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# Antioxidant Activity in Lingonberries (*Vaccinium vitis-idaea* L.) and Its Inhibitory Effect on Activator Protein-1, Nuclear Factor-κB, and Mitogen-Activated Protein Kinases Activation

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Lingonberry has been shown to contain high antioxidant activity. Fruits from different cultivars of lingonberry (*Vaccinium vitis-idaea* L.) were evaluated for fruit quality, antioxidant activity, and anthocyanin and phenolic contents. The fruit soluble solids, titratable acids, antioxidant capacity, and anthocyanin and phenolic contents varied with cultivars. Lingonberries contain potent free radical scavenging activities for DPPH•, ROO•, •OH, and $O_2^{\bullet -}$ radicals. Pretreatment of JB6 $P^+$ mouse epidermal cells with lingonberry extracts produced a dose-dependent inhibition on the activation of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) induced by either 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or ultraviolet-B (UVB). Lingonberry extract blocked UVB-induced phosphorylation of the mitogen-activated protein kinase (MAPK) signaling members ERK1, ERK2, p38, and MEK1/2 but not JNK. Lingonberry extract also prevented TPA-induced phosphorylation of ERK1, ERK2, and MEK1/2. Results of soft agar assays indicated that lingonberry extract suppressed TPA-induced neoplastic transformation of JB6 $P^+$ cells in a dose-dependent manner. Lingonberry extract also induced the apoptosis of human leukemia HL-60 cells in a dose-independent manner. These results suggest that ERK1, ERK2, and MEK1/2 may be the primary targets of lingonberry that result in suppression of AP-1, NF-κB, and neoplastic transformation in JB6 $P^+$ cells and causes cancer cell death by an apoptotic mechanism in human leukemia HL-60 cells.

**KEYWORDS: Antioxidant activity; activator protein-1; apoptosis; nuclear factor-κB; mitogen-activated protein kinase; neoplastic transformation; *Vaccinium vitis-idaea***

## INTRODUCTION

Free radicals (ROO•, $O_2^{\bullet -}$, •OH, and $^1O_2$) are formed during normal cell metabolism (*1*). There are mechanisms to detoxify these free radicals in plant cells. Small fruits have been shown to have a high content of antioxidant compounds and anticarcinogenic components (*2*−*4*). Consumption of these fruits has been associated with lower incidence and mortality rates of cancer in several human cohort and case control studies (*2*, *5*−*7*). A high intake of small fruits has also been reported to prevent urinary tract infections, enhance the immune function, and reduce blood pressure (*8*−*11*).

Lingonberry is a low shrub bearing bright red globular fruits between 0.75 and 1.0 cm in diameter. It is considered a minor crop in the United States and has thus far received little attention for research. There is evidence of growing interest in this crop as demonstrated by the recent increase in lingonberry acreage planted and an increased number of requests for growing information from people throughout the Pacific Northwest and the United States (*12*). There is wide genetic variability in lingonberry genotypes with respect to sugars, acids, and antioxidant content. These properties make lingonberry a possible crop for focusing efforts on optimizing fruit quality and antioxidant content. However, techniques for improving the levels of these compounds in lingonberry fruit do not currently exist.

Our preliminary studies have shown that lingonberries (*Vaccinium vitis-idaea* L.) have a higher antioxidant activity as compared to other berry fruits including blackberries, blueberries, raspberries, strawberries, and cranberries (*4*, *13*−*15*). Reports focusing on the chemopreventive effects of lingonberry fruit are limited. To elucidate the mechanism of

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the antitumorigenic effects of lingonberry, we studied the effects of lingonberry fruit extract on the activation of activator protein-1 (AP-1) and nuclear factor-$\kappa$B (NF-$\kappa$B) and cellular transformation in JB6 $P^+$ cells. AP-1 and NF-$\kappa$B are transcription factors associated with carcinogenesis (*16*). AP-1 is composed of homodimers and/or heterodimers of the JUN and FOS families (*17*). Many stimuli induce the binding of AP-1 to the promoter region of various genes that govern cellular processes such as inflammation, proliferation, and apoptosis (*18*). Inhibition of AP-1 activity has been shown to lead to suppression of cell transformation (*19*). Some chemopreventive agents, including aspirin, tea polyphenols, and retinoic acid, have been reported to inhibit cell transformation and tumor promotion by suppressing AP-1 transactivation (*19*−*21*).

NF-$\kappa$B is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. The NF-$\kappa$B-regulated genes are part of the larger Rel/NF-$\kappa$B family of transcription factors involved mainly in stress-induced, immune, and inflammatory responses. NF-$\kappa$B is also an important regulator in deciding cell fate, such as programmed cell death and proliferation control, and is critical in tumorigenesis (*18*). Reactive oxygen species (ROS) stimulate transcription by activating transcription factors such as AP-1 and NF-$\kappa$B. AP-1 and NF-$\kappa$B signal transduction pathways are important in transformation and tumor promotion (*16*). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and ultraviolet-B (UVB) are carcinogens and can produce ROS and stimulate AP-1 and NF-$\kappa$B activity by activating mitogen-activated protein kinases (MAPK) signaling pathways such as the extracellular signal-regulated kinases 1/2(ERK1/2), c-Jun amino-terminal kinases (JNKs), p38, and MEK1/2 MAP kinases (*22*, *23*).

JB6 $P^+$ mouse epidermal cells are sensitive to tumor promoter treatment and provide a cell culture-based model for studying the mechanism of tumor promotion (*24*). Transformation sensitive JB6 $P^+$ cells are preferred for probing early events in oxidative stress-related signaling leading to carcinogenesis and for identifying the molecular targets for chemoprevention (*25*). Antioxidants and extracts of apple peel have been shown to inhibit AP-1 activity in JB6 cells (*19*, *26*).

Little information is available on the chemopreventive effects of lingonberry fruit. The purpose of this study is to evaluate the antioxidant capacities in different genotypes and developmental stages of lingonberries and to study the potential inhibitory effects of lingonberry extract on TPA- or UVB-induced AP-1 and NF-$\kappa$B activities as well as the underlying signal kinase pathways. The potential of lingonberry extract on induction of human cancer cell death was also investigated. Our results demonstrate the possible chemopreventive activity of lingonberries and its mechanism of the inhibitory actions on tumor promotion.

## MATERIALS AND METHODS

**Chemicals.** 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-di-(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) were obtained from Aldrich Chemical Co. (Milwaukee, WI). 2′,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. (Richmond, VA). Eagle's minimum essential medium (EMEM) and Dulbecco's modified Eagle's medium were obtained from Whittaker Biosciences (Walkersville, MD). Fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were purchased from Life Technologies, Inc. (Gaithersburg, MD). Luciferase assay kits were obtained from Promega (Madison, WI). PhosphoPlus MAPK antibody kits were purchased from New England BioLabs (Beverly, MA). Chelex 100, $FeSO_4$, $H_2O_2$, xanthine, and xanthine oxidase were purchased from Sigma (St. Louis, MO). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich.

**Fruit Samples.** Fruits of different genotypes of lingonberry were harvested from the experimental plots at Fall Creek Farm and Nursery (Lowell, OR); the USDA lab (Corvallis, OR), and Faw Family Farm (Rainier, WA). Approximately 500−900 g of fruit was harvested per genotype. Three replicated plots were developed, and 13 different genotypes were sampled [Ammerland, Erntedank, Erntekrone, Erntesegen, European Red, Ida (8726-8), Koralle, Koralle-German, Red Pearl, Sanna, Scarlett, Splendor, and Sussi]. The green, pink (50% red), and red (mature ripe fruit with well-developed red color) fruits were selected and picked around 0900 h in the first week in August and October and shipped with dry ice overnight to the Beltsville, Maryland laboratory and used for chemical analyses.

Fresh ripe lingonberries were washed with a mild detergent and rinsed three times with Milli-Q deionized water to remove possible pesticide and preservative residues. Lingonberry fruit extract was prepared using 1 mL of distilled water per gram of washed lingonberry fruit and mixed and blended at high speed. The blended homogenate was strained, centrifuged, filter sterilized, and stored at −20 °C. All juice samples were centrifuged at 6000*g*, 4 °C, for 20 min, and the supernatant was filtered. The filtrate was stored at −20 °C until used for measurement of the effects on $^{\bullet}OH$ and $O_2^{\bullet-}$ scavenging, AP-1 and NF-$\kappa$B activation, MAPKs phosphorylation, and anchorage-independent transformation assay.

