**Oligomeric proanthocyanidins from rabbiteye blueberry leaves inhibits
the proliferation of human T-cell lymphotropic virus type 1-associated
cell lines via apoptosis and cell cycle arrest**

Kiyoko Nagahama ^a, Nozomu Eto ^{a,*}, Yoichi Sakakibara ^a, Yoh-ichi Matsusita ^a, Kazuhiro
Sugamoto ^a, Kazuhiro Morishita ^b, Masahito Suiko ^a

^aInterdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki,
1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan.

^bDivision of Tumor and Cellular Biochemistry, Department of Medical Sciences, Faculty
of Medicine, University of Miyazaki, 5200 Kihara Kiyotake, Miyazaki 889-1692, Japan.

*Corresponding author: Department of Biochemistry and Applied Biosciences, Faculty
of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192,
Japan.

Tel.: +81-985-58-7210; FAX: +81-985-58-7210; E-mail: neto@cc.miyazaki-u.ac.jp

Abstract

Adult T-cell leukemia/lymphoma (ATLL) is an aggressive disease caused by human T-cell lymphotropic virus type 1 (HTLV-1). As ATLL develops over many years, daily diet may play an important role in its occurrence. In this study, we investigated the growth inhibitory effect of extracts of blueberry leaf fractions on HTLV-1-associated cell lines. The most effective fraction contained highly oligomeric **proanthocyanidins** (OPA) with a mean degree of polymerization of 3.1. Cell growth inhibition by the OPA fraction was higher in all HTLV-1-associated cell lines than in the MOLT-4 HTLV-1-negative T-cells. It inhibited growth of the HTLV-1-infected T-cell line MT2 and ATLL-derived cell line Su9T01 by inducing apoptosis and G₂/M cell cycle arrest. OPA fraction-induced apoptosis of HTLV-1-associated cells required death receptor-mediated caspase-dependent pathways. Treatment-induced G₂/M arrest resulted from down-regulation of cyclin B1 and cdc2. The blueberry leaf OPA fraction may be a source of novel compounds for reducing the risk of developing ATLL.

Keywords: oligomeric **proanthocyanidins**; blueberry leaves; adult T-cell leukemia; HTLV-I; apoptosis; G₂/M cell cycle arrest

Abbreviations: ATLL, adult T-cell leukemia/lymphoma; HTLV-1, human T-cell lymphotropic virus type 1; OPA, oligomeric **proanthocyanidins**; BBL, blueberry leaves; HWE, hot water extracts; HPLC, high-performance liquid chromatography; PBS, phosphate buffered saline; PARP, poly (ADP-ribose) polymerase; XIAP, X-linked inhibitor of apoptosis protein; DR, death receptor; CDKs, cyclin-dependent kinases; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

1. Introduction

Adult T-cell leukemia/lymphoma (ATLL) is an aggressive malignant disease caused by clonal proliferation of human T-cell lymphotropic virus type 1 (HTLV-1)-infected mature CD4⁺ cells (Iwanaga, Watanabe, & Yamaguchi, 2012). An estimated 10-20 million people worldwide are infected with HTLV-1. The vast majority of infected people remain clinically asymptomatic, whereas 2-5% of infected patients develop ATLL after a very long latency period of at least 20-30 years (Iwanaga, Watanabe, & Yamaguchi, 2012). Despite the availability of various combination chemotherapy regimens, patients with aggressive ATLL, such as those with acute and lymphoma types, have a poor prognosis after ATLL development with a median survival time of less than 13 months. To date, the known therapies for ATLL are not very effective. Therefore, it is important to identify adequate therapies that are more effective at preventing the development of ATLL or prolonging survival after its occurrence. As ATLL develops over time, daily diet may play an important role in ATLL occurrence.

