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Protective effect of hawthorn extract against genotoxicity induced by benzo(< alpha >)pyrene in C57BL/6 mice

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ABSTRACT

Benzo(< alpha >)pyrene [B(< alpha >)P], widely originated from environmental pollution or food process such as roasting and frying, is a strong mutagen and potent carcinogen. Utilization of hawthorn has been reported against physical mutagens. Our study found that hawthorn extract (HE) contained abundant phenolic compounds, wherein chlorogenic acid was 2.78 mg/g, procyanidine B2 was 3.58 mg/g, epicatechin was 2.99 mg/g DW, which may contribute to anti-genotoxicity activity. So, the role of HE against B(< alpha >)P-induced genotoxicity in C57BL/6 mice was further assessed. Fifty mice were distributed into five groups: control group, B(< alpha >)P group (30 mg/kg, i.p.), B(< alpha >)P + HE-L group (100 mg/kg, i.g.), B(< alpha >)P + HE-M group (200 mg/kg, i.g.), B(< alpha >)P + HE-H group (400 mg/kg, i.g.). Mice were orally administered with solutions of HE for 10 days and injected intraperitoneally with B(< alpha >)P for 3 days from the 8th day. Results showed that B(< alpha >)P can induce significantly pathological damage in liver, lung and spleen, as well as decrease white blood cells (WBCs). Remarkably elevated levels of reactive oxygen species (ROS), DNA strand breaks (DSBs) and G1 cell cycle arrest were also found in B(< alpha >)P group, with up-regulated expressions of p-H2AX, p-p53 and p21 in bone marrow cells. With administration of HE, liver, lung and spleen injury significantly mitigated, while WBCs were evidently increased in B(< alpha >)P-treated mice. Consistently, HE markedly reduced level of ROS, DSBs and G1 cell cycle arrest accompanied by reducing expressions of p-H2AX, p-p53 and p21 in bone marrow cells. Combined, these results indicated a protective role of HE on B(< alpha >)P-induced genotoxicity.

1. Introduction

Benzo(< alpha >)pyrene [B(< alpha >)P] is the most characterized and toxic member of polycyclic aromatic hydrocarbons (PAHs) (Samanta et al., 2002). It has been reported that B(< alpha >)P is a carcinogen, which has been recognized as "carcinogenic substance-level 1" by International Agency for Research on Cancer (IARC) (Pokhariyal et al., 2019). Persistent exposure to B(< alpha >)P can increase the risk for lung carcinoma and colorectal adenoma, etc. (Kasala et al., 2015; Sinha et al., 2005). B(< alpha >)P production by human pollution is ineluctable and generally exist in burning coal, oil and natural gas, as well as smoked food and tobacco (Urbancova et al., 2017; Xia et al., 2010). On the one hand, during food processing, B(< alpha >)P can be formed in heat-treated foods owing to the thermal polymerization of coking product of fat and attached to the food surface, which is the main source of B(< alpha >)P in baked food. Besides, imperfect combustion of sugar and fat can also generate B(< alpha >)P in smoked fish and meat, and the level of B(< alpha >)P in the fumes is likely high (Singh et al., 2016). A study has evaluated various heat-treated foods in China, B(< alpha >)P level of 105 samples were 0.03–19.75 μg/kg. Especially in 12 animal foods, B(< alpha >)P contents were higher than the Chinese maximum permissible level (5 μg/kg), and the highest level was up to 19.75 μg/kg (Chen et al., 2012). On the other hand, B(< alpha >)P also can be produced by incomplete

Abbreviations: B(< alpha >)P, Benzo(< alpha >)pyrene; BPDE, benzo(a)pyrene-trans-7,8-dihydrodio1-9,10-epoxide; DSBs, DNA strand breaks; HE, hawthorn extract; WBCs, white blood cells; ROS, reactive oxygen species

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combustion of coal and oil, which will be absorbed through air pollution and accumulated in vegetables and grain, indicated that health risk of B(< alpha >)P is widely exist in foods (Muntean et al., 2013). Therefore, it is noteworthy to investigate functional foods to mitigate toxicity induced by B(< alpha >)P.

Despite human body possessed capacity of immunity and invertase system to inactivate exogenous toxic molecules and achieve detoxification purposes, exposure to hazardous environmental chemicals can lead to irreversible genotoxicity. A Study has shown that B(< alpha >)P can be transformed to benzo(a)pyrene-trans-7,8-dihydrodio1-9,10-epoxide (BPDE) and directly react with DNA to form BPDE-DNA adducts (Arlt et al., 2015). Besides, research indicated that B(< alpha >)P can lead to generation of reactive oxygen species (ROS) such as superoxide anion radicals and hydrogen peroxide (Zhu et al., 2014). With the rise of oxidative stress, DNA strand breaks (DSBs), a probable mechanism of genotoxicity may occur (Nwagbara et al., 2007; Shukla et al., 2011). Searching proper materials to inhibit genotoxicity is attracting much concern of researchers at present.

Fruits with abundant phenolic compounds have been reported with potential antimutagenic effects (Delgado-Vargas et al., 2018). Moreover, phenolic compounds showed beneficial properties including anticancer, cardiovascular protective and anti-inflammatory effects. It's believed that antioxidant activity of phenolic compounds probably contributed to the gorgeous physiological function of these fruits (Trumbeckaite et al., 2011). Hawthorn has a long history of use in traditional Chinese medicine and widely consumed as food. Studies showed that many biological effects of hawthorn extract (HE), such as cardiotonic, hypotensive, anti-atherosclerosis and immune-regulation effects, related to its antioxidant activities (Fujisawa et al., 2005; Kwok et al., 2010). Phytochemical study further revealed that HE contained abundant phenolic compounds including procyanidine B2, epicatechin, chlorogenic acid and catechin, which have powerful antioxidant activity (Liu et al., 2011). Moreover, it has been reported that the main antioxidant mechanisms of HE included increasing DPPH and H_2O_2 radical-scavenging activities, and elevating levels of glutathione and activity of superoxide dismutase etc. (Kwok et al., 2013; Rabiei et al., 2012; Shatoor et al., 2019). Research showed that utilization of hawthorn revealed protective effect against physical mutagenesis such as UVB (Liu et al., 2019). Therefore, it's essential to evaluate the effect of HE on B(< alpha >)P-induced genotoxicity. In this study, we employed mice treated with B(< alpha >)P to investigate the effect of HE on B(< alpha >)P-induced genotoxicity. In addition, bone marrow cells in B(< alpha >)P treated mice also have been investigated to further explore the detailed molecular mechanisms of HE resisted B(< alpha >)P-induced genotoxicity.

2. Meterials and methods

2.1. Chemicals and antibodies

The species of hawthorn is Dajinxing (C. pinnatifida Bge Var.major) that was cultivated and provided by Shandong Linqu County Piankouge Ecological Agriculture Company (Weifang, China). The hawthorn was stored in glass vials with dry, cool, and dark environment. B(< alpha >)P was purchased from sigma (St. Louis, MO, USA). Standars like chlorogenic acid, procyanidin B2, epicatechin, hyperin, isoquercitrin (Purity > 98%) were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Cell Cycle Analysis Kit, Reactive Oxygen Species Assay Kit and 4′, 6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488-labeled Goat Anti-Rabbit were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). BPDE-DNA Elisa Kit was applied from Jianglai Biotechnology Co., Ltd. (Shanghai, China). Anti-bodies against p-H2AX, H2AX, p-p53, p53, p21, β-actin and secondary antibodies were purchased from Cell Signaling Technology (USA).

