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Antioxidant Activity of *Vaccinium stamineum*: Exhibition of Anticancer Capability in Human Lung and Leukemia Cells

Abstract

Fruit of deerberry [*Vaccinium stamineum* L.] were evaluated for their antioxidant capacity and anticancer properties in JB6 P^+ mouse epidermal cells, human lung and leukemia cells. Deerberries contain potent free radical scavenging activities. Pretreatment of JB6 P^+ mouse epidermal cells with deerberry fruit extracts produced an inhibition on the activation of activator protein-1 (AP-1) and nuclear factor-kappaB (NF-κB) induced by either 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or ultraviolet-B (UVB). Deerberry fruit extracts also blocked TPA- or UVB-induced phosphorylation of ERKs and MEK $^1/_2$, two upstream regulators of AP-1 and inhibited proliferation of human leukemia HL-60 cancer cells and human lung epithelial cancer A549 cells and induced apoptosis of HL-60 cells. These results suggest that the inhibition of TPA- or UVB-induced AP-1 and NF-κB activity, inhibition of HL-60 cells and cancer A549 cells proliferation and induction of apoptotic in human leukemia HL-60 cancer cells may be mediated through the ERKs and MEK $^1/_2$ signal pathway.

Key words
Deerberry fruit · antioxidant activity · anticancer properties · mitogen-activated protein kinase · Ericaceae · *Vaccinium stamineum* L.

Introduction

Deerberry [*Vaccinium stamineum* L.) is a low, slender shrub. The fruits of deerberry resemble cranberries (*V. macrocarpum* Ait.) and have been utilized for flavored jelly, preserves and pie [1]. Deerberries contain a high amount of flavonoids [1], [2]. Many studies have suggested that these naturally occurring flavonoid compounds exhibit biological activities and show a remarkably high scavenging activity toward chemically generated radicals, thus making them effective in inhibiting oxidation of human low-density lipoproteins and preventing various human diseases [3], [4], [5], [6], [7]. Although deerberry is a member of the *Vaccinium* genus, it has been considered a minor crop in the United States and thus received little attention for research and little information is available on its health benefits.

The AP-1 signal transduction pathway is known to be important as molecular targets of chemopreventive strategies. AP-1, a ubiquitous transcriptional activator, is composed of members of the Jun and Fos families that form homodimers or heterodimers and bind to a distinct DNA response element. NF-κB is also an important regulator in deciding cell fate, such as programmed cell death and proliferation control, and is critical in tumorigenesis [8]. Reactive oxygen species (ROS) stimulate transcription by ac-

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Received September 5, 2006 · **Revised** February 12, 2007 · **Accepted** February 23, 2007

Bibliography
Planta Med 2007; 73: 451–460 © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2007-967164 · Published online March 29, 2007
ISSN 0032-0943

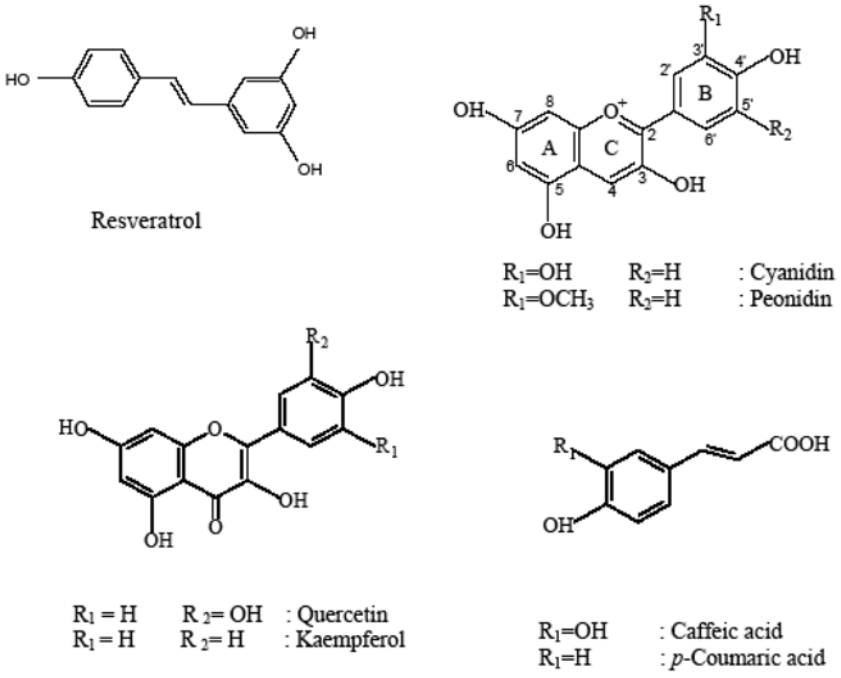


Fig. **1** The chemical structures of the major flavonoids in deerberries.

tivating transcription factors such as AP-1 and NF-κB. AP-1 and NF-κB signal transduction pathways are important in transformation and tumor promotion [9]. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) or ultraviolet-B (UVB) are carcinogens and can produce ROS and stimulate AP-1 and NF-κB activity by activating mitogen-activated protein kinases (MAPK) signaling pathways such as the extracellular signal-regulated kinases $^1/_2$ (ERK $^1/_2$), c-Jun amino-terminal kinases (JNKs), p38 and MEK $^1/_2$ MAP kinases [9], [10], [11].

The purpose of this study was to evaluate the antioxidant capacity that might be involved in oxygen detoxification in deerberries and to study the inhibitory effect of deerberry fruit extracts on activator protein-1 (AP-1), nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinase (MAPK) activation induced by either 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or ultraviolet-B (UVB). The potential therapeutic activity of deerberry fruit extracts on inhibition of cancer proliferation and induction of apoptosis in human cancer cells was also investigated.

Materials and Methods

Cell lines and reagents

The JB6 P$^+$ mouse epidermal cell line, stably transfected with AP-1-luciferase or NF-κB-luciferase reporter plasmid (JB6/AP/κB) [12], was cultured in Eagle's MEM (EMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin. Human lung carcinoma A549 and human leukemia HL-60 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin in 75 cm^2 T-flasks at 37 °C, 85% humidity, and 5% CO_2 atmosphere.

EMEM and DMEM were obtained from Whittaker Biosciences (Walkersville, MD, USA). EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate – $Na_2EDTA2\,H_2O$), FBS, gentamicin, L-glutamine, and trypsin were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). Luciferase assay substrate was obtained from Promega (Madison, WI, USA). PhosphoPlus MAPK antibody kits were purchased from New England BioLabs (Beverley, MA, USA). Chelex 100, $FeSO_4$, H_2O_2, xanthine, and xanthine oxidase were purchased from Sigma (St. Louis, MO, USA). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Aldrich (Milwaukee, WI, USA).