Natural products, including some foods and herbal medicines, have recently attracted attention as potential therapeutics because of their general low toxicities and costs compared with regular drugs. Epidemiological studies suggest dietary intake of flavonoids, compounds naturally present in many foods, may reduce the risk of tumors of the breast, colon, lung, prostate, and pancreas (Langner & Rzeski, 2012). Recent studies have reported that proliferation of HTLV-1-associated cell lines were inhibited by some constituents or extracts of natural products, such as curcumin (Tomita, et al., 2006), soy-derived isoflavones (Yamasaki, et al., 2007), brown algae fucoxanthin (Ishikawa, et al., 2008),

fucoidan extracts (Haneji, et al., 2005), and *Bidens pilosa* (Nakama, Ishikawa, Nakachi, & Mori, 2011). We have previously shown that *Vaccinium virgatum* Aiton (rabbiteye blueberry) leaf extracts have the strongest inhibitory effect on HTLV-1-associated cell lines growth among 52 agricultural plants (Kai, et al., 2011). However, the growth inhibitory potential of blueberry leaves (BBL) and their constituents on HTLV-1-associated cell lines is not well characterized.

The fruits and leaves of blueberry, a member of the Ericaceae family, contain high levels of polyphenols and have high antioxidant capacity. The content of polyphenols and the antioxidant activity of BBL are relatively high compared with fruits (Li, Feng, Huang, & An, 2013). Despite the fact that BBL have high polyphenols content and antioxidant activity, the leaves have only been used in a tea as a folk medicine treatment for diabetics in Europe. Analysis of the chemical constituents by Matsuo *et al.* has revealed that the most abundant polyphenols in rabbiteye BBL are oligomeric proanthocyanidins (Matsuo, et al., 2010). Since they can suppress hepatitis C virus replication (Nagahama, et al., 2011; Takeshita, et al., 2009), BBL have recently come to be used as a tea also in both North America and Japan. Recent studies show that the proanthocyanidins from rabbiteye BBL exert not only suppressive effect on hepatitis C virus replication but also some health-promoting effects *in vitro* or *in vivo* (Inoue, et al., 2011; Nagahama, et al., 2011; Takami, et al., 2010; Takeshita, et al., 2009). Noteworthy, orally administered proanthocyanidins fraction from rabbiteye BBL, which is separated by a similar method as our study, enhances lipolysis in the liver, resulting in the fraction exerts hypolipidemic effect on obese Otsuka Long-Evans Tokushima Fatty rats (Inoue, et al., 2011). Proanthocyanidins, compounds highly concentrated in dietary plants such as grapes and

cacaos, is known to inhibit the proliferation of various cancer cells including breast cancer, lung cancer, and colorectal carcinoma (Agarwal, Sharma, Zhao, & Agarwal, 2000; Carnésecchi, et al., 2002; Singh, Sharma, & Katiyar, 2011). However, to our knowledge, the growth inhibitory potential of BBL constituents has not been evaluated against HTLV-1-associated cell lines. Therefore, we evaluated the inhibition of cell growth by BBL fractions on HTLV-1-associated cell lines and the effects of those fractions on the cell cycle and induction of apoptosis. Moreover, we analyzed the molecular mechanisms responsible for the apoptotic effects and cell cycle arrest induced by the BBL fractions in HTLV-1-associated cell lines.

2. Materials and methods

2.1. Chemicals

Folin-Denis reagent, cyanidin chloride, chlorogenic acid, and rutin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid monohydrate, (-)-epicatechin, and quinic acid were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (+)-catechin was purchased from Kurita Kogyo (Tokyo, Japan).

2.2. BBL fractions

The BBL fractions were separated by a previously described method with some modifications (Fig. 1) (Inoue, et al., 2011). A lyophilized powder of fresh rabbiteye BBL was kindly supplied by Unkai Shuzo Co., Ltd. (Miyazaki, Japan). The lyophilized BBL

powder (300 g) was extracted for 30 min with boiling water (15 l) and then cooled in an ice bath. After centrifugation (10 min at 1,700×g), the supernatant was lyophilized and stored at -20°C until use. The lyophilized powder (57.7 g) of BBL hot water extracts (BBL-HWE) was suspended in distilled water (3 l), followed by sonication at 50°C for 30 min. Insoluble material was removed by filtration. The filtrate was directly subjected to Sephadex LH-20 column chromatography (5 cm i.d. × 100 cm), and eluted with water containing increasing proportions of methanol (100% water and then 20%, 40%, 60%, 80% and 100% methanol), and finally eluted with 60% acetone. The eluate was collected in test tubes (900 ml in each) to yield seven fractions (F1-F7), concentrated with a vacuum evaporator, and completely freeze dried.