**Analysis of Soluble Solids Content (SSC) and Titratable Acids (TA).** Lingonberries were pulverized with a cold mortar and pestle and pressed through four layers of cheesecloth to express the juice used for SSC and TA determination. The SSC of the fruit was determined on a digital refractometer Palette 100 PR-100 (ATAGO-Spectrum Technologies, Plainfield, IL) standardized with distilled water. TA was determined by diluting each 5 mL aliquot of lingonberry juice to 100 mL with distilled water and adjusting the pH to 8.2 using 0.1 N NaOH. The acidity was expressed as percent of citric acid equivalent.

**Total Anthocyanin and Total Phenolic Content.** Lingonberries were extracted with 80% acetone containing 0.2% formic acid using a Polytron (Brinkmann Instruments, Inc., Westbury, NY). The homogenized samples from acetone extract and juice (as described above) were then centrifuged at 14000*g* for 20 min at 4 °C. The supernatants were transferred to vials, stored at −80 °C, and later used for anthocyanin, total phenolic, and antioxidant analyses.

The total anthocyanin content in fruit extracts was determined using the pH differential method (*27*). The absorbance was measured in a Shimadzu Spectrophotometer (Shimadzu UV-160) at 510 and 700 nm in buffers at pH 1.0 and 4.5, using $A = [(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}]$ with a molar extinction coefficient of cyanidin-3-galactoside. Results were expressed as milligrams of cyanidin-3-galactoside equivalent per 100 g of fresh weight. Total soluble phenolics in the fruit extracts were determined with Folin−Ciocalteu reagent by the method of Slinkard and Singleton (*28*) using gallic acid as a standard. Results were expressed as milligrams gallic acid equivalent (GAE) per 100 g of fresh weight.

**Oxygen Radical Absorbance Capacity (ORAC) Assay.** The ORAC assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a microplate fluorescence reader (*29*). The automated sample preparation was performed using a Precision 2000 instrument. The sample series dilution sequence was programmed and controlled by the precision power software. The ORAC values were determined by calculating the net area under the curve (AUC) of the standards and samples. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Final ORAC values were calculated using the regression equation between the Trolox concentration and the net AUC and were expressed as micromoles Trolox equivalents (TE) per gram of fresh weight.

Then, the AUC was calculated as

$$\mathrm{AUC} = 0.5 + f_1/f_0 + \ldots f_i/f_0 + \ldots + f_{34}/f_0 + 0.5(f_{35}/f_0)$$

where $f_0$ = initial fluorescence reading at 0 min and $f_i$ = fluorescence reading at time *i*.

The data were analyzed by a Microsoft Excel macro program (Microsoft, Roselle, IL). The net AUC was obtained by subtracting

the AUC of the blank from that of a sample. The relative TE ORAC value was calculated as

$$\text{ORAC value} = [(AUC_{sample} - AUC_{blank})/(AUC_{Trolox} - AUC_{blank})] \text{ (molarity of Trolox/molarity of sample)}$$

**DPPH Assay.** To determine the antioxidant activity of different extracts, DPPH radicals were used. In the radical form, this molecule has an absorbance at 515 nm that disappears with acceptance of an electron from an antioxidant compound to become a stable diamagnetic molecule. The method described by Hatano et al. (*30*) was used with some modifications. For each extract, different concentrations were tested. An aliquot (0.5 mL) of the DPPH solution (50 mg/100 mL) was diluted in 4.5 mL of methanol. Then, 0.1 mL of a methanol solution of the extract was added. The mixtures were shaken vigorously and allowed to stand for 45 min in the dark. The decrease in absorbance was measured at 515 nm against a blank without extract using a spectrophotometer. Using a calibration curve with different amounts of DPPH, the $ED_{50}$ was calculated. The $ED_{50}$ is the concentration of an antioxidant that is required to quench 50% of the initial DPPH radicals under the experimental conditions given.

**Electron Spin Resonance (ESR) Measurement for $^{\bullet}OH$ and $O_2^{\bullet-}$.** ESR measurements were conducted using a Varian E9 ESR spectrometer (Bruker Instruments, Billerica, MA) 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 $^{\bullet}OH$ or $O_2^{\bullet-}$ radicals by lingonberry (cultivar Sanna) extracts was measured by ESR using DMPO as the spin trap according to the procedure described previously (*31*). Experiments were performed at room temperature and under ambient air.

**Cell Culture and Assay of AP-1 and NF-κB Activity in Vitro.** JB6 $P^+$ mouse epidermal cells that stably transfected with AP-1-luciferase or NF-κB−luciferase reporter plasmid (*32*, *33*) were cultured in EMEM containing 5% FBS, 2 mM L-glutamine, and 1% penicillin−streptomycin (10 000 U/mL penicillin and 10 mg/mL streptomycin). A confluent monolayer of JB6 $P^+$ cells was trypsinized, and $5 \times 10^4$ 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% $CO_2$. Twelve hours later, 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 lingonberry (cultivar Sanna) extracts at the concentrations indicated. The cells were then exposed to TPA (20 ng/mL) or UVB (4 kJ/m$^2$) irradiation in the same medium for additional hours to monitor the effects on AP-1 or NF-κB induction. The cells were extracted with 200 μL of lysis buffer provided in the luciferase assay kit by the manufacturer. The luciferase activity was measured using a Monolight luminometer, model 3010 (*34*). The results were expressed as relative AP-1 or NF-κB activity as compared with untreated controls.

**Protein Kinase Phosphorylation Assay.** Immunoblots for phosphorylation of ERKs, JNKs, p38, and MEK1/2 kinases were carried out using PhosphoPlus MAPK antibody kits as described in the protocol of New England BioLabs. Phosphospecific antibodies were used to detect phosphorylated sites of ERKs, JNKs, p38, and MEK1/2 kinase (*34*). Nonphosphospecific antibodies against ERKs, JNKs, p38, and MEK1/2 kinase proteins provided in each assay kit were used to normalize the phosphorylation assay by using the same transferred membrane blot. The protein contents in cell lysates were determined by Pierce Protein Assay Reagents (Rockford, IL).

**Anchorage-Independent Transformation Assay.** JB6 $P^+$ mouse epidermal cells ($10^4$) were exposed to TPA (20 ng/mL) in the presence or absence of lingonberry extracts (cultivar Sanna), in 0.33% Bacto agar containing 20% FBS over 0.5% agar medium containing 15% FBS EMEM medium. The cultures were maintained in a 37°C, 5% $CO_2$ incubator for 2 weeks, and then, the colonies were counted as described by Colburn et al. (*35*).

**Induction of Apoptosis in Cancer Cells.** HL-60 human leukemia cells from American Type Culture Collection (Manassas, VA) were used in this study. To testify if lingonberry (cultivar Sanna) extracts possess any apoptotic induction on cancer cells, human leukemia HL-60 cells were treated with lingonberry extracts for 18 h. Cells were assessed for typical apoptotic nuclear morphology (nuclear shrinkage, condensation, fragmentation, and apoptotic bodies) by staining with 10 μmol/L bis-benzimide Hoechst 33258 fluorochrome (Molecular Probes) for 30 min. The apoptotic morphology was determined with a fluorescence microscope and photographed using a digital video camera (Pixera, Los Gatos, CA). Approximately 200−400 cells per group were assessed randomly in selected fields to avoid experimental bias. The percent apoptotic cells was calculated by determining the number of cells with nuclear morphology change divided by the total number of cells.

**Statistical Analysis.** Data presented are the means ± standard error of values as compared and analyzed using one-way analysis of variance test to assess the statistical significance between treatments. Statistical significance was set at $p \leq 0.05$.

