was brought to pH 2.0 and blended with porcine pepsin (6000 U), followed by incubation for 2 h at 37 °C in a shaking water bath at 100 r/min simulating gastric digestion. Then, the mixture was adjusted to pH 6.0 and blended with pancreatin (20 mg) and bile salts (125 mg). Afterward, the pH was adjusted to 7.4 and the mixture was incubated at 37 °C for 2 h to simulate intestinal digestion. Thereafter, in vitro human GMF was conducted for gastrointestinal digested fresh jujube fruit homogenate following a previous method (Gowd et al., 2018). Three healthy diet and antibiotic-free donors provided stool samples. The stool samples were diluted to a concentration of 1% (0.01 g/mL) using 0.2 mol/L of carbonate phosphate buffer (pH 5.5), followed by filtration using a 1-mm sieve. Then, the digested jujube fruit homogenate was mixed with stool solution and kept in an anaerobic environment with oxygen-free gas (85% N_2, 10% CO_2, and 5% H_2; volume fraction), with constant stirring at different time intervals (0, 2, 6, 12, 24, and 48 h) at 37 °C. The supernatant was collected, filtered using 0.22 μm Millipore filter (Bedford, MA, USA), and finally preserved at −80 °C for experimental analysis.

2.3 Estimation of total phenolic, flavonoid, sugar, and reducing sugar contents

A Folin-Ciocalteu assay was conducted to determine the total phenolic contents of the jujube fruit

(Singleton and Rossi, 1965), and values were expressed as milligram gallic acid equivalent (GAE) per gram of fresh weight (FW). To determine the total flavonoid contents of the fruit, we applied a colorimetric method (Chang et al., 2002), and expressed values as milligram rutin equivalent (RE) per gram of FW. However, the total sugars and total reducing sugars were determined according to the Association of Official Analytical Chemists (AOAC) standard procedures (Miller, 1959; AOAC, 1995).

2.4 Phenolic compound analysis by HPLC

The phenolic compounds of the jujube fruit were measured by a Dionex Ultimate 3000 HPLC system (ThermoFisher Scientific, Waltham, MA, USA) which was equipped with a diode array detector (Li YT et al., 2018). The separations were operated on a C18 column (250 mm×4.60 mm, 5 μm), and the flow rate was fixed at 0.8 mL/min. In addition, the column temperature was set at 30 ℃, and injection volume of jujube samples was set at 20 μL. A mobile phase gradient (composed of two solvents such as 1.5% of aqueous formic acid solution (A) and methanol (B)) was used for the following linear gradient program: 0–15 min, 15% to 25% phase B; 15–25 min, 25% phase B; 25–60 min, 25% to 69% phase B; 60–62 min, 69% to 100% phase B; 62–67 min, 100% phase B; 67–70 min, 100% to 15% phase B. Absorbance was recorded at 280 and 360 nm.

2.5 In vitro antioxidant assay

2.5.1 FRAP assay

The antioxidant capacity of jujube samples was calculated using a ferric reducing antioxidant power (FRAP) assay (Chen W et al., 2013). In brief, 50 μL of the fermented jujube was incubated for 30 min with 150 μL of FRAP solution at 37 ℃. The absorbance was measured at 593 nm and the values were represented as milligram vitamin C equivalent per gram of FW.

2.5.2 ABTS assay

The 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) radical scavenging activity was calculated following a previously established method (Li et al., 2019). Fermented jujube (50 μL) was incubated for 6 min in the dark with 150 μL of $ABTS^{\cdot+}$ solution. The absorbance was measured at 734 nm and the values were expressed as half maximal inhibitory concentration (IC_{50}).

2.6 Cell culture

A human intestinal epithelial cell line (Caco-2) was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) and supplemented with fetal bovine serum (FBS; 10% (volume fraction)), streptomycin (0.1 mg/mL), and penicillin (100 U/mL). The cells were maintained at 37 ℃ with 5% CO_2 in an incubator (Chen et al., 2016a).

2.7 Cell viability assay

First, the Caco-2 cells were seeded into a 96-well plate with a density of 6×10^3 cells/well and pretreated with different concentrations of digested jujube fruit (0.25, 0.50, 1.00, or 2.00 mg/mL) for 24 h. After incubation, the cells were treated with 60 mmol/L of EC for another 24 h followed by washing with phosphate-buffered saline (PBS), and then incubated with MTT (0.5 mg/mL) for 4 h. The absorbance was recorded at 490 nm using a Tecan Infinite M200 microplate reader (Grodig, Austria) (Hu et al., 2018).

