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ORIGINAL RESEARCH PAPER

Cytosolic protection against ultraviolet induced DNA damage by blueberry anthocyanins and anthocyanidins in hepatocarcinoma HepG2 cells

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Abstract UV-induced DNA damage plays a key role in the etiology of certain diseases. The ability of blueberry anthocyanins and anthocyanidins (BA) to protect cellular DNA from UV-induced damage was investigated. BA were extracted by water (BAW), ethanol (BAE) or methanol (BAM). These extracts partially restored proliferation of UV-irradiated HepG2 cells as shown by MTT assay. Treatment with BA extracts at 75 μg/ml decreased reactive oxygen species and decreased DNA damage by tail moment of comet assay and expression of γH2AX in situ. BAM significantly decreased gene and protein expression of p53, phospho-p53 (Ser15), and p21 in UV-irradiated HepG2 cells. BA thus efficiently protects cells from DNA damage in vitro. Blueberry may potentially be used as a good source of naturally radioprotective agents.

Keywords Anthocyanins · Anthocyanidins · Blueberry · DNA damage · p53 · UV-irradiation

Introduction

DNA is the major target of UV-induced cellular damage and occurs through the formation of reactive oxygen species (ROS). Excessive ROS attack cellular components and cause damage to lipids, proteins, and DNA. This cellular damage initiates a chain of events resulting in the onset of a variety of diseases (Luo et al. 2011).

The occurrence of DNA double strand breaks (DSBs) triggers phosphorylation at serine residue 139 of H2AX protein (termed γH2AX) in DSB sites (a so-called "foci" structure). These foci can then recruit numerous checkpoint proteins to the damaged sites, including BRCA1, 53BP1, Werner syndrome protein (WRN), and others. Because of their close relationship with DSBs, γH2AX expression or γH2AX foci formation have been suggested as sensitive methods to detect DNA damage.

For maintenance of DNA integrity, p53 is a crucial protein in multicellular organisms in which it regulates the cell cycle and functions as a tumor suppressor to prevent DNA damage. Activation of p53-mediated

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transcription is a critical cellular response to DNA damage (Johnson et al. 2001). Therefore, p53 is regarded as a biomarker for DNA repair. The cyclin-dependent kinase inhibitor p21 is a key player in cell cycle control and more recently in apoptosis. Following DNA damage, p53 induces cell cycle arrest by increasing expression of p21. Transcription of and phosphorylation of p53 (Ser15) are important mechanisms that regulate p53 stability and protein levels (Zhou and Jia 2010).

Blueberry (*Vaccinium corymbosum*) effectively prevents DNA damage or carcinogenesis in mice because of the presence of large amounts of antioxidants, including anthocyanins, anthocyanidins and other undetermined compounds. In order to establish a standard measurement, we tested cytosolic protection of DNA by different fractions of blueberry extracts on UV-irradiated hepatocarcinoma HepG2 cells. The goal is to apply this measurement as a model to test DNA protection efficacy of ancillary drugs, functional foods, and cosmetics.

Materials and methods

Chemicals

Dulbecco's modified eagle medium (DMEM) and RevertAid First Strand cDNA Synthesis Kit were from Thermo (St. Louis, MO, USA). RNeasy Mini Kit was from Qiagen (Hilden, Germany). Antibodies of p53, phospho-p53, p21 and β-actin were from Sigma–Aldrich. Phospho-histone H2A.X (Ser139) rabbit antibody was from cell signaling technology (Boston, MA, USA). Horseradish peroxidase-conjugated secondary antibody and fluorescein-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Lab (West Grove, PA, USA).

Preparation of BA extracts

Fresh blueberries were crushed, defatted with petroleum ether at 80 °C, then lyophilized. Extractions of BA were obtained by three different mechanisms. Methanol extract (BAM) was obtained by maceration at room temperature for 1 week using 80 % (powder/solvent ratio = 1:10 w/v) methanol. Ethanol extract (BAE) was obtained through a Soxhlet extractor at 90 °C for 9 h using 80 % (1:10 w/v) ethanol. Finally, water extract (BAW) was obtained by boiling in water in the same powder/solvent ratio for 3 h. These samples were filtered through Whatman No. 1 filter paper and evaporated into concentrated solutions in a rotary evaporator at 40 °C. The solutions were further purified by adsorption of AB-8 macroporous resin, following 60 % (v/v) ethanol for desorption of BA. Finally, samples were lyophilized to obtain the three different types of BA extracts.