Fruit sample preparation

Deerberry fruit (*Vaccinium stamineum* L.) were grown in Jackson Spring, NC, USA, and were hand-harvested at a commercially mature stage. Approximately 900 g to 2 kg of fruit were harvested for each of the three genotypes sampled [B-59, B-76 and SHF-3A]. The fruits were sorted and selected for uniform size and color. Undamaged berries were randomized and frozen in liquid nitrogen, and then stored at –80 °C until they were used for analysis.

Extraction and isolation

For HPLC analysis, triplicate samples of 4 g deerberry fruit were extracted twice with 20 mL of 80% acetone-0.2% formic acid using a Polytron homogenizer (Brinkmann Instruments, Inc.; Westbury, NY, USA) for one min. Extracts were combined and concentrated to 1 mL using a Buchler Evapomix (Fort Lee, NJ, USA) in a water bath at 30 °C. The concentrated samples were dissolved in 4 mL of acidified water (3% formic acid) and then passed through a C_{18} Sep-Pak cartridge, which was previously activated with methanol followed by water and then 3% aqueous formic acid. Anthocyanins and other phenolics were then recovered with 2.0 mL of acidified methanol containing 3% formic acid. The methanol extract was passed though a 0.45 μm membrane filter (Millipore, MSI; Westboro, MA, USA) and 25 μL were analyzed by HPLC.

For the electron spin resonance (ESR) measurement of ·OH and $O_2^{\cdot-}$ and the assays of AP-1, NF-κB activation, MAPK phosphorylation, cell proliferation and cell apoptosis, deerberry fruit extracts were prepared by mixing 100 g of deerberries with 100 mL of distilled H_2O and blended at high speed. The blended homogenates were strained, centrifuged at 6000 × *g*, 4 °C for 20 min and the supernatants were filtered. The supernatants were transferred to vials, stored at –80 °C until analysis.

HPLC analysis

The separation and identification of individual anthocyanin and phenolic compounds were performed with an HPLC system. The samples were analyzed using a Waters (Waters Associated; Millipore; Milford, MA, USA) HPLC system equipped with two pumps (600 E system Controller) coupled with a photodiode array detector (Waters 990 Series). Samples were injected at ambient temperature (20 °C) onto a reverse phase NOVA-PAK C_{18} column (150 × 3.9 mm, particle size 4 μm) with a guard column (NOVA-PAK C_{18}, 20 × 3.9 mm, particle size 4 μm) (Sentry guard holder universal). The mobile phase was acidified water containing 2.5 % formic acid (A) and acetonitrile (B). The flow rate was 1 mL/min, with a gradient profile consisting of A with the following proportions (v/v) of B: 0 min, 3 %, 1 – 10 min, 3 – 6 % B; 10 – 15 min, 6 % B; 15 – 35 min, 6 – 18 % B; 35 – 40 min, 18 – 20 % B; 40 – 45 min, 20 – 100 % B; 45 – 50 min, 100 % B. The phenolic compounds in fruit extracts were identified by their UV spectra, recorded with a diode-array-detector and by chromatographic comparison with authentic markers. Individual flavonols and anthocyanins were quantified by comparison with an external standard of flavonols and anthocyanins. Scanning between 250 and 550 nm was performed and data were collected by the Waters 990 3-D chromatography data system. HPLC analysis showed that, in addition to anthocyanins, other phenolic compounds were also present (Fig. **1**). The content of flavonoids varied among the different genotypes. Caffeic acid ranged from 40.6 to 61.2 mg fresh wt (FW) kg^{-1} and *p*-coumaric acid ranged from 27.7 to 33.1 mg FW g^{-1}. Quercetin 3-galactoside ranged from 26.2 to 43.4 mg FW kg^{-1}, quercetin 3-rhamnoside contributed 77.9 to 94.7 mg FW kg^{-1} and kaempferol ranged from 26.2 to 43.4 mg FW kg^{-1}. Flavonols such as cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside and peonidin 3-glucoside in deerberries ranged from 32.2 to 987.5 mg FW kg^{-1}. Cyanidin 3-galactoside (744.2 to 987.5 mg FW kg^{-1}) and cyanidin 3-arabinoside (128.1 to 201.0 mg FW kg^{-1}) were the two predominant anthocyanins. Resveratrol (3,5,4′-trihydroxystilbene) was also detected in deerberries. The values of resveratrol for deerberry were 36.2 to 47.9 μg FW kg^{-1}.

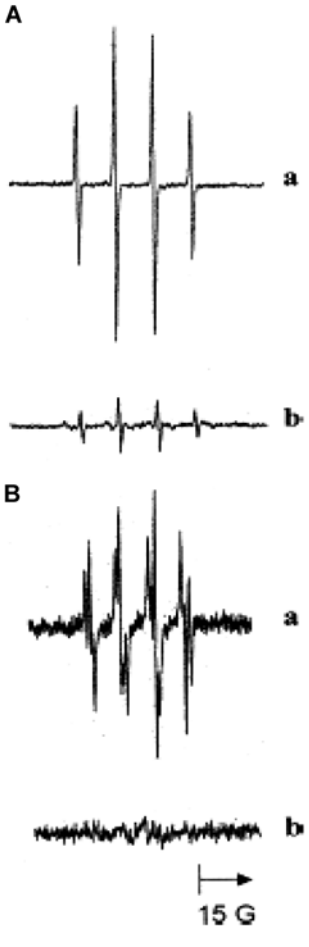


Fig. **2** **A** The scavenging effect of deerberry ('B-76') fruit extract on OH radicals. ESR spectra were recorded 1 min after reaction initiation from a phosphate-buffered solution (pH 7.4) containing 10 mM DMPO and the following reactants: **(a)** 1.0 mM $FeSO_4$ and 1.0 mM H_2O_2; **(b)** 1.0 mM $FeSO_4$, 1.0 mM H_2O_2, and deerberry extract (50 mg mL^{-1}). **B** The scavenging effect of deerberry ('B-76') fruit extract on $O_2^{\cdot-}$, ESR spectra recorded 1 min after reaction initiation from a phosphate-buffered solution (pH 7.4) containing 100 mM DMPO and the following reactants: **(a)** 3.5 mM xanthine and 2 U/mL xanthine oxidase; **(b)** 3.5 mM xanthine, 2 U/mL xanthine oxidase, and deerberry fruit extract (50 mg mL^{-1}). The ESR spectrometer settings were: receiver gain, 2.52×10^4; time constant, 20 ms; modulation amplitude, 0.5 G; (for ·OH radicals) and 1.0 G (for $O_2^{\cdot-}$ radicals); scan time, 60 s; and magnetic field, 3480 ± 100 G.