2.8 Nuclear staining analysis

Hoechst 33258 stain solution was used for nuclear staining as in a previously published report (Chen et al., 2017). Briefly, as described in Section 2.7, the cells were treated with jujube fruit samples and EC. Next, the treated cells were collected, followed by staining with Hoechst 33258 (10 μmol/L) at 37 ℃ for 30 min. Then, the cells were visualized under a fluorescence microscope.

2.9 Determination of intracellular ROS

A fluorescent probe dye, DCFH-DA, was used to determine intracellular ROS generation following a previous study (Chen et al., 2016b). Then, the Caco-2 cells were seeded into a 24-well plate (density 2.5×10^4 cells/well). The samples and EC were incubated with cells as described in Section 2.7. After that, 10 μmol/L of DCFH-DA was added to the treated cells for 30 min at 37 ℃. A fluorescence microscope was used to evaluate cellular fluorescence. The results were calculated from the fluorescence images with ImageProPlus6.0 (Media Cybernetics, Inc., Singapore).

2.10 Determination of cellular GSH

Cellular glutathione (GSH) content was analyzed using NDA as described previously (Chen et al., 2014). The samples and EC were incubated with cells as described in Section 2.9. Then, 50 μmol/L of NDA was incubated with cells for 30 min at 37 ℃. Afterwards, the cells were visualized under a fluorescence microscope. The values were calculated as described in Section 2.9.

2.11 Determination of mitochondrial membrane potential

The same treatment conditions were adopted as detailed in Section 2.9. The cells were then incubated with 10 μg/mL of Rh123 for 30 min at 37 ℃ (Gowd et al., 2019). The values were calculated as mean Rh123 fluorescence intensity, as described in Section 2.9.

2.12 Statistical analysis

All experimental data were analyzed as mean± standard deviation (SD) using SPSS (Version 22.0). One-way analysis of variance (ANOVA) with Tukey's post-hoc analysis was done for multiple comparison testing. $P<0.05$ was considered statistically significant.

3 Results

3.1 Determination of phenolic components in fermented jujube fruit

We analyzed phenolic, flavonoid, and sugar contents in fermented jujube fruit (0–48 h fermentation). As shown in Fig. 1, total phenolic compounds were sharply raised at 2 h (5.91 mg GAE/g FW) and gradually declined until 48 h (4.64 mg GAE/g FW) ($P<0.05$; Fig. 1a). The total flavonoid contents reached their maximum at 2 h (0.34 mg RE/g FW) of GMF, and then dropped steeply, reaching a minimum at 24 h (0.13 mg RE/g FW) and remaining unchanged until 48 h (0.13 mg RE/g FW) (Fig. 1b). The total sugar content (TSC) and reducing sugar content (RSC) were also detected throughout the GMF process. TSC declined gradually, from 417.06 to 205.82 mg glucose equivalent (GluE)/g FW ($P<0.05$; Fig. 1c); however, RSC increased significantly in the first 6 h ($P<0.05$), and then increased gradually, reaching a maximum at 24 h (124.65 mg GluE/g FW) and remaining unchanged until 48 h (122.84 mg GluE/g FW) ($P>0.05$; Fig. 1d). Taken together, these results showed that gut microbiota can decompose polyphenols and flavonoids in jujube. They also can decompose TSC and increase RSC.