The yields of extracts were 4.7, 4.4, and 4.1 % for BAM, BAE, and BAW, respectively, as determined by the pH differential method using cyanidin 3-glucoside as the standard (Jiang 2010). The percentage of extracts was determined spectrometrically to be 87, 88 and 88 % for BAM, BAE and BAW, respectively.

Cell culture and UV irradiation

The extracts were dissolved in DMSO and diluted in serum-free DMEM to the desired concentration. Final DMSO concentration was never higher than 0.05 % and DMSO was used as a control in each assay. HepG2, a human hepatocarcinoma cell line, was maintained in DMEM supplemented with 10 % (v/v) fetal bovine serum at 37 °C in a 5 % (v/v) CO_2 incubator.

Irradiation of cells was carried out using a standard UV radiation case at a dose of 4 mJ/cm^2. For synchronization, HepG2 cells were incubated overnight with BA extracts in serum-free DMEM. At the end of the incubation, the medium was removed and the cells were bathed in serum-free DMEM and then exposed to a fixed UV dose. After irradiation, the cells were incubated for an additional 12 h at 37 °C in an incubator containing 5 % CO_2 prior to use in different assays.

MTT assay

MTT assay was used to measure cell viability and proliferation. HepG2 cells at 10^5/ml were treated with 0.5 mg MTT/ml, prepared just before use and maintained in the dark for 4 h at 37 °C. Absorbance was measured at 570 nm after insoluble dark blue formazan crystals were dissolved with acid/2-propanol (40 mM HCl). The experiments were performed on three separate occasions.

Immunofluorescence staining

10^5 HepG2 cells were seeded into a 6-well culture plate containing a glass cover slip in each well. After UV irradiation and BA treatment, cells were fixed in 4 % (v/v) paraformaldehyde for 15 min, washed with PBS, and permeabilized in 0.2 % Triton-X 100. After blocking for non-specific binding with blocking serum for 0.5 h, samples were incubated with a rabbit monoclonal anti-γH2AX antibody (1:400) for 2 h, followed by FITC-conjugated goat anti-rabbit secondary antibody (1:50) for 1 h. Nuclei were counterstained with DAPI (10 μg/ml in PBS) for 15 min. Slides were mounted with coverslips and viewed using a confocal laser scanning microscope (Nikon).

Comet assay

DNA damage and protection were also evaluated through the alkaline single cell gel electrophoresis (Comet) assay, performed under alkaline conditions according to previous reports (Mozaffarieh et al. 2008) with a slight modification. Cells were plated at 2.2×10^6 cells per dish and left to adhere overnight. Cells were treated with BA extracts, UV-irradiated, and then trypsinized, resuspended in PBS, and counted. Normal melting agarose (0.5 %) in PBS (100 μl) prewarmed at 45 °C was dropped onto slides that were covered with a glass coverslip. The slides were allowed to set at 4 °C for 10 min, coverslips were removed, and 10 μl treated cells were mixed with 75 μl 0.7 % low melting agarose in PBS at 37 °C and spread onto slides for 10 min at 4 °C. The final layer of 75 μl of 0.7 % low melting agarose was applied in the same way. The slides without coverslips were immersed in frosted lysis buffer with 10 % (v/v) DMSO at 4 °C for 2 h, then placed in electrophoresis buffer (1 mM EDTA, 0.3 M NaOH) in a tank at 4 °C for 40 min to allow alkaline unwinding. Electrophoresis was carried out for 20 min under 25 V and 300 mA. Slides were then transferred to 0.4 mmol Tris/buffer (pH 7.5), washed three times, and gently dried. Comets were stained with PI (2 μg/ml) and analyzed under a confocal laser scanning microscope at 543 nm. Image analysis and tail moment were performed with the software CASP 1.2.2 (CaspLab). Twenty-five cells were randomly selected per sample.

Reactive oxygen species (ROS) assay

Intracellular ROS were examined utilizing H_2DCF-DA (Matsuo et al. 2005). UV-irradiated cells with or without BA extract-treatment were incubated with 10 μM H_2DCF-DA for 30 min at 37 °C to assess ROS-mediated oxidation of DCFH-DA to the fluorescent compound, 2′,7′-dichlorofluorescein (DCF). Cells were then harvested and the pellets were suspended in 500 μl PBS. Samples were analyzed by FACScan flow cytometry with excitation at 480 nm and emission at 525 nm.

