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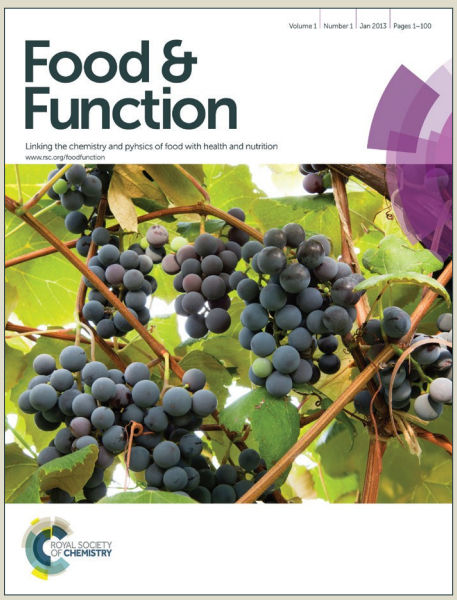

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Paper

Whole body radioprotective effect of phenolic extract from the fruits of *Malus baccata* (Linn.) Borkh.

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This work was designed to evaluate the radioprotective effect of the phenolics extracted from fruits of *Malus baccata* (Linn.) Borkh. (MBP-3b) against the damage induced by ^{60}Co γ - irradiation in vivo. MBP-3b could significantly improve the activity of endogenous antioxidant enzymes and T-AOC, as well as reduce the MDA level in the liver and kidney of irradiation mice. In addition, pretreatment with MBP-3b at dose of 150 mg/kg bw could significantly enhance immunomodulation activity through promoting the proliferation of spenocytes and monocyte phagocytosis. Administration of MBP-3b prevented the haematological parameters (WBC, RBC, PLT and HGB) decline induced by radiation. Furthermore, MBP-3b could protect spenocytes from radiation-induced damage by inhibiting the cell apoptosis. The results indicated that MBP-3b possesses strong whole body radioprotective and immunomodulatory activities. The main compositions in MBP-3b were tentatively identified as delphinidin-3,5-diglucoside, cyanidin-3-glucoside, chlorogenic acid, proanthocyanidins C1, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-xyloside/arabinoside, phloretin-2-xyloseglucoside, quercetin-3-rhamnoside and phlorizin. MBP-3b could be used as a probable radioprotector against gamma radiation induced oxidative damage.

Introduction

Ionizing radiation is commonly used to treat cancers. However, it was often limited in clinic due to lesion on normal tissues and organs. Ionizing radiation could induce the body to generate excessive free radicals, which disturb the redox dynamic equilibrium. In normal physiological metabolism, small amount of free radicals play an important role in electron transfer and molecular signal transduction in the body. Once the homeostasis was destructed, the redundant free radicals fast react with the biological substances, leading to the protein carbonylation, lipid peroxidation, and especially genetic toxicity.[1] Active free radicals sharply attack the chemical bonds of DNA, which results in the change of helical conformation, codon depletion, translocation, even single or double strand breaks, and produces genetic toxicity.[2] Moreover, the uncontrollable free radicals also injure and decrease activities of the DNA repair enzymes, and destruct the molecular signal connections in the body self-repair systems, finally causing the cell over apoptosis.[3] Therefore, long-term exposure to the radiation environments could induce the human body to generating acute or subacute radiation syndromes.

Most of the genetic damages induced by radiation are mediated through the mass formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) free radicals. The bursts of ROS and RNS may directly or indirectly affect proteins/genes that participate in oxidative metabolism.[4] Although living cells have an endogenous antioxidant defense mechanism, such as catalase, glutathione peroxidase, they can become overwhelmed with the fast increased production of ROS.[5] Radiation attenuates the endogenous antioxidant enzymes, which are considered as the first defense line in maintenance of redox balance and normal biochemical processes.[6] Homeostatic cellular functions require tight control of the redox environment.[7] Therefore, the agents which can protect normal tissue from radiation induced oxidative damages received extensive attention. Due to the toxicity of synthetic agents, researchers tried to find more safer and effective radioprotectors.[8] Many natural compounds have been proved to protect organism from radiation-induced damage without obvious side effects, thus they could be potential agents as radioprotectors.[9] Some *in vitro* and *in vivo* studies have confirmed that phenolic compounds have potential radioprotective effect on lymphocytes.[10-12] The effects may account for their antioxidant activities, such as OH· and O_2-· scavenging abilities. However, to the best of our knowledge, the radioprotective molecular signalling mechanism of the phenolics is still unclear, and need to further systematic research.