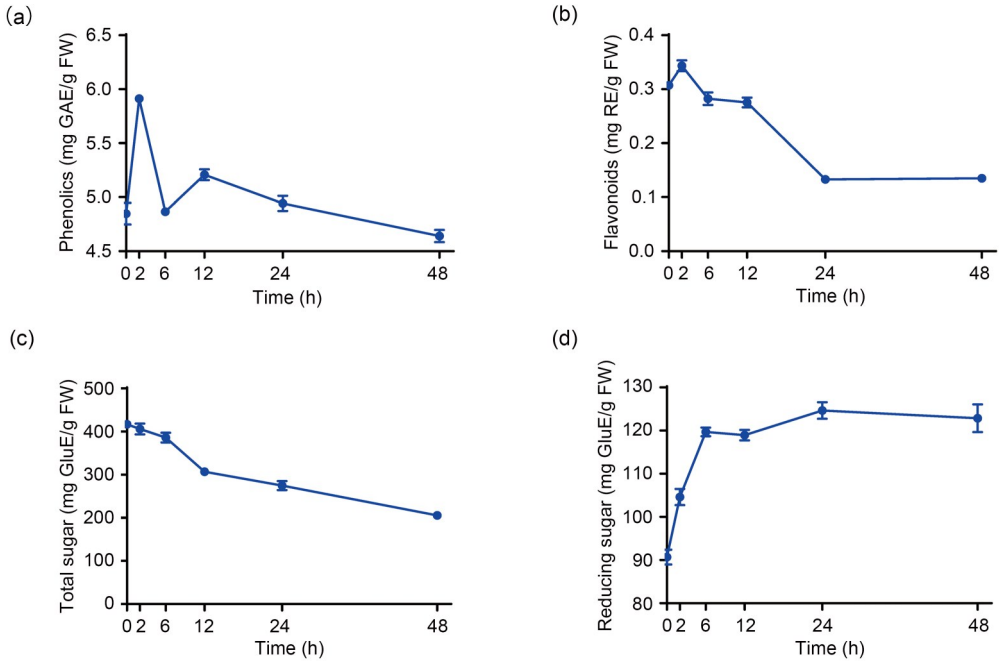

Fig. 1 Effects of gut microbiota on the contents of jujube phenolics (a), flavonoids (b), total sugar (c), and reducing sugar (d). Data were expressed as mean±standard deviation (n=3). GAE, gallic acid equivalent; FW, fresh weight; RE, rutin equivalent; GluE, glucose equivalent.

3.2 Identification of phenolics of jujube after gut microbiota fermentation

Phenolic compounds of fermented jujube fruit (0–48 h fermentation) were detected by HPLC (Table 1 and Fig. S1). We monitored the changes of 13 representative polyphenol compounds in jujube. Protocatechuic acid, catechol, catechin, chlorogenic acid, *p*-coumaric acid, ferulic acid, ellagic acid, and cinnamic acid reached their maximum contents at 2 h of GMF, and then stayed at a low level with the prolongation of GMF. *p*-Hydroxybenzoic acid and caffeic acid had the highest contents when GMF reached 24 h. The contents of epicatechin, rutin, and quercetin decreased with prolonged GMF. Epicatechin, rutin, and cinnamic acid were not detected after 12 h of GMF, indicating that these three compounds were either easily metabolized and decomposed by gut microbiota, or that they were structurally unstable. Catechol content remained at a high level compared to other polyphenol compounds after digested jujube fruit underwent GMF. Therefore, we believe that the antioxidant capacity of digested jujube fruit may be dominated by catechol. In a word, GMF could somewhat increase the content of phenolic compounds in jujube fruit, while catechol had the highest content in digested jujube fruit after GMF.

3.3 Determination of antioxidant activity in fermented jujube fruit

The antioxidant activity of jujube (0–48 h fermentation) was further studied using in vitro antioxidant assays (ABTS and FRAP). According to ABTS assay (Fig. 2a), the inhibitory effect of fermented jujube increased significantly with the extension of GMF, exhibiting the best inhibitory activity (0.0139 mg/mL) at 2 h, at which point inhibitory activity reduced steeply. The IC_{50} values were 0.0150, 0.0139, 0.0160, 0.0197, 0.0213, and 0.0209 mg/mL. The antioxidant activity

Table 1 Phenolic components (mg/kg FW) of jujube fruit during gut microbiota fermentation

Time (h)	Protocatechuic acid	Catechol	Catechin	*p*-Hydroxybenzoic acid	Chlorogenic acid	Caffeic acid
0	5.28±0.13^{e}	24.49±1.44^{d}	3.48±0.32^{c}	2.35±0.22^{b}	13.11±0.58^{d}	1.46±0.19^{bc}
2	6.13±0.25^{f}	27.89±2.32^{e}	4.05±0.05^{d}	2.63±0.09^{b}	15.42±1.52^{e}	1.50±0.23^{bc}
6	2.87±0.01^{c}	10.64±0.11^{b}	2.03±0.20^{b}	1.87±0.12^{a}	8.76±0.15^{bc}	1.28±0.07^{ab}
12	1.74±0.11^{a}	7.38±0.17^{a}	1.38±0.10^{a}	2.28±0.28^{b}	7.97±0.46^{b}	1.18±0.03^{a}
24	2.27±0.07^{b}	14.04±0.54^{c}	2.03±0.22^{b}	5.11±0.31^{d}	9.99±0.23^{c}	1.68±0.02^{c}
48	4.55±0.29^{d}	9.93±0.47^{b}	1.67±0.06^{a}	4.63±0.12^{c}	4.33±0.02^{a}	1.33±0.01^{ab}

Time (h)	Epicatechin	*p*-Coumaric acid	Ferulic acid	Rutin	Ellagic acid	Cinnamic acid	Quercetin
0	5.84±0.16^{c}	0.86±0.05^{d}	2.25±0.22^{c}	0.83±0.12^{c}	1.24±0.12^{a}	0.52±0.01^{b}	2.09±0.09^{f}
2	5.31±0.27^{b}	0.95±0.10^{d}	3.00±0.35^{d}	0.26±0.04^{a}	1.82±0.07^{b}	0.58±0.04^{c}	0.77±0.03^{c}
6	3.25±0.20^{a}	0.48±0.05^{c}	1.35±0.14^{b}	0.44±0.03^{b}	1.75±0.18^{b}	0.40±0.04^{a}	1.24±0.04^{e}
12		0.31±0.01^{b}	0.56±0.06^{a}		1.23±0.01^{a}		0.88±0.01^{d}
24			0.66±0.01^{a}		1.70±0.02^{b}		0.21±0.01^{b}
48		0.03±0.01^{a}	0.50±0.03^{a}		1.64±0.10^{b}		0.02±0.00^{a}

Data were expressed as mean±standard deviation (n=3). Values in the same column with different superscript letters are significantly different at P<0.05 according to the Duncan test. FW, fresh weight.