2.2. Preparation of HE

Fresh hawthorn was denucleated and cut into flakes, then freeze-dried and crushed to attain fresh hawthorn powder by sieving. Aqueous ethanol (75%) was added to the powdered (20 g) and homogenized with ice bath for 10 min. Then the precipitate was separated by centrifugation at 3000 r/min for 10 min and repeated extraction 3 times. Merged the extracts, and dried them via rotary evaporation. Ethanol was evaporated under reduced pressure at 40 ˚C and the remained water extract was freeze-dried. In this way, extract powder was obtained.

2.3. High performance liquid chromatography (HPLC) analysis

The phenolic compounds of HE were analyzed by HPLC method. Before HPLC analysis, 0.5 mL of the HE was diluted with HPLC-grade methanol to 1 mL. The diluted HE were filtered through a 0.22 μm Millipore filter for analysis. The identification of phenol in hawthorn was performed on Shimadzu HPLC system. Chromatographic separations were conducted with a C18 column (4.6 mm × 250 mm, 5 μm) with flow rate of 1 mL/min and injection volume of 10 μL at 30 ˚C. The detection wavelength was set at 280 nm and the mobile phase was composed of 0.1% aqueous formic acid (A) and acetonitrile (B). The following gradient procedures were used: 0 min, 5% B; 5 min, 10% B; 6 min, 14% B; 31 min, 26% B; 35 min, 26% B; 38 min, 60% B; 41 min, 60% B; 43 min, 80% B; 48 min, 80% B; 55 min, 5% B. Chlorogenic acid, procyanidin B2, epicatechin, hyperin, isoquercitrin were identified and quantified by their retention time and peak area.

2.4. Animals and treatment

Male C57BL/6 mice (n = 50; 6–8 weeks old; weighing 18–22 g) were supplied from Guangdong Medical Laboratory Animal Center (Guangzhou, China).All the animal housed in standard cages (5 mice/cage) under the experimental condition of temperature (~24 ˚C), humidity (~45%), 12 h light–dark cycle, and free access to deionized drinking water. Treatments were followed the “Guideline for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85–23, 1996). The animal experiments were approved by the Laboratory Animal Ethics Committee of South China Agricultural University. Mice were divided into 5 groups including the control group, B(< alpha >)P group, B(< alpha >)P + HE-L group (100 mg/kg), B(< alpha >)P + HE-M group (200 mg/kg), B(< alpha >)P + HE-H group (400 mg/kg). All animal experiments were conducted in accordance with standards of humane animal care. During 10 consecutive days of treatment, the control and B(< alpha >)P groups were received physiological saline by intragastric (i.g.) administration, B(< alpha >)P + HE-L group, B(< alpha >)P + HE-M group and B(< alpha >)P + HE-H group were respectively treated with HE (100/200/400 mg/kg, i.g.). From the 8th day of treatment, B(< alpha >)P group, B(< alpha >)P + HE-L group, B(< alpha >)P + HE-M group and B(< alpha >)P + HE-H group were treated by intraperitoneal (i.p.) injection of B(< alpha >)P (30 mg/kg) for 3 consecutive days, while the control group was treated with solvent (corn oil, i.p.) on the same schedule.

2.5. Calculation of liver, lung and spleen indexes

After animal treatment, body weight of mice was recorded and then they were sacrificed by cervical dislocation. The liver, lung and spleen were removed and weighed. Based on previous studies (Ma et al., 2016; Zhou et al., 2019), liver, lung and spleen indexes were calculated according to the equation: index (%) = organ weight (g)/body weight (g) × 100.

2.6. Hematoxylin and eosin staining assay

At the end of the treatment period, food was removed for 12 h. Prior to sacrifice by cervical dislocation, mice were adequately anesthetized with ether. Liver, lung and spleen tissues were collected to fix with 4% paraformaldehyde. Hematoxylin and eosin staining were performed on liver, lung and spleen fixed sections. The tissues were dehydrated, dipped in paraffin, sectioned and stained with hematoxylin and eosin. Slides were observed by a microscope (Axio Observer A1, Carl Zeiss, Germany).

2.7. Detection of white blood cells (WBCs)

After anesthetized, blood (1 mL) was collected from the orbit of mice and transferred to tubes with 100 μL EDTA-Na2 (1.5 mg/mL). Then blood was mixed upside down and detected the WBCs counts by a Sysmex XS-800i automated hematology analyzer (Sysmex Co., Kobe, Japan) in the First Affiliated Hospital of Jinan University. The analyzer measured WBCs counts by a semiconductor red-diode laser to collect forward and side scattered light information.

2.8. Determination of ROS level

The hindlimb femur and tibia were obtained, and bone marrow cells were collected by flushing with PBS and filtrated through a 40 μm nylon cell strainer (BD Falcon, USA), which were then centrifuged at 400 g for 5 min, and resuspended in PBS. Bone marrow cells collected from 10 mice in each group were divided into 3 groups. Reactive Oxygen Species Assay Kit was applied to detect ROS level in bone marrow cells. Then, cells were incubated with DCFH-DA solution in the dark for 20 min at 37 ˚C. Bone marrow cells were then washed three times with serum-free cell culture medium. The green fluorescence (480–530 nm) intensity was measured at the FL1-A channel with a flow cytometer (BD Accuri C6, USA). The experiment was performed three times.

2.9. Assessment of p-H2AX using fluorescent microscopy

The expression of p-H2AX, a specific marker of DSBs, was determined by immunofluorescence assay using an antibody against p-H2AX. Bone marrow cells collected from 10 mice in each group were divided into 3 groups and cytospun onto glass slides, then bone marrow cells were fixed with 1:1 MeOH, acetone for 20 min at −20 ˚C. The fixed cells were incubated with anti-p-H2AX antibody at a concentration of 1:1000 diluted in Tris-buffered saline with Tween 20 (TBST) containing 1% BSA at 4 ˚C overnight. Cells were washed 3 times with TBST and then incubated with Alexa 488-conjugated goat anti-rabbit for p-H2AX at room temperature for 1 h. The slides were continually washed three times and stained with DAPI (500 ng/mL) for 3 min at room temperature. The slides were subsequently washed three times following anti-fade solution mounted. Images were taken by a confocal microscope (Leica TCS SP8, Germany).

2.10. Cell cycle analysis

Cell Cycle Analysis Kit was applied to detect DNA content. Bone marrow cells collected from 10 mice in each group were divided into 3 groups, which were then re-suspended in PBS and filtered through cell strainer (40 μm). Cells were centrifuged at 1000 g for 5 min, and fixed with 75% ethanol at 4 ˚Cfor 2 h. Then the cells were incubated with PI staining solution in the dark for 30 min at 37 ˚C, and PI fluorescence was analyzed by the FL2-A channel of a flow cytometry (BD Accuri C6, USA) with a 580 nm laser and 610 nm filter equipped.