**Table 1.** SSC, TA, and Ratio of SSC to TA in Various Cultivars of Ripe Lingonberry Grown at Different Locations and Harvested at Different Times (August or October)

| cultivar | season | location | SSC% juice | TA% juice | SSC/TA juice |
|---|---|---|---|---|---|
| Ammerland | Oct | Fall Creek | 16.9 | 0.40 | 42.25 |
| Erntedank | Oct | Fall Creek | 13.6 | 0.36 | 37.78 |
| Erntekrone | Oct | Fall Creek | 13.4 | 0.32 | 41.88 |
| Erntesegen | Aug | Fall Creek | 14.4 | 0.33 | 43.64 |
| Erntesegen | Oct | Fall Creek | 13.5 | 0.32 | 42.19 |
| European Red | Aug | Fall Creek | 15.6 | 0.34 | 45.88 |
| Koralle | Aug | Walker | 15.7 | 0.35 | 44.86 |
| Koralle | Aug | Fall Creek | 15.6 | 0.35 | 44.57 |
| Koralle | Oct | Whitham | 16.9 | 0.33 | 51.21 |
| Koralle-German | Oct | Fall Creek | 15.2 | 0.31 | 49.03 |
| Red Pearl | Aug | Walker | 15.9 | 0.38 | 41.84 |
| Red Pearl | Oct | Whitham | 16.1 | 0.39 | 41.28 |
| Sanna | Aug | Walker | 13.3 | 0.31 | 42.90 |
| Scarlett | Oct | Fall Creek | 12.9 | 0.33 | 39.09 |
| Splendor | Aug | Fall Creek | 14.5 | 0.41 | 35.37 |
| Splendor | Oct | Fall Creek | 14.3 | 0.40 | 35.75 |
| Sussi | Aug | Fall Creek | 14.1 | 0.35 | 40.29 |
| 8726–8 | Oct | Fall Creek | 13.9 | 0.33 | 42.12 |
| mean | | | 14.7 | 0.35 | 42.33 |
| significance[a] | | | | | |
| cultivar | | | * | * | * |
| season | | | ns | ns | ns |
| location | | | ns | ns | ns |

[a] * and ns, significant or nonsignificant, respectively, at $p \leq 0.05$.

## RESULTS AND DISCUSSION

**SSC and TA.** The effect of different cultivars, harvest months, and growth locations on SSC, TA, and ratios of SSC/TA is presented in **Table 1**. The fruit SSC, TA, and SSC/TA varied among cultivars. The SSCs were in the range of 12.9−16.9%, the TA was in the range of 0.31−0.41%, and the ratio of SSC/TA ranged from 35.37 to 51.21. Among the cultivars, Ammerland and Koralle had the greatest SSC, Ammerland and Splendor had the most TA, and Koralle had the highest ratio of SSC/TA (**Table 1**). The general flavor selection criteria for berry fruits have been combined in sensory-perceived high sweetness and high acidity. All of the 13 cultivars tested have good and pleasing flavors, suggesting that there are many combinations of SSC and TA that confer good flavor. Examples are high solids and high acids (Ammerland and Red Pearl); high solids and average acids (European Red and Koralle); and average solids and high to medium acidity (Erntesegen and Splendor). The cultivars with high fruit quality could be selected for use in a breeding program. Harvest time (August vs October) and berry growth location in Oregon resulted in no significant effect on fruit SSCs, TAs, and SSC/TA ratios.

**Table 2.** Anthocyanin Content, Total Phenolic Content, and Antioxidant Activity (Scavenging ROO• Radical; ORAC) from Juices and Acetone Extracts in Various Cultivars of Lingonberry at Different Maturities Grown at Different Locations and Harvested at Different Times (August or October)[a]

| | | | | anthocyanin[b] (mg/100 g) | | total phenolic[c] (mg/100 g) | | antioxidant activity[d] ORAC ($\mu$mol TE/g) | |
|---|---|---|---|---|---|---|---|---|---|
| cultivar | season | maturity | location | juice | acetone | juice | acetone | juice | acetone |
| Ammerland | Oct | green | Fall Creek | 3.2 ± 0.3 | 8.1 ± 0.2 | 540.0 ± 12.0 | 1306.4 ± 28.2 | 105.9 ± 6.7 | 223.6 ± 6.5 |
| Ammerland | Oct | red | Fall Creek | 65.2 ± 4.3 | 95.5 ± 11.5 | 443.7 ± 16.2 | 974.3 ± 18.4 | 97.1 ± 2.5 | 159.1 ± 4.8 |
| Erntedank | Oct | green | Fall Creek | 2.7 ± 0.3 | 5.8 ± 1.0 | 481.6 ± 15.8 | 1207.0 ± 25.6 | 106.2 ± 7.9 | 187.8 ± 6.4 |
| Erntedank | Oct | red | Fall Creek | 46.4 ± 5.2 | 57.3 ± 8.1 | 443.2 ± 11.4 | 719.2 ± 18.1 | 81.7 ± 5.2 | 110.7 ± 3.7 |
| Erntekrone | Oct | red | Fall Creek | 29.4 ± 2.4 | 37.9 ± 5.4 | 100.7 ± 10.4 | 540.2 ± 9.5 | 57.9 ± 2.8 | 89.3 ± 4.3 |
| Erntesegen | Aug | green | Fall Creek | 1.7 ± 1.1 | 3.7 ± 1.2 | 579.3 ± 13.2 | 1094.4 ± 15.4 | 94.7 ± 6.7 | 155.9 ± 8.1 |
| Erntesegen | Aug | pink | Fall Creek | 17.4 ± 2.1 | 18.4 ± 3.4 | 481.6 ± 15.3 | 494.0 ± 7.2 | 82.5 ± 4.6 | 110.0 ± 3.5 |
| Erntesegen | Aug | red | Fall Creek | 25.7 ± 3.7 | 57.2 ± 6.7 | 248.3 ± 11.8 | 572.0 ± 3.2 | 66.4 ± 3.5 | 85.4 ± 2.7 |
| Erntesegen | Oct | green | Fall Creek | 1.9 ± 0.5 | 3.1 ± 0.3 | 539.9 ± 16.8 | 1038.7 ± 12.4 | 97.8 ± 6.5 | 162.7 ± 4.2 |
| Erntesegen | Oct | red | Fall Creek | 45.9 ± 6.2 | 50.8 ± 5.6 | 361.3 ± 13.5 | 655.0 ± 11.8 | 74.8 ± 4.7 | 104.2 ± 5.7 |
| European Red | Aug | green | Fall Creek | 2.5 ± 0.4 | 1.8 ± 0.6 | 570.4 ± 16.3 | 931.7 ± 13.1 | 106.3 ± 8.2 | 133.1 ± 6.2 |
| European Red | Aug | pink | Fall Creek | 20.4 ± 3.2 | 21.2 ± 2.7 | 656.6 ± 18.2 | 744.7 ± 12.6 | 93.4 ± 3.4 | 123.8 ± 5.4 |
| European Red | Aug | red | Fall Creek | 49.7 ± 6.2 | 55.1 ± 6.7 | 291.3 ± 12.7 | 721.5 ± 14.2 | 70.5 ± 4.1 | 110.0 ± 3.2 |
| Koralle | Aug | green | Walker | 1.8 ± 0.2 | 2.2 ± 0.7 | 590.0 ± 14.7 | 739.4 ± 11.1 | 118.9 ± 9.2 | 122.2 ± 5.6 |
| Koralle | Aug | pink | Walker | 18.4 ± 3.1 | 25.3 ± 3.3 | 392.4 ± 13.2 | 714.4 ± 12.3 | 71.0 ± 5.3 | 128.6 ± 4.9 |
| Koralle | Aug | red | Walker | 42.4 ± 6.5 | 54.4 ± 5.2 | 403.2 ± 12.2 | 566.5 ± 13.5 | 79.7 ± 5.6 | 95.3 ± 2.7 |
| Koralle | Aug | green | Fall Creek | 1.0 ± 0.9 | 1.5 ± 1.8 | 544.6 ± 15.1 | 725.0 ± 6.3 | 107.6 ± 7.5 | 136.6 ± 5.6 |
| Koralle | Aug | pink | Fall Creek | 21.4 ± 2.2 | 26.4 ± 3.2 | 416.3 ± 13.7 | 862.4 ± 13.3 | 79.8 ± 4.3 | 118.3 ± 5.4 |
| Koralle | Aug | red | Fall Creek | 52.3 ± 4.6 | 57.3 ± 6.6 | 411 ± 11.6 | 505.2 ± 12.4 | 83.4 ± 5.1 | 108.2 ± 6.1 |
| Koralle | Oct | red | Whitham | 59.9 ± 7.6 | 87.4 ± 5.2 | 419.2 ± 10.5 | 891.5 ± 14.7 | 92.0 ± 6.4 | 136.7 ± 4.2 |
| Koralle-German | Oct | red | Fall Creek | 43.8 ± 4.3 | 63.8 ± 4.1 | 444.6 ± 15.4 | 879.0 ± 12.5 | 86.5 ± 4.8 | 120.2 ± 3.8 |
| Red Pearl | Aug | green | Walker | 2.1 ± 0.8 | 2.1 ± 1.4 | 300.4 ± 12.5 | 722.7 ± 12.3 | 79.7 ± 5.2 | 108.1 ± 5.4 |
| Red Pearl | Aug | pink | Walker | 14.2 ± 1.1 | 17.9 ± 2.7 | 432.0 ± 13.2 | 440.3 ± 8.1 | 76.8 ± 4.6 | 92.9 ± 2.7 |
| Red Pearl | Aug | red | Walker | 48.7 ± 7.6 | 54.7 ± 6.1 | 406.0 ± 12.5 | 513.0 ± 9.1 | 64.9 ± 5.8 | 73.2 ± 4.3 |
| Red Pearl | Oct | red | Whitham | 52.2 ± 8.2 | 62.1 ± 5.9 | 450.7 ± 11.4 | 540.7 ± 7.2 | 81.5 ± 4.2 | 91.3 ± 5.4 |
| Sanna | Aug | green | Walker | 0.5 ± 2.0 | 2.7 ± 0.4 | 168.2 ± 12.4 | 560.6 ± 8.1 | 65.5 ± 4.1 | 94.3 ± 2.7 |
| Sanna | Aug | pink | Walker | 12.9 ± 4.8 | 19.4 ± 2.3 | 196.1 ± 13.1 | 345.0 ± 7.2 | 60.7 ± 2.6 | 70.8 ± 6.2 |
| Sanna | Aug | red | Walker | 28.1 ± 2.6 | 30.8 ± 3.4 | 95.4 ± 12.7 | 285.1 ± 8.3 | 54.8 ± 4.5 | 58.5 ± 2.3 |
| Scarlett | Oct | red | Fall Creek | 42.4 ± 3.9 | 40.5 ± 4.3 | 274.3 ± 6.8 | 551.1 ± 11.8 | 71.2 ± 2.9 | 80.8 ± 2.9 |
| Splendor | Aug | green | Fall Creek | 1.6 ± 1.1 | 7.1 ± 0.5 | 482.6 ± 14.2 | 1140.9 ± 21.0 | 85.7 ± 3.4 | 158.0 ± 6.2 |
| Splendor | Aug | pink | Fall Creek | 25.3 ± 3.0 | 28.1 ± 1.5 | 756.3 ± 21.7 | 842.5 ± 13.5 | 94.8 ± 5.8 | 135.2 ± 8.2 |
| Splendor | Aug | red | Fall Creek | 34.3 ± 8.7 | 59.6 ± 5.0 | 425.3 ± 12.7 | 549.3 ± 6.7 | 79.4 ± 3.2 | 98.9 ± 3.6 |
| Splendor | Oct | red | Fall Creek | 45.4 ± 1.9 | 55.8 ± 4.6 | 445.0 ± 15.4 | 508.8 ± 13.9 | 82.6 ± 2.6 | 93.1 ± 4.2 |
| Sussi | Aug | green | Fall Creek | 3.1 ± 0.9 | 8.3 ± 1.2 | 985.5 ± 25.4 | 1199.4 ± 20.4 | 135.8 ± 9.7 | 199.6 ± 5.8 |
| Sussi | Aug | pink | Fall Creek | 22.1 ± 2.2 | 25.3 ± 2.1 | 774.3 ± 15.6 | 1273.2 ± 16.3 | 99.4 ± 4.2 | 170.1 ± 6.5 |
| Sussi | Aug | red | Fall Creek | 60.8 ± 6.8 | 92.2 ± 12.8 | 765.4 ± 12.8 | 1067.1 ± 18.8 | 98.4 ± 3.6 | 154.3 ± 5.9 |
| Ida (8726-8) | Oct | red | Fall Creek | 47.5 ± 5.6 | 62.9 ± 6.1 | 386.3 ± 12.7 | 789.1 ± 12.9 | 78.6 ± 4.2 | 112.5 ± 3.2 |
| mean | | | | | | | | | |
| green | | | | 2.1 | 4.2 | 525.6 | 969.5 | 100.3 | 151.4 |
| pink | | | | 18.8 | 23.0 | 513 | 688.6 | 82.3 | 115.9 |
| red | | | | 45.4 | 60.1 | 378.6 | 663.0 | 78 | 106.7 |
| significance[e] | | | | | | | | | |
| cultivar | | | | * | * | * | * | * | * |
| maturity | | | | * | * | * | * | * | * |
| season | | | | ns | ns | ns | ns | ns | ns |
| location | | | | ns | ns | ns | ns | ns | ns |
| extraction (juice vs acetone extract) | | | | * | * | * | * | * | * |