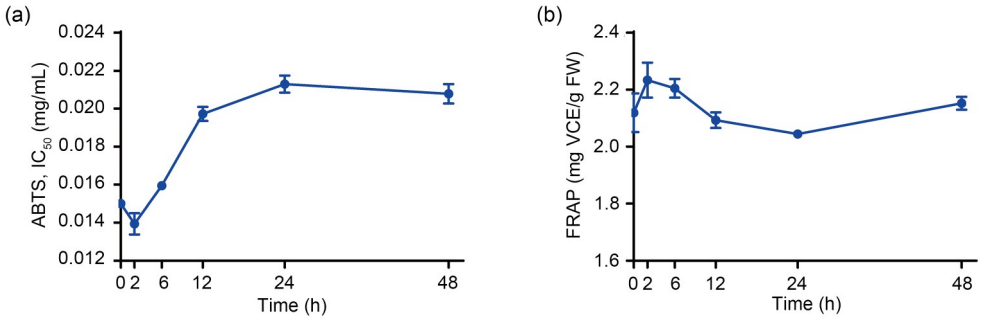

Fig. 2 Effect of gut microbiota on the antioxidant activity of jujube. (a) ABTS; (b) FRAP. Data were expressed as mean± standard deviation (n=3). ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate); FRAP, ferric reducing antioxidant power; IC_{50}, half maximal inhibitory concentration; VCE, vitamin C equivalent; FW, fresh weight.

of fermented jujube was further assessed by FRAP assay (Fig. 2b). The ferric ion reduction ability of samples significantly increased after GMF at 2 h, then gradually decreased until 24 h, and returned to the initial level at 48 h. Overall, gut microbiota can enhance the free-radical scavenging capacity of fermented jujube in a time-dependent manner, but only temporarily improves ferric reducing ability, and then returns to normal levels.

3.4 Inhibitory effects of digested jujube fruit on EC-induced toxicity and DNA damage

EC-induced toxicity has been found in both in vitro cellular and in vivo animal studies (Chun et al., 2013). We collected metabolites of fermented jujube under GMF treatment at 48 h and employed the Caco-2 cell model to further identify differences in the effectiveness of jujube fruit against EC impairment based on antioxidant capacity. Different fermented jujube fruit samples (0.25, 0.50, 1.00, and 2.00 mg/mL) did not induce any toxicity to Caco-2 cells (P>0.05; Fig. 3a) and cell viability was significantly decreased by treatment with EC (P<0.05; Fig. 3b). Caco-2 cells pretreated with 0.50 mg/mL samples of fermented jujube fruit with 60 mmol/L EC showed increased viability from 63% to 70%, while 1.00 mg/mL of fermented jujube fruit samples increased viability to 74% (P<0.001; Fig. 3b). Next, we investigated the efficacy of metabolites of fermented jujube (0.25, 0.50, and 1.00 mg/mL) against EC-treated DNA damage in Caco-2 cells. In the EC 60 mmol/L group, a relatively large number of cell nuclei were visible with bright blue dots, indicating that DNA was affected by nuclear fragmentation and/or chromatin condensation. However, cells treated with 1.00 mg/mL of fermented jujube samples for 24 h showed fewer cell nuclei with dark blue than the EC 60 mmol/L group (P<0.05), which was almost the same as the control (Fig. 3c). Taken together, metabolites of fermented jujube at 0.50 or 1.00 mg/mL concentration under GMF can enhance cell viability and reduce cytotoxicity caused by EC.