Gene expression of p53 and p21 by RT-PCR

For reverse transcription polymerase chain reaction (RT-PCR) analysis, total RNA was isolated by using Qiagen's RNeasy Mini Kit from BA extract-treated and/or UV-irradiated cells according to the manufacturer's instructions. The RT was carried on isolated RNA out as follows: 1 μl Ribolock RNase Inhibitor, 1 μl Oligo $(dT)_{18}$ Primer, 2 μl 10 mM dNTP Mix, 4 μl 5 × reaction buffer, 2 μl template RNA (100 ng/μl), 1 μl RevertAid Reverse Transcriptase, and Nuclease-free water to make 20 μl were mixed and incubated with RNA at 42 °C for 1 h, then at 70 °C for 5 min for termination of reaction. The cDNA was kept at −20 °C. The primers for p53, p21 and the housekeeping β-actin gene were designed with Primers online program and are listed in Supplementary Table 1. The PCR reaction consisted of 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s, followed by a final extension of 10 min at 72 °C. Electrophoresis was performed in 2 % agarose gel. Band intensity was quantified with ImageJ Launcher software (National Institutes of Health). The correct fragment of PCR was confirmed by a commercial sequencing service company (BGI, Beijing, China).

Protein expression of p53, phospho-p53, and p21 by Western blotting

Western blotting analysis was performed as previously described (Kim et al. 2011) with slight modification. HepG2 cells were treated with BA extracts for 12 h before irradiation. Cells were washed with ice-cold PBS and lysed for 10 min in lysis buffer. Equal amounts (60 μg) of protein were separated using 10 % SDS-PAGE and then transferred to a PVDF

membrane. After blotting in 5 % (v/v) non-fat milk in TBST buffer, the membrane was soaked in blocking buffer and then incubated overnight with primary antibodies against p53, phospho-p53, or p21, followed by horseradish peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ, USA).

Statistics analysis

All data are presented as mean ± SEM. Statistical significance of changes in the test responses was assessed using independent-samples *t* test. Significance was as assigned at $p < 0.05$. All statistical procedures were performed using SPSS software, version 17.0 (SPSS, Inc.).

Results

Effects of BA extracts on HepG2 cell viability and proliferation following UV-irradiation

To avoid the influence of reduced cell number on the following experiment, we first performed cell viability assay to select a concentration that did not affect cell viability. As shown in Table 1, there was no effect on cell viability at any concentration at 24 h. Therefore, 10, 25, 50, 75, or 100 μg/ml of the three extracts were used in the following experiments.

Cell viability was significantly decreased in UV-irradiated cells as measured by cell proliferation. Pretreatment of cells with each type of BA extract significantly increased cell proliferation compared to UV-irradiated control cells (Fig. 1). Cell viability was slightly decreased at 100 μg/ml compared with 75 μg/ml of each extract. Therefore, for subsequent experiments, HepG2 cells were incubated with 75 μg/ml of each extract for 12 h.

Reduction of UV-induced DNA damage in HepG2 cells by BA extracts

Phosphorylation of H2AX is one of the major and early responses to DSBs. γH2AX foci are not observed in normally proliferating HepG2 cells; however, UV exposure induced H2AX phosphorylation (Oh et al. 2011). As shown in Fig. 2, γH2AX staining became visible after exposure to UV without BA extracts, and the fluorescence intensity became stronger after treatment. Compared with the UV control group, BA extracts, in particular BAE and BAM, protected the cells from DNA damage caused by UV.

Protective effects of BA extracts on UV-induced DNA damage by comet assay

Significant protection from DNA damage by BA extract pre-treatment was also observed at the single cell level as measured by the comet assay using confocal laser scanning microscopy (Fig. 3A). The resulting fluorescence was digitally analyzed by CASP and plotted as % tail moment (Fig. 3B). The results indicate that pre-treatment with BAM significantly decreased the level of DNA damage compared with control.

Antioxidative effects of BA extracts on UV-induced ROS

Intracellular ROS was detected using a fluorescence sensitive probe (H_2DCR-DA). Overnight pre-treatment of

Table 1 Cell viability[a] after treatment with extracts of blueberry (as μg dry wt/ml)

Compounds[b]	0	10	25	50	75	100
BAW	100	98.5 ± 0.5	98.9 ± 0.4	99.5 ± 0.3	100.4 ± 0.5	100 ± 0.2
BAE	100	98.6 ± 0.6	98.7 ± 0.6	99.0 ± 0.7	98.8 ± 0.5	99.3 ± 0.4
BAM	100	99.2 ± 0.4	99.1 ± 0.2	99.2 ± 0.5	100.2 ± 0.2	100.7 ± 0.3

