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Research Article

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Polysaccharide isolated from wax apple suppresses ethyl carbamate-induced oxidative damage in human hepatocytes

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Abstract: Wax apple (*Syzygium samarangense*) has received growing research interest for its high nutritional and medicinal value due to its constituents such as polysaccharide, organic acids, flavonoids, minerals, and other substances. In this study, wax apple polysaccharide (WAP) was isolated from this plant and its protective effect against ethyl carbamate (EC)-induced oxidative damage was evaluated in human hepatocytes (L02 cells). Firstly, a series of analyses such as high-performance liquid chromatography (HPLC), high-performance gel permeation chromatography (HPGPC), Fourier transform infrared spectroscopy (FT-IR), gas chromatography/mass spectrometry (GC/MS), and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) were conducted to identify the structure of WAP. Thereafter, in vitro cell experiments were performed to verify the protective effects of WAP against EC-induced cytotoxicity, genotoxicity, and oxidative damage in L02 cells. Our results revealed that WAP is composed of mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, arabinose, and fucose in a molar ratio of 2.20:3.94:4.45:8.56:8.86:30.82:39.78:1.48. Using a combination of methylation and NMR spectroscopic analysis, the primary structure of WAP was identified as Ara*f*-(1→, Glc*p*-(1→, →2)-Ara*f*-(1→, →3)-Gal*p*-(1→, →3)-Ara*f*-(1→, and →6)-Gal*p*-(1→. Cell experiments indicated that WAP exhibited significant protective effects on EC-treated L02 cells via suppressing cytotoxicity and genotoxicity, reducing reactive oxygen species (ROS) and $O_2^{\cdot-}$ formation, as well as improving mitochondrial membrane potential (MMP) and glutathione (GSH). In a nutshell, WAP has the potential as an important therapeutic agent or supplement for hepatic oxidative damage. Meanwhile, further studies are needed to prove the above effects in vivo at the biological and clinical levels.

Key words: Wax apple polysaccharide; Polysaccharide characterization; Ethyl carbamate; Hepatic oxidative stress

1 Introduction

Liver is the primary organ affected by reactive oxidants from food, the environment, and chemicals. In severe cases, these agents first cause hepatocyte apoptosis via initiating oxidative-dependent cell signaling pathways, and then induce hepatic pathologies such as non-alcoholic fatty liver disease, cirrhosis, and even hepatocellular carcinoma (Cichoż-Lach and Michalak, 2014; Meng et al., 2020; Karim et al., 2022). Reactive oxygen species (ROS) including superoxide anion ($O_2^{\cdot-}$), hydroxyl (·OH), hydrogen peroxide (H_2O_2), peroxyl ($RO_2^{\cdot}$), alkoxyl (RO·), and others have important roles in balancing cell homeostasis (Gowd et al., 2018a, 2019; Shishir et al., 2019). However, excessive production of ROS has been associated with the imbalance of the cellular redox system and was further linked to oxidative stress (Karim et al., 2018, 2022). Ethyl carbamate (EC) is known as a by-product in several fermented foods, alcoholic beverages, and others like bread or soy sauce (Gowd et al., 2018b). Some studies have suggested the potential carcinogenic effect of EC in experimental animals (Liu et al., 2017; Wang et al., 2021), while there is limited evidence of such effect in humans. In 2010, the International Agency for Research on Cancer (IARC) recognized EC as a group 2A carcinogenic substance (IARC, 2010). Previous studies by our lab and others indicated that EC treatment induces ROS overproduction, mediates the imbalance of the cellular redox

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system, and thereby generates toxicity in cells (Chun et al., 2013; Chen et al., 2016b, 2016c, 2017).

Accumulating evidence indicates that natural antioxidants such as polyphenols, sterols, peptides, and polysaccharides, have positive effects against toxicant-induced oxidative damage (Sun et al., 2004; Chen et al., 2013; da Silva Marineli et al., 2014; Jin et al., 2016; Wang et al., 2016; Zhang et al., 2017; Lu et al., 2021). Owing to their antioxidant properties, several natural polysaccharides from plants, marine flora, bacteria, fungi, fauna, etc. were shown to exert potential protective effects against oxidative damage (Zhang et al., 2003; Ng et al., 2006; Kodali and Sen, 2008; Guo et al., 2010; Chen W et al., 2011). The above studies also revealed that the reduction of capacity, radical scavenging activity, and messenger RNA (mRNA) expression of some antioxidant enzymes (glutathione peroxidase (*GPx*) and superoxide dismutase (*SOD*)) was associated with the antioxidant activity of polysaccharides (Zhang et al., 2003; Ng et al., 2006; Kodali and Sen, 2008; Guo et al., 2010). Wax apple (*Syzygium samarangense*), originating from the Greater Sunda Islands, Malay Peninsula, Andaman and Nicobar Islands, is now cultivated in Guangdong and Guangxi Provinces, China. It has been shown to contain an abundance of phytochemicals, such as flavonoids, ellagitannins, proanthocyanidins, anthocyanidins, triterpenoids, and chalcones (Srivastava et al., 1995; Resurreccion-Magno et al., 2005; Simirgiotis et al., 2008; Huang et al., 2016), which have potential health benefits such as antioxidant, liver protection, hypoglycemic, antibacterial, and anti-tumor (Wang et al., 2019; Li et al., 2020; Yang, 2022). Wax apple and jambo (*Syzygium jambos* (L.) Alston) belong to the same genus. Recently, an arabinogalactan was found in jambo (Tamiello et al., 2018); however, there have been no reports on polysaccharides in wax apple.

In this work, we isolated a new polysaccharide from wax apple and evaluated its protective effect against EC-induced oxidative damage in normal human hepatocytes (L02 cells). Firstly, we characterized the chemical properties of this polysaccharide, such as molecular weight (MW), monosaccharide composition, linkages, functional groups, and internal chemical bonds. Thereafter, we evaluated the in vitro antioxidant activity (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical scavenging activity and ferric reducing antioxidant power (FRAP)). Subsequently, we investigated the protective effect of wax apple polysaccharide (WAP) against EC-induced oxidative damage in L02 cells.

