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REVIEW
In Vitro-Screening for Cancer-Suppressive Effect of Food Components

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Abstract

Some diets have been shown to be correlated with the development and progression of cancer. In contrast, many kinds of foods are expected to prevent the development and progression of cancer. To identify such cancer-preventive food components and elucidate their cancer-preventive functions, we investigated important anti-cancer effects, that is, growth-inhibitory and apoptosis-inducing effects of some food and food components on cancer cells. Among the vegetables studied, bitter gourd was the most effective in inhibiting the growth of HL60 human leukemia cells. Bitter gourd extract induced apoptosis in the cells. The bitter gourd extract also inhibited growth and induced apoptosis in B16 mouse melanoma 4A5 cells, whereas it inhibited the growth of BALBc/3T3 mouse fibroblast cells only to a small extent. Berries are rich sources of the naturally occurring phenolic pigments anthocyanins, which show a potent antioxidant activity. Bilberry ethanol extract strongly inhibited the growth of HL60 and HCT116 human colon carcinoma cells, and also induced apoptosis in HL60 cells but not in HCT116 cells. Of the berry extracts tested, that from bilberry contained the largest amount of phenolic compounds, including anthocyanins, and exhibited the highest DPPH radical-scavenging activity. The anthocyanidins, delphinidin and malvidin, inhibited cell growth and induced apoptosis in HL60 cells. Delphinidin, but not malvidin inhibited the HCT116 cell growth. These effects of food components probably contribute to cancer prevention.

Discipline: Food
Additional key words: apoptosis, growth-inhibition, cancer cells, bitter gourd, berries

Introduction

Food and nutrient intake has been examined in relation to many types of cancer. It is generally recognized that some diets are correlated with the development and progression of cancer. In contrast, vegetables and fruits have been reported to reduce the risk of cancer in epidemiological studies. Some food factors, such as vitamins and polyphenols, are listed as possible cancer suppressors. They display antioxidant activities, which prevent the formation of highly reactive lipid peroxidation products and reduce the deleterious effects of reactive oxygen species, in addition to anti-mutagenicity, anti-tumor activity or other anti-cancer effects.

Tumors proliferate beyond the constraints limiting growth in normal tissues[3]. Therefore, the inhibition of cancer cell growth and the induction of apoptotic cancer cell death are important mechanisms of cancer prevention. Apoptosis is one of the major mechanisms of cancer suppression[5,7,10]. The apoptotic cells are fragmented and removed by phagocytosis. To elucidate the cancer preventive effects of food components, we investigated the growth-inhibiting and apoptosis-inducing effects of some food and food components in cultured cancer cells.

Materials and methods

1. Materials

Carrot (*Daucus carrot* L.), Welsh onion (*Allium tistulosum* L.), Chinese radish (*Raphanus sativus* L.), bitter gourd (*Momordica charantia* L.), cabbage (*Brassica oleracea* L.) and sweet potato (*Ipomoea batatas* Poir.) were cultivated in Kagoshima prefecture. Lowbush blueberry

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(*Vaccinium angustifolium*), highbush blueberry (*Vaccinium corymbosum*), bilberry (*Vaccinium myrtillus*), cowberry (*Vaccinium vitis-idaea*), cranberry (*Vaccinium oxycoccos*), black currant (*Ribes nigrum*), red currant (*Ribes sativum*), raspberry (*Rubus idaeus*), blackberry (*Rubus mesogaeus*) and strawberry (*Fragaria × ananassa*) were purchased from trading companies. Components from vegetables and frozen berries were extracted in ethanol. The vegetable extracts were concentrated using an evaporator. The berry extracts were concentrated using an evaporator and then lyophilized. Anthocyanidins (delphinidin, malvidin, peonidin, cyanidin and pelargonidin) and flavonols (myricetin, quercetin and kaempferol) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