Cell viability assay assessed by the MTT reduction test after BA extracts exposure to HepG2 cells in a time course of 24 h. Each value represents the mean of three experiments. * $p < 0.05$, ** $p < 0.01$ compared with UV control group

Data are reported as mean ± SEM of triplicate experiments

[a] HepG2 cells at 10^5/ml were exposed to different concentrations of the extracts for 24 h and then the cell viability was determined by the MTT assay

[b] The lyophilized blueberries were extracted for blueberry anthocyanins and anthocyanidins with methanol (BAM), ethanol (BAE) and water (BAW) as described in the 'Materials and methods'. The extracts were then added at the concentrations indicated

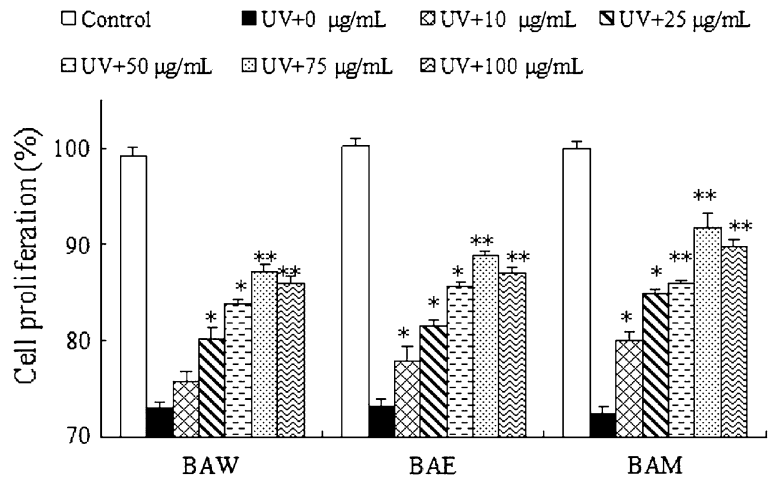

Fig. 1 Proliferation of UV-irradiated HepG2 cells after pretreatment with different concentrations of BAW, BAE, or BAM for 24 h. Each value represents the mean of three experiments. $*p < 0.05$, $**p < 0.01$ compared with UV control group

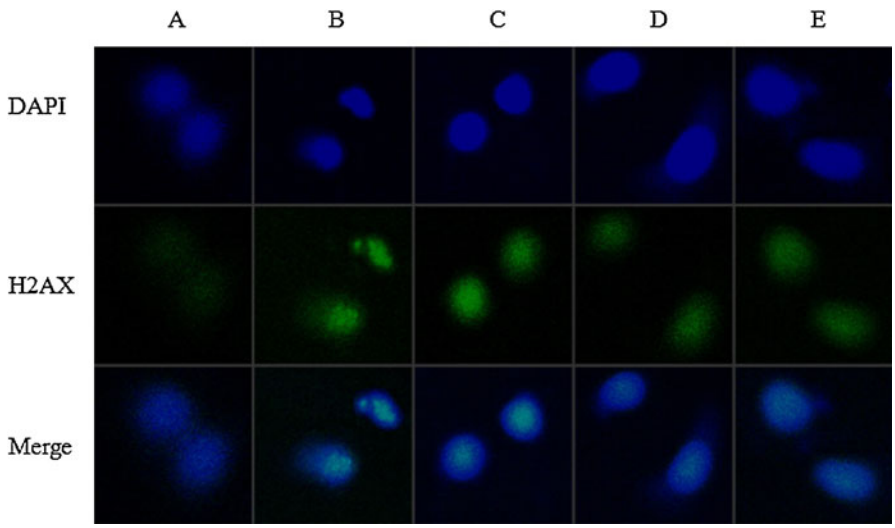

Fig. 2 Immunocytochemical detection of γH2AX in UV-irradiated cells after pretreatment for 12 h with 75 μg/ml BA extracts. Cells were stained with anti-γH2AX antibody and visualized by laser scanning confocal microscopy (*upper panels*). Nuclei and chromosomes were stained with DAPI (lower panels). **a** Control (without UV radiation), **b** UV-irradiation without extracts, **c**, **d** and **e** are UV + BAW, UV + BAE, UV + BAM, respectively

HepG2 cells with BAM significantly decreased ROS production by 79 % in UV-irradiated cells compared with UV-irradiated control cells (Fig. 4).