2 Materials and methods

2.1 Materials and reagents

Fresh wax apples (cultivar: Heijingang (in Chinese)) were collected in July 2017 from Guangxi Province, China. Based on weight uniformity, shape, and visual color, suitable fruits (average weight (120± 30) g) were selected for experimental analysis. Fruits with immature size or shape or color defect were excluded. The selected fruits were thoroughly washed with distilled water, left to dry in a clean dark place, and then stored at −80 °C.

The chemicals 2,4,6-tris(2-pyridyl)-*S*-triazine (TPTZ), ABTS, 3-(4,5-dimethyl-2-thiazolyl)-2,-5-diphenyl-2H-tetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), dihydroethidium (DHE), Rhodamine 123 (Rh123), Mito-Tracker Green (MTG), naphthalene-2,3-dicarboxaldehyde (NDA), and Hoechst 33258 were purchased from Sigma Chemical (St. Louis, MO, USA). Glucose, fucose, arabinose, rhamnose, mannose, galacturonic acid, galactose, glucuronic acid, erythritol, and glycerin were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). All reagents used in this study were of analytical grade unless stated otherwise.

2.2 Extraction of polysaccharide from wax apple

The peeled wax apples (1 kg) were homogenized and the dissolved contents were extracted using 4 L distilled water at 90 °C for 2 h. The extraction process was repeated three times. The supernatant was collected and mixed with ethanol (final concentration of 95%, volume fraction) to obtain the crude polysaccharide. And then it was dissolved in distilled water and mixed with 1/4 volume of Sevag solution to remove the proteins. The mixture was shaken for 20 min and then centrifuged at 8000*g* for 10 min. The supernatant was collected and the centrifugation process was repeated six times. Next, the polysaccharide solution was placed in a dialysis bag (MW cut-off: 3.50 kDa), and then dialyzed for 48 h in tap water and then for 24 h in ultra-pure water. The obtained WAP solution was lyophilized and stored at −80 °C until further use.

2.3 Determination of total sugar

The total sugar content of WAP was measured using the phenol-sulfuric acid method, with arabinose acting as the standard (DuBois et al., 1956).

2.4 Analysis of monosaccharide composition and molecular weight

Briefly, trifluoroacetic acid (2 mol/L) was employed to hydrolyze the WAP (5 mg) at 110 ℃ for 8 h, followed by complexation with 1-phenyl-3-methyl-5-pyrazolone (PMP; 0.5 mol/L, dissolved in methanol). Afterwards, high-performance liquid chromatography (HPLC) equipped with a Promosil C18 column (4.6 mm×250 mm, 5 μm) was used to analyze the obtained product. Phosphate buffer (KH_2PO_4-NaOH, 0.05 mol/L, pH 6.9) and acetonitrile were employed as mobile phases A and B, respectively. A linear gradient program was set as follows: 17% (mobile phase B) from 0 to 15 min, 17% to 25% (mobile phase B) from 15 to 18 min, 25% (mobile phase B) from 18 to 23 min, 25% to 17% (mobile phase B) from 23 to 25 min, and 17% (mobile phase B) from 25 to 30 min. The sample injection volume was 10 μL and the flow rate was fixed at 1.0 mL/min. The chromatograms were detected at the wavelength of 245 nm. Commercially available standards were used for the qualitative and quantitative determination of monosaccharides in the samples.

In order to identify the MW of WAP, this study employed high-performance gel permeation chromatography (HPGPC) equipped with a Waters-1525 HPLC system matched with Waters ultra-hydrogel 500 column (7.8 mm×300 mm, column temperature 30 ℃). The sample was detected by the differential refraction index detector (RID) at 40 ℃. A sample volume of 50 μL was injected into the HPGPC system and operated at 30 ℃ with the 0.5 mL/min flow rate using 0.2 mol/L NaCl solution. Dextran standard with different molecular weights (1, 5, 12, 50, 80, 150, 270, 410, and 670 kDa) was used for the calibration curve.

2.5 FT-IR analysis

The Fourier transform infrared spectroscopy (FT-IR) spectra of WAP were analyzed within the range of 4000–400 cm^{-1} with 2 cm^{-1} resolution (Nicolet iS 10, Thermo Fisher Scientific, Massachusetts, USA) (Ren et al., 2017).

2.6 Characterization of WAP using methylation and NMR

The reduction of WAP was achieved following the method of Song et al. (2018). Firstly, 20 mg of sample was methylated following the protocol of Ciucanu and Kerek (1984). Then, the dried methylated sample was hydrolyzed with 2 mol/L trifluoroacetic acid at 110 ℃ for 8 h. Methanol was used to remove the excess trifluoroacetic acid from the sample, which was then reduced by sodium borohydride at room temperature for 2 h. Subsequently, acetylation of the sample was performed with acetic anhydride to yield alditol acetates via interacting between pyridine and acetic anhydride at 100 ℃ for 2 h. Then, gas chromatography-mass spectrometry (GC-MS; GC7890B-MS7000C, Agilent Technologies Co., USA) was used to analyze the partially methylated alditol acetates (PMAAs). The obtained data were used to determine the methylated sugar linkages according to the standard data in the Complex Carbohydrate Research Center (CCRC) Spectral Database (https://glygen.ccrc.uga.edu/ccrc/specdb/ms/pmaa/pframe.html). The acetylated derivatives were loaded into an HP-5 capillary column, and the temperature program was set as follows: the initial column temperature was set at 80 ℃ and kept constant for 1 min, increased to 220 ℃ at the rate of 10 ℃/min kept constant for 1 min, and then increased from 220 to 300 ℃ at the rate of 20 ℃/min. The temperature of the interface was 250 ℃. Helium was used as the carrier gas. WAP was dissolved in deuterium oxide (D_2O) and examined at 30 ℃. The chemical shift of the sample was expressed in ppm (1×10^{-6}).