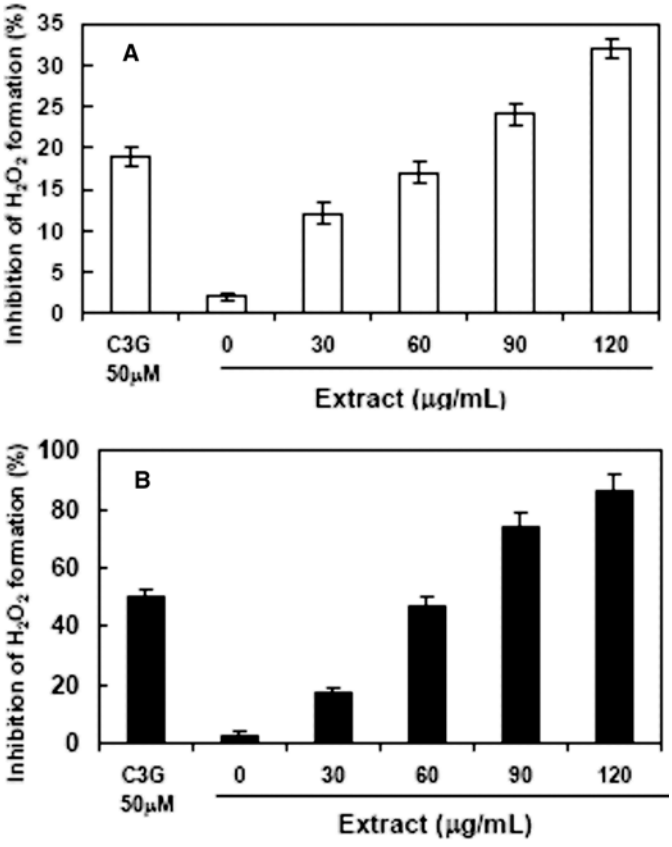


Fig. **3** Deerberry fruit extracts suppressed hydrogen peroxide formation in the *in vitro* H_2O_2-Ti(IV) system (**A**) and HL-60 cells (**B**). **A** *In vitro* H_2O_2-Ti(IV) system, the titanium reagent was added to 200 μL of various concentrations of deerberry fruit extracts (0 to 120 μg/mL) in the presence of NH_4OH and H_2O_2. The absorbance of the supernatant from the Ti-H_2O_2 complex was measured at 410 nm and the inhibition of H_2O_2 production by deerberry fruit extracts was determined against blank control. **B** HL-60 cells were incubated with various concentrations of deerberry fruit extracts (0 to 120 μg/mL) for 1 hour. The intracellular H_2O_2 levels were determined by the red-phenol method as described in Materials and Methods. Cyanidin 3-glucoside (C 3G) at 50 μM was used as positive control.

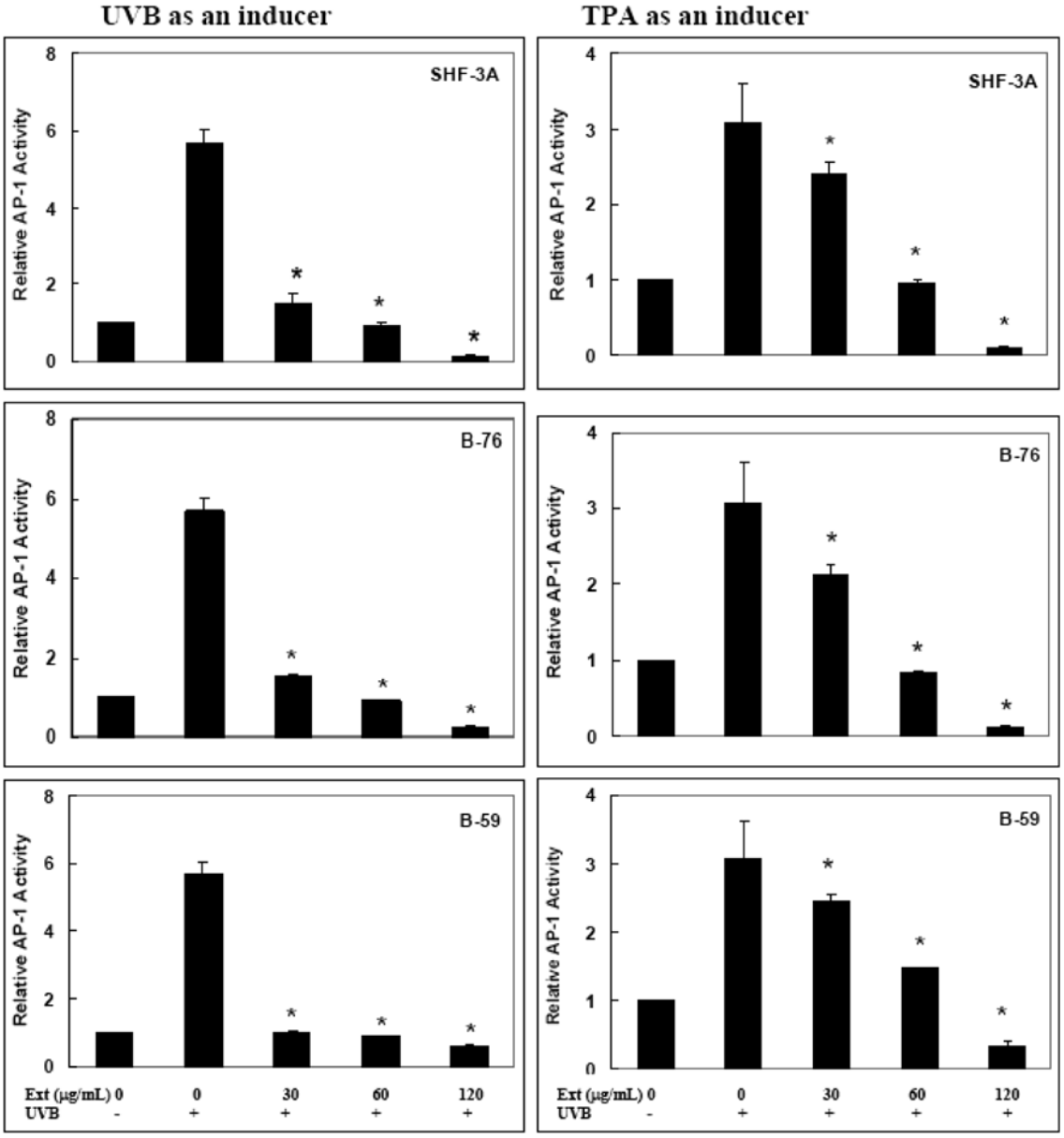


Fig. **4** Deerberry fruit extracts suppress UVB- or TPA-induced AP-1 activity (**A**) and NF-κB activity (**B**).
A JB6 P^+ mouse epidermal cells that were stably transfected with an AP-1 luciferase or a NF-κB luciferase reporter plasmid were cultured as described in Materials and Methods. The cells were pretreated with or without various concentrations of deerberry fruit extracts as indicated for 1 h, and then were exposed to UVB (4 kJ/m^2) or TPA (20 ng/mL) and cultured for an additional 48 h. AP-1or NF-κB activity was determined by luciferase assay. Results, presented as relative AP-1 induction or relative NF-κB induction compared to untreated control cells, were expressed as means and standard errors from three wells. The experiment was repeated three times. * Indicates a significant inhibition of UVB- or TPA-induced AP-1 or NF-κB activation by deerberry ('B-59', 'B-76', 'SHF-3A') extracts ($p \leq 0.05$).