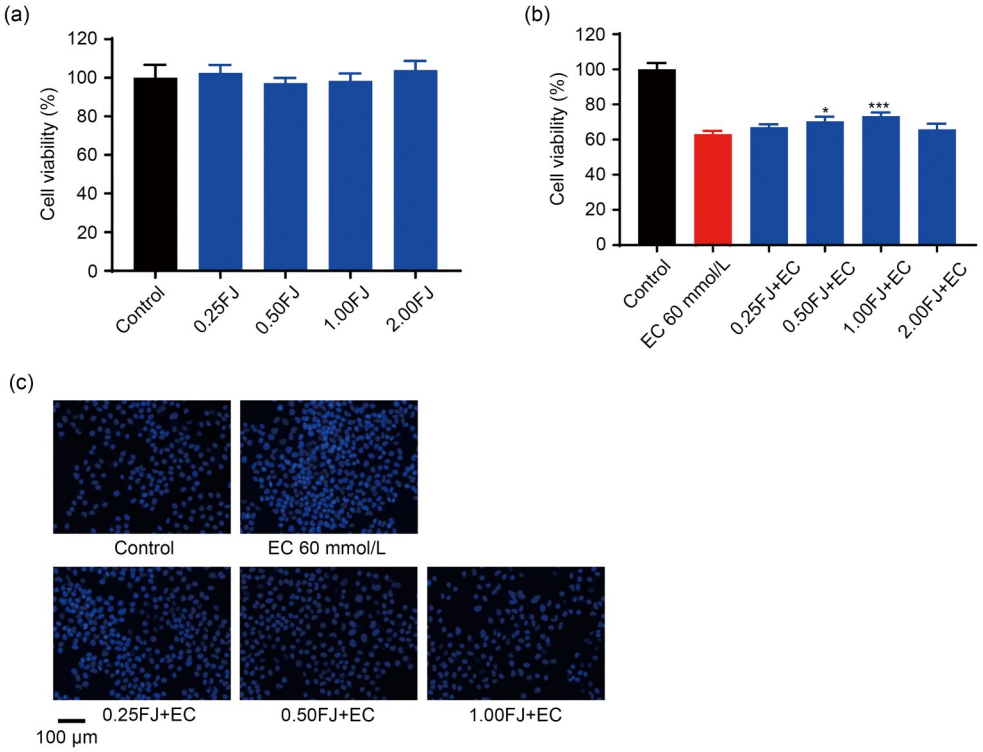

Fig. 3 Effects of fermented jujube samples on ethyl carbamate (EC)-induced cytotoxicity determined by MTT assay. (a) Effect of fermented jujube samples on cytotoxicity in Caco-2 cells. (b) Effect of fermented jujube samples on EC-induced cytotoxicity. (c) Effect of fermented jujube samples on EC-induced DNA damage. The cells were treated for 24 h with fermented jujube samples (0.25, 0.50, 1.00, or 2.00 mg/mL) followed by incubation with EC (60 mmol/L) for another 24 h. Data were expressed as mean±standard deviation (n=3). * P<0.05 and *** P<0.001 represent significant differences compared to the EC group. FJ: concentration of fermented jujube sample in mg/mL.

3.5 Mitigation capacity of digested jujube fruit in EC-induced ROS generation

We further determined the antioxidant capacity of jujube after GMF in Caco-2 cells. According to Figs. 4a and 4b, the fluorescence intensity of the EC 60 mmol/L group was increased by 149% over the control group (normalized to 100%) (P<0.001). However, in fermented jujube fruit samples (0.25, 0.50, or 1.00 mg/mL), fluorescence intensity was lessened in a dose-dependent fashion (P<0.05) compared to the EC 60 mmol/L group (Fig. 4b). Fermented jujube fruit samples at a concentration of 1.00 mg/mL showed better ROS scavenging activity (116%). The fluorescence intensities of fermented jujube sample groups at 0.25, 0.50, and 1.00 mg/mL were 130% (P<0.01), 117% (P<0.001), and 116% (P<0.001), respectively. Thus, jujube fruit fermented by gut microbiota could protect against EC-treated oxidative stress by hindering ROS overproduction.

3.6 Amelioration of EC-induced glutathione reduction by fermented jujube fruit

Since fermented jujube samples could effectively scavenge ROS induced by EC, we speculated that fermented jujube samples could also influence EC-treated GSH depletion. According to Figs. 4c and 4d, a marked reduction of fluorescence intensity was noticed in the EC 60 mmol/L group (49% of control) (P< 0.001), and cells supplemented with fermented jujube at three concentrations (0.25, 0.50 and 1.00 mg/mL) significantly ameliorated cellular GSH depletion in a concentration-dependent fashion. The fluorescence intensities of fermented jujube sample groups at 0.25, 0.50, and 1.00 mg/mL were 59% (P<0.05), 72% (P< 0.001), and 78% (P<0.001), respectively. Thus, we