A Bruker DRX-600 nuclear magnetic resonance (NMR) spectrometer (Bruker, Rheinstetten, Germany) was implemented to record the ^{1}H and ^{13}C NMR spectra of sample. WAP was dissolved in D_2O and examined at 30 ℃.

2.7 Antioxidant properties of WAP

2.7.1 ABTS radical scavenging activity of WAP

The radical scavenging effect of WAP was evaluated using ABTS radical cation ($ABTS^{+}$) assay with a slight modification (Bao et al., 2016, 2021). Briefly, WAP solution (50 μL) was incubated with $ABTS^{+}$ working solution (150 μL), and then kept at room temperature for 20 min in the dark. The absorbance was measured at 734 nm. The ABTS radical scavenging rate (RSR) of WAP was calculated using Eq. (1):

$$\text{RSR}=\frac{A_{\text{control}}-A_{\text{sample}}}{A_{\text{control}}}\times 100\%, \quad (1)$$

where A_{control} is absorbance value of the control and A_{sample} is absorbance value of the sample. The ABTS radical scavenging activity was expressed as the concentration of WAP that yields 50% of radical scavenging (IC_{50}).

2.7.2 FRAP of WAP

FRAP assay was conducted according to Bao et al. (2016) with a slight modification. In brief, 100 μL of WAP solution was added to 100 μL of FRAP solution, and then incubated at 37 ℃ for 5 min. The absorbance was measured at 593 nm. $FeSO_4$ was selected as standard. The data were expressed as μmol Fe^{2+}/mg.

2.8 Culture of normal human hepatocytes (L02 cells)

Normal human hepatocytes (L02 cells) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured with 1640 medium containing 10% (volume fraction) of the calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. They were kept in an incubator at 37 ℃ with 5% CO_2 (Chen et al., 2014).

2.9 Cell viability assay and nuclear detection

MTT assay was performed according to a method described by Zhang et al. (2017) to evaluate cell viability. At first, cells were seeded at a density of 6×10^3 cells/well into a 96-well plate. After 24 h of incubation, different concentrations (2.5, 5, 10, 25, 50, 100, 200, and 250 μg/mL) of WAP were added to cells and incubated for another 24 h. Thereafter, cells were washed and added with MTT solution (0.5 mg/mL) for 4 h. After the removal of medium and washing the cells, dimethyl sulfoxide (DMSO, 200 μL) was added to cells and the absorbance was measured at 490 nm using Tecan Infinite M200 microplate reader (Molecular Devices, California, USA).

The protective effect of WAP on EC-induced cytotoxicity was estimated as follows. After seeding cells into a 96-well culture plate at a density of 6×10^3 cells/well, the plate containing the cells was incubated for 24 h and then treated with different concentrations of WAP. Following 24 h of treatment, EC was added for another 24 h, and absorbance was recorded at 490 nm using Tecan Infinite M200 microplate reader (Molecular Devices).

For nuclear detection, Hoechst 33528 (fluorescence probe) was used according to a protocol described by Zhang et al. (2017). Firstly, cells were seeded into a 24-well plate at a density of 4×10^4 cells/well. After 24 h of cell culture, different concentrations (2.5, 5, 10, 25, 50, 100, 200, and 250 μg/mL) of WAPs were added, followed by incubation for 24 h. Thereafter, cells were treated with EC (60 mmol/L) for another 24 h. Then, all cells were washed and incubated with Hoechst 33258 (10 μmol/L) for 30 min at 37 ℃. After washing properly, cell nuclei were detected under a Nikon Eclipse Ti-S inverted fluorescence microscope (Nikon, Natori, Japan).

2.10 Determination of intracellular ROS and $O_2^{\cdot-}$

An oxidation-sensitive fluorescence probe, DCFH-DA, was implemented to determine the intracellular ROS production (Chen et al., 2016a). Briefly, L02 cells were treated exactly as described in Section 2.9 (nuclear detection part). Subsequently, cells were washed with phosphate-buffered saline (PBS) and then incubated with DCFH-DA (10 μmol/L) for 30 min at 37 ℃. Thereafter, cells were washed with PBS immediately before examination through fluorescence microscope (Nikon). $O_2^{\cdot-}$ production in L02 cells was examined using a DHE probe. The data were obtained from five microscopic images evaluated using ImageProPlus 6.0 software (Media Cybernetics, Inc., Maryland, USA) and were expressed as mean fluorescence intensity.

2.11 Evaluation of mitochondrial mass and MMP

Mitochondrial mass and mitochondrial membrane potential (MMP) were assessed according to an earlier protocol (Chen et al., 2016a). In short, L02 cells were treated exactly as in Section 2.9 (nuclear detection part). Subsequently, cells were washed with PBS and then incubated with MTG (1 μmol/L) for 30 min at 37 ℃. Thereafter, cells were washed with PBS immediately before examination through a fluorescence microscope (Nikon). For MMP analysis, L02 cells were treated with 10 μmol/L Rh123 probe. The data were obtained from five microscopic images evaluated by ImageProPlus 6.0 software (Media Cybernetics, Inc.) and were expressed as mean fluorescence intensity.

2.12 Analysis of cellular glutathione

Cellular glutathione (GSH) was detected using NDA probe according to a slightly modified method by Li et al. (2018). In brief, L02 cells were treated exactly as in Section 2.9 (nuclear detection part). Subsequently, cells were washed with PBS and then incubated with NDA (50 μmol/L) for 30 min at 37 ℃. Thereafter, cells were washed with PBS immediately before examination through a fluorescence microscope (Nikon). The data were obtained from five microscopic images evaluated by ImageProPlus 6.0 software (Media Cybernetics, Inc.) and were expressed as mean NDA fluorescence intensity.