[a] Data expressed as means ± SEM. [b] Data expressed as milligrams of cyanidin 3-glucoside equivalents per 100 grams of fresh weight. [c] Data expressed as milligrams of GAEs per 100 grams of fresh weight. [d] Data expressed as micromoles of TE per gram of fresh weight. [e] * and ns, significant or nonsignificant, respectively, at $p \leq 0.05$.

**ORAC, Total Anthocyanin, and Total Phenolic Concentrations.** The antioxidant capacity of lingonberry fruit is expressed as ORAC. The ORAC assay depends on free radical damage to a fluorescent probe manifested by a change in its fluorescence intensity. This change in fluorescence intensity is an index of the severity of free radical damage. Therefore, the inhibition of free radical damage by an antioxidant is reflected in its protection against the change of probe fluoresence in the ORAC assay and is a measure of its antioxidant capacity against the free radical (*29*). The ORAC assay is unique in that the reaction is driven to completion, and quantitation is achieved using the net AUC. The AUC technique allows ORAC to combine both inhibition time and inhibition percentage of the free radical damage by the antioxidant into a single quantity. The advantage of the ORAC assay over other methods has been reviewed by Cao and Prior (*36*).

Significant differences were found in ORAC, total anthocyanin, and total phenolic contents among different cultivars and various maturity of lingonberry (**Table 2**). Lingonberry fruits harvested during their green stage consistently yielded the highest ORAC values and total phenolic contents. This may be due to procyanidin contents in the green fruit. From previous research, we also found that blackberries, black raspberries, and strawberries had the highest ORAC values and total phenolic contents during the green stages (*4*). The acetone extraction yielded fruit with higher ORAC, total anthocyanin, and total phenolic contents. The ORAC values from acetone extraction for lingonberries ranged from 58.5 to 223.6 $\mu$mol of TE/g fresh

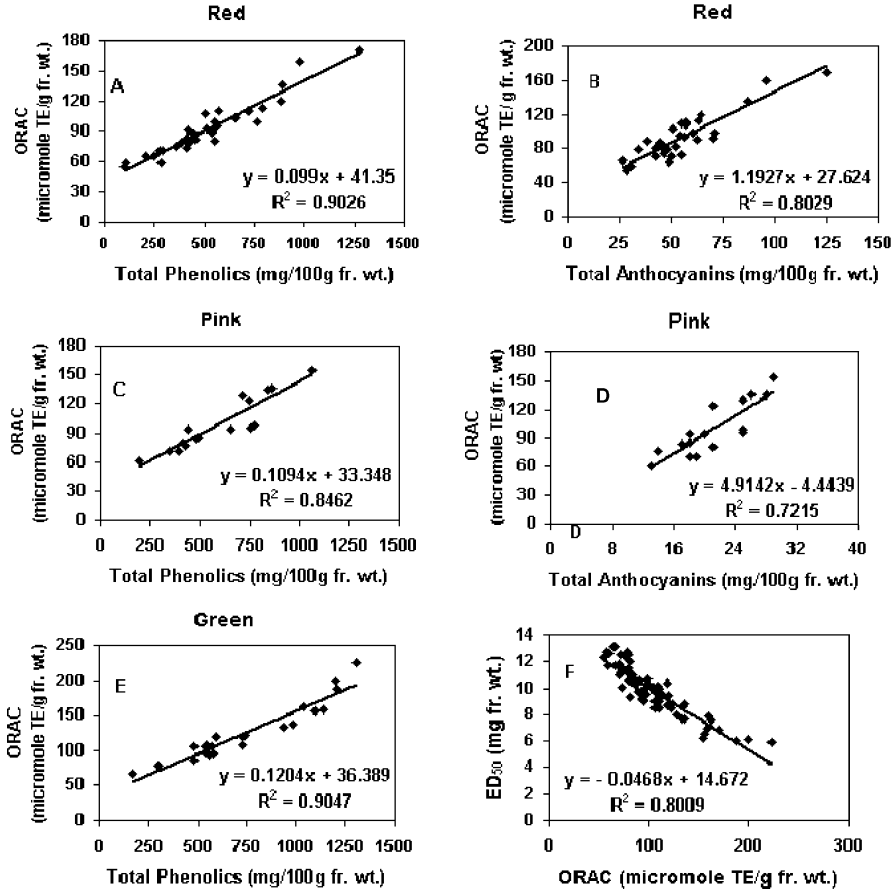


**Figure 1.** Correlation coefficient between (i) antioxidant (ORAC) and total phenolics for (**A**) red fruit ($R^2 = 0.9026$), (**C**) pink fruit ($R^2 = 0.8462$), and (**E**) green fruit ($R^2 = 0.9047$); (ii) antioxidant (ORAC) and total anthocyanins for (**B**) red fruit ($R^2 = 0.8029$) and (**D**) pink fruit ($R^2 = 0.7215$); and (iii) antioxidant (ORAC) and $ED_{50}$ for lingonberry fruit extracts in all different developmental stages (**F**) ($R^2 = 0.8009$).

berries, with the cultivar Ammerland yielding the highest ORAC value and total phenolic content at green and red stages of maturity (**Table 2**).

