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Comparison of the protective effect of black and white mulberry against ethyl carbamate-induced cytotoxicity and oxidative damage

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ABSTRACT

Increasing evidence indicates that crude extracts derived from mulberry confer protection against oxidative stress. However, the antioxidant capacity of mulberry extract among different cultivars remains elusive. The main objective of the present study was therefore to investigate the effect of black mulberry extract (BMB) and white mulberry extract (WMB) on ethyl carbamate (EC)-induced cytotoxicity and oxidative stress. This study showed that the contents of total phenolics, total flavonoids, total procyanidins, cyanidin-*3*-*O*-glucoside, cyanidin-*3*-*O*-rutinoside and pelargonidin-*3*-*O*-glucoside of BMB were higher than that of WMB. Moreover, our results showed that phenolics-abundant BMB was stronger than WMB in scavenging ABTS and DPPH free radicals. BMB was more effective in ameliorating EC-induced cytotoxicity by inhibiting excessive ROS generation, suppressing mitochondrial dysfunction and increasing GSH concentration in HepG2 cells than WMB. Taken together, our study revealed that BMB afforded better protection against EC-caused cytotoxicity than WMB.

1. Introduction

Mulberry (*Morus* spp.), belonging to the genus *Morus* of the family *Moraceae*, is widely distributed (Ercisli & Orhan, 2007). The chemical composition and biological activities of mulberry fruit vary, depending on the type of cultivar used. For instance, white mulberry (*Matropurpurea Roxb.*) and black mulberry (*Morus alba* L.), well-known species in the *Morus* genus, are different in terms of their bioactive compound contents. Black mulberry fruits have a higher content of total phenolics, total flavonoids and total monomeric anthocyanins than have white mulberry fruits (Bao et al., 2016). Phenolics exhibit various bioactivities, such as anti-inflammatory, anti-obesity and anti-cancer properties (Kim, Nam, & Friedman, 2015; Munagala et al., 2017). Since black mulberry has higher contents of phenolic compounds than has white mulberry, the bioactivities of these two cultivars may exhibit great differences.

Oxidative stress exerts a significant influence on the development of arthritis, aging and cancer (Doppler & Storz, 2017; Grune, 2000; Hultqvist et al., 2004). Growing attention has been paid to the fact that bioactive phytochemicals from vegetables and fruits can reduce the negative effect of oxidative damage resulting from environmental toxicants (Chen, Zhuang, Li, Shen, & Zheng, 2013). Mulberry has been recognized as both an edible fruit and herbal medicine for thousands of years in China. Accumulating evidence indicates that mulberry is capable of providing protection against oxidative stress (Bao et al., 2016; Lee et al., 2015). Studies reveal that both black mulberry and white mulberry provide positive effects on scavenging free radicals (Isabelle, Lee, Ong, Liu, & Huang, 2008; Khan et al., 2013). However, more convincing evidence on the differences of anti-oxidative capacities between these two cultivars needs further investigation.

Ethyl carbamate (EC) is generated as a by-product of fermentation in various foods and beverages (Battaglia, Conacher, & Page, 1990). The classification of EC was upgraded to group 2A (probably carcinogenic to humans) by the International Agency for Research on Cancer (IARC) in 2007. Metabolites of EC lead to oxidative damage in DNA molecules and the formation of adducts and C-hydroxylation, which generates vinyl carbamate (VC) and then converts to an epoxide that interacts with nucleic acid (Colombo et al., 2015). EC treatment was reported to lead to an increase of ROS generation in RAW 264.7 macrophages and A549 lung epithelial cells (Chun, Cha, & Kim, 2013). The present study, therefore, was aimed to investigate whether phenolics-abundant mulberry could exert ROS-scavenging ability under EC treatment and to carry out a systematic assessment of the potential differences of anti-oxidative capacity between different mulberry cultivars.

2. Materials and methods

2.1. Chemicals and materials

3-(4,5-Dimthyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide

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Table 1
Bioactive components of black and white mulberry.

	Total phenolics (mg GAE/kg FW)	Total flavonoids (mg QE/kg FW)	Procyanidins (mg CE/kg FW)	C3G (mg/kg FW)	C3R (mg/kg FW)	P3G (mg/kg FW)	Quercetin-*3*-*O*-rutinoside (mg/kg FW)
BMB	6585 ± 146	1292 ± 52.7	2716 ± 24.5	1698 ± 63.9	693 ± 19.2	141 ± 7.21	89.9 ± 2.30
WMB	879 ± 19.0	663 ± 28.5	217 ± 18.2	ND	ND	ND	13.0 ± 1.95

The values are expressed as means ± SD (n = 3); ND, not detected; C3G, Cyanidin-*3*-*O*-glucoside; C3R, Cyanidin-*3*-*O*-rutinoside; P3G, Pelargonidin-*3*-*O*-glucoside; BMB, black mulberry extract WMB, white mulberry extract; FW, fresh weight; GAE, gallic acid equivalents; QE, quercetin-*3*-*O*-rutinoside equivalents; CE, catechin equivalents.

(MTT), nonyl acridine orange (NAO), Hochest 33258, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 2-2′azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), cyanidin-*3*-*O*-glucoside (C3G), cyanidin-*3*-*O*-rutinoside (C3R), pelargonidin-*3*-*O*-glucoside (P3G), and quercetin-*3*-*O*-rutinoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, monobasic potassium phosphate, ascorbic acid, Folin–Ciocalteu reagent, ethyl carbamate (EC), chromatographic grade methanol and acetonitrile were purchased from Aladdin (Shanghai, China). Dichlorodihydrofluorescein diacetate (DCFH-DA), Rhodamine 123 (Rh123) and naphthalene-2,3-dicarboxaldehyde (NDA) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). All other reagents used were of analytical grade.