2.13 Statistical analysis

Data are expressed as mean±standard deviation (SD), whereas multiple comparisons were performed by two-tailed unpaired Student's *t*-tests with GraphPad Prism version 7.0 (GraphPad Software, California, USA). $P<0.05$ was considered as indicative of statistical significance. All analyses were carried out in triplicates.

3 Results and discussion

3.1 Chemical properties of WAP

Our total carbohydrates assay (phenol-sulfuric acid method) revealed that WAP contained (81.23±2.25)% carbohydrates. After acid hydrolysis and subsequent PMP derivatization, the monosaccharide composition of WAP was analyzed by HPLC. WAP was composed of mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, arabinose, and fucose in a molar ratio of 2.20:3.94:4.45:8.56:8.86:30.82:39.78:1.48. Among all monosaccharides in WAP, arabinose and galactose were the major constituents. The MW of WAP were 5.34 and 40.34 kDa. Based on the results of carbohydrate content and monosaccharide composition, WAP was acknowledged as an ideal polysaccharide.

3.2 FT-IR spectral and linkage analysis of WAP

In order to characterize the functional groups and chemical bonds of polysaccharides, we conducted the FT-IR of WAP within the wavelength of 4000–400 cm^{-1} (Meng et al., 2017). As shown in Fig. 1, a major broad stretching peak was found at 3388.12 cm^{-1}, which indicated the hydroxyl group with stretching vibration. The characteristic peaks of methyl and methylene C–H stretching vibration were found at 2921.02 and 2851.33 cm^{-1}, respectively. Moreover, the characteristic peaks of C=O of acetyl and carboxylic acid ester bonds were found at 1738.12 and 1639.56 cm^{-1}, respectively. The presence of pyranose was confirmed by the peaks of 1141.39 and 1074.27 cm^{-1}, while the presence of β-type and α-type glycosidic linkages in WAP was confirmed by the bands of 893.77 and 832.14 cm^{-1}, respectively (Liu et al., 2016; Luo et al., 2016). The above results indicated that WAP represents the typical absorption groups of an ideal polysaccharide.

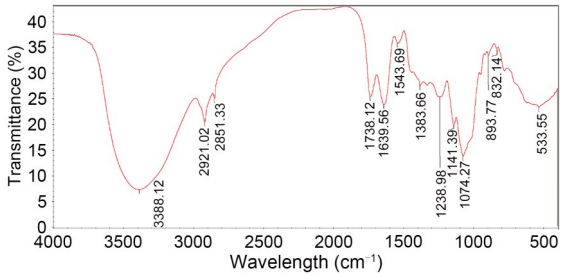

Fig. 1 Fourier transform infrared spectroscopy (FT-IR) spectra of wax apple polysaccharide (WAP).

3.3 Methylation results and NMR data of WAP

At first, the fully methylated product of WAP was hydrolyzed via acid and then converted to PMAA, which was analyzed by GC-MS. Table 1 showed the related linkage patterns and corresponding molar ratios in WAP. From the peak of GC chromatogram, the molar ratios of all sugar residues were calculated. According to the results, WAP mainly consisted of Ara*f*-(1→, Glc*p*-(1→, →2)-Ara*f*-(1→, →3)-Gal*p*-(1→, →3)-Ara*f*-(1→, and →6)-Gal*p*-(1→ in a molar percentage of 9.3%, 6.2%, 19.4%, 16.6%, 26.0%, and 22.5%, respectively. The data were consistent with the monosaccharide composition results of HPLC.

The ^{1}H and ^{13}C NMR spectra of WAP were shown in Fig. S1. The standardization of chemical shifts of H_2O (δ 4.79 ppm) was performed according to a previous report of ^{1}H NMR spectrum (Gottlieb et al., 1997). As shown in Fig. S1a, in the anomeric region of the ^{1}H NMR spectrum, δ 4.97, 5.00, 5.02, 5.04, 5.05, 5.06, 5.07, 5.10, 5.11, 5.12, 5.14, 5.15, 5.16, 5.17, 5.18, 5.20, 5.21, 5.28, and 5.32 ppm were recognized for α-anomeric protons, and the others (δ 4.31,

Table 1 Results of wax apple polysaccharide (WAP) methylation analysis

Methylated sugar	Linkage type	Molar ratio (%)	Mass fragment (*m*/*z*)
2,3,5-Me_3-Ara*f*	Ara*f*-(1→	9.3	43, 57, 71, 85, 101, 117, 131, and 161
2,3,4,6-Me_4-Glc*p*	Glc*p*-(1→	6.2	43, 57, 71, 85, 101, 117, 129, 145, 161, and 205
3,5-Me_2-Ara*f*	→2)-Ara*f*-(1→	19.4	43, 57, 71, 85, 101, 117, 129, 145, 161, and 190
2,4,6-Me_3-Gal*p*	→3)-Gal*p*-(1→	16.6	43, 57, 71, 85, 101, 117, 129, 143, 149, 161, 173, and 233
2,5-Me_2-Ara*f*	→3)-Ara*f*-(1→	26.0	43, 57, 71, 85, 99, 117, 129, 149, 161, 173, and 233
2,3,4-Me_3-Gal*p*	→6)-Gal*p*-(1→	22.5	43, 49, 57, 71, 87, 99, 101, 117, 129, 161, 189, and 233

Ara: arabinose; Gal: galactose; Glc: glucose; Me: methyl.