Different antioxidants have different antioxidant abilities to scavenge different ROS (*13*). Lingonberries had a high content of antioxidants, and cyanidin 3-galactoside was the most dominant anthocyanin, contributing the most antioxidant activity in lingonberries (*15*). Anthocyanins and phenolics are secondary plant metabolites. They protect the plant against damaging photodynamic reactions by quenching the excited state of active oxygen species (*37*). Anthocyanins are probably the largest group of phenolic compounds in the human diet and have been used for several therapeutic purposes including the treatment of diabetic retinopathy, fibrocystic disease, and vision disorders (*38*, *39*). Anthocyanins also have the potential to serve as radiation protective agents, vasotonic agents, and chemo-protective agents (*40*) and decrease the fragility of capillaries, inhibit blood platelet aggregation, and strengthen the collagen matrix of connective tissues (*41*).

There is a correlation between total phenolic contents and free radical scavenging activity (**Figure 1**). The ORAC value correlated with the total phenolic content at ripe, pink, and green stages with $R^2 = 0.9026$, 0.8462, and 0.9047, respectively (**Figure 1A**,**C**,**E**). No significant correlation existed between the ORAC value and the anthocyanin content at the green stage. However, there appeared to be an increasing correlation between the ORAC value and the anthocyanin content as the fruit matured from the pink stage to the ripe stages. The $R^2$ value between the ORAC value and the anthocyanin at the pink stage was 0.7215 (**Figure 1D**) and increased to 0.8029 at the ripe stage (**Figure 1B**). Harvest time (August vs October) and berry growth location in Oregon did not affect the fruit ORAC value, total anthocyanin, and total phenolic contents. This may be due to little variation of environmental conditions (such as soil, precipitation, solar radiation, and temperature) within the state of Oregon where the lingonberry was grown and collected for use in this study. The effect of environmental conditions and the interactions between genotype and environmental conditions on the antioxidant properties of strawberries has been reported. Wang and Zheng (*42*) found that the growing temperature substantially influenced the antioxidant capacity of strawberry (*Fragaria* × *ananassa* Duch.). They evaluated the effect of four day/night temperature conditions, including 18/12, 25/12, 25/22, and 30/22 °C, on the production of phenolic acids, flavonols, and anthocyanins and on antioxidant capacities against peroxyl radicals, superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, in Earliglow and Kent strawberry cultivars. The highest day/night temperature condition (30/22 °C) resulted in the greatest production of phenolics and antioxidant activities in strawberry fruits.

**DPPH Radical Scavenging Activity.** We compared the results of the ORAC assay with another rapid test for antioxidant activity. This test involves the use of DPPH radicals (*30*). Significant DPPH radical scavenging activity was detected in all lingonberry extracts, although the tested lingonberry varieties differed in their activity to react and quench DPPH radicals (**Table 3**). The $ED_{50}$ value is used to express the concentration of an antioxidant required to quench 50% of the initial DPPH radicals under the experimental conditions given. A smaller $ED_{50}$ value corresponds to a greater DPPH radical scavenging activity.

**Table 3.** Antioxidant Activity ($ED_{50}$ and % Inhibition of DPPH Radical Scavenging Activity) in Various Cultivars of Lingonberry at Different Maturities Grown at Different Locations and Harvested at Different Times (August or October)

| | | | | antioxidant activity (DPPH radical scavenging activity)[a] | | | |
|---|---|---|---|---|---|---|---|
| | | | | $ED_{50}$[b] (mg fr. wt) | | % inhibition | |
| culitvar | month | maturity | location | juice | acetone | juice | acetone |
| Ammerland | Oct | green | Fall Creek | 8.52 ± 0.45 | 5.91 ± 0.08 | 43.61 | 95.1 |
| Ammerland | Oct | red | Fall Creek | 9.55 ± 0.64 | 7.88 ± 0.12 | 29.18 | 71.22 |
| Erntedank | Oct | green | Fall Creek | 9.03 ± 0.32 | 6.01 ± 0.15 | 42.65 | 83.83 |
| Erntedank | Oct | red | Fall Creek | 10.62 ± 0.31 | 8.54 ± 0.21 | 23.8 | 62.16 |
| Erntekrone | Oct | red | Fall Creek | 12.76 ± 0.62 | 9.76 ± 0.14 | 19.5 | 53.06 |
| Erntesegen | Aug | green | Fall Creek | 9.09 ± 0.37 | 6.53 ± 0.19 | 37.89 | 78.35 |
| Erntesegen | Aug | pink | Fall Creek | 10.88 ± 0.63 | 8.99 ± 0.37 | 27.46 | 59.89 |
| Erntesegen | Aug | red | Fall Creek | 11.72 ± 0.58 | 10.57 ± 0.45 | 21.14 | 40.8 |
| Erntesegen | Oct | green | Fall Creek | 10.03 ± 0.63 | 7.62 ± 0.23 | 35.99 | 68.34 |
| Erntesegen | Oct | red | Fall Creek | 11.44 ± 0.42 | 10.10 ± 0.36 | 19.65 | 61.91 |
| European Red | Aug | green | Fall Creek | 8.98 ± 0.32 | 7.67 ± 0.11 | 39.56 | 72.24 |
| European Red | Aug | pink | Fall Creek | 9.54 ± 0.29 | 8.80 ± 0.14 | 42.93 | 69.64 |
| European Red | Aug | red | Fall Creek | 11.83 ± 0.62 | 9.97 ± 0.16 | 19.1 | 64.75 |
| Koralle | Aug | green | Walker | 8.76 ± 0.36 | 7.98 ± 0.21 | 53.26 | 69.19 |
| Koralle | Aug | pink | Walker | 11.25 ± 0.67 | 8.53 ± 0.32 | 21.85 | 65.52 |
| Koralle | Aug | red | Walker | 12.57 ± 0.87 | 9.04 ± 0.15 | 21.52 | 50.01 |
| Koralle | Aug | green | Fall Creek | 9.63 ± 0.23 | 8.84 ± 0.24 | 42.03 | 63.84 |
| Koralle | Aug | pink | Fall Creek | 11.55 ± 0.54 | 10.41 ± 0.34 | 24.8 | 61.14 |
| Koralle | Aug | red | Fall Creek | 11.01 ± 0.59 | 10.20 ± 0.21 | 25.8 | 61.76 |
| Koralle | Oct | red | Whitham | 9.80 ± 0.36 | 8.80 ± 0.17 | 32.56 | 68.28 |
| Koralle-German | Oct | red | Fall Creek | 10.31 ± 0.35 | 9.46 ± 0.31 | 30.23 | 60.26 |
| Red Pearl | Aug | green | Walker | 11.28 ± 0.29 | 9.27 ± 0.36 | 23.92 | 63.48 |
| Red Pearl | Aug | pink | Walker | 12.37 ± 0.74 | 9.15 ± 0.27 | 31.48 | 50.64 |
| Red Pearl | Aug | red | Walker | 13.18 ± 0.92 | 10.08 ± 0.28 | 17.11 | 42.98 |
| Red Pearl | Oct | red | Whitham | 12.02 ± 0.21 | 10.54 ± 0.16 | 25.8 | 42.42 |
| Sanna | Aug | green | Walker | 13.19 ± 0.43 | 9.39 ± 0.13 | 18.22 | 55.98 |
| Sanna | Aug | pink | Walker | 12.62 ± 0.26 | 10.95 ± 0.21 | 19.93 | 39.09 |
| Sanna | Aug | red | Walker | 12.37 ± 0.42 | 11.77 ± 0.34 | 17.78 | 30.81 |
| Scarlett | Oct | red | Fall Creek | 12.54 ± 0.51 | 9.31 ± 0.11 | 23.02 | 41.28 |
| Splendor | Aug | green | Fall Creek | 10.67 ± 0.27 | 6.97 ± 0.08 | 37.63 | 76.28 |
| Splendor | Aug | pink | Fall Creek | 9.47 ± 0.14 | 8.56 ± 0.27 | 25.3 | 65.57 |
| Splendor | Aug | red | Fall Creek | 10.53 ± 0.35 | 10.39 ± 0.42 | 24.43 | 42.48 |
| Splendor | Oct | red | Fall Creek | 10.50 ± 0.24 | 9.24 ± 0.09 | 25.11 | 52.47 |
| Sussi | Aug | green | Fall Creek | 7.67 ± 0.21 | 6.17 ± 0.08 | 53.05 | 86.16 |
| Sussi | Aug | pink | Fall Creek | 10.65 ± 0.42 | 6.25 ± 0.11 | 54.02 | 78.94 |
| Sussi | Aug | red | Fall Creek | 10.35 ± 0.28 | 6.85 ± 0.07 | 42.02 | 76.93 |
| Ida (8726–8) | Oct | red | Fall Creek | 12.71 ± 0.31 | 9.56 ± 0.21 | 20.97 | 56.73 |
| mean | | | | | | | |
| green | | | | 9.71 | 7.49 | 38.89 | 73.89 |
| pink | | | | 11.04 | 8.96 | 30.97 | 61.30 |
| red | | | | 11.43 | 9.56 | 24.37 | 54.46 |
| significance[c] | | | | | | | |
| cultivar | | | | * | * | * | * |
| maturity | | | | * | * | * | * |
| season | | | | ns | ns | ns | ns |
| location | | | | ns | ns | ns | ns |
| extraction (juice vs acetone extract) | | | | * | * | * | * |