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Table 1 Effect of MBP-3b pretreatment on antioxidant enzymes activities, MDA and T-AOC in liver and kidney of mice.

Group [a]	Liver			
	CAT (U/mg protein)	SOD (U/mg protein)	MDA (nmol/mg protein)	T-AOC (U/mg protein)
□	50.52±4.95**	266.52±11.51	1.91±0.19$^{**\#\#}$	0.90±0.12$^{*\#}$
□	68.02±4.83$^{**\#\#}$	276.23±22.23$^{\#}$	1.35±0.25$^{\#\#}$	1.1±0.21$^{\#\#}$
□	68.51±6.19$^{**\#\#}$	273.51±15.42	1.62±0.2$^{\#\#}$	1.13±0.15$^{\#\#}$
□	55.40±6.13$^{**\#}$	275.35±20.62$^{\#}$	1.77±0.25$^{*\#\#}$	0.70±0.11**
□	45.50±2.30**	236.03±25.20*	2.83±0.28**	0.57±0.12**
□	82.15±5.15	287.59±22.71	1.23±0.19	1.28±0.17
	Kidney			
	CAT (U/mg protein)	SOD (U/mg protein)	MDA (nmol/mg protein)	T-AOC (U/mg protein)
□	71.29±10.62$^{\#\#}$	119.41±8.17	1.42±0.15$^{*\#\#}$	0.48±0.12$^{**\#}$
□	73.49±4.71$^{\#\#}$	122.63±14.69	1.17±0.18$^{\#\#}$	0.71±0.08$^{\#\#}$
□	78.75±11.34$^{\#\#}$	131.38±13.24$^{\#}$	1.25±0.20$^{\#\#}$	0.77±0.11$^{\#\#}$
□	70.33±8.73$^{*\#\#}$	124.30±11.24	1.32±0.26$^{\#\#}$	0.38±0.06**
□	45.73±12.44**	102.39±15.85**	2.19±0.28**	0.29±0.1**
□	88.90±6.00	142.44±12.95	0.93±0.17	0.67±0.12

[a] Values are mean ± SD (n=6). Group □: 50 mg/kg bw of MBP-3b; Group □: 100 mg/kg bw of MBP-3b; Group □: 150 mg/kg bw of MBP-3b; Group □: Leucogen; Group □: Radiation control; Group □: Normal control. * p<0.05 compare with Normal control group; ** p<0.01 compare with Normal control group; $^{\#}$ p<0.05 compare with Model group; $^{\#\#}$ p<0.01 compare with Model group.

Malus baccata (Linn.) Borkh. (*Malus Mill*, *Rosaceae*) is widely known in China as "Shangdingzi", which is a fast growing wild plant, native to the Greater Higgnan Mountains in northern China. *M. baccata* belongs to a kind of cold-field fruit. The tree of *M. baccata* has strong tolerance to cold circumstance even below -40 °C that makes it producing special substances to enhance the cold resistance. Our previous study studies have confirmed that the fruit of *M. baccata* is rich in phenolic compounds, which are valuable secondary metabolites for human health care. In addition, it exhibited antioxidant and immunomodulation activities. This study was undertaken to investigate the possibility that administration of *M. baccata* extracts was able to protect the body from radiation injury through improving immune function and endogenous antioxidant defense mechanism.

Results and discussion

Effects of MBP-3b on the activities of antioxidant enzymes, and the contents of MDA and T-AOC

The changes in antioxidant levels, MDA levels, as well as total antioxidant capacity (T-AOC) in liver and kidney tissues of mice exposed to whole body γ-radiation are presented in Table 1. Superoxide dismutase (SOD) is an effective antioxidant enzyme that converts dismutated superoxide anions into H_2O_2, and then catalase (CAT) continues to metabolize H_2O_2 to oxygen and water. CAT is a hemoprotein, which combines with SOD playing a key role in the antioxidant defense system.[13]