2. Cells and cell culture

HL60 human promyelocytic leukemia cells (JCRB0085) were provided by the Human Science Research Resources Bank (Osaka, Japan). B16 mouse melanoma cells (RCB557) were obtained from RIKEN Cell Bank (Tsukuba, Japan). BALBc/3T3 cells were kindly provided by Dr. J. Yuan. HCT116 human colon carcinoma cells (ATCC CCL247) were purchased from Dainippon Pharmaceutical Corp. (Osaka, Japan). HL60 cells were maintained in RPMI1640 medium (Invitrogen, USA). B16 and BALBc/3T3 cells were maintained in DMEM medium (Invitrogen, USA). HCT116 cells were grown in McCoy's 5A medium (Invitrogen, USA). Cells were cultured at 37ºC in 5% CO_2 in air in a medium supplemented with 10% heat-inactivated fetal calf serum (FCS; ICN Biomedicals, USA). Viable cells were counted with a hemocytometer by trypan blue exclusion. To analyze the nuclear morphology, the cells were stained with 1mM bisbenzimide (Hoechst) 33258 in PBS.

3. DNA extraction and agarose gel electrophoresis

Cells were lysed in lysis buffer (50mM Tris-HCl (pH 8.0), 100mM EDTA, 0.5% SDS), incubated with RNase and then incubated with Proteinase K. The samples were electrophoresed in Tris-borate buffer (pH 8.0) on a 2% agarose gel and the DNA was stained with ethidium bromide.

4. Determination of contents of total phenolic compounds and anthocyanins and of DPPH-radical scavenging activity

Total phenolic compounds in the berry ethanol extract were determined by the Folin-Ciocalteu's method[9] and the contents were expressed as milligram of D-catechin equivalents per 1 g of extract. Anthocyanins in the berry ethanol extract were analyzed by the HPLC method of Ando et al.[1] and the contents were expressed as milligram of cyanidin equivalents per 1 g of extract. To measure the DPPH-radical scavenging activity, the berry ethanol extract was added to the DPPH solution (the final concentration of the DPPH solution was 400 μM DPPH (Nacalai Tesque, Japan), 200 μM MES buffer (pH 6.0) and 20% ethanol), mixed well and left for 20 min, after which the absorbance of the solution was measured at 520 nm. The DPPH radical-scavenging activity was compared with that of Trolox (Sigma-Aldrich, USA)[11].

Results and discussion

First, we examined the effects of ethanol extracts of vegetables, which were grown in Kagoshima prefecture in the Kyushu area, on cancer cell growth[2]. We prepared extracts from 5 vegetables (carrot, Welsh onion (2 varieties), Chinese radish, bitter gourd and cabbage) and 4 varieties of sweet potato, and investigated the inhibitory effects of the vegetable extracts on the growth of HL60 human leukemia cells. The HL60 cells were seeded at a concentration of 2×10^5 cells/mL on 24 well plates. Each extract was added to the medium of HL60 cells, incubated for 24 h and then the viable cell number was counted. Among the extracts, the bitter gourd extract was the most effective inhibitor of HL60 cell growth (Fig. 1). The bitter gourd extract decreased the viable cell number by 60% at a concentration of 200 μg/mL (Fig. 1). The extract induced apoptotic cell bodies and oligonucleosomal DNA fragmentation typical of apoptosis in HL60 cells (data not shown, Fig. 2a). The results indicated that the bitter gourd extract induced apoptosis in HL60 cells. The bitter gourd extract inhibited the growth of B16 melanoma cells at a concentration of 200–400 μg/mL after 24 h incubation. The extract induced nuclear and DNA fragmentations typical of apoptosis in the B16 cells (Fig. 2b). Thus the bitter gourd extract inhibited cancer cell growth and induced apoptosis in cancer cells, whereas it hardly inhibited the growth of BALBc/3T3 mouse fibroblast cells. The extract decreased the viable cell number only by 20% at a concentration of 400 μg/mL after 24 h incubation (Fig. 3).