2.3. Chemical assays

The total phenols content was determined by modified Folin-Denis method (Folin & Denis, 1915). Folin-Denis reagent (1 ml) was added to either appropriate dilution of the fractions (1 ml) or standard solution (1 ml). After 5 min, 1 ml of 10% (w/v) Na₂CO₃ was mixed. After 60 min of incubation at room temperature, the test solution was centrifuged at 10,000×g for 5 min. The absorption value was determined at 700 nm, with gallic acid monohydrate used as a standard. The total phenols were expressed as mg gallic acid equivalent/g dry weight.

For quantification of proanthocyanidins, the fractions were measured using the HCl/butanol method (Takeshita, et al., 2009). Each fraction was quantitated from 0.5 mg/ml (for F0-F4) or 0.2 mg/ml (for F5-F7) in methanol. Briefly, 400 µl of the solution

was mixed with 3.5 ml of *n*-butanol/HCl (95:5, v/v) and 100 µl of 2% (w/v) NH₄Fe(SO₄)₂·12H₂O in 2 M HCl, heated in an oil bath at 105°C for 30 min. After heating, the reaction mixture was cooled in water for 15 min, and its absorbance at 550 nm was measured. The **proanthocyanidins** content was expressed in mg of cyanidin chloride equivalent/g dry weight.

The degree of polymerization of proanthocyanidins is estimated by thiolysis (Takeshita, et al., 2009), then reverse-phase high-performance liquid chromatography (HPLC). A solution of 10 mg/ml of a fraction in methanol (500 µl) was mixed with 1,000 µl of 5% (v/v) benzyl mercaptan in methanol and 500 µl of 3.3% (v/v) HCl in methanol, heated at 50°C for 30 min, and then kept at room temperature for 3 h. **The resulting thiolytic products were filtered and then the filtrate (1,000 µl) was injected into the HPLC system.** The HPLC was carried out on a Shimadzu HPLC system (Kyoto, Japan) equipped with a SPD-20A photodiode array detector and an Atlantis T3 column (4.6 mm × 150 mm, 3 µm, Waters, Millford, MA, USA). The separation conditions were as follows: flow rate, 1 ml/min; elution solvent, A (water) and B (methanol); and the gradient program, 15% to 25% B from 0 to 10 min, 25% to 100% B from 10 to 50 min, 100% B from 50 to 65 min. **UV detection was performed at 280 nm. The mean degree of polymerization was calculated as follows: mean degree of polymerization = [sum of (benzylthio adducts × n) + sum of (free flavan-3-ol × n)]/[total free flavan-3-ol] where n represents degree of polymerization of detected flavan-3-ol by thiolysis.**

2.4. Cell culture

We used five HTLV-1-associated cell lines: two HTLV-1-infected T-cell lines and three ATLL-derived cell lines. HTLV-1-infected T-cell lines MT2 and HUT-102 (obtained from the Fujisaki Cell Center, Hayashibara Biochemical Laboratories, Okayama, Japan) were established from HTLV-1-transformed umbilical cord blood T-cell lines (Miyoshi, et al., 1981) and a patient with cutaneous T-cell lymphoma (Poiesz, et al., 1980), respectively. ATLL-derived cell lines Su9T01 and S1T (Arima, et al., 1991) (kindly provided by Dr. N. Arima, Kagoshima University, Japan) and ED-40515 (Maeda, et al., 1985) (kindly provided by Dr. M. Maeda, Kyoto University, Japan) were originally established from peripheral blood lymphocytes of a patient with ATLL. HTLV-1-infected T-cell lines, ATLL-derived cell lines, and the human T-cell acute lymphoblastic leukemia cell line MOLT-4 (obtained from the Fujisaki Cell Center) were cultured in RPMI 1640 medium (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Sigma-Aldrich Co.). All cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

2.5. Cell growth assay

The effect of the extract of BBL on cell growth was examined by the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] cell-counting kit (Dojindo, Kumamoto, Japan), based on the fact that the water soluble formazan reaction occurs only in viable cells. Briefly, cells were inoculated into a 96-well microtiter plate at 9×10^3 cells/well, and cultured for 24 h. The cells were treated with various concentrations of BBL extracts or fractions or compounds (quinic acid,

chlorogenic acid, rutin, (+)-catechin, and (-)-epicatechin) or vehicle control (dimethyl sulphoxide). After 72 h of incubation, WST-8 (10 µl) was added for 4 h of incubation, and the absorbance was measured at 450 nm with a reference wavelength of 650 nm using a multichannel microtiter plate reader (Emax; Molecular Devices, Redwood City, CA, USA). The effect of BBL extract on cell growth was calculated as the percent of control (vehicle-treated cells), which was arbitrarily assigned a value of 100% growth.