4.32, 4.36, 4.39, 4.41, 4.43, 4.46, 4.48, 4.50, 4.51, 4.54, 4.56, and 4.57 ppm) were identified as β-anomeric protons (Tamiello et al., 2018). The anomeric ^{1}H signals mainly occurred within the range of δ 4.31–4.57 ppm, which indicated that the configurations of WAP were mostly β form. Fig. S1b presented the ^{13}C NMR spectrum of WAP. The existence of the C1 signal (δ 102.87, 100.46, 96.22, 96.07, 95.90, 95.87, 95.36, 92.02, and 91.73 ppm) indicated the presence of pyranose forms in WAP (Gorin and Mazurek, 1975; Agrawal, 1992). Furthermore, the ^{13}C signal range of δ 64–70 ppm indicated the presence of →6) glycosidic linkages in WAP. The signals at δ 102.87/4.48 (C1/H1) were related to β-D-Gal*p* units (Yan et al., 2013). Overall, the data indicated that WAP represents all characteristics of an ideal polysaccharide.

3.4 In vitro antioxidant effects of WAP

Previous studies have revealed the good antioxidant effect of wax apple extracts (Simirgiotis et al., 2008; Sulaiman and Ooi, 2014; Esua et al., 2017). Moreover, polysaccharides from plants and fungi showed remarkable antioxidant activity (Zeng et al., 2014; Xie et al., 2016; Yan et al., 2016). Therefore, the antioxidant activity of WAP was verified by two in vitro antioxidant assays, while vitamin C was used as positive control. Regarding the ABTS scavenging activity, the IC_{50} values of WAP and vitamin C were (0.29±0.01) and (0.01±0.00) mg/mL, respectively. In terms of FRAP, the antioxidant activity levels of WAP and vitamin C were (0.51±0.01) and (14.15±0.18) μmol Fe^{2+}/mg, respectively. These results indicated that WAP from wax apple has potent antioxidant effects.

3.5 Effects of WAP on EC-induced cytotoxicity and genotoxicity in L02 cells

In our study, L02 cells were used to evaluate the effect of WAP on EC-induced toxicity. Firstly, different concentrations (2.5, 5, 10, 25, 50, 100, 200, and 250 μg/mL) of WAP were analyzed for cytotoxicity on L02 cells. According to Fig. 2a, no cytotoxicity was detected for the different concentrations of WAP on L02 cells; hence, it was confirmed that WAP was suitable for further investigation. EC was selected at a concentration of 60 mmol/L due to its cytotoxicity of 55.45%, which was suitable for the subsequent investigation of the protective effect. When L02 cells were pretreated with different concentrations (2.5, 5, 10, 25, 50, 100, 200, and 250 μg/mL) of WAP for 24 h, the cell viability recovered dose-dependently compared to EC-treated group (Fig. 2b). Specifically, when L02 cells were treated with lower concentrations of WAP (2.5, 5, 10, and 25 μg/mL), no effective protection was observed against EC-induced cytotoxicity. Higher concentrations of WAP (50, 100, 200, and 250 μg/mL) significantly improved the cell viability at 60.77%, 63.75%, 65.10%, and 62.10%, respectively, compared to the EC (60 mmol/L) group. These data revealed that WAP could resist EC-induced cytotoxicity at a suitable concentration (50, 100, 200, and 250 μg/mL).

Previous studies found that EC exposure can cause genotoxicity in cells (Jiao et al., 2014; Chen et al., 2016b), which is an important perspective of cytotoxicity, as shown by the condensation of chromatin and nuclear fragmentation. Therefore, we evaluated the protective effect of WAP against EC-induced genotoxicity. Hoechst 33258 is a specific fluorescent dye that can bind with genomic DNA at high sensitivity; therefore, it was used in the present study. According to Fig. 2c, the fluorescence intensity of EC-treated cell nuclei was brighter than that of control group. In the WAP-treated group, the decrease in fluorescence intensity (by contrast) was observed compared to the EC-treated group. These results suggested that WAP can prevent the EC-induced DNA damage in L02 cells.