Berries are known to contain the phenolic pigments, anthocyanins, which display an antioxidant activity. We also screened the growth inhibitory effects of berry ethanol extracts in human cancer cells[6]. Berry extracts were added to the culture medium of HL60 and HCT116 human carcinoma cells and incubated for 24 and 48 h, respectively. The bilberry extract markedly inhibited the growth of HL60 and HCT116 cells. The bilberry extract decreased the number of viable HL60 cells by 84 and 88% at concentrations of 4 and 6 mg/mL, respectively,

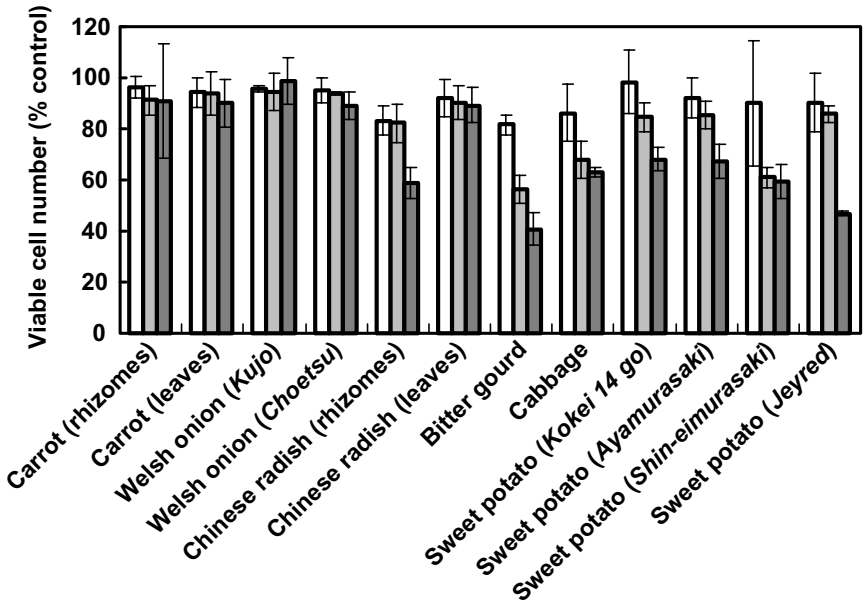


Fig. 1. Effect of vegetable extracts on the growth of HL60 human leukemia cells

HL60 cells were incubated with the vegetable extracts (□ , 50 μg/mL ; ▒ , 100 μg/mL; ■ , 200 μg/mL) for 24 h. Viable cells were counted with a hemocytometer by trypan blue exclusion (n = 3). Vertical bars indicate SD.

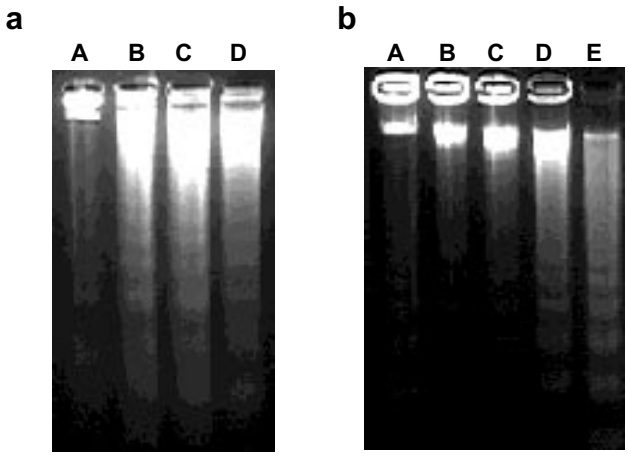


Fig. 2. Analysis of DNA fragmentation patterns by agarose gel electrophoresis

a : DNA was extracted from the HL60 cells treated with the bitter gourd extract (A, none; B, 50 μg/mL; C, 100 μg/mL; D, 200 μg/mL) for 24 h.

b : DNA was extracted from the B16 cells treated with the bitter gourd extract (A, none ; B, 100 μg/mL; C, 200 μg/mL; D, 300 μg/mL; E, 400 μg/mL) for 24 h.