Measurement of ROS

For electron spin resonance (ESR) determination of $^{\cdot}OH$ and $O_2^{\cdot -}$, measurements were conducted using a Varian E9 ESR spectrometer (Bruker Instruments; Billerica, MA, USA) and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using K_3CrO_8 and DPPH as reference standards. An EPRDAP 2.0 program was used for data acquisition and analysis. The scavenging efficiency of $^{\cdot}OH$ or $O_2^{\cdot -}$ radicals by deerberries was measured by ESR using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as the spin trap according to the procedures described previously [13]. Experiments were performed at room temperature and under ambient air.

The *in vitro* assay for inhibition of H_2O_2 levels by deerberry fruit extracts was carried out as described earlier [14]. The final results were expressed as percent inhibition of H_2O_2 levels in a Ti(IV)-H_2O_2 complex system in the presence of deerberry fruit extracts.

For determining the inhibition of deerberry fruit extracts on intracellular H_2O_2 production the analysis was carried out in HL-60 cells. HL-60 cells were incubated with deerberry fruit extracts at the designated dose for 1 hour. The intracellular H_2O_2 production was determined based on the horseradish peroxidase-dependent oxidation of phenol red as described by Pick and Keisari [15].

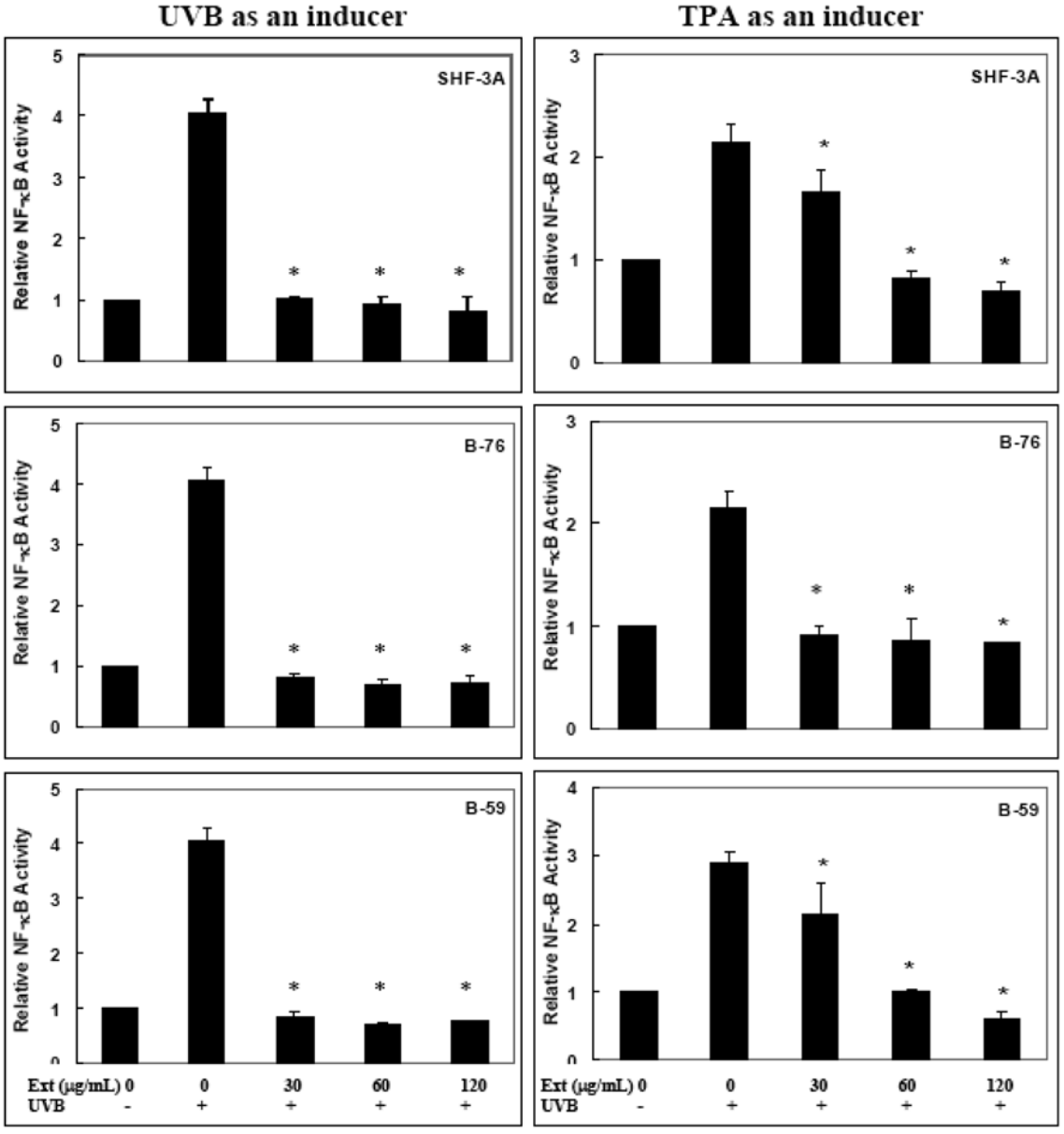


Fig. **4** Deerberry fruit extracts suppress UVB- or TPA-induced AP-1 activity (**A**) and NF-κB activity (**B**).
B JB6 P^+ mouse epidermal cells that were stably transfected with an AP-1 luciferase or a NF-κB luciferase reporter plasmid were cultured as described in Materials and Methods. The cells were pretreated with or without various concentrations of deerberry fruit extracts as indicated for 1 h, and then were exposed to UVB (4 kJ/m^2) or TPA (20 ng/mL) and cultured for an additional 48 h. AP-1or NF-κB activity was determined by luciferase assay. Results, presented as relative AP-1 induction or relative NF-κB induction compared to untreated control cells, were expressed as means and standard errors from three wells. The experiment was repeated three times. * Indicates a significant inhibition of UVB- or TPA-induced AP-1 or NF-κB activation by deerberry ('B-59', 'B-76', 'SHF-3A') extracts ($p \leq 0.05$).