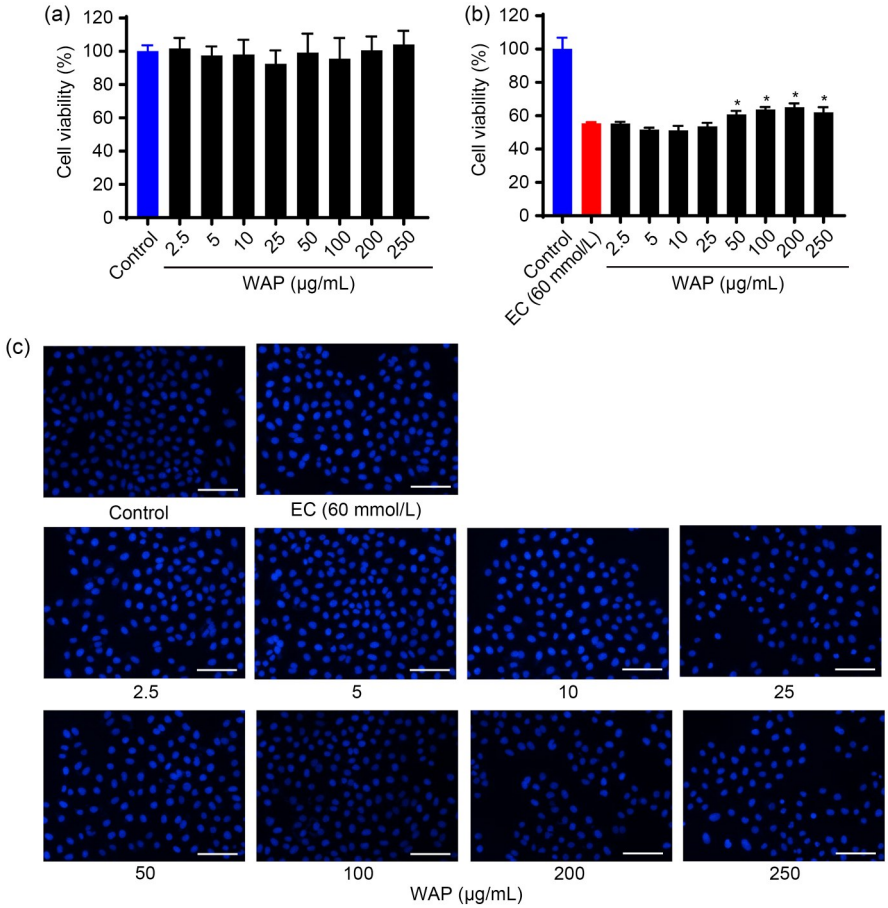

Fig. 2 Effects of WAP on EC-induced cytotoxicity and genotoxicity in L02 cells. (a) Quantitative determination of cell viability after treatment of L02 cells with different concentrations of WAP for 24 h using MTT assay. (b) Quantitative determination of cell viability after EC treatment for another 24 h using MTT assay after pre-treatment of L02 cells with different concentrations of WAP for 24 h. (c) Qualitative determination of cell viability based on nuclear staining with Hoechst 33258. Scale bar=100 μm. Data are expressed as mean±standard deviation (SD), n=4. * P<0.05 vs. control. EC: ethyl carbamate; WAP: wax apple polysaccharide; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,-5-diphenyl-2H-tetrazolium bromide; SD: standard deviation.

3.6 Protective effect of WAP against EC-induced ROS and $O_2^{\cdot-}$ generation

Previous studies associated EC cytotoxicity with ROS (Chen et al., 2016b; Cui et al., 2016). Accumulating evidence indicates that cellular redox disorders are caused by ROS overproduction, and treatment with antioxidant-rich components could suppress the ROS by regulating the cellular redox system (Uttara et al., 2009; Rodríguez-Ramiro et al., 2011). ROS production in L02 cells can be analyzed by the 2',7'-dichlorofluorescein (DCF) fluorescence assay. According to Figs. 3a and 3b, EC treatment applied to L02 cells significantly increased the ROS production (124.02% mean fluorescence intensity) compared to the control (100.00%). On the contrary, pretreatment with WAP at 5, 10, 25, 50, 100, 200, and 250 μg/mL resulted in the significantly decreased mean fluorescence intensity of ROS, namely, 112.01%, 113.72%, 105.69%, 101.67%, 106.75%, 98.77%, and 90.65%, respectively (Figs. 3a and 3b). As a whole, WAP exhibited good performance in scavenging intracellular ROS.

Cellular $O_2^{\cdot-}$, a one-electron reduced product from the reduction of mitochondrial electron transport in the respiratory chain, was evaluated in previous studies (Pervaiz and Clement, 2007; Chen W et al., 2011). As compared with the control (100.00%), EC-treated L02 cells showed significantly higher fluorescence intensity of $O_2^{\cdot-}$ (122.96%), which was suppressed dose-dependently in response to WAP treatment (Figs. 3c and 3d). WAP at 200 and 250 μg/mL significantly decreased the mean fluorescence intensity,