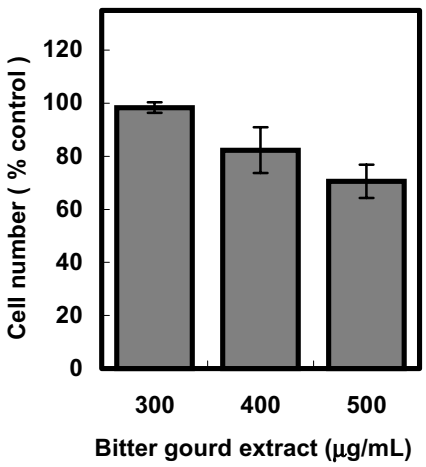


Fig. 3. Effect of the bitter gourd extract on the growth of BALBc/3T3 mouse fibroblast cells

BALBc/3T3 cells were incubated with the extract of bitter gourd for 24 h. Viable cells were counted with a hemocytometer by trypan blue exclusion (n = 3). Vertical bars indicate SD.

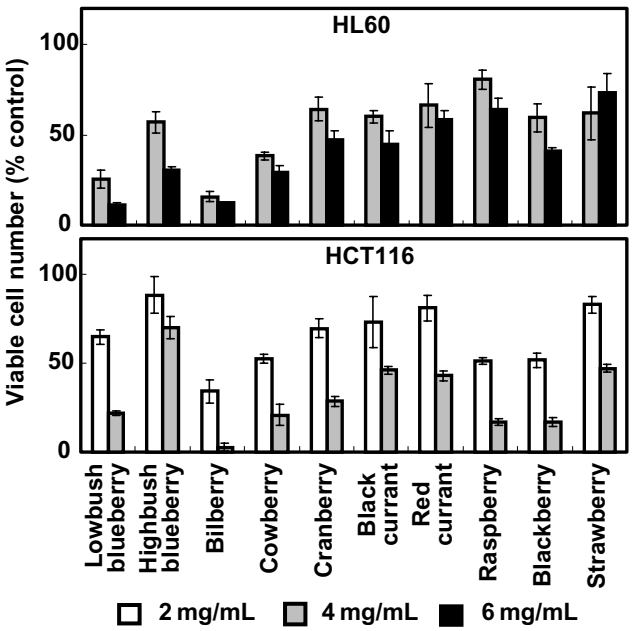


Fig. 4. Effects of berry ethanol extracts on the growth of HL60 and HCT 116 cells

HL60 cells were incubated with 4 or 6 mg/mL berry ethanol extract for 24 h. HCT 116 cells were incubated with 2 or 4 mg/mL berry extract for 48 h. Each value is the mean ± SD of triplicate cultures.

and the number of viable HCT116 cells by 66 and 97% at 2 and 4 mg/mL (Fig. 4). The bilberry extract induced apoptotic cell bodies and nuclear fragmentation typical of apoptosis in HL60 cells at the concentration of 4 mg/mL after 6 h incubation (Figs. 5a & 5c). Apoptotic cells with fragmented nuclei were also observed in HCT116 cells treated with the 4 mg/mL bilberry extract, but the proportion of apoptotic cells was much lower than in HL60 cells (Figs. 5b & 5d).

Among the 10 berry extracts, the bilberry extract showed the highest DPPH radical-scavenging activity and the highest total phenolic and anthocyanin contents (Figs. 6a & 6b). We next fractionated the bilberry extract by OASIS HLB column chromatography. Our results suggested that the anthocyanins contained in the bilberry extract inhibited the growth of HL60 and HCT116 cancer cells and induced apoptosis in HL60 cells (data not shown). Therefore, we investigated the growth-inhibitory and apoptosis-inducing effects of pure anthocyanidins on cancer cells. Anthocyanidins are aglycons of anthocyanins. Fig. 7 shows the structure of the anthocyanidins contained in the berries. Delphinidin markedly inhibited the growth of HL60 and HCT116 cells over a concentration range of 50–200 μM. The number of viable HL60 and HCT116 cells was reduced by 100 μM del-