Cell culture and assay of AP-1 and NF-κB activities *in vitro*

JB6 P^+ mouse epidermal cells were cultured as described above and a confluent monolayer of cells was trypsinized, and 5×10^4 viable cells (suspended in 1 mL of EMEM supplemented with 5% FBS) were added to each well of a 24-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for twelve hours. After incubation, cells were cultured in EMEM supplemented with 0.5% FBS for 12 – 24 h to minimize basal AP-1 activity or NF-κB activity before treatment for 1 h with or without deerberry extracts at the concentrations indicated. The cells were then exposed to TPA (20 ng mL^{-1}) or UVB (4 kJ m^{-2}) irradiation in the same medium for additional hours to monitor the effects on AP-1 or NF-κB activation. The cells were extracted with 200 μL of lysis buffer provided in the luciferase assay kit by the manufacturer. Luciferase activity was measured using a Monolight luminometer, model 3010. The results were expressed as relative AP-1 or NF-κB activity compared with untreated controls.

Protein kinase phosphorylation assay

Immunoblots for phosphorylation of ERKs, JNKs, p38 and MEK $^1/_2$ kinases were carried out using PhosphoPlus MAPK antibody kits as described in the protocol of New England BioLabs, using both phosph-specific and non-phospho-specific antibodies [16]. The protein contents in cell lysates were determined by Pierce Protein Assay Reagents (Rockford, IL, USA).

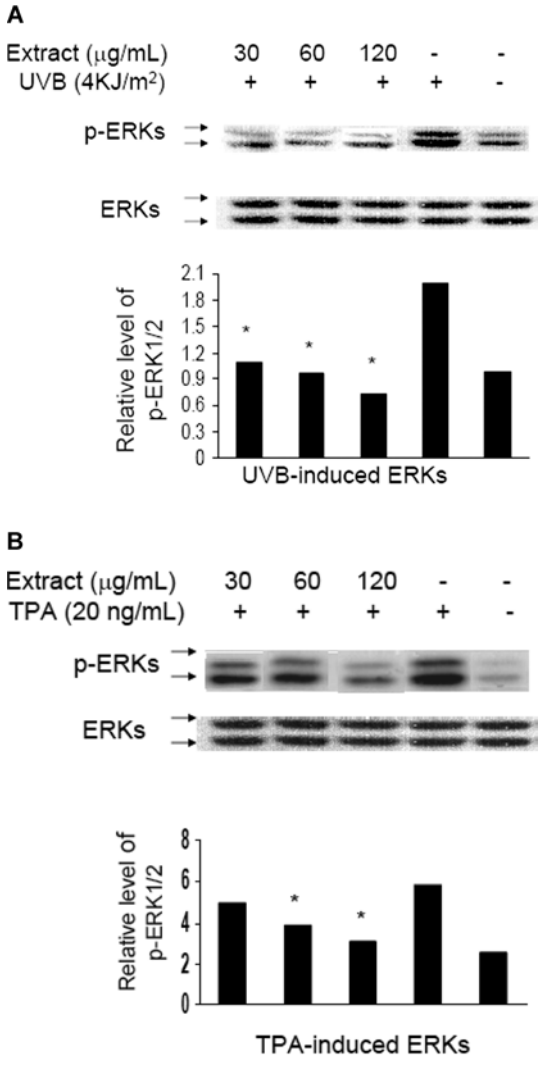


Fig. **5** Inhibition of UVB (**A**)-or TPA (**B**)-induced induced activation of ERKs by deerberry fruit extracts. JB6 P^+ mouse epidermal cells were pretreated with various concentrations of deerberry ('B-76') fruit extracts for 1 h and then exposed to UVB radiation (4 kJ/m^2) for 30 min or exposed to TPA (20 ng/mL) for 30 min. The lysates were separated by SDS-PAGE and analyzed by Western blot with phospho-specific antibody against phosphorylated sites of ERKs. Phosphorylated and non-phosphorylated proteins were detected by using the same transferred membrane blot following a stripping procedure. One representative of three similar results is shown. The histogram shows the densitometric analysis of phosphorylated protein expression normalized to total ERKs.

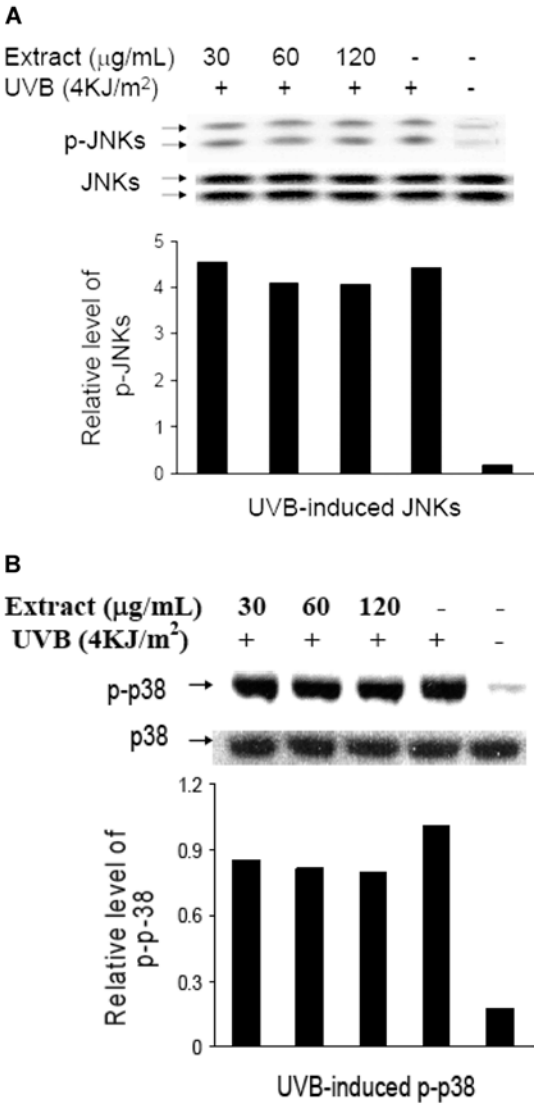


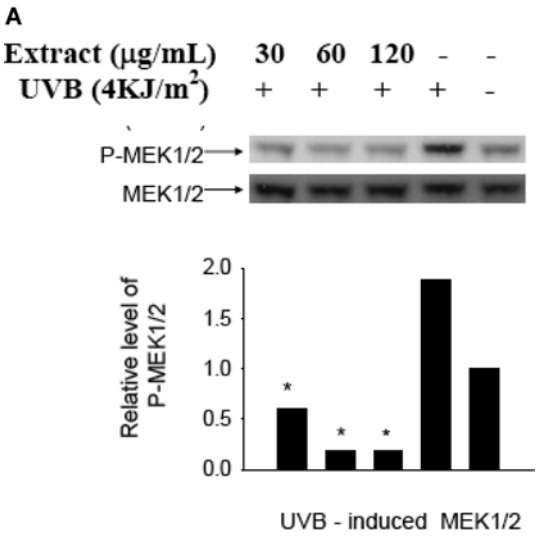
Fig. **6** Inhibition of UVB-induced activation of JNKs (**A**) or p-38 (**B**) by deerberry fruit extracts. JB6 P^+ mouse epidermal cells were pretreated with various concentrations of deerberry ('B-76') fruit extracts for 1 h and then exposed to UVB radiation (4 kJ/m^2). The cell lysates were separated by SDS-PAGE and analyzed by Western blot with phospho-specific antibody against phosphorylated sites of JNKs. Data were representative of three experiments. Histograms show the densitometric analysis of phosphorylated protein expression normalized to total MAPK.