The results indicated a significant reduction of SOD activity in liver (p<0.05) and kidney (p<0.01) in radiation treated group. Similarly, the CAT activity was decreased in liver (p<0.01) and kidney (p<0.01) after treated with irradiation. The decrease of CAT may result in several deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide, which also could induce the oxidation of the biological components in the cells.[14]

Thus, exposure with radiation damaged the enzymatic antioxidant defense system. In contrast, we observed a significant increase in SOD in MBP-treated group (100 or 150 mg/kg bw) after radiation compared to radiation control group both in liver and kidney (p<0.05). Also, administration MBP at the doses of 50~150 mg/kg bw could cause a significant increase in the activities of CAT (all p<0.01). Administration the positive control (Leucogen) could significantly increase SOD and CAT activities relative to radiation treatment alone (p<0.05 or p<0.01). The reduction of antioxidant enzymes activities in liver and kidney of gamma radiated mice might be due to the denaturation of enzymes structures induced by gamma radiation.[15]

MDA is the major product of LPO. It could bring the loss of integrity of cell membranes. Radiation treatment significantly (p<0.01) increased the MDA level in the liver and kidney tissues. However, oral administration of Leucogen and MBP at different doses all could significantly decrease the MDA levels both in liver and kidney in a comparison with model group (p<0.01).

T-AOC reflects the capacity of the non-enzymatic antioxidant defense system. T-AOC levels in liver and kidney decreased remarkably with gamma radiation treatment (p<0.01). However, MBP administration greatly elevated T-AOC in the tissues with a dose dependent mode. The obtained results