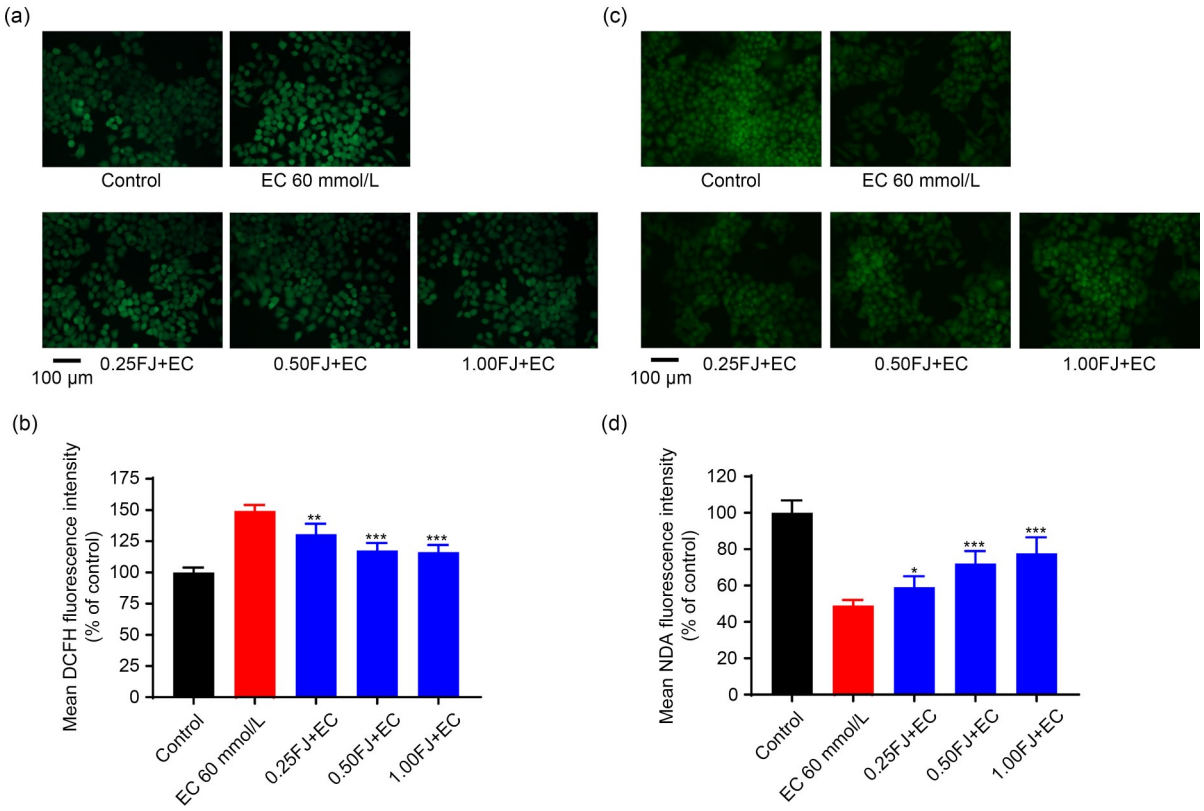

Fig. 4 Effects of fermented jujube samples on ethyl carbamate (EC)-treated reactive oxygen species (ROS) accumulation (a, b) and glutathione (GSH) depletion (c, d) in Caco-2 cells. The cells were treated with fermented jujube samples (0.25, 0.50, or 1.00 mg/mL) for 24 h, followed by incubation with 60 mmol/L EC for another 24 h. The quantitative data (b, d) in panels (a, c) were calculated as mean DCFH fluorescence intensity and mean NDA fluorescence intensity (mean± standard deviation (n=3)), respectively. * P<0.05, ** P<0.01, and *** P<0.001 represent significant differences compared to the EC group. DCFH: 2',7'-dichlorofluorescin; NDA: naphthalene-2,3-dicarboxal-dehyde. FJ: concentration of fermented jujube sample in mg/mL.

found that jujube fruit fermented by gut microbiota possessed potential cellular antioxidant activity.

3.7 Effect of fermented jujube fruit on EC-induced mitochondrial membrane impairment

ROS is mainly produced by mitochondria and affects mitochondrial metabolism (Reddy, 2006). Hence, we analyzed the function of fermented jujube samples on the mitochondrial membrane in the presence of EC. As shown in Figs. 5a and 5b, the fluorescence intensity of the EC 60 mmol/L group decreased to 71% of control, showing that exposure to EC leads to the reduction of mitochondrial membrane potential (MMP). Then, we evaluated the ameliorative effect of fermented jujube samples against EC-treated MMP collapse. All samples significantly augmented MMP of Caco-2 cells in a concentration-dependent fashion (0.25, 0.50, and 1.00 mg/mL). The fluorescence intensities of fermented jujube samples at 0.25, 0.50, and 1.00 mg/mL were 84% (P<0.001), 85% (P<0.001), and 92% (P<0.001), respectively. Taken together, jujube fruit fermented by gut microbiota has potential against EC-induced mitochondrial dysfunction.