2.6. Cell cycle and apoptosis analysis

MT2 and Su9T01 cells (1×10^5 cells/ml) were treated with or without F7 for 24 h. Retrieved cells were then washed with cold phosphate buffered saline (PBS) and fixed in ice-cold methanol at -20°C for at least 30 min. Cells were then treated with 10 µg/ml propidium iodide and 10 µg/ml Ribonuclease A (Sigma-Aldrich Co.), and incubated in the dark at room temperature. Analysis of the cell cycle and detection of the sub-G₁ population were performed using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with MultiCycle software (San Diego, CA, USA). Cells were excited with a 488 nm argon laser line and the fluorescence of propidium iodide was analyzed on FL3 (610 nm), counting 10,000 events per sample.

2.7. Western blot analysis

MT2 and Su9T01 cells (1×10^5 cells/ml) were treated with or without F7 for 24 h. The cells were harvested and collected by centrifugation at $180 \times g$ for 5 min. The cell pellet was washed with ice-cold PBS, and then lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 0.5% Nonidet-P40, 5 mM EDTA and 150 mM NaCl], supplemented with Complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). After vortexing, the cell lysates were centrifuged at $8,000 \times g$ for 20 min at 4°C , and the supernatant was stored at -80°C

until required. Stepwise extraction of the cytosolic fraction and organelle/membrane fractionation was performed with a subcellular proteome extraction kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. The total protein content was determined by the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of the protein samples were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). For blocking, 5% skim milk in PBS containing 0.1% TritonX-100 was used. The following primary antibodies were applied: cleaved caspase-3, cleaved caspase-8, cleaved caspase-9 (Asp 315 and 330), cleaved poly (ADP-ribose) polymerase (PARP), p53, p21^{Waf1/Cip1}, cyclin B1, and cdc2 (Cell Signaling, Danvers, MA, USA), Bax (Biolegend, San Diego, CA, USA), Bcl-2, survivin, X-linked inhibitor of apoptosis protein (XIAP) (R&D Systems, Minneapolis, MN, USA), and death receptor (DR) 4 and DR5 (ProScience Inc., Poway, CA, USA). β -Actin (Sigma-Aldrich Co.) was used as a loading control. The antibodies were incubated overnight at 4°C. Secondary horseradish peroxidase-conjugated anti-mouse, and rabbit antibodies (Cell Signaling), and anti-goat antibody (Pierce, Rockford, IL, USA) were applied for 1 h at room temperature. Primary and secondary antibodies were diluted in Canget signal solution (TOYOBO, Tokyo, Japan). The immunoblots were visualized using Immunostar Zeta (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and a Chemi Doc XRS-J digital densitometer (Bio-Rad Laboratories). Band intensities were quantified with Quantity One Software (Bio-Rad Laboratories).

2.8. Statistical analysis

All results are expressed as the means $\pm$ standard error (SE) of at least three independent experiments. A one-way analysis of variance (ANOVA) and Dunnett's post test were used to evaluate the differences between the means of the results for non-HTLV-1-infected leukemic Molt-4 T cells and HTLV-1-related cells or the control and treated samples. A p -value of <0.05 was considered significant. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Inhibition of the proliferation of HTLV-1-associated cell lines by BBL-HWE and fraction

We have recently shown that an 80% ethanol extract of BBL inhibits the proliferation of HTLV-1-associated cell lines (Kai, et al., 2011). As BBL have been used in Europe as a tea, we first examined whether BBL-HWE also inhibited the proliferation of several HTLV-1-associated cell lines. The BBL-HWE suppressed the cell growth of two HTLV-1-infected T-cell lines (MT2 and HUT-102) and three ATLL-derived cell lines (S1T, ED-40515, and Su9T01) in a dose-dependent manner (Fig. 2). Cell growth of a control HTLV-1-negative T-cell line, MOLT-4, was also inhibited, but MOLT-4 cells were less susceptible to BBL-HWE than HTLV-1-associated cell lines. Inhibition of cell growth was significantly greater in all HTLV-1-associated cell lines than in MOLT-4 cells at 100 μ g/ml of BBL-HWE.