[a] Data expressed as means ± SEM. [b] The $ED_{50}$ is that concentration of an antioxidant (mg fresh weight of lingonberry), which is required to quench 50% of the initially DPPD radicals under the experimental conditions given. [c] * and ns, significant or nonsignificant, respectively, at $p \leq 0.05$

The $ED_{50}$ values ranged from 5.91 to 11.77 mg fresh weight when extracted with acetone, while they ranged from 8.52 to 13.19 mg fresh weight without acetone. Thus, the acetone extraction may contribute to a higher radical scavenging activity. The fruit in the green stage had the highest DPPH radical scavenging activity, followed by the pink stage. The ripe fruit had the lowest. The green fruit of the Ammerland acetone extract had the greatest free radical scavenging activities against the DPPH radical with an $ED_{50}$ of 5.91 mg fresh weight, which equaled 95.1% of inhibition (**Table 3**). Harvest time (August vs October) and berry growth location showed no significant effect on DPPH radical scavenging activity in lingonberry fruits. The DPPH radical scavenging activity is correlated to the ORAC value with $R^2$ equal to 0.8009 (**Figure 1F**). This indicated that the antioxidant capacity of lingonberry could be measured by either the ORAC or the DPPH radical scavenging assay. The advantage of the ORAC assay over other methods has been reviewed by Cao and Prior (*36*).

**Scavenging of ROO•, •OH, and $O_2^{\bullet -}$ by Lingonberry Extract.** Flavonoids are potent quenchers of ROO•, $O_2^{\bullet -}$, •OH, and $^1O_2$ and have been shown to play a protective role against carcinogenesis by reducing the bioavailability of carcinogens (*43*). **Figure 2** and **Table 2** display the effectiveness of lingonberry as ROO•, •OH, and $O_2^{\bullet -}$ scavengers. The AAPH was used as a peroxyl radical (ROO•) generator. The Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$) was used as a source of •OH radicals. Superoxide radicals ($O_2^{\bullet -}$) were generated using a xanthine/xanthine oxidase system and measured by ESR. **Figure 2Aa** shows a typical ESR spectrum of •OH radicals generated by a 1:2:2:1 quartet indicating the

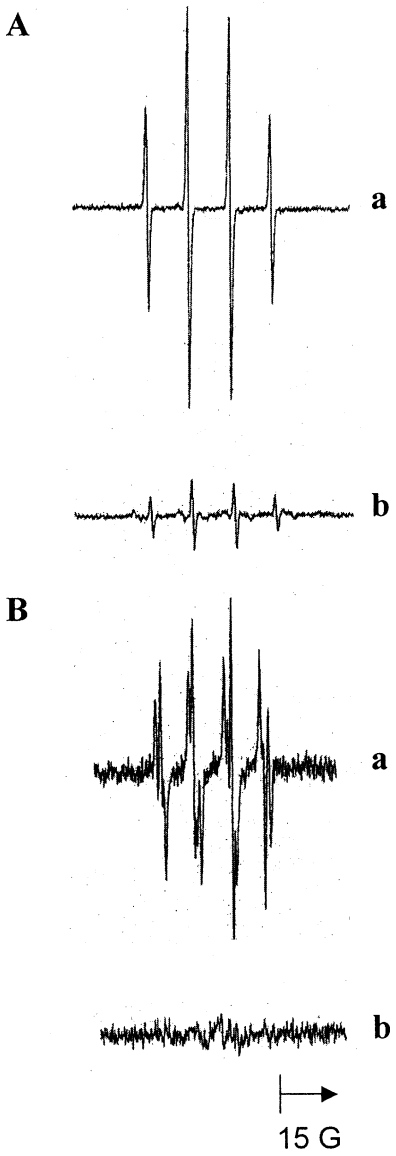


**Figure 2.** (**A**) Scavenging effect of lingonberry (cultivar Sanna) fruit extract on •OH radical. ESR spectra were recorded after reaction initiation from a phosphate-buffered solution (pH 7.4) containing 10 mM DMPO and the following reactants: (**a**) 1.0 mM $FeSO_4$ and 1.0 mM $H_2O_2$; (**b**) 1.0 mM $FeSO_4$, 1.0 mM $H_2O_2$, and lingonberry fruit extract (1:50). The ESR spectrometer settings were as follows: receiver gain, $2.52 \times 10^4$; time constant, 20 ms; modulation amplitude, 0.5 G; scan time, 60 s; and magnetic field, 3480 ± 100 G. (**B**) Scavenging effect of lingonberry (cultivar Sanna) fruit extract on $O_2^{\bullet -}$ radical. 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 lingonberry fruit extract (1:50). The ESR spectrometer settings were as follows: receiver gain, $2.52 \times 10^4$; time constant, 20 ms; modulation amplitude, 1.0 G; scan time, 60 s; and magnetic field, 3480 ± 100 G.

DMPO/•OH adduct. The addition of lingonberry (cultivar Sanna) extracts reduced the signal intensity (**Figure 2Ab**). This effect on observed •OH radical peak heights resulted in an 83% decrease with 50 mg/mL of lingonberry extract.

**Figure 2B** shows the effect of lingonberry extract on $O_2^{\bullet -}$ radicals. The spectrum recorded from a solution containing xanthine and xanthine−oxidase reaction alone is shown in **Figure 2Ba**. With the addition of lingonberry (cultivar Sanna) extract, the intensity of $O_2^{\bullet -}$ radical peaks was reduced (**Figure 2Bb**). The inhibition of $O_2^{\bullet -}$ radicals peak heights shows a 99% decrease with 50 mg/mL of lingonberry extract. These results indicate that free radicals such as ROO•, •OH, and $O_2^{\bullet -}$ probably constitute the ROS that may be responsible for the induction of AP-1 and NF-κB since they both are transcription factors activated by oxidative stress. The antioxidant properties of lingonberry may play an important role in protecting cells against the oxidative damage caused by free radicals.

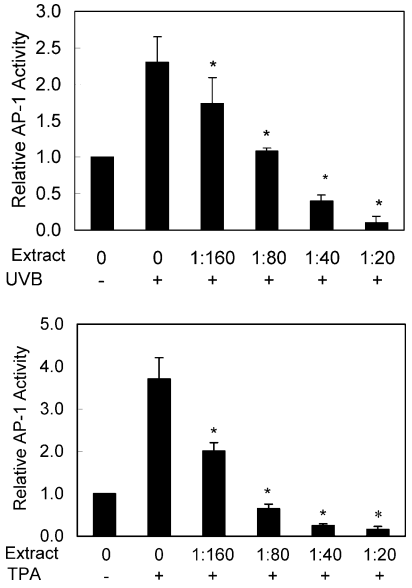


**Figure 3.** Lingonberry extract suppresses UVB- or TPA-induced AP-1 activity. JB6 P+ mouse epidermal cells that stably transfected with AP-1 luciferase reporter plasmid were cultured as described in the Materials and Methods. The cells were pretreated with or without various diluted lingonberry (cultivar Sanna) 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-1 activity was determined by luciferase assay. Results, presented as relative AP-1 induction as compared to untreated control cells, are 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 activation by lingonberry fruit extracts ($p \leq 0.05$).