2.11. Western blot analysis

The bone marrow cells were harvested and resuspended in lysis buffer on ice for 20 min. The lysates were centrifuged at 12,000 g for 10 min, and the supernatant containing total protein was collected. Protein concentrations were determined by spectrophotometry at A280 using NanoDrop 2000 (Thermo Scientific, Wilmington, NC). The protein samples were then mixed with × 5 loading buffer, and heated at 100 ˚C for 10 min to achieve complete denaturation. Then total protein (50 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% BSA and incubated with rabbit monoclonal antibodies specific for p-H2AX, H2AX, p-p53, p53, p21 (1:1000) and β-actin (1:3000) at 4 ˚C overnight. Then the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody at room temperature for 1 h. The bound antibodies were visualized using Imaging System (GE Healthcare, USA).

2.12. Statistics

Data analyses were performed using GraphPad Prism 5. Statistical differences were determined by one-way ANOVA analysis followed by Tukey multiple comparison test. Values were represented as means ± SEM. Different letters indicate significant differences at $p < 0.05$.

3. Results and discussion

3.1. Polyphenol content of HE

The phenolic compounds in many fruits can protect from side effects induced by destructive chemicals (Gholamine et al., 2019). It's well known that phenolic compounds are main antioxidant components among extraction of deseeded whole hawthorn fruit. Chemical analysis showed that procyanidine B2, epicatechin and chlorogenic acid are major phenolic components, among which chlorogenic acid (13.5%), procyanidin B2 (19.2%), and epicatechin (18.8%) collectively accounted for 51.4% of total phenol content and represent the active ingredients of hawthorn (Liu et al., 2018; Zheng et al., 2018). Conformably, in this study, HPLC analyses of the extracts confirmed chlorogenic acid, procyanidine B2 and epicatechin were the most prominent phenolic compounds identified. As shown in Fig. 1, the polyphenol substances were respectively calculated that chlorogenic acid was 2.78 mg/g DW, procyanidine B2 was 3.58 mg/g DW, epicatechin was 2.99 mg/g DW.

The unique antioxidant activities of HE may come from these phenolic compounds. Consistent to our results, studies have reported that chlorogenic acid has vicinal hydroxyl groups on an aromatic residue, which exerted activity of scavenging ROS (Rice-Evans et al., 1996). Besides, cellular study showed that procyanidins extracted from grape seeds have free radical scavenging activity, and inhibit low-density lipoprotein oxidation induced by metal ions or radical generators, which further protect the membrane peroxidation induced by UVB in erythrocytes (Carini et al., 2000; Mazur et al., 1999). In addition, epicatechin displayed anti-aging effect. Significant decrease of epicatechin uptake by human erythrocytes during age has been found, probably due to the alteration of plasma membrane redox system activity (Kumar et al., 2018). Though researches have shown that HE and its main phenolic substances possess excellent antioxidant activities, studies on the effect of genotoxicity induced by B(< alpha >)P haven't been progressed. In this study, protective effects of HE on B(< alpha >)P generated DNA damage and subsequent tissue injury were further evaluated.

3.2. Protective function of HE on B(< alpha >)P-induced tissue pathological damage

Current studies have confirmed that exposure to high levels of environmental B(< alpha >)P was associated with elevated rates of

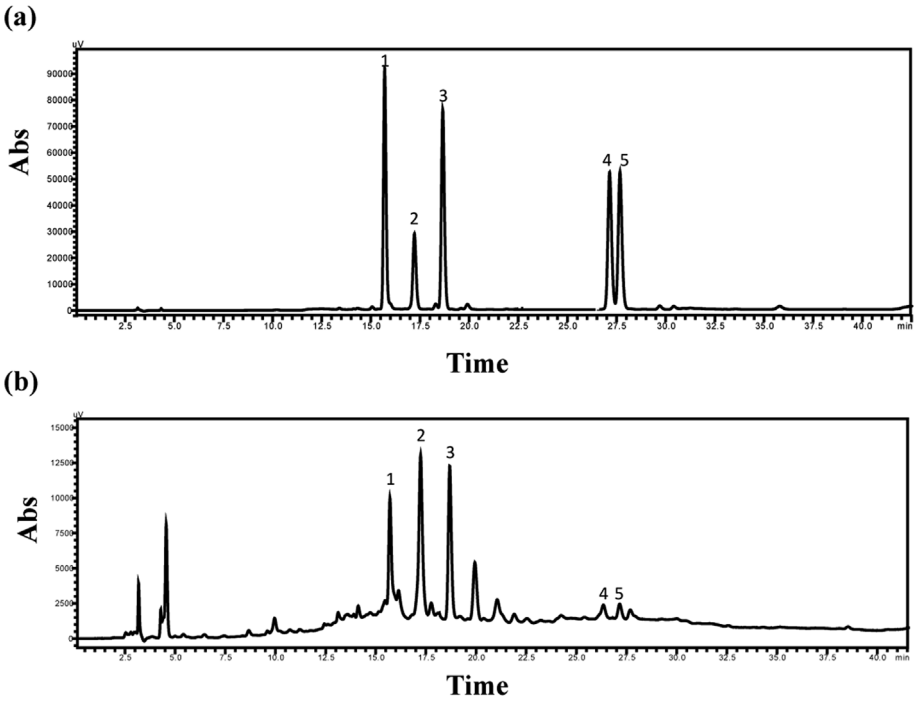

Fig. 1. HPLC of the aqueous ethanol (75%) soluble fractions (supernatants) of hawthorn. (a) Standards analytes involved 1, chlorogenic acid; 2, procyanidine B2; 3, epicatechin; 4, hyperin; 5, isoquercitrin. (b) The polyphenol content of HE.

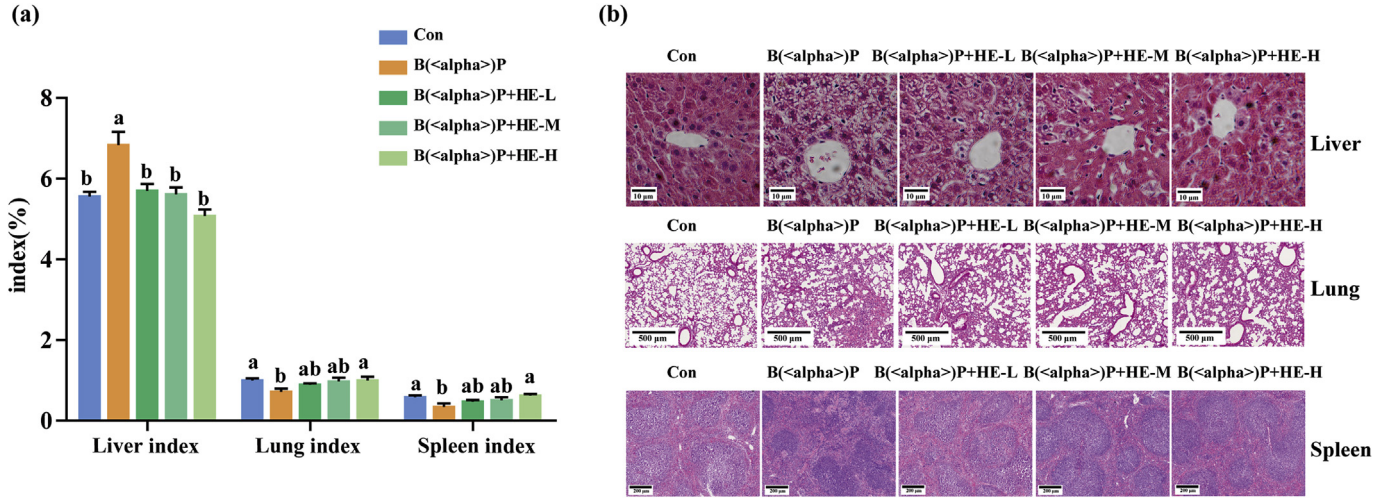

Fig. 2. Protective effects of HE in B(< alpha >)P-induced tissues pathological damage. (a) Liver, lung and spleen index in the groups. (b) The morphology of liver, lung and spleen (magnification, × 200). Values are expressed as the means ± SEM (n = 10). Different letters indicate significant difference ($p < 0.05$).