We next separated BBL-HWE by modifications of the method of Inoue *et al.* (Fig. 1) (Inoue, et al., 2011). Then, the effect of the BBL-HWE fractions on MT2 cell proliferation

was examined by the WST-8 assay. As shown in Table 1, the F7 (60% acetone fraction) exhibited lower IC₅₀ values than the other fractions (F0-F6), showing the strongest inhibitory effect. Previous studies reported that acetone (50-60%) fraction from BBL by Sephadex LH-20 contained more total phenols and condensed tannins (proanthocyanidins) than the other fractions (Inoue, et al., 2011; Naczek, Grant, Zadernowski, & Barre, 2006). We then performed chemical analysis of the fractions including total phenols content, proanthocyanidins content, and the mean degree of polymerization. Total phenols and proanthocyanidins contents varied greatly among all the fractions, the highest value being measured for F7 (Table 1). Although F6 and F7 showed very similar values of total phenols content, proanthocyanidins content of F7 was 2.1-fold higher than that of F6. The F7 may contain lower content of monomeric proanthocyanidins such as (–)-epicatechin and (+)-catechin than F6, because the HCl/butanol method is applied to detect the oligomeric forms in proanthocyanidins. The mean degree of polymerization for F6 and F7 was 2.4 and 3.1, respectively. F7 was found to be a complex mixture of proanthocyanidins, namely, oligomeric proanthocyanidins (OPA) with mean degree of polymerization of 3.1. Then, as proanthocyanidins from BBL were mainly composed of epicatechin and catechin units (Inoue, et al., 2011), we examined the growth inhibitory effects of (–)-epicatechin and (+)-catechin in MT2 cells. However, these compounds did not have the effects in the cells (Fig. S1). These results suggest that OPA (except for monomer) from BBL exhibit the growth inhibitory effects on HTLV-1-associated cells. Moreover, A recent study by Matsuo *et al.* has reported that freeze-dried BBL contain 11.34% OPA, 6.16% quinic acid, 2.28% chlorogenic acid, and 0.92% rutin (Matsuo, et al., 2010). Then, we also examined the growth inhibitory effects of quinic acid, chlorogenic acid and rutin in three ATLL-derived

cell lines (S1T, ED-40515, and Su9T01). IC₅₀ values of quinic acid, chlorogenic acid and rutin for three cell lines were >100 µg/ml, showing lack of inhibitory effects (Supplemental Table S1).

The growth inhibitory effects of the OPA-F7 were also evaluated against five HTLV-1-associated cell lines and the HTLV-1-negative T-cell line MOLT-4 (Fig. 3). Although the growth of all tested cell lines was inhibited by the fraction in a dose-dependent manner, MOLT-4 cells (IC₅₀, 32.4 µg/ml) had a lower sensitivity to the fraction than HTLV-1-associated cell lines (IC₅₀, 2.8-22.2 µg/ml). The ATLL-derived cell lines, ED-40515, S1T, and Su9T01, were more sensitive to the OPA fraction compared with the HTLV-1-infected T-cell lines, MT2 and HUT-102. In HTLV-1-infected T-cell lines, MT2 cell growth was more strongly inhibited by the OPA fraction than HUT-102 cell growth. Moreover, Su9T01 cells (IC₅₀, 2.8 µg/ml) had the highest sensitivity to the fractions among all of the tested cell lines. Therefore, we focused on the defining the mechanism of action of the OPA fraction against MT2 and Su9T01 cell growth.