**Inhibition of AP-1 and NF-κB Activity by Lingonberry Extract.** AP-1 and NF-κB are two transcription factors that regulate genes involved in ROS-induced responses, and both factors are targets of oxidative stress (*44*, *45*). AP-1 and NF-κB signal transduction pathways are important in transformation and tumor promotion (*16*). TPA or UVB are both carcinogens and can produce ROS and stimulate AP-1 and NF-κB activities. Increased AP-1 and NF-κB activities have been shown to be involved in the tumor promotion and progression of various types of cancers, such as skin, lung, and breast cancer (*22*, *23*). In vivo mouse data also demonstrate that AP-1 activity is required for tumor promotion (*46*, *47*). Therefore, we tested the effects of lingonberry on TPA- or UVB-induced AP-1 and NF-κB activities by using a reporter gene assay.

Pretreatment of JB6 P+ mouse epidermal cells with lingonberry (cultivar Sanna) extracts produced a dose-dependent inhibition of AP-1 and NF-kB activity induced by either UVB or TPA (**Figures 3** and **4**). The AP-1 activity induced by TPA or UVB was inhibited by 82−96 or 23−95%, respectively, by lingonberry extract dilution range of 1:20 to 1:160 (**Figure 3**). The NF-κB activity induced by TPA or UVB was inhibited by 55−87 or 46−97%, respectively, with the tested dose range of 1:20 to 1:160 dilution (**Figure 4**). In addition, lingonberry extract alone had no effect on AP-1 and NF-κB activities (data not

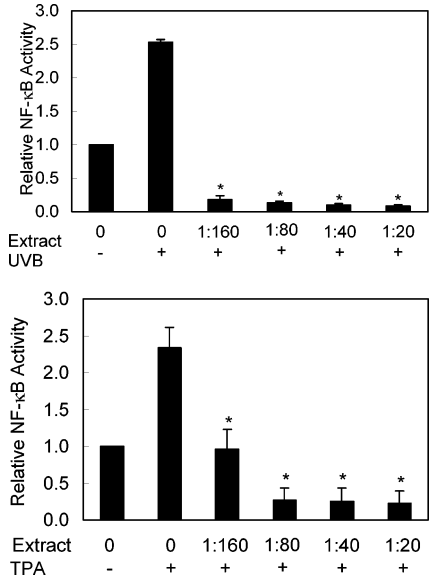


**Figure 4.** Lingonberry extract suppresses UVB- or TPA-induced NF-$\kappa$B activity. JB6 P$^+$ mouse epidermal cells that stably transinfected with NF-$\kappa$B luciferase reporter plasmid were cultured as described in the Materials and Methods. The cells were pretreated with or without various diluted lingonberry (cultivar Sanna) 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. NF-$\kappa$B activity was determined by luciferase assay. Results, presented as relative NF-$\kappa$B induction as compared to untreated control cells, are 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 activation by lingonberry fruit extracts ($p \leq 0.05$).

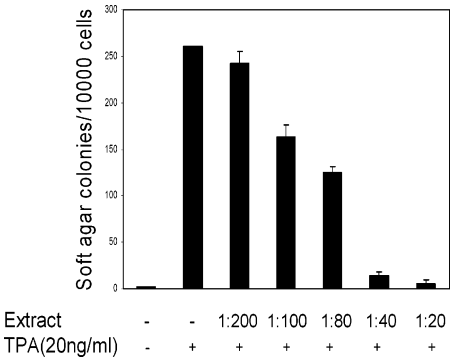


**Figure 5.** Inhibition of TPA-induced transformation by lingonberry extracts. JB6 P$^+$ mouse epidermal cells ($1 \times 10^4$) were exposed to TPA (20ng/mL) with or without lingonberry (cultivar Sanna) fruit extract in 0.33% agar for 14 days and scored for colonies at the end of the experiments. Results, presented as the number of colonies per $10^4$ cells, are the means and standard errors from twice independent assay. * Indicates a significant different from TPA-treated positive control ($p \leq 0.05$).

shown). These results suggest that lingonberry extracts are effective inhibitors of TPA- and UVB-induced signal transduction pathways and may be an effective strategy for chemoprevention.

**Effect of Lingonberry Extract on TPA-Induced Cell Transformation.** Because both AP-1 and NF-$\kappa$B play an important role in neoplastic transformation and that lingonberry inhibited TPA- or UVB-induced AP-1 and NF-$\kappa$B activation, we hypothesized that lingonberry may also suppress the tumor promoter TPA-induced cell transformation. To test this hypothesis, soft agar analysis was performed in the presence or absence of lingonberry (cultivar Sanna) extract. As shown in **Figure 5**,

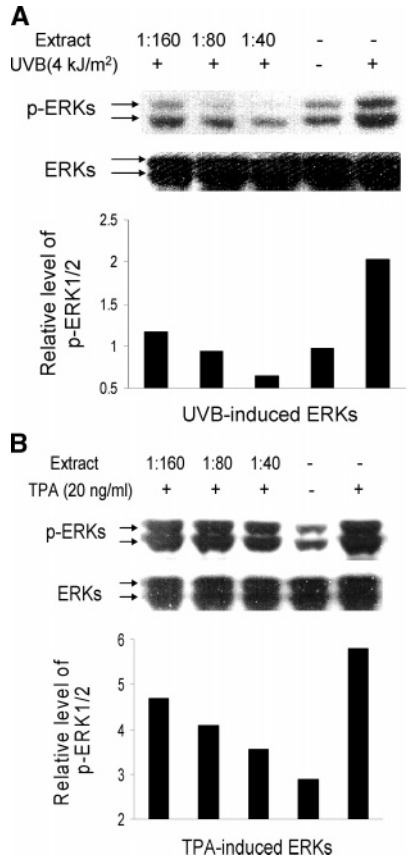


**Figure 6.** Inhibition of UVB- or TPA-induced activation of ERKs by lingonberry extracts. JB6 P$^+$ mouse epidermal cells were pretreated with lingonberry (cultivar Sanna) fruit extracts for 1 h and then exposed to (**A**) UVB radiation (4 kJ/m$^2$) or (**B**) TPA (20 ng/mL) for 30 min. Proteins (20 $\mu$g) in the JB6 cell lysate were separated by SDS–PAGE and analyzed by Western blot with phosphospecific antibody against phosphorylated sites of ERKs. Phosphorylated and nonphosphorylated proteins were detected by using the same transferred membrane blot following a stripping procedure. One representative of three similar results was shown. The histogram shows the densitometric analysis of phosphorylated protein expression normalized to total ERKs.

TPA efficiently induced JB6 P$^+$ mouse epidermal cell transformation after 2 weeks of culture. The addition of lingonberry extracts significantly suppressed TPA-induced cell transformation in a concentration-dependent manner ($p \leq 0.05$). Lingonberry extract alone had no effect on spontaneous cell transformation (data not shown). A previous study suggested that transcription factors of AP-1 and NF-$\kappa$B are able to cross-talk (*48*), and both factors may play a role in cell transformation (*49*). Furthermore, it was also reported that NF-$\kappa$B inhibitors (such as pyrrolidine dithiocarbamate treatment or I$\kappa$B$\alpha$ mutant expression) could suppress JB6 P$^+$ cell transformation, indicating the critical role of NF-$\kappa$B activation in neoplastic cell transformation (*32*, *50*). Inhibition of AP-1 and NF-$\kappa$B activation by a variety of chemopreventive agents has been shown to reduce neoplastic transformation (*16*, *19*, *20*, *32*). Therefore, our data suggest that the inhibition of TPA-induced cell transformation by lingonberry extract may be a result of inhibition of AP-1 and/or NF-$\kappa$B activation. These data suggest that the inhibitory effects of lingonberry extract on AP-1 and/or NF-$\kappa$B activation may have a beneficial role in preventing carcinogenesis induced by environmental carcinogens.