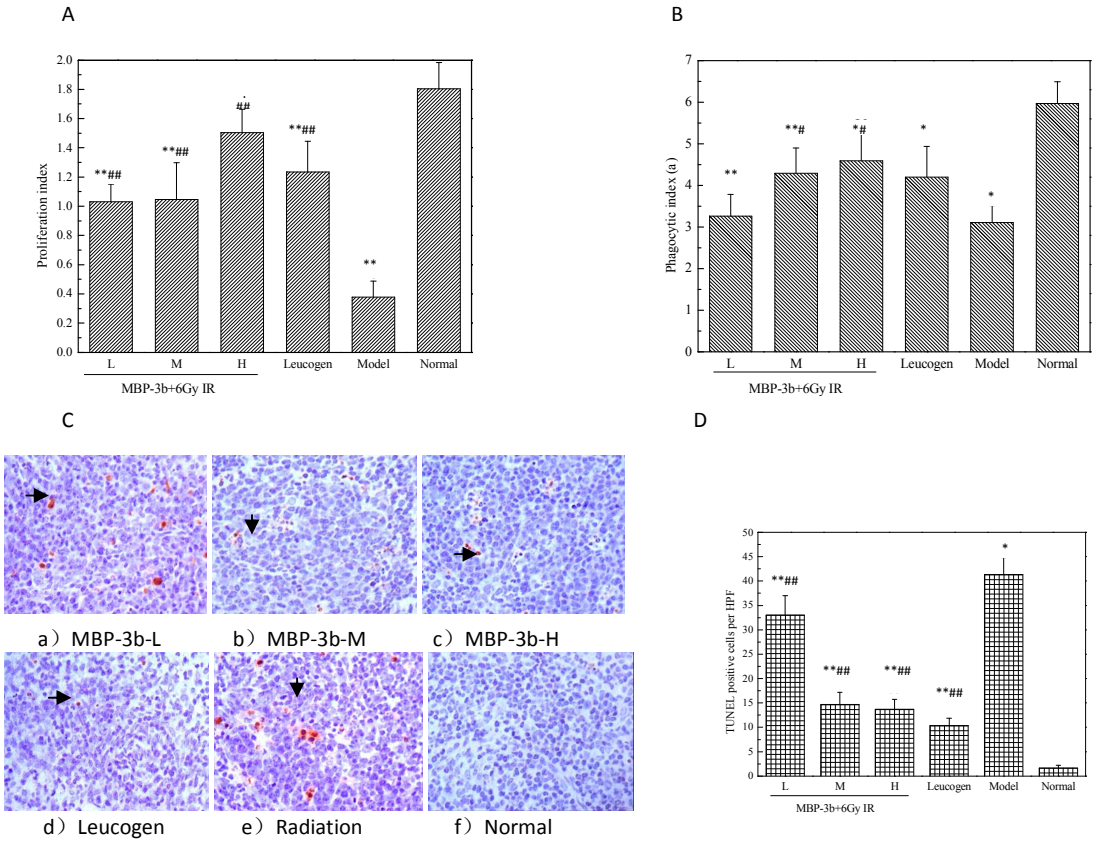

Fig. 1. Effects of MBP-3b on immunomodulatory activities of mice after radiation. (A) Splenocytes proliferation of the mice was analyzed by MTT method. (B) Phagocytosis of monocytes of mice was determined by measuring carbon particles clearance. (C) The spleen was fixed, sectioned, and stained using a TUNEL kit. The pictures above were representative micrographs of TUNEL staining (a-f). (D) The bar graph represented the average numbers of TUNEL-positive cells per HPF in each group. The arrows showed the TUNEL-positive cells. Values are mean ± SD (n=6). (* p<0.05 compare with Normal control group; ** p<0.01 compare with Normal control group; $^{\#}$ p<0.05 compare with Model group; $^{\#\#}$ p<0.01 compare with Model group).

suggested that extract might ameliorate oxidative response through increasing levels of T-AOC in the tissues. Our preliminary studies have found that phenolics were major antioxidant components in MBP-3b. The potential health of foods may account for the benefits of flavonoids and phenolics.[16] Several *in vitro* studies have demonstrated the preventive efficacy of phenolic extracts against radiation-induced damage.[17-18]

Effect of extract on splenocyte proliferation

Lymphocyte proliferation is extremely crucial in the activation cascade of immune responses in organisms.[19] Radiation often inhibits the immune system through inhibition of immune cell proliferation, suppression of bone marrow, microcirculatory disturbances, interleukin decline, as well as microenvironment injuries. ConA induced lymphocyte proliferation *in vitro*, which could be used to evaluate T lymphocyte viability. As shown in Fig. 1A, the proliferation of splenocytes in radiation group was significantly decreased when compared with normal control (p<0.01). MBP-3b administration obviously increased the proliferation of splenocytes in a concentration-dependent manner compared to that of model control (p<0.01). The 150 mg/kg bw extract combined with ConA significantly promoted the proliferation of splenocytes, implying a more potent stimulating activity. Above results indicated MBP-3b had T-cell mediated immunostimulating effect of mice, which were consistent with other reports.[20-21]

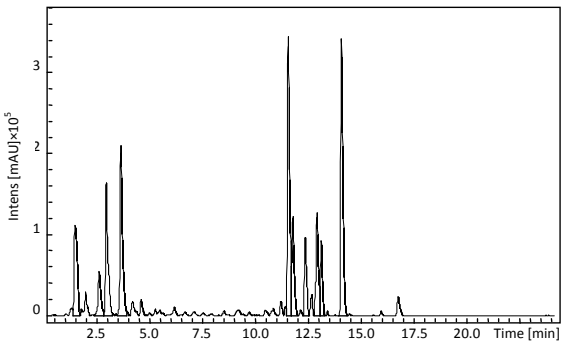

Fig. 2. UPLC spectrum of MBP-3b recorded at 254 nm.

Table 2 Effects of MBP-3b pretreatment on the counts of white blood cells (WBC), red blood cells (RBC) and platelet (PLT), and the concentration of haemoglobin (HGB) in mice.

Group [a]	WBC (10^9/L)	RBC (10^{12}/L)	PLT (10^9/L)	HGB (G/L)
Ⅰ	1.81±0.30$^{**\#\#}$	8.03±1.21$^{\#}$	563.63±21.34$^{\#\#}$	171.14±10.55$^{\#\#}$
Ⅱ	2.71±0.21$^{**\#\#}$	9.25±0.83$^{\#\#}$	511.25±33.58$^{**\#\#}$	155.56±11.20$^{\#\#}$
Ⅲ	2.73±0.11$^{**\#\#}$	9.18±1.50$^{\#\#}$	672.63±53.02$^{\#\#}$	155.86±20.65$^{\#\#}$
Ⅳ	3.49±0.32$^{**\#\#}$	9.54±0.71$^{\#\#}$	522.41±27.66$^{**\#\#}$	161.72±25.11$^{\#\#}$
Ⅴ	1.10±0.40**	5.48±0.87**	379.40±61.20**	99.08±10.20**
Ⅵ	5.62±0.11	9.94±1.26	615.87±44.10	175.56±21.33