Inhibition of proliferation of cancer cells

Two cell lines, human lung cancer A549 cells, and HL-60 human leukemia cells, were cultured as described above. Subcultures were carried out every 2 – 3 days using a 0.25% trypsin and 0.02% EDTA solution. Briefly, A549 and HL-60 cells were plated in their growth medium at a density of 1×10^4 cells/well in 96 well-flat bottomed well cell culture plates and incubated at 37 °C. Twenty-four hours after plating, 0.33 μL of indicated doses of deerberry fruit extracts (30 to 120 μg/mL) was added to each well (except for control wells). Following 48 h incubation, 10 μL MTT solution were added in each well to form formazan salt crystals and the plates were further incubated for 4 h. Then 100 μL solubilization solution (10% SDS in 0.01 M HCl) were added and the plate was incubated overnight at 37 °C. The amount of formazan produced was proportional to the number of viable cells [17]. After incubation, the MTT-formazan was solubilized in 2-propanol and the optical density was measured at a wavelength of 575 nm and a reference wavelength of 690 nm using a Microplate Spectrophotometer (Spectra MAX™ 250; Molecular Devices, Inc.; Sunnyvale, CA, USA), where higher OD values indicated more cell proliferation. Proliferation was expressed as a percentage of cell growth in wells that received no extract. Cyanidin 3-glucoside (50 μM) was used as a positive control. Data are expressed as mean ± S.E. of three samples.

Induction of apoptosis in cancer cells

HL-60 human leukemia cells and JB6 P^+ mouse epidermal cell line were used in this study. To test whether deerberry extracts



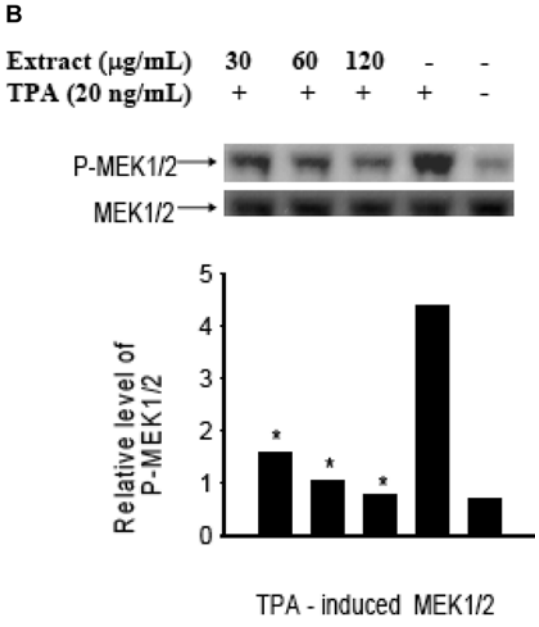


Fig. **7** Inhibition of UVB (**A**)- or TPA (**B**)-induced phosphorylation of MEK 1/2 deerberry fruit extracts. JB6 mouse epidermal cells were pretreated with different concentrations of deerberry ('B-76') fruit extracts for 1 h and exposed to UVB (4 kJ/m^2) or TBA (20 ng/mL). Phosphorylated MEK 1/2 in cell lysate was determined, using a phospho-specific antibody from Cell Signaling Technology. The phosphorylated proteins and non-phosphorylated proteins were detected using the same transferred membrane blot following a stripping procedure. Data were representative of three experiments. Histograms show the densitometric analysis of phosphorylated protein expression normalized to total MEK 1/2.

possessed any apoptotic induction on cancer cells, HL-60 cells and JB6 P$^+$ were treated with deerberry extracts (30 to120 μg/mL) for 18 h. Cells were assessed for typical apoptotic morphology by staining with 10 μmol/L bis-benzimide Hoechst 33258 fluorochrome (Molecular Probes) for 30 min. Apoptotic cells were counted with a fluorescence microscope, and photographed using a digital video camera (Pixera; Los Gatos, CA, USA). Approximately 200 – 400 cells per group were assessed in randomly selected fields to avoid experimental bias.

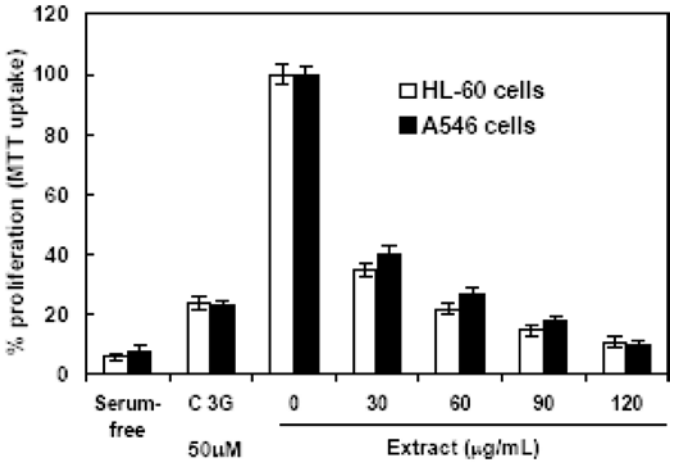


Fig. **8** Inhibition of proliferation of cancer cells by deerberry fruit extracts. (**A**) HL-60 human leukemia cells and (**B**) human lung cancer cells, A549 (1×10^4), were incubated with various concentrations of deerberry ('B-76') fruit extracts (0 to 180 μg/mL) for 48 h and cell proliferation was determined by the MTT assay. Proliferation was expressed as a percentage of cell growth in wells that received no extract. Cyanidin 3-glucoside (C 3G) at 50 μM was used as positive control. Data are expressed as mean ± S.E. of three samples.

Statistical analysis

Data presented are the means ± S.E. values. All statistical analyses were performed with NCSS Statistical Analysis System (StatisticalSystem for Windows; Kaysville, UT, USA) [18]. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered significant at $p \leq 0.05$.

Results and Discussion

ESR was utilized to measure the ability of deerberry extract to scavenge $^{\cdot}OH$ and $O_2^{\cdot -}$ radicals. Addition of deerberry (B-76) extracts (50 mg mL^{-1}) reduced the signal intensity by 88% for $^{\cdot}OH$ radicals (Fig. **2A**) and 90% for $O_2^{\cdot -}$ radicals (Fig. **2B**). In the standard *in vitro* assay, we found that deerberry fruit extracts demonstrated concentration-dependent inhibition of H_2O_2 production in a Ti(IV)-H_2O_2 complex system (Fig. **3A**). When human leukemia HL-60 cells were treated with deerberry fruit extracts, there was also a reduction of intracellular of H_2O_2 level in a dose-dependent manner (Fig. **3B**).