**Lingonberry Extract Blocks TPA- or UVB-Induced MAPK Activation.** The activation of AP-1 is linked to MAPK signaling

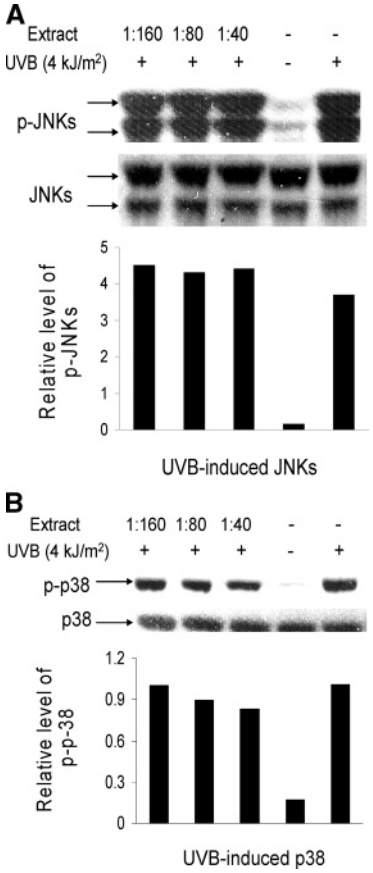


**Figure 7.** Inhibition of UVB-induced activation of JNKs and p-38 by lingonberry extract. JB6 $P^+$ mouse epidermal cells were pretreated with lingonberry (cultivar Sanna) extracts for 1 h and then exposed to UVB radiation (4 kJ/$m^2$). Proteins (20 $\mu$g) in the JB6 cell lysate were separated by SDS–PAGE and analyzed by Western blot with phosphospecific antibody against phosphorylated sites of (**A**) JNKs and (**B**) p38. Data are representative of three experiments. Histograms show the densitometric analysis of phosphorylated protein expression normalized to total MAPK.

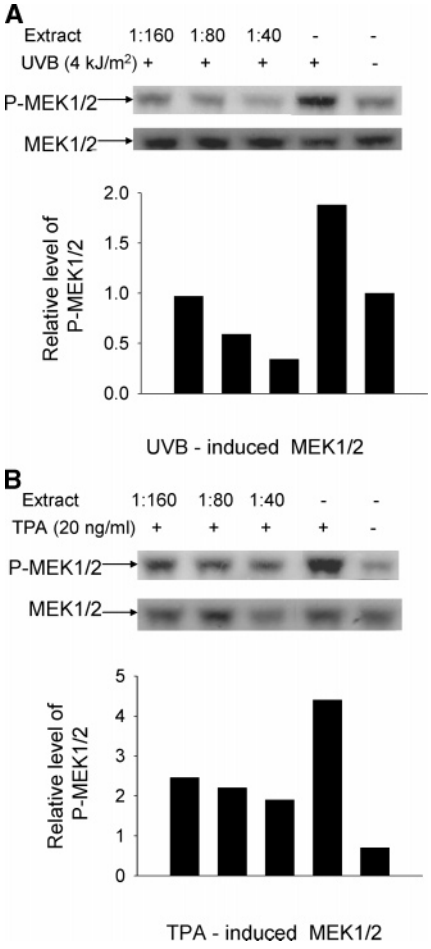


**Figure 8.** Inhibition of UVB- or TPA-induced phosphorylation of MEK1/2 by lingonberry. JB6 mouse epidermal cells were pretreated with different dilution of lingonberry (cultivar Sanna) extracts for 1 h and exposed to (**A**) UVB (4 kJ/m2) or (**B**) TPA (20 ng/mL). Phosphorylated MEK1/2 in cell lysate was determined, using a phosphospecific antibody from Cell Signaling Technology. The phosphorylated proteins and nonphosphorylated proteins were detected, using the same transferred membrane blot following a stripping procedure. Data are representative of three experiments. Histograms show the densitometric analysis of phosphorylated protein expression normalized to total MEK1/2.

pathways (*23*, *51*). To testify if the inhibition of AP-1 activity by lingonberry was due to the blockage of the MAPK pathway, we studied the effect of lingonberry extract on tumor promoter-induced activation of MAPKs. Within 30 min, exposure of JB6 $P^+$ mouse epidermal cells to UVB irradiation stimulated the phosphorylation of ERKs, JNKs, p38, and MEK1/2 kinase, whereas TPA treatment for 30 min only stimulated the phosphorylation of ERKs and MEK1/2 (**Figures 6**−**8**). Pretreatment of cells with lingonberry (cultivar Sanna) extract blocked UVB-induced phosphorylation of ERKs and MEK1/2 in a dose-dependent manner (**Figures 6A** and **8A**). However, lingonberry fruit extract had no effect on JNKs (**Figure 7A**) and little effect on p38 kinase activation (**Figure 7B**). Lingonberry fruit extract also inhibited TPA-induced phosphorylation of ERKs and MEK1/2 (**Figures 6B** and **8B**). Given the fact that JB6 $P^+$ cell transformation and AP-1 activation may be blocked by PD98059, a specific inhibitor of ERK and MEK1/2 pathway (*52*, *53*), our present data indicate that the ERKs and MEK1/2 may be the primary targets of lingonberry that lead to suppression of AP-1 activation and cell transformation in JB6 $P^+$ cells.

**Lingonberry Extract Induces Human Leukemia HL-60 Cell Apoptosis.** Cancer cell apoptosis is a novel target for chemoprevention study (*54*). We found that lingonberry extract induced the apoptosis of human HL-60 leukemia cells in a dose-dependent manner, as indicated by morphology characteristics of apoptosis via Hoechst 33258 staining (**Figure 9**) (*55*). These

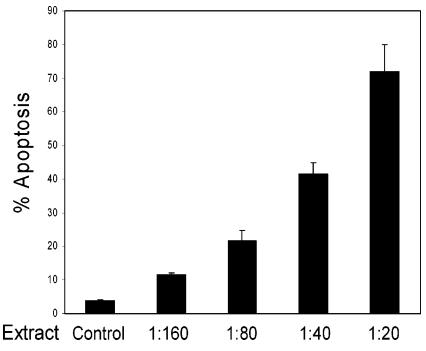


**Figure 9.** Apoptosis induction of human leukemia HL-60 cells by lingonberry extract. Human leukemia HL-60 cells were treated with indicated doses of lingonberry (cultivar Sanna) extracts for 18 h. The typical apoptotic changes of nuclear morphology of HL-60 cells were indicated by Hoechst 33258 staining. The percent apoptotic cells was calculated by determining the number of cells with nuclear morphology change divided by the total number of cells.

data suggest that the treatment of HL-60 cells with lingonberry extract causes cancer cell death by an apoptotic mechanism.

We assume that several fractions in the extract might be responsible for the induction of apoptosis, but the possibility that the antioxidant property of the extract may enhance the apoptosis of cancer cells should not be ruled out. As compared with nonneoplastic cells, cancer cells constitutively generate large but tolerable 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 (*55*). From these perspectives, Toyokuni et al. (*56*) proposed the concept of "persistent oxidative stress in cancer cells". Reducing oxidative stress may suppress the proliferation of tumor cells and enhance cancer cell apoptosis (*57*, *58*). Oxidative stress interferes with cancer chemotherapy: inhibition of lymphoma cell apoptosis and phagocytosis. The results of the current study demonstrate that lingonberry extract exhibited a strong antioxidant capacity (**Figures 1** and **2**). The inductive effect of lingonberry extracts on the apoptosis of human leukemia HL-60 cells may partially be due to its antioxidant properties by perturbing the favorable redox condition in cancer cells.

In the present study, we show that lingonberry fruit soluble solids, TA, anthocyanins, and total phenolics vary among cultivars. Lingonberries also contain potent free radical scavenging activities for DPPH$^{\bullet}$, ROO$^{\bullet}$, $^{\bullet}$OH, and $O_2^{\bullet-}$ radicals. Different cultivars exhibited varying degrees of scavenging capacity. Lingonberries also significantly suppressed TPA-induced cell transformation and blocked UVB-induced phosphorylation of ERKs, p38, and MEK1/2 kinase (not JNK), as well as TPA-induced phosphorylation of ERKs and MEK1/2 in cell cultures. These results suggest that the blocking of UVB- and TPA-induced AP-1-MAPKs and NF-$\kappa$B activation by lingonberries may be due to its high antioxidant properties. The lingonberries may be highly effective as a chemopreventive agent that acts by targeting specific oncogenes, such as AP-1 and NF-$\kappa$B, suppressing cell neoplastic transformation, and inducing cancer cell apoptosis. The antioxidant compounds in lingonberry may make them possible candidates for a role in cancer chemoprevention and treatment. However, the correlation between the antioxidant activity of various lingonberry cultivars and the inhibition of AP-1, NF-$\kappa$B, cell transformation, MAPKs, as well as induction of cancer cell apoptosis warrants further study.

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