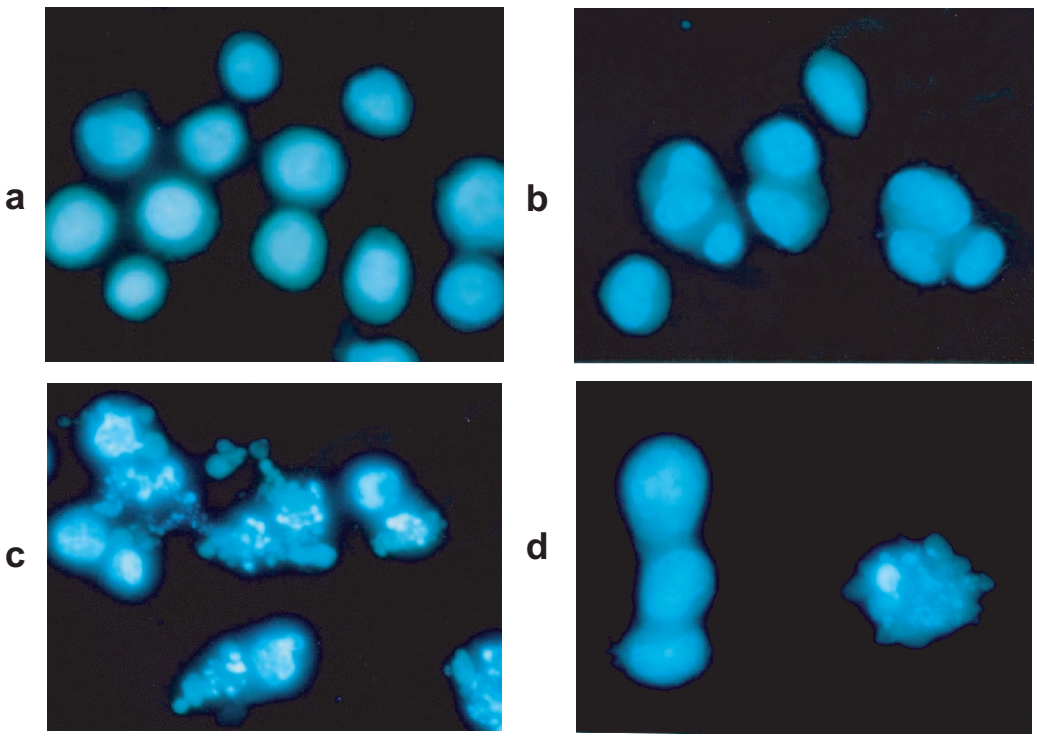


Fig. 5. Nuclear morphology of HL60 and HCT116 cells

HL60 and HCT116 cells were incubated with or without 4 mg/mL bilberry extract and then stained with Hoechst 33258. a: HL60 cells, b: HCT116 cells, c: HL60 cells treated with 4 mg/mL bilberry extract for 6 h, d: HCT116 cells treated with 4 mg/mL bilberry extract for 24 h.

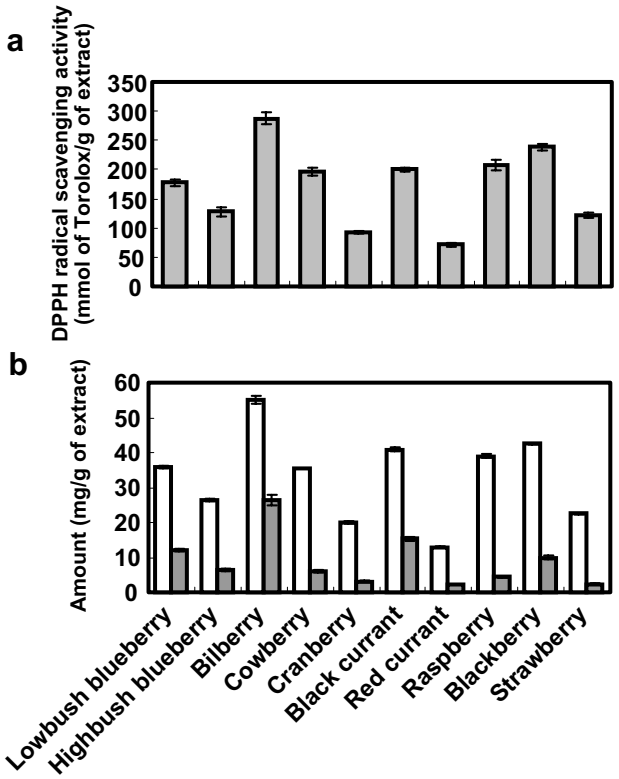
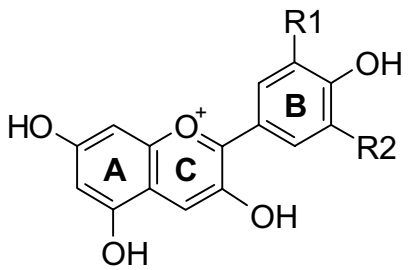