cancer (Zhu et al., 2019). Animal experiments revealed that B (< alpha >)P can cause advanced fibrosis in liver specimens, tumor nodules in lung and reduction of spleen cells (Chen et al., 2011). Encouragingly, radioprotective and cardioprotective effect of HE has been found (Hosseinimehr et al., 2007; Swaminathan et al., 2010), and several studies have shown protective effects of HE against atherosclerosis and hypertension (Zhang et al., 2014; Zheng et al., 2019), which indicated that HE may act as a natural protective agent resist to environmental toxic exposure. To investigate the protective function of HE on B(< alpha >)P-induced impairment, liver, lung and spleen were weighted and microscopically analyzed with a microscope. As illustrated in Fig. 2a, consistent to the previous studies, the average liver index in B(< alpha >)P group significantly increased, while lung and spleen index were evidently lowered than the control group. Compared with the B(< alpha >)P group, liver index was significantly reduced by HE treatment ($p < 0.05$), while lung index was remarkably high in B (< alpha >)P + HE-H group ($p < 0.05$). Spleen index also showed markedly increased in B(< alpha >)P + HE-H group ($p < 0.05$). To further confirmed the protective effect of HE on B(< alpha >)P induced tissue pathological damage, hematoxylin and eosin staining was performed on liver, lung and spleen. Similar to previous research (Near et al., 1999), our results showed that B(< alpha >)P lead to tissues impair in mice as compared to the control group. The tissues pathological damage by B(< alpha >)P represented as dissociable hepatic cord structure, swelling, turbidity and diffuse steatosis of hepatocyte cytoplasm. Inflammatory infiltration of alveolar wall and interstitial tissue, as well as diffuse alveolar damage were also found in lung tissues. Besides, the structure of spleen corpuscles was damaged, the splenic cord and sinusoid were disordered in B(< alpha >)P group. Mice treated with HE showed markedly reduced hepatocellular vacuolization, decreased inflammatory lung cells and mitigated splenic cord and sinusoid injury induced by B(< alpha >)P (Fig. 2b).

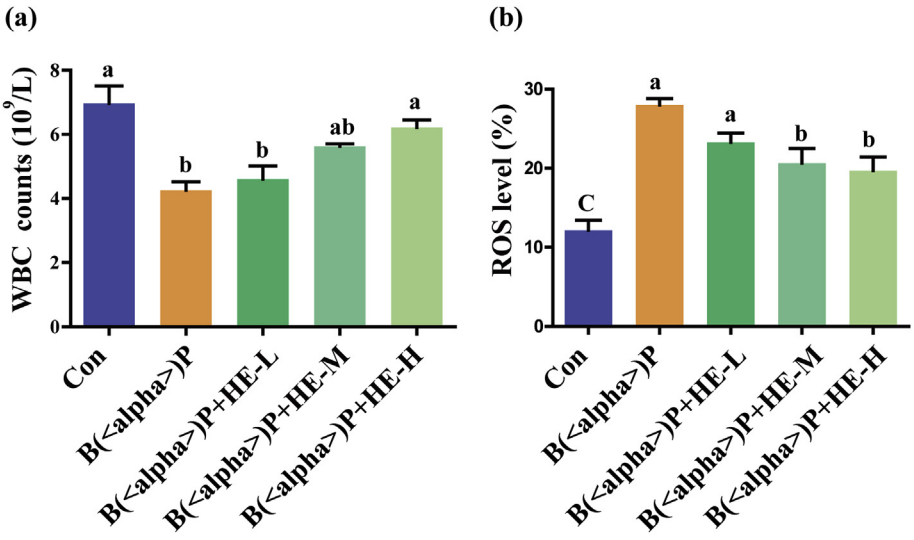

Fig. 3. Effects of HE on WBCs counts and ROS level in B(< alpha >)P-treated mice. (a) WBCs counts in the groups. Values are expressed as the means ± SEM (n = 10) (b) ROS level was determined by flow cytometry. Values are expressed as the means ± SEM, the experiment was repeated in triplicate. Different letters indicate significant difference ($p < 0.05$).

It is well known that properties of immune cells can protect human body against mechanical injury. However, overwhelming uptake of dangerous chemicals is likely to lead to reduction of neutrophils, lymphocytes and monocytes, which are intrinsic to the injury tissue as defense. Consistent with our results, previous studies showed that loss of human lymphocytes induced by gamma irradiation or methyl methanesulfonate was significantly modulated by HE treatment (Hosseinimehr et al., 2009, 2011), which suggested that liver, lung and spleen injury were relieved by HE treatment may attribute to immunologic enhancement.

3.3. Effect of HE on WBCs and ROS levels

B(< alpha >)P can suppress systemic immunity, including significant reduction of WBCs and apoptosis of bone marrow cells. (Knuckles et al., 2001; Near et al., 1999). To investigate the effect of HE on immunologic disfunction induced by B(< alpha >)P, WBCs were evaluated. In accordance with previous results, B(< alpha >)P can significantly reduce WBCs (4.26×10^9/L vs 6.96×10^9/L in control group, $p < 0.01$), while the decline of WBCs was significantly ameliorated by HE-H treatment (4.26×10^9/L, $p < 0.05$) (Fig. 3a). WBCs were originated from bone marrow cells, and generally generate neutrophils, lymphocytes, monocytes and other cells, which play a major role in tissues injury repair, while bone marrow cells serve as a backup rescue system (Butterfield et al., 2006). This indicated that bone marrow cells may play a key role in immune system and support the normality of tissues' function. However, B(< alpha >)P treatment can cause a significant induction of chromosomal aberration and micronuclei in mice bone marrow cells, which served as the factor of overall tissue injury and genotoxicity (Prasad et al., 2008). Our results indicated that the protective effect of HE against B(< alpha >)P may associate with its beneficial activity of systemic immunity, and further studies need to be done to investigate the detail mechanism.