[a] Values are mean ± SD (n=6). Group Ⅰ: 50 mg/kg bw of MBP-3b; Group Ⅱ: 100 mg/kg bw of MBP-3b; Group Ⅲ: 150 mg/kg bw of MBP-3b; Group Ⅳ: Leucogen; Group Ⅴ: Radiation control; Group Ⅵ: Normal control. * p<0.05 compare with Normal control group; ** p<0.01 compare with Normal control group; $^{\#}$ p<0.05 compare with Model group; $^{\#\#}$ p<0.01 compare with Model group.

Phagocytosis of monocyte

Macrophage could protect the host through phagocytosis. The primary role of phagocytosis is removal of microorganisms, foreign bodies, as well as the dead or injured cells.[22] Phagocytic defects are closely associated with many pathological lesions in organisms.[23] The faster eliminate of carbon particles could directly reflect the enhanced phagocytic activity of the macrophage. Results in Fig. 1B indicated that oral administration of extract for 14 days exhibited a dose-dependent increase in the phagocytic index, as 3.26±0.52, 4.29±0.61 and 4.59±0.66, respectively. The mice administrated with the dose of 150 mg/kg bw showed significant greater phagocytic index compared to gamma radiation group (p<0.05). Therefore, the results suggested MBP-3b appeared to enhance the phagocytic function by elevation of the carbon clearance in the cells of the reticuloendothelium system.

MBP-3b protects mouse spleen from radiation-induced apoptosis

To determine whether MBP-3b could be used to prevent the mice spleen tissues from the radiation-induced apoptosis, the spleen was analyzed using TUNEL staining. In the spleen of irradiated mice, we observed a significant increase in TUNEL-positive cells at spleen (p<0.01). MBP-3b pretreatment significantly inhibited radiation-induced apoptosis, as shown in Fig. 1C and 1D. Among the three different concentrations of MBP-3b, High-dose group possessed the least apoptosis cells (p<0.01). All the TUNEL-positive cells numbers in MBP-3b groups were a little more than the ones in Leucogen group. The mechanism of protection was likely based on the ability of MBP-3b to inhibit oxidative stress and scavenge ionizing radiation induced excessive radicals.[24]

Effect of MBP-3b pretreatment on haematological parameters

The haematological parameters of mice against gamma radiation damage were listed in Table 2. The WBC, RBC, PLT counts and HGB concentration decreased significantly (p<0.01) in irradiation group as compared to the normal control. However, extract pretreatment prior to gamma radiation showed the recovery of haematological parameters. Among the three doses, the counts of WBC and PLT in high dose group were closest to the ones in normal group. Results indicated the extract could significantly decrease the damages induced by radiation in blood system. The leucocytes and erythrocytes belong to rapidly dividing cells of the blood system, which are sensitive to gamma irradiation. Gamma irradiation could induce blood cell damage as ROS decreases blood cellular components, such as reticulocytes. While the decrease of reticulocytes is a response to the early damage of the bone marrow haematopoietic function.[15]

UPLC-Q-TOF-MS analyses of MBP-3b

The phenolic compounds were separated by UPLC, and then the characterization was carried out through analysis of mass spectral data obtained by Q-TOF. Identification of the compounds was dependent on the calculations of the [M-H]- deprotonated molecular formulas together with the explanation of MS fragmentations.[25] Fig. 2 showed the HPLC chromatograms of MBP-3b. At a wavelength of 254 nm, there were existed several peaks that correspondingly belonged to phenolic compounds. Fig. S1 showed the BPC spectrum of MBP-3b in positive mode. Peak identification was performed by comparing retention times, UV-Vis spectra and mass spectra with those of reference standards or literature data. The results are summarized and listed in Table 3. Ten phenolic compounds were tentative characterized. Fragment ion m/z: 465.1028, 465.1032, 435.0935 and 449.1092 respectively corresponded to $C_{21}H_{21}O_{12}$, $C_{21}H_{21}O_{12}$, $C_{20}H_{19}O_{11}$ and $C_{21}H_{21}O_{11}$, which indicated the residues of quercetin with different glycosides. Among the derivatives, quercetin-3-galactoside and quercetin-3-glucoside was a pair of isomers leading to exhibit similar fragment ions.