Fig. 6. DPPH radical-scavenging activity and total phenolic and anthocyanin contents in berry extracts

a: DPPH radical-scavenging activity of berry extracts. Each value is the mean ± SD of triplicate experiments.

b: Total phenolic and anthocyanin contents in berry extracts. Total phenolic compounds (□) in the berry ethanol extract were determined by the Folin-Ciocalteu's method[9] and the contents were expressed as milligram of D-catechin equivalents per g of extract. Anthocyanins (■) in the berry extract were analyzed by the HPLC method of Ando et al.[1]. Total anthocyanin content was expressed as milligram of cyanidin equivalents per 1 g of extract. Each value is the mean ± SD of triplicate experiments.



Anthocyanidin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Fig. 7. Structure of anthocyanidins contained in berries

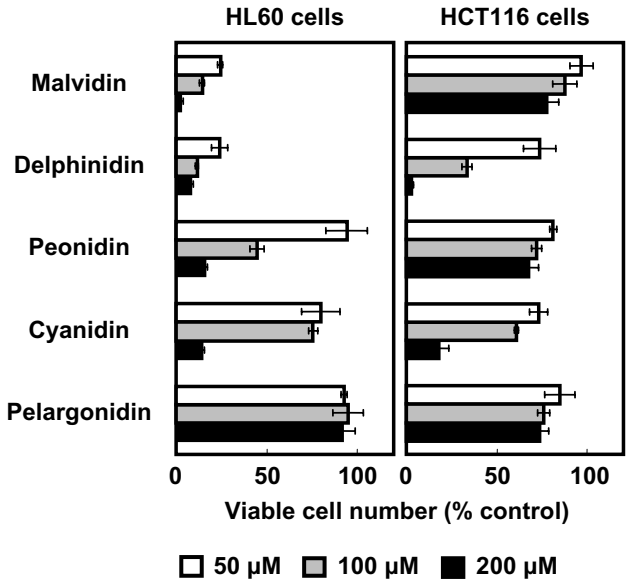


Fig. 8. Effects of anthocyanidins on the growth of HL60 and HCT116 cells

Anthocyanidins were incubated with HL60 cells for 24 h or with HCT116 cells for 48 h. Each value is the mean ± SD of triplicate cultures.

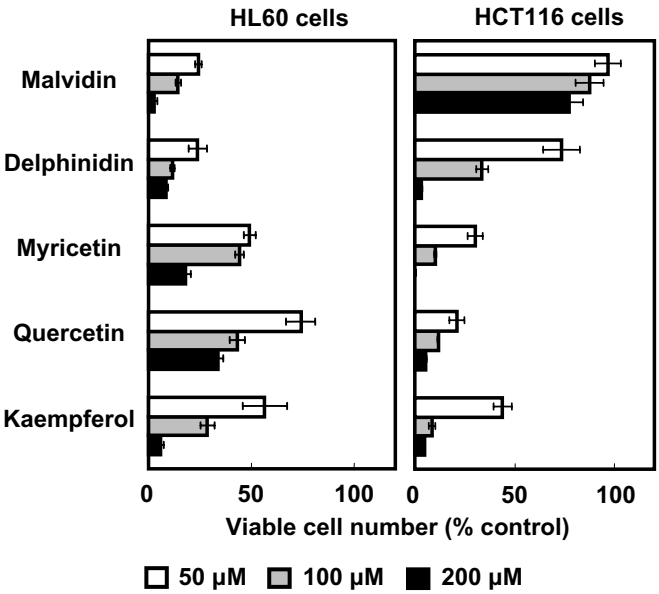


Fig. 10. Effects of flavonols on growth of HL60 and HCT116 cells

The flavonoids were incubated with HL60 cells for 24 h or HCT116 cells for 48 h. Each value is the mean ± SD of triplicate cultures.