Toxic metabolites of B(< alpha >)P such as BPDE was capable of binding to nucleic acids and forming BPDE-DNA, which has been indicated as one of the risk factors for generation of ROS (Briede et al., 2004). Meanwhile, DNA has become the target of ROS during carcinogenesis process. Therefore, it's believed that supplement of natural antioxidants with activity of reducing DNA dysfunction may considered as effective strategy to protect cells from B(< alpha >)P-mediated genotoxicity. In our study, we have shown high content of antioxidants like chlorogenic acid, procyanidine B2 and epicatechin in HE. To further study antioxidative effect of HE against B(< alpha >)P, we measured the levels of ROS by flow cytometry in bone marrow cells of mice treated with B(< alpha >)P. Consistent with previous result (Perumal Vijayaraman et al., 2012), B(< alpha >)P treatment significantly increased the level of ROS compared with the control group. When B(< alpha >)P-treated mice were simultaneously supplemented with HE-M and HE-H, a significant decrease in the level of ROS was observed as shown in Fig. 3b ($p < 0.05$). Similar to our results, study has revealed that HE loaded with antioxidant properties to activate antioxidant enzymes, which can alleviate oxidative stress and inflammation (Han et al., 2016). It appears that HE, particularly its phenolic components with strong DNA adducts reducing and ROS scavenging activity may alleviate genotoxicity induced by B(< alpha >)P in mice bone marrow cells. Due to the discovery, the potential effect of HE on B(< alpha >)P induced genotoxicity has been attracting attention and requires further investigation.

3.4. HE reduced DSBs and cell cycle arrest of bone marrow cells in B(< alpha >)P -treated mice

B(< alpha >)P can induce ROS generation and cause oxidization of DNA bases through the orthoquinone pathway that may result in DSBs (Toyooka et al., 2006). Histone H2AX phosphorylated at serine 139 is very sensitive to bind with DSBs, which has been recognized as a specific indicator for the existence of DSBs. To further explore the effect of HE on B(< alpha >)P induced DSBs, immunofluorescent assay was applied to determine the expression of p-H2AX in bone marrow cells. As shown in Fig. 4a and B(< alpha >)P noticeably increased p-H2AX positive staining in mice bone marrow cells, which was in accordance with previously reported results (Yan et al., 2011). By contrast, HE treatment can markedly reduce expression of p-H2AX activated by B(< alpha >)P. Among the types of DNA damage, DSBs is the most lethal type of damage to cells, which can lead to cell cycle arrest (Karagiannis and El-Osta, 2004). In the process of DSBs, p-H2AX can cause elevated expression of cell cycle arrest related proteins (Fragkos et al., 2009). Current research showed that B(< alpha >)P or BPDE can stimulate human prostate carcinoma cell line arrested at the G1 phase (Onyinye et al., 2007). In this study, flow cytometry was performed to determine the effect of HE on cell cycle arrest induced by B(< alpha >)P in bone marrow cells. Consistently, as shown in Fig. 4b, B(< alpha >)P induced an obvious G1 cell cycle arrest (75.48% vs. 58.97% in control group). HE treatment can greatly attenuate the G1 cell cycle arrest induced by B(< alpha >)P. These findings indicated that HE can exert protective effect on G1 cell cycle arrest, which probably related to lessen of DSBs.

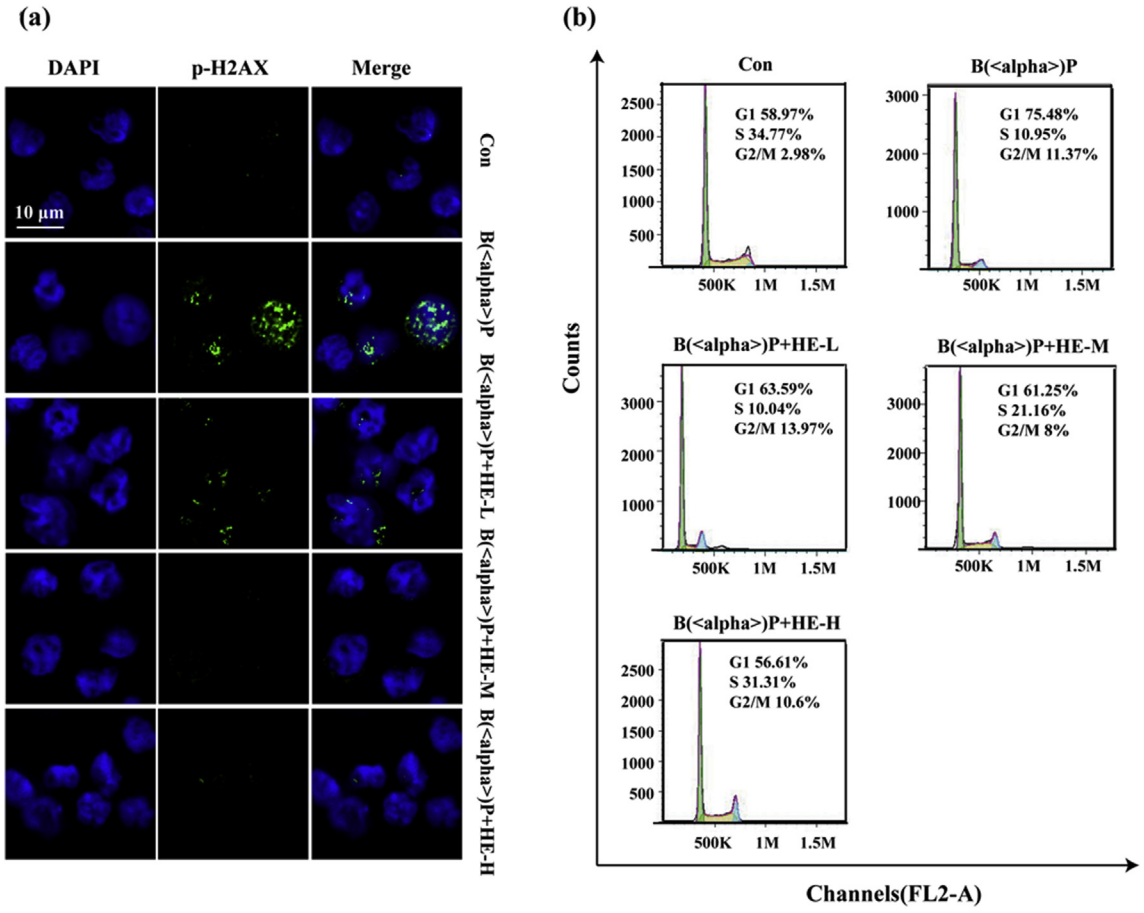

Fig. 4. HE inhibited B(< alpha >)P-induced increase in DSBs marker of p-H2AX and attenuated G1 cell cycle arrest of bone marrow cells in B(< alpha >)P-treated mice. (a) Expression of p-H2AX was detected by immunofluorescence. The confocal images were captured at a magnification of 400 × . (b) G1, S, and G2/M populations analysis in bone marrow cells was detected by flow cytometry assay. The experiment was repeated in triplicate.