Previous study found that the oxidative stress was the main type of damages induced by radiation. Many researches have focused on the antioxidant activities of flavonoid polyphenols with multiple hydroxyl structure, especially with hydroxyl in B-ring. The polyphenols of *Malus baccata* (Linn.) Borkh were mainly consisted by quercetin derivatives, which had the significant antioxidant effects through the donation of electron or hydrogen atom by the typical hydroxyl in B-ring.[26]

Table 3 Composition of monomers in MBP-3b.

No.	Retention time (min)	m/z [M+H]+	err (mDa)	Fragments	Molecular formula	Proposed compound	Relative content (%)
1	3.0	627.1581	-1.56	465	$C_{27}H_{31}O_{17}$	delphinidin-3,5-diglucoside	3.60
2	3.2	449.1083	-0.20	287	$C_{21}H_{21}O_{11}$	cyanidin-3-glucoside	9.29
3	3.7	355.1031	-1.20	163	$C_{16}H_{19}O_9$	chlorogenic acid	11.96
4	4.7	867.2057	0.12	579/289	$C_{45}H_{38}O_{18}$	proanthocyanidins C1	1.64
5	11.6	465.1028	-0.71	303	$C_{21}H_{21}O_{12}$	quercetin-3-galactoside	18.74
6	11.8	465.1032	-0.65	303	$C_{21}H_{21}O_{12}$	quercetin-3-glucoside	6.63
7	12.4	435.0935	1.40	303	$C_{20}H_{19}O_{11}$	quercetin-3-xyloside/arabinoside	4.75
8	12.8	569.1865	0.55	275/169	$C_{26}H_{33}O_{14}$	phloretin-2-xyloseglucoside	7.16
9	13.1	449.1092	0.48	303	$C_{21}H_{21}O_{11}$	quercetin-3-rhamnoside	4.32
10	14.1	437.1442	-0.37	275/169	$C_{21}H_{25}O_{10}$	phlorizin	18.24

In addition, anthocyanins, phlorizin and its derivatives were reported to possess obvious antioxidative activities.[27] Therefore, based on the notable protective function on oxidative damages, the phenolic compounds in MBP-3b could potentially act synergistically in providing the radioprotection and immunomodulation activities.

Experimental section

Materials and regents

Fruits of *Malus baccata* (Linn.) Borkh. were collected during the autumn season from a local farm in Greater Higgnan Mountains of Heilongjiang province, P. R. China. The fruits were selected on the basis of skin color (ripe red stage) and size (average 0.8 cm in diameter). The fresh fruits were washed with distilled water and dried out using blotting paper, then frozen with liquid nitrogen and stored at -20 °C prior to further analysis.

Preparation of crude *M. baccata* extract

The fruits were ground using a grinder to yield a fine paste. The sample was soaked in 13-fold volume of 80% acetone (v/v) solvent under ultrasonic wave treatment for 10 min, then 1 h at thermostat-controlled water bath (47 °C). The extract was separated from the cake by filtration on a Buchner funnel, then centrifuged at 10000 rpm for 10 min and supernatants were collected. The samples were extracted three times in a row and combined the extracts for each sample, then concentrated under reduced pressure at 50 °C. Enrichment of phenolics from *M. baccata* extract was carried out in glasscolumns packed with NKA-9 macroporous resin. After the adsorption equilibrium was reached, most of the phenolic compounds were absorbed onto the resin column while sugars, acids and other water-soluble compounds were removed by washing the column with distilled water. The phenolic compounds were eluted by adding 40% ethanol (v/v) with a constant flow velocity of 2 BV/h until the phenolics can not be detected. The collected fraction was evaporated to remove the ethanol with a rotary evaporator under reduced pressure at 45 °C, then followed by freeze-drying and stored at -20 °C until use. And this collected fraction was named MBP-3b.