Since AP-1 and NF-κB play a critical role in tumorgenesis induced by carcinogens, we assessed the effects of deerberry extract on AP-1 and NF-κB activation. Pretreatment of JB6 cells with deerberry extracts (30 to 120 μg mL^{-1}) produced an inhibition on AP-1 activation induced by either UVB or TPA (Fig. **4A**). AP-1 activity induced by either UVB or TPA was inhibited by 74 – 97% or 20 – 98%, respectively, with deerberry extracts in the dose range of 30 to 120 μg mL^{-1}. Similarly, the NF-κB activity induced by UVB or TPA was also inhibited by deerberry extracts (Fig. **4B**).

MAP kinases have been implicated in the activation of AP-1. Therefore, we investigated the effect of deerberry extracts on UVB- or TPA-induced MAP kinase activity by assessing the phosphorylation of MAPK family members and the upstream regula-

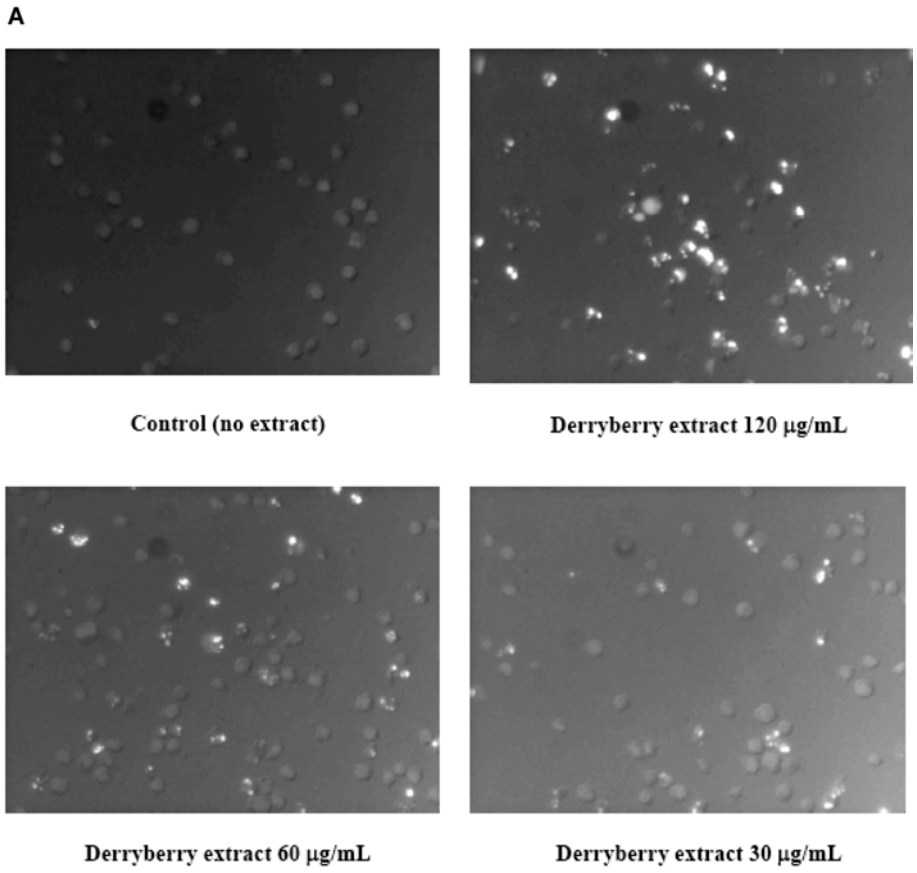


Fig. **9** Deerberry fruit extracts induced human leukemia HL-60 cells apoptosis but JB6 P^+ mouse epidermal cells were resistant to deerberry fruit extracts induced apoptosis. Human leukemia HL-60 cells and JB6 cells were treated with indicated doses of deerberry ('B-76') fruit extracts (0 to 180 μg/mL) for 18 h. The apoptotic HL-60 cells were detected by Hoechst 33 258 staining (**A**). The percent apoptotic cells were calculated by determining the number of cells with nuclear morphology change divided by the total number of cells (**B**). Cyanidin 3-glucoside (C 3G) at 50 μM was used as positive control. Data are expressed as mean ± S.E. of three samples.

tor MEK $^1/_2$ kinase. Pretreatment of JB6 cells with deerberry extracts resulted in a significant inhibition of UVB-induced phosphorylation of ERKs in a dose-dependent manner (Fig. **5A**). However, deerberry extracts showed no effect on JNKs activation and little effect on p38 kinase activation (Figs. **6A** and **6B**), respectively. Deerberry extracts also inhibited phosphorylation of MEK $^1/_2$ kinase, the upstream regulator of MAPKs (Fig. **7A**). Similar results were obtained in TPA-induced phosphorylation of ERKs and MEK $^1/_2$ kinase (Figs. **5B** and **7B**).

The proliferation of human lung cancer A549 cell and HL-60 human leukemia cells were inhibited in a dose-dependent fashion after exposure to the deerberry fruit extracts (30 to 180 μg mL^{-1}) (Fig. **8**). Furthermore, deerberry extracts induced apoptosis in human promyelocytic leukemia HL-60 cells. As shown in Fig. **9A** and **9B**, deerberry extracts induced apoptosis in HL-60 cells in a dose-dependent manner**.** In contrast, the extracts did not cause apoptosis in non-tumor JB6 cells (Fig. **9B**).

The results from this study showed that deerberries had high free radical scavenging capacity and also had anti-cancer properties in human lung carcinoma A549 and human leukemia HL-60 cell lines. At the concentration of 30 to 180 μg mL^{-1} of deerberry extract, AP-1 and NF-κB activity induced by TPA or UVB was de-

creased. Inhibition of AP-1 and NF-κB activity has been shown to lead to suppression of cell transformation [19]. Therefore, deerberry extracts showed inhibition against tumor promoter-induced carcinogenic processes and the inhibitory effect of deerberry extracts on AP-1 and NF-κB activation noted in this study may have a beneficial role in chemoprevention.

Studies indicate that ERKs, JNKs, and p38 kinase are key molecules activated in response to oxidant injury, such as that induced by UVB and TPA. AP-1 is a downstream target of these three MAP kinases. We found that deerberry extracts could scavenge free radicals and inhibit UVB- or TPA-induced phosphorylation of ERKs and MEK $^1/_2$ kinase. These observations suggest that blocking UVB- and TPA-induced AP-1 MAPK activation by deerberry extracts may be due to their antioxidant properties.