3.5. Effect of HE on cell cycle related proteins expression

Studies have illustrated that the cellular response to genotoxicity involve a series of events that lead to cell cycle arrest (Ando et al., 2014; Juan-Garcia et al., 2018). The role of p-H2AX foci in the DNA damage signaling pathway induced by DSBs is to recruit signaling molecules and cause further cell cycle arrest. It is essential that elevated expression of p53 can enhance p21 transcription, which in turn inhibited cyclin-dependent kinase activity and facilitated cell cycle arrest at G1 phase (Fragkos et al., 2009). Previous researches showed that B(< alpha >)P can induce DNA damage and lead to reduction of cell viability, which may due to the elevated level of p53 and subsequent up-regulate expression of p21 protein (Park et al., 2006).In order to explore the underlying mechanism of HE inhibited G1 cell cycle arrest induced by B(< alpha >)P, the cell cycle-regulatory proteins were evaluated in bone marrow cells of B(< alpha >)P-treated mice. In conformity with previous studies, our results revealed significantly elevated ratios of p-H2AX/H2AX and p-p53/p53 and remarkably increased expression of p21 in mice treated with B(< alpha >)P ($p < 0.05$), while HE treatment evidently reduced ratios of the p-H2AX/H2AX and p-p53/p53 and decreased the expression of p21($p < 0.05$) (Fig. 5). Similarly to our results, previous study found polyphenol of HE as protective agent against UVB-induced skin damage by attenuating oxidative stress and DNA damage, as well as modulating the p53 mitochondrial pathway (Liu et al., 2019). These results suggested that HE may attenuate B(< alpha >)P induced G1 cell cycle arrest by regulating p-H2AX/p-p53/p21 pathway.

4. Conclusion

The results of this study suggested that B(< alpha >)P exposure can cause pathological damage in liver, lung and spleen, as well as decreasing WBCs. Besides, increasing levels of ROS, DSBs and G1 cell cycle arrest were found with upregulated expressions of p-H2AX, p-p53 and p21 in bone marrow cells of B(< alpha >)P-treated mice. In addition, our results showed that treatment of HE with high contents of phenolic compounds can mitigate damaged morphology of liver, lung and spleen tissues in B(< alpha >)P-treated mice, which is accompanied with reducing levels of ROS and WBCs. Further results appeared that HE possessed activity of alleviating B(< alpha >)P-induced DSBs and G1 cell cycle arrest probably by regulating p-H2AX/p-p53/p21 pathway. Combined, our findings indicated favorable prospects for dietary applications of HE to inhibit genotoxicity induced by B(< alpha >)P.

CRediT authorship contribution statement

Jie Deng: Conceptualization, Data curation, Formal analysis, Writing - original draft. **Ximiao Chen:** Conceptualization, Investigation. **Da Wang:** Methodology. **Ya Song:** Project administration. **Yongchun Chen:** Software. **Dongmei Ouyang:** Resources. **Yuxuan Liang:** Visualization. **Yuanming Sun:** Supervision, Validation. **Meiying Li:** Funding acquisition, Writing - review & editing.

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

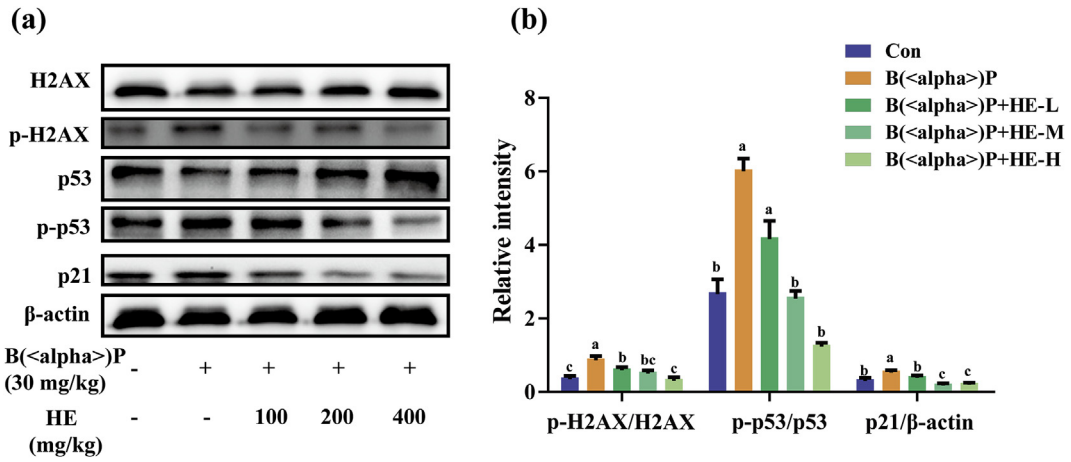

Fig. 5. Analysis of cell cycle arrest signaling related proteins expression. (a) Proteins expression of p-H2AX, H2AX, p-p53, p53, p21 and β-actin were evaluated by Western blot. (b) The relative intensities of p-H2AX/H2AX, p-p53/p53 and p21. Values are expressed as the mean ± SEM, the experiment was repeated in triplicate. Different letters indicate significant difference ($p < 0.05$).

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References

Ando, M., Yoshikawa, K., Iwase, Y., Ishiura, S., 2014. Usefulness of monitoring gamma-H2AX and cell cycle arrest in HepG2 cells for estimating genotoxicity using a high-content analysis system. J. Biomol. Screen 19, 1246–1254. https://doi.org/10.1177/1087057114541147.

Arlt, V.M., Krais, A.M., Godschalk, R.W., Riffo-Vasquez, Y., Mrizova, I., Roufosse, C.A., et al., 2015. Pulmonary inflammation impacts on CYP1A1-mediated respiratory tract DNA damage induced by the carcinogenic air pollutant benzo a pyrene. Toxicol. Sci. 146, 213–225. https://doi.org/10.1093/toxsci/kfv086.

Briede, J.J., Godschalk, R.W.L., Emans, M.T.G., de Kok, T., van Agen, E., van Maanen, J.M.S., et al., 2004. In vitro and in vivo studies on oxygen free radical and DNA adduct formation in rat lung and liver during benzo a pyrene metabolism. Free Radic. Res. 38, 995–1002. https://doi.org/10.1080/10715760400000976.

Butterfield, T.A., Best, T.M., Merrick, M.A., 2006. The dual roles of neutrophils and macrophages in inflammation: a critical balance between tissue damage and repair. J. Athl. Train. 41, 457–465.

Carini, M., Aldini, G., Bombardelli, E., Morazzoni, P., Facino, R.M., 2000. UVB-induced hemolysis of rat erythrocytes: protective effect of procyanidins from grape seeds. Life Sci. 67, 1799–1814. https://doi.org/10.1016/s0024-3205(00)00771-2.

Chen, Y.H., Xia, E.Q., Xu, X.R., Li, S., Ling, W.H., Wu, S., et al., 2012. Evaluation of benzo [a] pyrene in food from China by high-performance liquid chromatography-fluorescence detection. Int. J. Environ. Res. Publ. Health 9, 4159–4169.

Chen, Z., Zhang, Y., Yang, J., Jin, M., Wang, X.W., Shen, Z.Q., et al., 2011. Estrogen promotes benzo a pyrene-induced lung carcinogenesis through oxidative stress damage and cytochrome c-mediated caspase-3 activation pathways in female mice. Canc. Lett. 308, 14–22. https://doi.org/10.1016/j.canlet.2011.04.007.