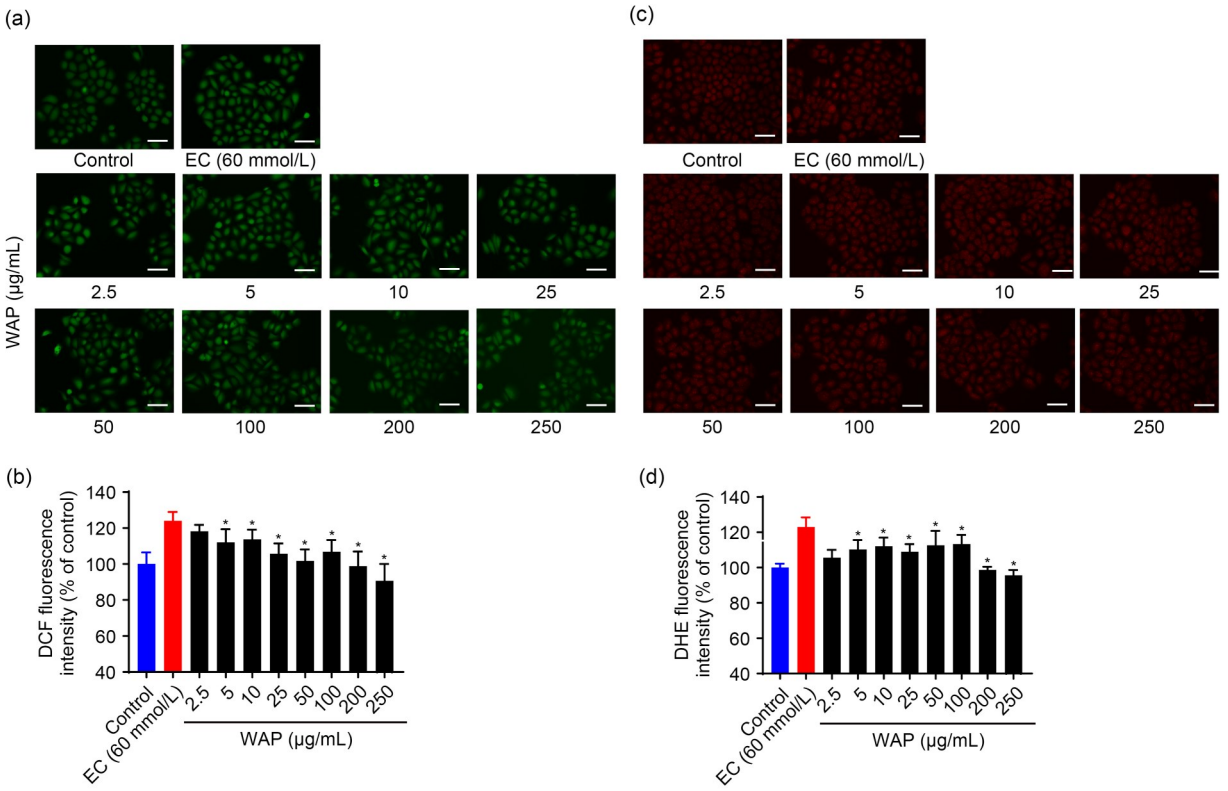

Fig. 3 Effects of WAP on EC-induced ROS and $O_2^{\cdot-}$ accumulation in L02 cells. (a) Qualitative determination of ROS based on DCFH-DA fluorescence intensity after EC treatment with or without WAP pretreatment for 24 h. (b) Quantitative determination of ROS (data were expressed as mean DCF fluorescence intensity±SD, *n*=4). (c) Qualitative determination of $O_2^{\cdot-}$ based on DHE fluorescence intensity after EC treatment with or without WAP pretreatment for 24 h. (d) Quantitative determination of $O_2^{\cdot-}$ (data were expressed as mean DHE fluorescence intensity±SD, *n*=4). * *P*<0.05 vs. control. Scale bar= 100 μm. DCF: 2',7'-dichlorofluorescein; DCFH-DA: 2',7'-dichlorofluorescein diacetate; DHE: dihydroethidium; EC: ethyl carbamate; ROS: reactive oxygen species; WAP: wax apple polysaccharide; SD: standard deviation.

with values of 98.60% and 95.64%, respectively. It can be concluded that WAP could provide protection against EC-induced intracellular ROS and $O_2^{\cdot-}$ overproduction and accumulation in L02 cells.

3.7 Prevention of EC-induced mitochondrial dysfunctions in L02 cells by WAP pretreatment

Mitochondria, as crucial organelles for the regulation of cellular energy production and metabolism, are associated with ROS production and regulating the cell redox potential (Zorov et al., 2014). The number, structure, and compartments of mitochondria are indicative of cell state. Therefore, the protective effects of WAP on mitochondrial mass and MMP in the EC-treated L02 cells were evaluated. EC-treated L02 cells lowered mitochondrial mass (79.35%) compared to the control (100.00%), while pretreatment with different concentrations of WAP did not improve the mitochondrial mass (Figs. 4a and 4b).

As is known, mitochondrial membrane dysfunctions such as MMP decline and mitochondrial membrane lipid peroxidation are associated with ROS generation (Lin and Beal, 2006). Thus, we further investigated the protective effect of WAP on MMP. According to our work, the exposure of L02 cells to EC (60 mmol/L) for 24 h significantly suppressed the MMP level, whereas the mean fluorescence intensity of EC-treated cells was only 71.71% compared to the control (100.00%) (Figs. 4c and 4d). In contrast, the pretreatment of L02 cells with different concentrations of WAP (10, 25, 50, 100, 200, and 250 μg/mL) dose-dependently increased the mean fluorescence intensity of Rh123, namely, with 79.57%, 83.35%, 99.73%, 101.29%, 99.96%, and 94.82%, respectively. These results suggested that EC treatment induced the collapse of MMP, which was effectively ameliorated by WAP (Fig. 4d).