4 Discussion

Jujube fruit is widely used in traditional Chinese medicine because of its high nutritional value and various pharmacological effects (Guo et al., 2009). We have earlier reported the biologically active ingredients and antioxidant properties of dried jujube fruit (Bao et al., 2021). However, knowledge of gastrointestinal and gut microbiota modifications, along with bioavailability and their impact on bioactivity, is lacking due to the many analytical challenges faced when analyzing jujube fruit. This study aimed to investigate the main active ingredients of jujube fruit after GMF and their mechanisms related to antioxidant activity.

$ABTS^{\bullet+}$ is commonly used to assess free radical scavenging ability (Gu et al., 2019). Thus, this study compared the antioxidant capacity of jujube fruit after GMF through $ABTS^{\bullet+}$ assay. Using traditional in vitro experiments to detect the contents of phenolics, flavonoids, and sugar, as well as the free radical scavenging ability of jujube fruit after GMF, we observed a reduction of all of these in vitro, except that the content of reducing sugars increased. We speculate that total sugar is decomposed into reducing sugars due to the metabolism of intestinal flora. In another study on vegetables, correlation statistical analysis between the $ABTS^{\bullet+}$ value and the phenolic contents indicated significant correlations (Dudonné et al., 2009). Jujube still possessed considerable antioxidant activity (FRAP) after GMF compared with dried jujube fruit (Bao et al., 2021). At the same time, our findings are consistent with a previous published report (Choi et al., 2011), in which FRAP is not significantly correlated with the duration of GMF that jujube fruit undergoes. These results indicate that measurements of total polyphenol content and free radical scavenging potential cannot be considered accurate in terms of the assumed beneficial health effects if the effects of digestion and fermentation processes in the gastrointestinal tract are

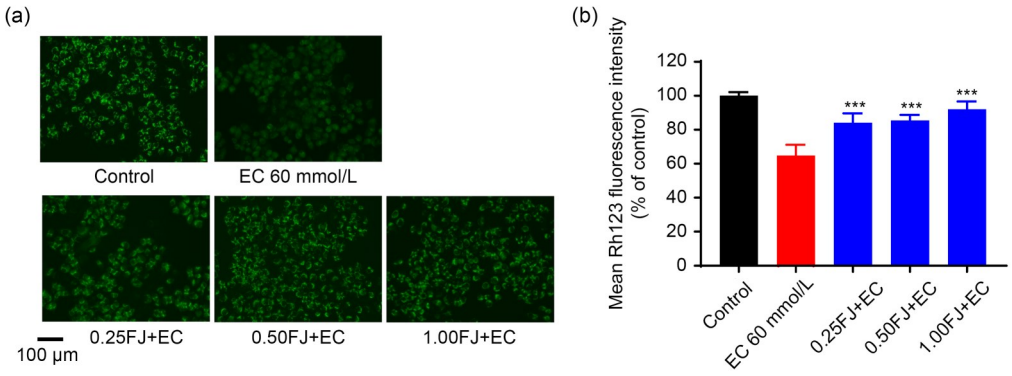

Fig. 5 Effects of fermented jujube samples on mitochondrial membrane damage in Caco-2 cells. The cells were treated for 24 h with fermented jujube samples (0.25, 0.50, or 1.00 mg/mL) followed by incubation with 60 mmol/L of ethyl carbamate (EC) for another 24 h. The quantitative data (b) in panel (a) were calculated as mean Rh123 fluorescence intensity (mean±standard deviation (n=3)). *** P<0.001 represents a significant difference compared to the EC group. FJ: concentration of fermented jujube sample in mg/mL.

not considered. In GMF, dietary polyphenols can act as substrates for various enzymes in the small intestine and stomach when exposed to specific pH conditions (Singh et al., 2017). The polyphenols in the colon may undergo further transformations, and the enzymes of the intestinal flora break down the complex polyphenolic structure into smaller units. Bacterial enzymes can catalyze many reactions, including hydrolysis, demethylation, dehydroxylation, ring cleavage, and decarboxylation (Fraga et al., 2019).