Delgado-Vargas, F., Sicairos-Medina, L.Y., Luna-Mandujan, A.G., López-Angulo, G., Salazar-Salas, N.Y., Vega-García, M.O., et al., 2018. Phenolic profiles, antioxidant and antimutagenic activities of Solanum lycopersicum var. cerasiforme accessions from Mexico. CyTA - J. Food 16, 715–722.

Fragkos, M., Jurvansuu, J., Beard, P., 2009. H2AX is required for cell cycle arrest via the p53/p21 pathway. Mol. Cell Biol. 29, 2828–2840. https://doi.org/10.1128/mcb.01830-08.

Fujisawa, M., Oguchi, K., Yamaura, T., Suzuki, M., Cyong, J.C., 2005. Protective effect of hawthorn fruit on murine experimental colitis. Am. J. Chin. Med. 33, 167–180. https://doi.org/10.1142/s0192415x05002849.

Gholamine, B., Houshmand, G., Hosseinzadeh, A., Kalantar, M., Mehrzadi, S., Goudarzi, M., 2019. Gallic acid ameliorates sodium arsenite-induced renal and hepatic toxicity in rats. Drug Chem. Toxicol. https://doi.org/10.1080/01480545.2019.1591434.

Han, X., Li, W., Huang, D., Yang, X., 2016. Polyphenols from hawthorn peels and fleshes differently mitigate dyslipidemia, inflammation and oxidative stress in association with modulation of liver injury in high fructose diet-fed mice. Chem. Biol. Interact. 257, 132–140. https://doi.org/10.1016/j.cbi.2016.08.002.

Hosseinimehr, S.J., Azadbakht, M., Mousavi, S.M., Mahmoudzadeh, A., Akhlaghpoor, S., 2007. Radioprotective effects of hawthorn fruit extract against gamma irradiation in mouse bone marrow cells. J. Radiat. Res. 48, 63–68. https://doi.org/10.1269/jrr.06032.

Hosseinimehr, S.J., Azadbakht, M., Tanha, M., Mahmodzadeh, A., Mohammadifar, S., 2011. Protective effect of hawthorn extract against genotoxicity induced by methyl methanesulfonate in human lymphocytes. Toxicol. Ind. Health 27, 363–369. https://doi.org/10.1177/0748233710387010.

Hosseinimehr, S.J., Mahmoudzadeh, A., Ahmadi, A., Mohamadifar, S., Akhlaghpoor, S., 2009. Radioprotective effects of hesperidin against genotoxicity induced by gamma-irradiation in human lymphocytes. Mutagenesis 24, 233–235. https://doi.org/10.1093/mutage/gep001.

Juan-Garcia, A., Taroncher, M., Font, G., Ruiz, M.J., 2018. Micronucleus induction and cell cycle alterations produced by deoxynivalenol and its acetylated derivatives in individual and combined exposure on HepG2 cells. Food Chem. Toxicol. 118, 719–725. https://doi.org/10.1016/j.fct.2018.06.024.

Karagiannis, T.C., El-Osta, A., 2004. Double-strand breaks: signaling pathways and repair mechanisms. Cell. Mol. Life Sci. 61, 2137–2147. https://doi.org/10.1007/s00018-004-4174-0.

Kasala, E.R., Bodduluru, L.N., Barua, C.C., Sriram, C.S., Gogoi, R., 2015. Benzo (a) pyrene induced lung cancer: role of dietary phytochemicals in chemoprevention. Pharmacol. Rep. 67, 996–1009.

Knuckles, M.E., Inyang, F., Ramesh, A., 2001. Acute and subchronic oral toxicities of benzo(a)pyrene in F-344 rats. Toxicol. Sci. 61, 382–388. https://doi.org/10.1093/toxsci/61.2.382.

Kumar, P., Wadhwa, R., Gupta, R., Chandra, P., Maurya, P.K., 2018. Spectroscopic determination of intracellular quercetin uptake using erythrocyte model and its implications in human aging. Biotech 3https://doi.org/10.1007/s13205-018-1524-4. 8.

Kwok, C.Y., Li, C., Cheng, H.L., Ng, Y.F., Chan, T.Y., Kwan, Y.W., et al., 2013. Cholesterol lowering and vascular protective effects of ethanolic extract of dried fruit of Crataegus pinnatifida, hawthorn (Shan Zha), in diet-induced hypercholesterolaemic rat model. Journal of Functional Foods 5, 1326–1335. https://doi.org/10.1016/j.jff.2013.04.020.

Kwok, C.Y., Wong, C.N.Y., Yau, M.Y.C., Yu, P.H.F., Au, A.L.S., Poon, C.C.W., et al., 2010. Consumption of dried fruit of Crataegus pinnatifida (hawthorn) suppresses high-cholesterol diet-induced hypercholesterolemia in rats. Journal of Functional Foods 2, 179–186. https://doi.org/10.1016/j.jff.2010.04.006.

Liu, P., Kallio, H., Lue, D., Zhou, C., Yang, B., 2011. Quantitative analysis of phenolic compounds in Chinese hawthorn (Crataegus spp.) fruits by high performance liquid chromatography-electrospray ionisation mass spectrometry. Food Chem. 127, 1370–1377. https://doi.org/10.1016/j.foodchem.2011.01.103.

Liu, S., Sui, Q., Zou, J., Zhao, Y., Chang, X., 2019. Protective effects of hawthorn (Crataegus pinnatifida) polyphenol extract against UVB-induced skin damage by modulating the p53 mitochondrial pathway in vitro and in vivo. J. Food Biochem. 43. https://doi.org/10.1111/jfbc.12708.

Liu, S.W., You, L., Zhao, Y.X., Chang, X.D., 2018. Hawthorn polyphenol extract inhibits UVB-induced skin photoaging by regulating MMP expression and type I procollagen production in mice. J. Agric. Food Chem. 66, 8537–8546. https://doi.org/10.1021/acs.jafc.8b02785.

Ma, W.-K., Li, H., Dong, C.L., He, X., Guo, C.R., Zhang, C.-F., et al., 2016. Palmatine from Mahonia bealei attenuates gut tumorigenesis in Apc(Min/+) mice via inhibition of inflammatory cytokines. Mol. Med. Rep. 14, 491–498. https://doi.org/10.3892/mmr.2016.5285.

Mazur, A., Bayle, D., Lab, C., Rock, E., Rayssiguier, Y., 1999. Inhibitory effect of procyanidin-rich extracts on LDL oxidation in vitro. Atherosclerosis 145, 421–422.

Muntean, N., Muntean, E., Duda, M., 2013. Contamination of some plant origin food products with polycyclic aromatic hydrocarbons. Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca - Agric. 70, 383–386.

Near, R.I., Matulka, R.A., Mann, K.K., Gogate, S.U., Trombino, A.F., Sherr, D.H., 1999. Regulation of preB cell apoptosis by aryl hydrocarbon receptor/transcription factor-expressing stromal/adherent cells. Proc. Soc. Exp. Biol. Med. 221, 242–252.