Animals

SPF-level male Kunming mice (6~8 weeks old, 20±2 g) were purchased from the Laboratory Animal Centre of Harbin Medical University. The animals were kept in a regulated environment at 25±1 °C and 45~55% relative humidity under 12 h light/12 h dark conditions. The animals were housed in sanitized polypropylene cages containing sawdust as bedding. They had free access to rodent laboratory chow pellets and water. The mice were randomly divided into six groups after 7 days of acclimatization. All the animal experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Experimental Animals approved by the Heilongjiang Province People's Congress. The specific rules were referred to the URL: (http://www.nicpbp.org.cn/sydw/CL0249/2730.html). The Ethics Research Committee of Harbin Institute of Technology approved the study protocol. All efforts were made to minimize suffering.

Irradiation

Mice were placed in well-ventilated plastic box and were exposed to whole body gamma radiation (6 Gy) from ^{60}Co gamma teletherapy facility (Heilongjiang Academy of Agricultural Sciences，China) at a dose rate of 1.33 Gy/min and source to surface distance of 140 cm. Radiation dose was fixed according to the previous standardizations.

Experimental design for radioprotection studies

Animals were divided into 6 groups (n=10) and treated as follows:

Group I~ III: The animals in these three groups were respectively administered 50 mg/kg bw (MBP-3b-L), 100 mg/kg bw (MBP-3b-M) and 150 mg/kg bw (MBP-3b-H) of MBP-3b once daily for 14 consecutive days prior to whole body γ-radiation;

Group IV: The animals in this group were administered 12 mg/kg bw of Leucogen once daily for 14 consecutive days prior to whole body γ-radiation;

Group V: The animals in this group were administered equal volume of sterile double distilled water instead of MBP as described above for 14 consecutive days prior to whole body γ-radiation;

Group VI: The animals in this group were administered equal volume of sterile double distilled water for 14 consecutive days.

After irradiation, the mice were returned to climate-controlled cages and were sacrificed by a cervical dislocation at 24 h postirradiation for further experiments.

Measurement of the activities of antioxidant enzymes, and the concentrations of MDA and T-AOC

Liver and kidney homogenates (10%, w/v) were prepared with ice-cold sodium chloride (150 mM). The resulting suspension was centrifuged at 4000 rpm for 10 min at 4 °C. The activities of catalase (CAT) and superoxide dismutase (SOD), and the concentrations of malondialdehyde (MDA) and the total antioxidant capacity (T-AOC) in the supernatant of the organs were respectively measured using CAT, SOD, MDA and T-AOC commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions. The determination of SOD activity was dependent on its inhibitory ability on hydroxylamine oxidation induced by O^{2-}, which was generated from the xanthine-xanthineoxiase system. The measuring principle of CAT activity was referred that the surplus H_2O_2 after the catalysis by CAT could react with ammonium molybdate to form a comoles compound with special colour. The content of MDA was analyzed according to the reaction between MDA with the thiobarbituric (TBA) that formed a stable chromophoric substance. The T-AOC was measured based on the antioxidative capacity of the antioxidant in organisms, which could reduce the Fe^{3+} to Fe^{2+}, and then react with phenanthroline, producing the salmon pink phenanthroline ferrous complex.

Splenocyte proliferation index

Spleens from sacrificed mice were removed for preparation of splenocytes. Spleen was chopped in PBS (pH 7.4) and passed through a stainless steel mesh. The obtained cell suspensions were centrifuged at 1000 rpm for 10 min, and then sediment was treated with red blood cell lysis solution for 5 min to remove erythrocytes and washed twice with PBS. Then spleen cells were resuspended in RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL) as well as 10 % fetal bovine serum. Then the cell suspension (1×10^6 cells/ml) was distributed in 96-well plates (95 μL/well) and cultured with 5 μL of ConA (7.5 μg/mL) or PBS for 48 h at 37 °C in a humidified 5% CO_2 incubator. Splenocyte proliferation activity was tested by MTT assay at 570 nm. The splenocyte proliferation index was calculated by the following equation: [28-29]