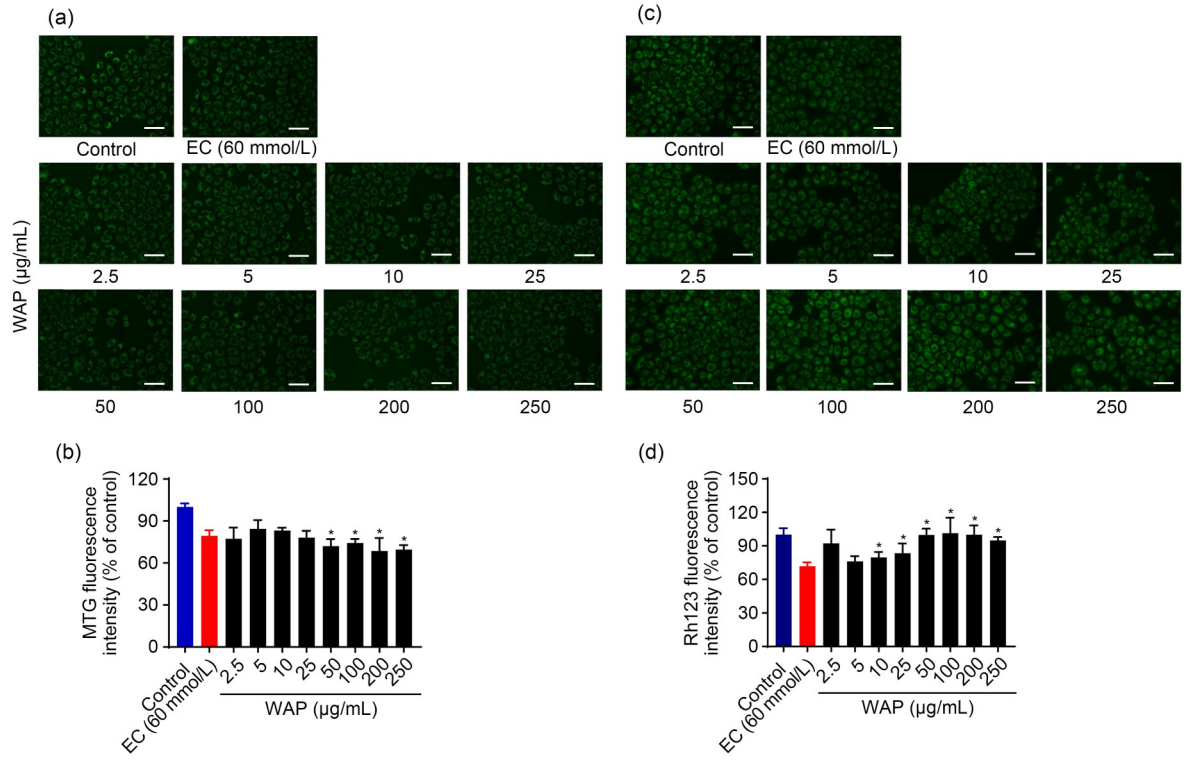

Fig. 4 Effects of WAP on EC-induced mitochondrial dysfunctions (mitochondrial mass and MMP) in L02 cells. (a) Qualitative determination of mitochondrial mass based on MTG fluorescence intensity after EC treatment with or without WAP pretreatment for 24 h. (b) Quantitative determination of mitochondrial mass (data were expressed as mean MTG fluorescence intensity±SD, *n*=4). (c) Qualitative determination of MMP based on Rh123 fluorescence intensity after EC treatment with or without WAP pretreatment for 24 h. (d) Quantitative determination of MMP (data were expressed as mean Rh123 fluorescence intensity±SD, *n*=4). * *P*<0.05 vs. control. Scale bar=100 μm. EC: ethyl carbamate; MMP: mitochondrial membrane potential; MTG: Mito-Tracker Green; Rh123: Rhodamine 123; WAP: wax apple polysaccharide; SD: standard deviation.

3.8 Effect of WAP on EC-treated GSH depletion in L02 cells

GSH is known as an antioxidant molecule with a potential role of maintaining the cellular redox system (Shila et al., 2005; Bhat et al., 2015). Excessive ROS production is associated with the depletion of intracellular GSH. This is because a decreased level of intracellular GSH cannot eliminate an excessive ROS level. Thus, intracellular GSH level was measured using an NDA fluorescence probe in our research in the presence or absence of WAP. As confirmed by Fig. 5, cellular GSH depletion occurred after EC treatment, while the mean fluorescence intensity of EC-treated group was only 50.76% compared to the control (100.00%) (Fig. 5a). On the other hand, WAP pretreatment significantly increased the intracellular GSH content compared to the EC-induced group. Different concentrations of WAP (25, 50, 100, 200, and 250 μg/mL) yielded dose-dependent fluorescence intensity, such as 69.72%, 73.38%, 79.28%, 79.14%, and 80.37%, respectively (Fig. 5b). The above data indicated that WAP had a positive effect against EC-induced GSH depletion, which helped to maintain the redox balance in L02 cells.

4 Conclusions

Oxidative stress plays an important causative role in hepatic oxidative diseases such as nonalcoholic fatty liver disease, alcoholic liver disease, and hepatitis type C, which can be ameliorated by the intake of polysaccharides or polysaccharide-rich foods. WAP has recently attracted increasing attention regarding its potential therapeutic effect on oxidative dysfunction. This study isolated a polysaccharide from wax apple and evaluated its protective effect against hepatic

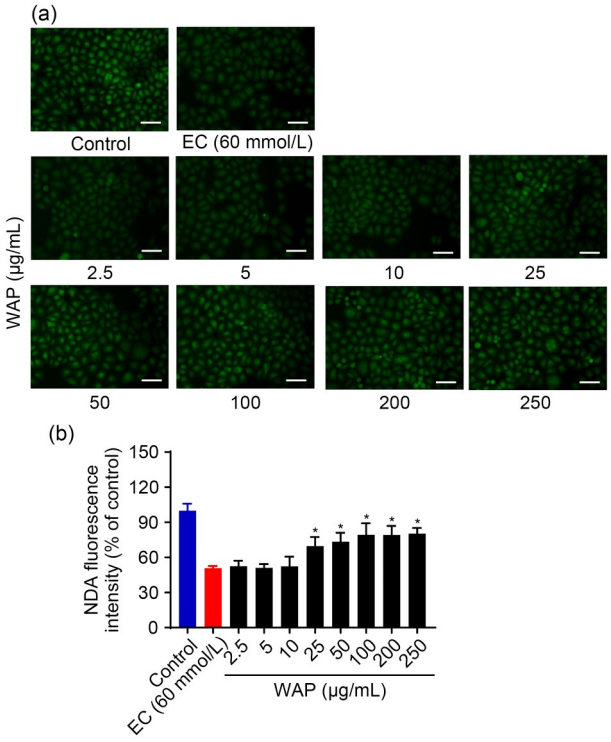

Fig. 5 Effect of WAP on EC-induced GSH depletion in L02 cells. (a) Qualitative determination of GSH content based on NDA fluorescence intensity after EC treatment with or without WAP pretreatment for 24 h. Scale bar=100 μm. (b) Quantitative determination of GSH content (data were expressed as mean NDA fluorescence intensity±SD, n=4). * P< 0.05 vs. control. EC: ethyl carbamate; GSH: glutathione; NDA: naphthalene-2,3-dicarboxal-dehyde; WAP: wax apple polysaccharide; SD: standard deviation.

oxidative disease. WAP was shown to contain several monosaccharides such as mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, arabinose, and fucose. The molar ratios of these monosaccharides were determined to be 2.20:3.94:4.45:8.56:8.86:30.82:39.78:1.48. Cell-level studies indicated that WAP pretreatment could suppress EC-induced oxidative damage in human hepatocytes via preventing cytotoxicity and DNA damage, scavenging intracellular ROS and $O2^{\cdot-}$, attenuating mitochondrial membrane potential collapse, and reducing GSH depletion. Collectively, our findings suggest that WAP could be an important component or supplement for the treatment of oxidative stress-mediated clinical complications such as EC-induced hepatic oxidative damage. At the same time, further studies are necessary to prove the effect in vivo at the biological and clinical levels.

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Author contributions

Tao BAO performed the experimental research and data analysis, wrote and edited the manuscript. Naymul KARIM contributed to the study design, writing and editing of the manuscript. Huihui KE performed the experimental research. Jitbanjong TANGPONG contributed to the writing and editing of the manuscript. Wei CHEN contributed to the study design, data analysis, writing and editing of the manuscript, and provided the funding. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Tao BAO, Naymul KARIM, Huihui KE, Jitbanjong TANGPONG, and Wei CHEN declare that there are no conflicts 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