Nwagbara, O., Darling-Reed, S.F., Tucker, A., Harris, C., Abazinge, M., Thomas, R.D., et al., 2007. Induction of cell death, DNA strand breaks, and cell cycle arrest in DU145 human prostate carcinoma cell line by benzo a pyrene and benzo a pyrene-

7,8-diol-9,10-epoxide. Int. J. Environ. Res. Publ. Health 4, 10–14. https://doi.org/10.3390/ijerph2007010002.
Onyinye, N., Darling-Reed, S.F., Alicia, T., Cynthia, H., Michael, A., Thomas, R.D., et al., 2007. Induction of cell death, DNA strand breaks, and cell cycle arrest in DU145 human prostate carcinoma cell line by benzo[a]pyrene and benzo[a]pyrene-7,8-diol-9,10-epoxide. Int. J. Environ. Res. Publ. Health 4, 10–14.
Park, S.Y., Lee, S.M., Ye, S.K., Yoon, S.H., Chung, M.H., Choi, J., 2006. Benzo[a]pyrene-induced DNA damage and p53 modulation in human hepatoma HepG2 cells for the identification of potential biomarkers for PAH monitoring and risk assessment. Toxicol. Lett. 167, 27–33. https://doi.org/10.1016/j.toxlet.2006.08.011.
Perumal Vijayaraman, K., Muruganantham, S., Subramanian, M., Shunmugiah, K.P., Kasi, P.D., 2012. Silymarin attenuates benzo(a)pyrene induced toxicity by mitigating ROS production, DNA damage and calcium mediated apoptosis in peripheral blood mononuclear cells (PBMC). Ecotoxicol. Environ. Saf. 86, 79–85. https://doi.org/10.1016/j.ecoenv.2012.08.031.
Pokhariyal, J., Mandal, A., Aggarwal, S.G., 2019. Measurement of benzo (a) pyrene in PM 10 collected in New Delhi. MAPAN 34, 465–471.
Prasad, S., Yadav, V.K., Srivastava, S., Shukla, Y., 2008. Protective effects of lupeol against benzo a pyrene induced clastogenicity in mouse bone marrow cells. Mol. Nutr. Food Res. 52, 1117–1120. https://doi.org/10.1002/mnfr.200700420.
Rabiei, K., Bekhradnia, S., Nabavi, S.M., Nabavi, S.F., Ebrahimzadeh, M.A., 2012. Antioxidant activity of polyphenol and ultrasonic extracts from fruits of Crataegus pentagyna subsp elburensis. Nat. Prod. Res. 26, 2353–2357. https://doi.org/10.1080/14786419.2012.658799.
Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20, 933–956. https://doi.org/10.1016/0891-5849(95)02227-9.
Samanta, S.K., Singh, O.V., Jain, R.K., 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol. 20, 243–248.
Shatoor, A.S., Al Humayed, S., Alkhateeb, M.A., Shatoor, K.A., Aldera, H., Alassiri, M., et al., 2019. Crataegus Aronia protects and reverses vascular inflammation in a high fat diet rat model by an antioxidant mechanism and modulating serum levels of oxidized low-density lipoprotein. Pharmaceut. Biol. 57, 38–48. https://doi.org/10.1080/13880209.2018.1564930.
Shukla, R.K., Sharma, V., Pandey, A.K., Singh, S., Sultana, S., Dhawan, A., 2011. ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. Toxicology 25, 231–241. https://doi.org/10.1016/j.tiv.2010.11.008. In Vitro.
Singh, L., Varshney, J.G., Agarwal, T., 2016. Polycyclic aromatic hydrocarbons' formation and occurrence in processed food. Food Chem. 199, 768–781.
Sinha, R., Kulldorff, M., Gunter, M.J., Strickland, P., Rothman, N., 2005. Dietary benzo [a] pyrene intake and risk of colorectal adenoma. Cancer Epidemiology and Prevention Biomarkers 14, 2030–2034.
Swaminathan, J.K., Khan, M., Mohan, I.K., Selvendiran, K., Devaraj, S.N., Rivera, B.K., et al., 2010. Cardioprotective properties of Crataegus oxycantha extract against ischemia-reperfusion injury. Phytomedicine 17, 744–752. https://doi.org/10.1016/j.phymed.2010.01.009.
Toyooka, T., Ibuki, Y., Takabayashi, F., Goto, R., 2006. Toyooka T, Ibuki Y, Takabayashi F, Goto R.. Coexposure to benzo[a]pyrene and UVA induces DNA damage: first proof of double-strand breaks in a cell-free system. Environ. Mol. Mutagen. 47, 38–47.
Trumbeckaite, S., Benetis, R., Bumblauskiene, L., Burdulis, D., Janulis, V., Toleikis, A., et al., 2011. Achillea millefolium L. s.l. herb extract: antioxidant activity and effect on the rat heart mitochondrial functions. Food Chem. 127, 1540–1548. https://doi.org/10.1016/j.foodchem.2011.02.014.
Urbancova, K., Lankova, D., Rossner, P., Rossnerova, A., Svecova, V., Tomaniova, M., et al., 2017. Evaluation of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns. Sci. Total Environ. 577, 212–219. https://doi.org/10.1016/j.scitotenv.2016.10.165.
Xia, Z., Duan, X., Qiu, W., Liu, D., Wang, B., Tao, S., et al., 2010. Health risk assessment on dietary exposure to polycyclic aromatic hydrocarbons (PAHs) in Taiyuan, China. Sci. Total Environ. 408, 5331–5337. https://doi.org/10.1016/j.scitotenv.2010.08.008.
Zhang, Y., Zhang, L., Geng, Y., Geng, Y., 2014. Hawthorn fruit attenuates atherosclerosis by improving the hypolipidemic and antioxidant activities in apolipoprotein E-deficient mice. J. Atherosclerosis Thromb. 21, 119–128. https://doi.org/10.5551/jat.19174.
Zheng, G., Deng, J., Wen, L., You, L., Zhao, Z., Zhou, L., 2018. Release of phenolic compounds and antioxidant capacity of Chinese hawthorn “Crataegus pinnatifida” during in vitro digestion. Journal of Functional Foods 40, 76–85. https://doi.org/10.1016/j.jff.2017.10.039.
Zheng, X., Li, X., Chen, M., Yang, P., Zhao, X., Zeng, L., et al., 2019. The protective role of hawthorn fruit extract against high salt- induced hypertension in Dahl salt- sensitive rats: impact on oxidative stress and metabolic patterns. Food & Function 10, 849–858. https://doi.org/10.1039/c8fo01818a.
Zhou, M.Z., Ge, X.Q., Ke, D.M., Tang, H., Zhang, J.Z., Calvaresi, M., et al., 2019. The Bioavailability, Biodistribution, and Toxic Effects of Silica-Coated Upconversion Nanoparticles in vivo. Frontiers in Chemistry 7. https://doi.org/10.3389/fchem.2019.00218.
Zhu, W., Cromie, M.M., Cai, Q., Lv, T., Singh, K., Gao, W., 2014. Curcumin and vitamin E protect against adverse effects of benzo a pyrene in lung epithelial cells. PloS One 9. https://doi.org/10.1371/journal.pone.0092992.
Zhu, Y., Duan, X., Qin, N., Lv, J., Wu, G., Wei, F., 2019. Health risk from dietary exposure to polycyclic aromatic hydrocarbons (PAHs) in a typical high cancer incidence area in southwest China. Sci. Total Environ. 649, 731–738. https://doi.org/10.1016/j.scitotenv.2018.08.157.