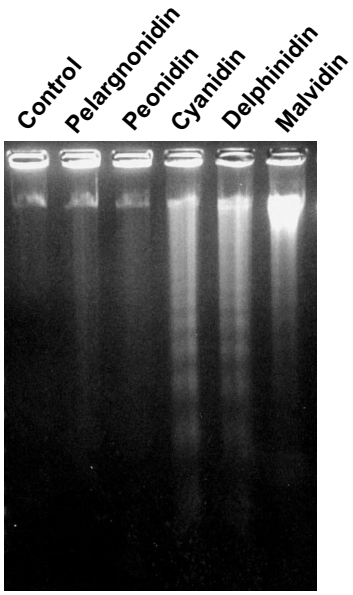


Fig. 9. Analysis of DNA fragmentation patterns by agarose gel electrophoresis

DNA was extracted from HL60 cells treated with 200 μM anthocyanidins for 24 h.

phinidin to 12% and 36% of the control, respectively (Fig. 8). Malvidin more selectively inhibited the growth of HL60 cells than that of HCT116 cells. Thus 50–200 μM malvidin reduced the number of viable HL60 cells to 3–25% of the control, whereas at 200 μM, this value was only 78% of the control for HCT116 (Fig. 8). Similarly, the growth inhibitory effect of peonidin was high in HL60 but low in HCT116 cells. In contrast, cyanidin reduced the number of both HL60 and HCT116 viable cells by 82–85% of the control at 200 μM (Fig. 8). Pelargonidin had no effect on the number of viable HL60 cells but slightly decreased the number of HCT116 viable cells at 50–200 μM (Fig. 8).

Induction of apoptotic cell bodies and nuclear fragmentation was observed after 6 h of treatment with 200 μM malvidin, delphinidin or cyanidin, but not with peonidin or pelargonidin (data not shown). Malvidin, delphinidin and cyanidin, but not peonidin and pelargonidin induced the DNA fragmentation typical of apoptosis in HL60 cells (Fig. 9). Induction of apoptosis in HCT116 cells was minimal following treatment with 200 μM pure anthocyanidins.

The flavonols, myricetin, quercetin and kaempferol, were reported to be constituents of edible berries and to induce apoptosis in cancer cells[4,8,10]. The growth inhibitory effects of malvidin and delphinidin on cancer cells were compared with those of flavonols. Myricetin, quercetin and kaempferol inhibited the growth of HL60 and HCT116 cells in the concentration range of 50–200 μM (Fig. 10). The inhibitory effects of malvidin and del-

phinidin were more pronounced than those of the flavonols in HL60 cells (Fig. 10). In contrast, the latter were more effective than the anthocyanidins in inhibiting HCT116 cells (Fig. 10). Bilberry has been reported to contain flavonols but only at about 1% of the anthocyanin content[4]. The growth inhibitory and apoptosis-inducing effects of bilberry on cancer cells are, therefore, likely to be attributable to the anthocyanins.

We then isolated all the anthocyanins contained in the bilberry extract and investigated the growth-inhibitory and apoptosis-inducing effects on HL60 and HCT116 cells. The anthocyanins, with delphinidin or malvidin as the aglycon, inhibit the growth of HL60 cells through the induction of apoptosis (data not shown). On the other hand, only delphinidin-glycosides isolated from the bilberry extract, but not malvidin-glycosides inhibited the growth of HCT116 cells (data not shown). Thus, berries such as bilberry that contain a large amount of anthocyanins are likely to suppress cancer through the induction of apoptosis and/or inhibition of cancer cell growth.

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