Expression of p53, phospho-p53, and p21 gene and protein was influenced by BA extracts on UV-irradiated HepG2 cells

By RT-PCR analysis, p53 and p21 gene expression were significantly increased in UV-irradiated HepG2 cells (Fig. 5A). Pre-treatment with BAW, BAE, or BAM at 75 μg/ml significantly decreased p53 and p21 gene expression (Fig. 5B) and also significantly decreased protein expression of p53, phosphorylated p53, and p21 as measured by Western blotting, consistent with effects on gene expression (Fig. 6).

Discussion

Blueberries, which contains numerous anthocyanins and anthocyanidins, have been tested in numerous animal models of cancer and other diseases, such as diabetes (Adams et al. 2010). Anthocyanidins are the

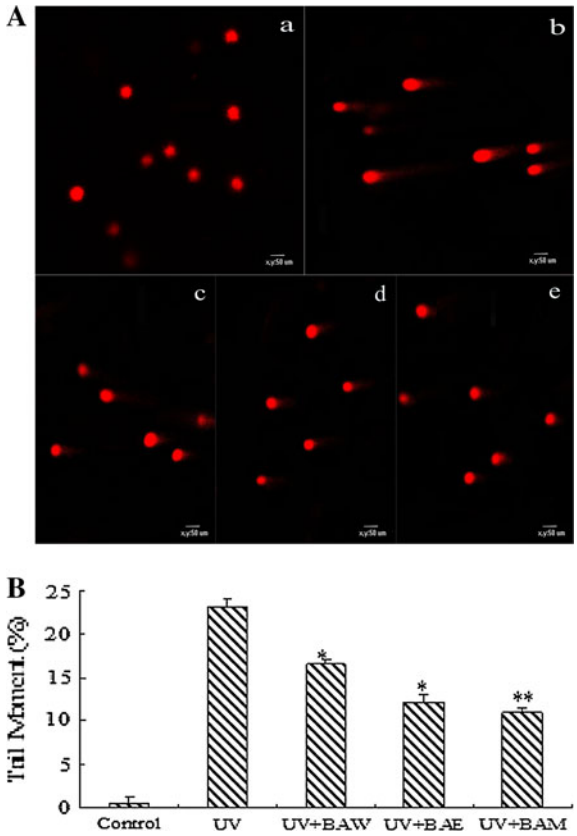

Fig. 3 Evaluation of protection by BA extracts against UV-induced DNA damage detected by Comet assay. HepG2 cells were pretreated for 12 h with 75 μg/ml BA extracts and then exposed to UV. **A** Comet images, *a* control, *b* UV-irradiated HepG2 cells, *c* UV + BAW, *d* UV + BAE, *e* UV + BAM; **B** quantification of DNA damage was analyzed by CASP software. Each value represents the mean of three experiments.*$p < 0.05$, **$p < 0.01$, compared with the UV group

major active ingredients in blueberry. The inhibition of cancer cell proliferation and metastasis by whole fraction of blueberry powder has been reported (Liu et al. 2011; Adams et al. 2001). It is, however, important to understand the precise dietary function of blueberry, particularly the whole fraction and different fractions, since blueberry has been widely used in supplements in different kinds of beverages or functional foods. Here we show DNA protection by blueberry extracts, especially the BAM fraction, which is believed to be enriched with both anthocyanins and anthocyanidins. This was also seen from the peaks around 280 nm in full wavelength scanning (data not shown). However, the BAW and BAE fractions also provided DNA protection from UV damage; an important finding for the food processing

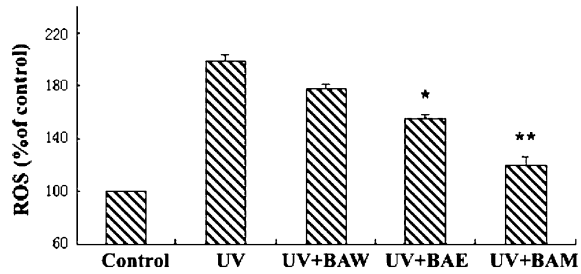

Fig. 4 Effects of BA extracts on UV-induced intracellular ROS in HepG2 cells. ROS production was detected with the fluorescent indicator H_2DCR-DA in a flow cytometry. Each value represents the mean of three experiments. *$p < 0.05$, **$p < 0.01$, compared with the UV-irradiated group

industry, especially for further purification of major active ingredients.

p53 is critical for nucleotide excision repair activity. Additionally, p21 has been identified as a universal inhibitor of cyclin kinases and inhibits cell cycle progression through both G1/S and G2/M phases. The mechanisms underlying DNA damage-induced p53 and p21 expression have been extensively studied (Ding et al. 2003).