Black mulberry (cultivar “J33”) and white mulberry (cultivar “Zhenzhubai”) fruits (Fig. S1) were collected from Jinhua in Zhejiang Province, China. For each mulberry cultivar, the materials were categorized into three groups for uniformity in shape, colour and weight. After being washed with sterile distilled water, the fruits were dried in a clean and dust-free environment at room temperature. After that, samples were stored at −80 °C for further analysis.

2.2. Mulberry extract preparation

Black mulberry or white mulberry fruits (each 100 g) were homogenized with 300 ml of ethanol/water (70:30, v/v). The extraction procedure was conducted twice, then the extracts were filtered. Collected samples were further concentrated by rotary evaporation to remove ethanol under reduced pressure at 40 °C. The final samples of black mulberry and white mulberry were named BMB and WMB, respectively.

2.3. Determination of total phenolics, total flavonoids and total procyanidin contents in BMB and WMB

The total phenolic content (TPC) was determined according to a previous method (Bao et al., 2016). Mulberry samples were diluted with distilled water (1:10, *v/v*). Then 100 µl of sample was mixed with 0.1 ml of Folin-Ciocalteu reagent, followed by adding 0.2 ml of sodium carbonate solution (15%). After incubation for 2 h at room temperature, the absorbance of the mixture was measured at 760 nm. Gallic acid was used as a calibration standard, and total phenolic content was expressed as mg gallic acid equivalents (GAE)/kg of fresh weight (FW).

Total flavonoid contents (TFC) of BMB and WMB were determined, using the aluminium chloride colorimetric method with slight modification (Chen, Su, Xu, Bao, & Zheng, 2016). 90 µl of BMB or WMB were mixed with 0.04 ml of 5% $NaNO_2$ and 0.41 ml of distilled water, respectively. After incubation for 5 min, the mixture was treated with 0.04 ml of 10% of $Al(NO_3)_3$ and kept at room temperature for 6 min. After that, 0.4 ml of 4% (v/v) NaOH and 0.02 ml of distilled water were added. After being incubated for 15 min, the absorbance of the mixture was read at 510 nm. Quercetin-*3*-*O*-rutinoside was used as a reference standard for the quantification of TFC. The TFC of BMB or WMB was expressed as g quercetin-3-O-rutinoside equivalents (QE)/kg fresh weight (FW).

Total procyanidin content was tested by a previous study with some modifications (Bao et al., 2016). In brief, 4% vanillin was dissolved in methanol solution and then incubated with 300 µl of concentrated hydrochloric acid and 100 µl of BMB or WMB (with a 1:5 dilution) at 20 °C for 20 min. The absorbance of the mixture was read at 500 nm, and total procyanidin content was expressed as g catechin equivalents (CE) per kg fresh weight (FW).

2.4. Analysis of phenolic compounds by HPLC

An HPLC (Dionex ultimate 3000, ThermoFisher Scientific, USA) system with a diode array detector was used to quantify the phenolic compounds in mulberry extract by corresponding standards. The mobile phase A was 1.5% aqueous formic acid solution and mobile phase B consisted of formic acid/acetonitrile/methanol/water (1.5:22.5:22.5:48.5, v/v/v/v). A linear gradient programme was carried out as follows: 0–35 min, 7–25% phase B; 35–45 min, 25–65% phase B; 45–46 min, 65–100% phase B; 46–50 min, 100% phase B; 50–57 min, 100–7% phase B; 57–60 min, 7% phase B. 10 µl of mulberry extract were injected into a Promosil C18 (4.6 × 250 mm, 5 m) column and eluted at 30 °C with the flow rate at 1.0 ml/min. Absorbance was detected at 280 nm. Cyanidin-*3*-*O*-glucoside, cyanidin-*3*-*O*-rutinoside, pelargonidin-*3*-*O*-glucoside and quercetin-*3*-*O*-rutinoside were used as standards.

2.5. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assay

The capacity of the cationic radical $ABTS^{\bullet+}$ was determined according to the previous report with slight modification (Chen, Zhuang, et al., 2013). Briefly, 10 ml of 7 mM 2,2-azobis (2-amidinopropane) dihydrochloride were added to 179 ml of 140 mM aqueous potassium persulfate in order to prepare the ABTS stock solution, then the mixture was kept for 12 h without light at room temperature. The solution was then diluted 20 times with sodium PBS for further use. The diluted working ABTS solution (700 µl) was mixed with BMB or WMB (20 µl) and incubated for 6 min in the dark. The variation in absorbance was read at 734 nm. $ABTS^{\bullet+}$ scavenging ability (%) = (Absorbance of control-Absorbance of sample)/Absorbance of control × 100.

The DPPH free radical-scavenging activities of BMB and WMB were evaluated according to a previous study (Chen, Zhao et al., 2016) with minor modification. 20 µl of the test sample were added to 700 µl of 0.1 mM solution of DPPH in ethanol. The mixture was vortexed and incubated for 30 min in the dark at room temperature, and then the absorbance was determined at 517 nm. All analyses were performed in triplicate. $DPPH^{\bullet}$-scavenging ability (%) = (Absorbance of control-Absorbance of sample)/Absorbance of control × 100.

Ascorbic acid was employed as positive control. Then ABTS and DPPH radical-scavenging activities of BMB and WMB were expressed as vitamin C equivalents (VCE)/kg fresh weight (FW), respectively.

2.6. Cell culture

Human HepG2 cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml of penicillin and 100 units/ml of streptomycin in a humidified cell incubator with an atmosphere of 5% CO_2 at 37 °C. Cells without any treatment (medium only) were used as control in the following experiment.