$$\text{Splenocyte proliferation index} = OD_{experimental}/OD_{control}$$

Phagocytosis of monocyte assay

The phagocytosis function of monocyte was evaluated by measuring the carbon particles clearance. At 24 h postirradiation, Indian ink (100 mL/kg body weight) was injected by tail intravenous injection. Blood samples (20 μL) was respectively collected through orbit plexuses of individual animals at an interval of 2 min (t_1) and 10 min (t_2) after the injection of carbon suspension, and then immediately added to 2 mL of 0.1% Na_2CO_3 to lyse the erythrocytes. The absorbance of blood after 2 min (A_1) and after 10 min (A_2) was measured at 600 nm. Then, the mice were sacrificed by cervical dislocation and the liver and spleen were weighted. Rate of carbon clearance (K) and phagocytic index (α) were calculated as following formula:[30]

$$K = \frac{\lg A_1 - \lg A_2}{t_2 - t_1} \qquad \alpha = \frac{Bodyweight}{Liverweight + Spleenweight} \times \sqrt[3]{K}$$

Immunohistochemistry

Mice were sacrificed at 24 h after irradiation by cervical dislocation. The spleen was fixed intact in 4% paraformaldehyde fixative for 24 h. The spleen was cut into five segments, embedded vertically, and sectioned to provide transverse section. Two-micrometer sections were then taken and placed on slides. Tissue sections were stained with the TUNEL System (NanJing JianCheng Bioengineering Institute, Nanjing, China) and counterstained with hematoxylin and eosin in the standard fashion. At least three animals were used in each experimental group. TUNEL-positive cells were counted under a light microscope (400×). At least three high power fields (HPF) per animal were scored. The average number of TUNEL-positive cells per HPF was calculated.

Haematological analysis

Haematological analysis was performed using automated haematology analyzer (Mindray BC-1800, Shenzhen Mindray Hi-Tech Electric Co., Ltd).

Tentative analyses of MBP-3b using UPLC-Q-TOF-MS

HPLC analysis was performed using Agilent 1290 series Rapid Resolution LC (Agilent Technologies, CA, USA) consisting of a vacuum degasser, autosampler, a binary pump and a diode array detector (DAD). This instrument was equipped with an Agilent Zorbax SB C18 column (2.1×150 mm, 1.8 μm particle size) from Agilent Technologies. Acidified water (0.1% formic acid, v/v) and acetonitrile (0.1% formic acid, v/v) were used as mobile phases A and B, respectively. The HPLC-pump was programmed to elute 10% B for 5 min, and gradient elution of B to 70% within 5 to 30 min; and isocratic elution of 70% B to 40 min. The flow rate was set at 0.25 mL/min throughout the gradient. The column temperature was maintained at 30 °C and the injection volume was 10 μL. UV-Vis absorption spectra were monitored by DAD at 254 nm. The HPLC system was coupled to micrOTOF-Q II (Bruker Daltonic, Germany), equipped with an ESI source. Parameters for analysis were set using positive ion mode with spectra acquired over a mass range from m/z 100 to 1200. The ESI-MS parameters were set to: capillary voltage, +4.5 kV; drying gas temperature, 180 °C; drying gas flow, 4.0 L/min.

Statistical analysis

All the values were expressed as means ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS (version 16.0). Differences at $p<0.05$ and $p<0.01$ were considered statistically significant by Duncan's new multiple-range test.

Conclusions

In this report, we investigated the radioprotective effect of the phenolics extracted from the fruits of *Malus baccata* (Linn.) Borkh. (MBP-3b) against the damage induced by ^{60}Co γ-irradiation in vivo. The results suggested that MBP-3b could effectively improve SOD, CAT activities, enhance T-AOC and reduce the level of MDA in liver and kidney of mice compared to the radiation controls. In addition, MBP-3b could significantly strengthen the immunomodulatory activities through promoting the splenocyte proliferation and phagocytosis of monocyte, and increasing the number of the blood WBC in mice. Furthermore, MBP-3b could also protect the spenocytes by inhibiting the cell apoptosis. The biological activities of MBP-3b may correlate to their specific compositions, such as delphinidin-3,5-diglucoside, cyanidin-3-glucoside, chlorogenic acid, proanthocyanidins C1, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-xyloside/arabinoside, phloretin-2-xyloseglucoside, quercetin-3-rhamnoside and phlorizin. Therefore, further research is needed to analyze the mechanism of anti-radiation activity and the relationship between the function and composition. Efficacy of MBP-3b provided the evidence that this functional plant extract could be developed as a potential anti-radiation agent.

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