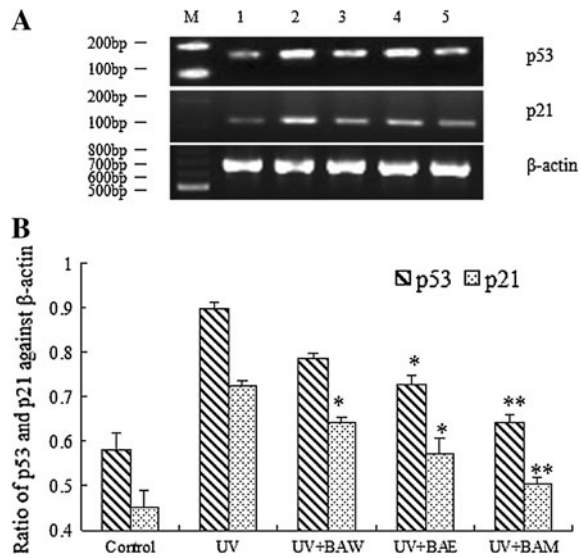

Fig. 5 Gene expression of p53 and p21 by RT-PCR in UV-irradiated HepG2 cells pre-treated with BA extracts. **A** *Lane M* marker, *lane 1* control (no UV radiation), *lane 2* UV radiation, *lane 3* UV + BAE, *lane 4* UV + BAW, *lane 5* UV + BAM. **B** The relative expression of p53 and p21 value after normalizing to β-actin

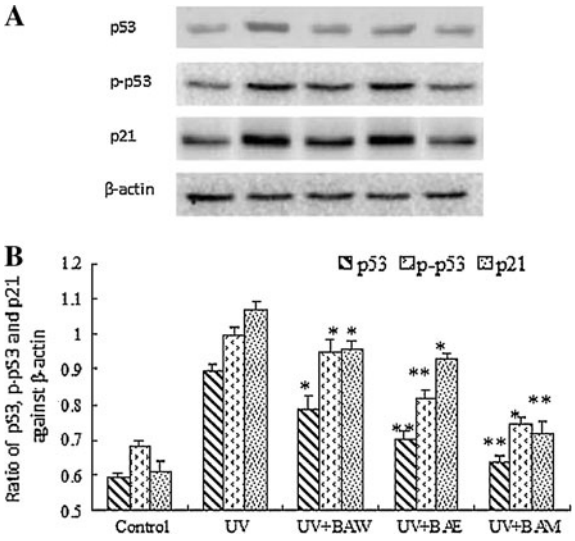

Fig. 6 Western blot detection of p53, phospho-p53, and p21 protein expression in UV-irradiated HepG2 cells pre-treated with BA extracts. **A** *Lane 1* control (no UV radiation), *lane 2* UV, *lane 3* UV + BAE, *lane 4* UV + BAW, *lane 5* UV + BAM; **B** the relative expression value after normalizing to β-actin

Here we have shown that UV irradiation induced a high level of p53 and p21 accumulation and phosphorylation at Ser15 in HepG2 cells. Suppressor p53 is stabilized and activated in response to DNA damage by two at least partially independent pathways, one of which responds to DNA double-strand breaks and the other of which is activated by bulky lesions in DNA such as those caused by exposure to UV light. The mechanism of DNA protection by p53 has been reported (Bae et al. 2009), but to our knowledge this is the first report that BA extracts, especially BAM, can significantly restore p53 gene and protein expression in UV-irradiated HepG2 cells. We also demonstrate the participation of γH2AX, a specific variant of a histone protein involved in DNA damage rescue, in protection of DNA from UV-irradiation (Kinner et al. 2008).

We believe that the underlying mechanism of DNA damage restoration primarily involves ROS scavenging capability by BA extracts, as shown in the ROS assay (Tang et al. 2005). Particularly the Comet assay directly showed DNA percentage in tail can be significantly decreased by BAE and BAM extracts, which strongly indicated that DNA fragmentation, or large deletions or mutations, can be inhibited by BA extracts.

In conclusion, we have shown that BA extracts, especially the BAM fraction, significantly reduced DNA damage in UV-irradiated HepG2 cells and that this protection of DNA is related to ROS scavenging regulation of p53 and p21. These findings suggest that BAM may be an excellent addition to cancer preventive diets and anti-ageing cosmetics ingredients.

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Conflict of interest The authors declare no conflicts of interest.

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