Cell proliferation is one of the key factors in the development of cancer. Aberrations in the regulation of a number of key pathways controlling cell proliferation are necessary for the establishment of all tumors. Deregulation of cell proliferation together with suppressed apoptosis is the minimal common platform for all cancer evolution and progression [20]. We found that proliferation of human lung cancer A549 cells and human leukemia HL-60 cells were inhibited after exposure to deerberry extracts. Dietary freeze-dried strawberries were shown to effectively inhibit *N*-nitrosomethylbenzylamine-induced tumorigensis in the rat esophagus [20]. Several other fruits have also shown inhibition of $HepG_2$ human liver cancer cell proliferation [21], [22], [23], [24], [25].

Deerberries also induced apoptosis in human leukemia HL-60 cells in a dose-dependent manner. The induction of apoptosis in HL-60 cells by deerberry extracts suggested that some compounds contained in deerberries possess therapeutic activity for human leukemia. As compared with non-neoplastic cells, cancer cells constitutively generate large but non-lethal amounts of ROS that apparently function as signaling molecules in the MAPK pathway to constantly activate redox-sensitive transcription factors and responsive genes. These gene products are involved in the survival of cancer cells as well as their proliferation. From these perspectives, Toyokuni et al. [26] proposed the concept of "persistent oxidative stress in cancer cells". Reducing oxidative stress may suppress the proliferation of tumor cells. The results of the current study demonstrate that deerberry extracts exhibited a strong antioxidant capacity. The induction of apoptosis of HL-60 cancer cells by deerberry extracts may be due to its antioxidant properties by inhibiting the favorable redox-induced ERK and MEK $^1/_2$ activation in cancer cells. All these data suggest that deerberries function as a potential chemopreventive and chemotherapeutic agent. The antioxidant activity and the apoptosis induction of human leukemia HL-60 cells were higher in deerberries 'B-76' than in lingonberries 'Sanna' or strawberries 'Earliglow' [24], [25].

Thus, in this study, we further elucidate the mechanisms by which phytochemicals may prevent carcinogenesis. Deerberry fruit extracts scavenged ROS and inhibited AP-1 and NF-κB activation, possibly by interfering with signal transduction events involving MAP kinases. These studies open a promising area of investigation in understanding the molecular mechanisms responsible for the beneficial effects of phytochemicals on health.

Acknowledgements

We thank Sue Kim for technical assistance

References

[1] Ballinger WE, Maness EP, Ballington JR. Anthocyanin and total flavonol content of *Vaccinium stamineum* L. Fruit. Scientia Hortic 1981; 15: 173 – 8.
[2] Ballington JR, Kirkman WB, Ballinger WE, Maness E. Anthocyanin, aglycone, and aglycone-sugar content in the fruits of temperate North American species of four sections in *Vaccinium*. J Am Soc Hortic Sci 1988; 113: 746 – 9.
[3] Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR. Resveratrol, pterostilbene, and piceatannol in *Vaccinium* berries. J Agric Food Chem 2004; 52: 4713 – 9.
[4] Ames BM, Shigena MK, Hagen TM. Oxidants, antioxidants and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993; 90: 7915 – 22.
[5] Heinonen IM, Meyer AS, Frankel EN. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. J Agric Food Chem 1998; 46: 4107 – 12.
[6] National Cancer Institute. Diet, nutrition and cancer prevention. The Good News. Review Edition. Bethesda: NCI; 1992.
[7] Rice-Evans CA, Miller NJ. Antioxidant activities of flavonoids as bioactive components of food. Biochem Soc Trans 1996; 24: 790 – 5.
[8] Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 1996; 14: 649 – 83.
[9] Bode AM, Dong Z. Signal transduction pathways: targets for chemoprevention of skin cancer. Lancet Oncol 2000; 1: 181 – 8.
[10] Hou DX, Kai K, Li JJ, Lin S, Terahara N, Wakamatsu et al. Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure-activity relationship and molecular mechanisms. Carcinogenesis 2004; 25: 29 – 36.
[11] Schulze-Osthoff K, Ferrari D, Riehemann K, Wesselborg S. Regulation of NF-kappa B activation by MAP kinase cascades. Immunobiology 1997; 198: 5 – 49.
[12] Li JJ, Westergaard C, Ghosh P, Colburn NH. Inhibitors of both nuclear factor-kappaB and activator protein-1 activation block the neoplastic transformation response. Cancer Res 1997; 57: 3569 – 76.
[13] Leonard SS, Xia C, Jiang BH, Stinefelt B, Klandorf H, Harris GK et al. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. Biochem Biophys Res Commun 2003; 309: 017 – 26.
[14] Patterson BD, MacRae EA, Ferguson IB. Estimation of hydrogen peroxide in plant extracts using titanium(IV). Anal Biochem 1984; 139: 487 – 92.
[15] Pick E, Keisari Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods 1980; 38: 61 – 70.
[16] Ding M, Dong Z, Chen F, Pack D, Ma WY, Ye J et al. Asbestos induces activator protein-1 transactivation in transgenic mice. Cancer Res 1999; 59: 884 – 9.
[17] Mosmann T. Rapid colorimetric assay for cellular growth and survival – Application to proliferation and cyto-toxicity assays. J Immunol Methods 1983; 65: 55 – 63.
[18] NCS S. Statistical analysis and graphics. Statistical software. Kaysville, Utah: 2007.
[19] Dong Z, Ma W, Huang C, Yang CS. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (–)-epigallocatechin gallate, and theaflavins. Cancer Res 1997; 57: 4414 – 9.
[20] Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature 2001; 411: 342 – 8.

[21] Carlton PS, Kresty LA, Siglin JC, Morse MA, Lu J, Morgan C et al. Inhibition of *N*-nitrosomethylbenzylamine-induecd tumorigensis in the rat esophagus by dietary freeze-dried strawberries. Carcinogensis 2001; 3: 441 – 6.
[22] Sun J, Chu YF, Wu X, Liu RH. Antioxidant and antipropliferation activities of fruits. J Agric Food Chem 2002; 50: 7449 – 754.
[23] Meyers KJ, Watkins CB, Pritts MP, Liu RH. Antioxidant and antiproliferative activities of strawberries. J Agric Food Chem 2003; 51: 6887 – 92.
[24] Wang SY, Feng R, Bowman L, Penhallegon R, Ding M, Lu Y. Antioxidant activity in lingonberries (*Vaccinium vitis-idaea* L) and its inhibory effect on activator protein 1, nuclear factor-κB, and MAPK-activated protein kinases activation. J Agric Food Chem 2005; 53: 3156 – 66.
[25] Wang SY, Feng R, Lu Y, Bowman L, Ding M. Inhibitory effect on activator protein-1, nuclear factor-kappaB, and cell transformation by extracts of strawberries (*Fragaria* x *ananassa* Duch.). J Agric Food Chem 2005; 53: 4187 – 93.
[26] Toyokuni S, Okamoto K, Yodoi J, Hiai H. Persistent oxidative stress in cancer. FEBS Lett 1995; 358: 1 – 3.