A growing body of work involving intervention in vivo and epidemiological study of dietary polyphenols has provided new evidence on a variety of health-promoting activities. These data have been examined from in vitro data records, including anti-inflammatory (Yahfoufi et al., 2018), antioxidant (Mojzer et al., 2016), anticancer (Niedzwiecki et al., 2016), antidiabetic (Cao et al., 2019), and neuroprotective potential (Spagnuolo et al., 2016), suggesting a positive correlation between the consumption of polyphenol-rich foods and lower risk of chronic diseases. In order to explore the changes of polyphenol contents in jujube fruit under GMF, we quantified polyphenol compounds in fermented jujube and found that the contents of most polyphenols increased briefly after GMF and then decreased, which may be due to the decomposition of jujube fruit by gut microbiota; however, the instability of the digestive system may result in a decrease in polyphenol contents. Our previous study along with others indicates that jujube contains abundant phenolic compounds such as rutin, chlorogenic acid, catechol, catechin, *p*-hydroxybenzoic acid, caffeic acid, and ferulic acid (Gao et al., 2013; Zhao et al., 2014; Bao et al., 2021). Rutin (142.05 to 292.33 mg/kg dry weight) is the predominant phenolic compound in dried and fresh jujube samples (Bao et al., 2021). However, the rutin content in jujube fruit ranges from 0.26 to 0.83 mg/kg FW after GMF for 0–6 h, and is undetectable after 12–48 h of GMF. This could be due to the gastrointestinal digestion procedure performed before GMF, which caused the degradation of rutin (Siracusa et al., 2011). Fermented jujube fruit still possesses considerable in vitro antioxidant activity, possibly because of the presence of multiple phenolic substances such as catechol, protocatechuic acid, *p*-hydroxybenzoic acid, and chlorogenic acid. We found the highest content of catechol in jujube fruit after GMF. It has also been reported that catechol has antioxidant activity and has shown beneficial effects in diseases such as diabetes (Cremonini et al., 2019), Alzheimer disease (Kim et al., 2020), and obesity (Wang et al., 2017). Some in vitro and in vivo studies have revealed EC-induced toxicity (Field and Lang, 1988; Cui et al., 2016). Additionally, in the present study, we used Caco-2 cells to explore the effectiveness of jujube fruit against EC-induced cytotoxicity and DNA damage after GMF. Although the contents of phenolics and flavonoids in fermented jujube are significantly reduced, the fruit still has a certain amount of concentration-dependent activity in resisting EC-induced DNA damage and improving cell viability. This indicates that the metabolites of jujube fruit fermented by gut microbiota may have biological activity against DNA damage and improve cell growth.

Disturbance of the redox balance between ROS and antioxidants in cells is related to cytotoxicity (Tseng et al., 2017). Jujube can reduce the overproduction of ROS induced by EC after GMF and shows a concentration-dependent effect. It has been reported that phenolic compounds can inhibit intracellular OS induced by exogenous substances (Lin et al., 2016). Thus, the inhibitory effect of jujube on EC-treated oxidative stress after GMF may be associated with the phenolic content. GSH is the core of the intracellular antioxidant defense system (Lv et al., 2019), and can scavenge ROS, thereby helping to control redox homeostasis (Bansal and Simon, 2018). Supplementing the diet with fermented jujube fruit significantly restores GSH, thereby enhancing the endogenous antioxidant defense mechanism against OS.

ROS is generated primarily by mitochondria, affecting mitochondrial metabolism (Cadenas, 2018). The maintenance of MMP is necessary for normal cell function and has a significant function in maintaining cell ROS homeostasis. Overproduction and accumulation of ROS are also related to mitochondrial dysfunction and MMP collapse (Murphy, 2016). Here, we report that the metabolites of jujube that has undergone GMF have the potential to resist EC-induced, ROS-related mitochondrial dysfunction, preventing oxidative stress induced by mitochondrial dysfunction. Consequently, this study uncovers some important insights about the activity of bioactive components of jujube after GMF, provides a basis for further research on the bioactive components of jujube in vivo, and helps to clarify the mechanism of action in GMF.

5 Conclusions

This study unveiled the effects of GMF on the antioxidant capacity of bioactive substances obtained from jujube fruit. As far as we know, there are several studies on the polyphenols of jujube and their biological activity, but none on the antioxidant activity of polyphenols in jujube fruit after GMF. The addition of jujube fruit samples significantly increases the cell viability of Caco-2 cells and reduces DNA damage after GMF; it also lessens the excessive ROS production caused by EC and significantly restores GSH levels and mitochondrial membrane potential in Caco-2 cells. In short, jujube polyphenols show potent antioxidant activity after GMF, and jujube can thus be considered a potential ingredient for development of functional foods.

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Author contributions

Tao BAO performed fermentation experiments, HPLC analysis, in vitro antioxidant activity and cell experiments, and wrote the initial manuscript. Ming ZHANG performed in vitro antioxidant activity experiments and wrote the initial manuscript. Yuanqing ZHOU performed fermentation experiments and HPLC analysis. Wei CHEN conceived the project, designed the study, and supervised the experiments. All authors have read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for its integrity and security.

Compliance with ethics guidelines

Tao BAO, Ming ZHANG, Yuanqing ZHOU, and Wei CHEN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Fig. S1