3.2. Induction of apoptosis and cell cycle arrest in MT2 and Su9T01 cells by OPA fraction 7

To investigate whether induction of apoptosis and cell cycle arrest account for the cell growth inhibition observed in HTLV-1-associated cell lines (MT2 and Su9T01), cells were treated with OPA-F7 for 24 h. Then, the cellular DNA content was measured by flow cytometric analysis. After OPA-F7 treatment at high concentrations (15 and 45 µg/ml), the G₂/M cell population in MT2 cells was significantly increased with a decrease in G₁ distribution (Fig. 4A). Treatment with OPA-F7 (7.5 µg/ml) caused the accumulation of

Su9T01 cells at G₂/M which was accompanied by a simultaneous decrease in the G₁ and S populations (Fig. 4B). These results indicate that the OPA-F7 inhibits G₂/M progression. The amount of dying and dead cells in the cell populations was also determined by flow cytometric analysis and corresponded to the percentage of hypodiploid cells present in the sub-G₁ region. In MT2 cells, OPA-F7 induced a significant dose-dependent increase in the sub-G₁ population, regarded as apoptotic cells (Fig. 4C). As shown in Fig. 4D, OPA-F7 also significantly induced cell death in Su9T01 cells at high concentration. These results show that OPA-F7 treatment induces a G₂/M cell cycle arrest together with induction of apoptosis in HTLV-1-associated cell lines (MT2 and Su9T01), which likely contributes to the observed growth inhibitory effects.

3.3. Effects of OPA-F7 from BBL on cell cycle and apoptosis regulatory proteins

To investigate the molecular mechanism of OPA-F7-induced inhibition of cell growth of the HTLV-1-associated cell lines MT2 and Su9T01, we examined the effect of OPA-F7 on the expression of several intracellular regulators of cell cycle and apoptosis by western blot analysis. We first investigated whether the observed apoptosis was due to caspase activation. Indeed, in both MT2 and Su9T01 cells, OPA-F7-induced apoptosis was associated with caspase activation, as shown by PARP cleavage into its death-associated fragment (Fig. 5A). Moreover, OPA-F7 treatment resulted in increases of cleaved/activated caspase-3, -8, -9 in MT2 and Su9T01 cells (Fig. 5A). We then examined the protein expression levels of the pro- and antiapoptotic proteins Bax, survivin, XIAP and Bcl-2. As shown in Fig. 5A, the protein expression levels of Bax and XIAP remained unchanged in MT2 cells. OPA-F7 treatment significantly decreased the expression of survivin, whereas it significantly increased the expression of Bcl-2 in MT2 cells. In contrast, OPA-F7 did not

alter Bax, survivin, XIAP and Bcl-2 expression levels in Su9T01 cells (Fig. 5A). Apoptosis is induced via two main routes, involving either the mitochondrial pathway (the intrinsic pathway) or the death receptor-dependent pathway (the extrinsic pathway). Each is initiated by an individual initiator caspase, caspase-9 in the mitochondrial pathway, and caspase-8 and -10 in the death receptor-dependent pathway (Pop & Salvesen, 2009). OPA-F7 resulted in activation of caspase-8 and -9 in MT2 and Su9T01 cells (Fig. 5A). Therefore, both the mitochondrial and death receptor-dependent pathways may be involved in OPA-F7-induced apoptosis. However, the expression of Bcl-2 in MT2 cells was elevated by OPA-F7 (Fig. 5A). Overexpression of Bcl-2 blocks the mitochondrial pathway (Scaffidi, et al., 1998). We then investigated whether the death receptor-dependent pathway was involved in the observed apoptosis. After OPA-F7 treatment, cells were separated into cytosolic and membrane fractions. The expression level of DR4 and DR5 were analyzed by western blot analysis. As shown in Fig. 5B, the up-regulation of DR5 expression by OPA-F7 mainly occurred in the membrane fraction (right) and not in the cytosolic fraction (left) in MT2 and Su9T01 cells. The protein expression levels of DR4 remained mostly unchanged in both the cytosolic and membrane fractions. These results likely suggest that OPA-F7-induced apoptosis occurs through the death receptor-mediated caspase-dependent pathway of cell death.

We then examined the effect of OPA-F7 treatment on cell cycle regulators. The cell cycle is regulated at different check points by the interaction of a variety of cyclins and cyclin-dependent kinases (CDKs). One of the checkpoints, the G₂/M, is regulated by cyclin B1 and the cdc2/CDK1 complex. Our results showed that OPA-F7 treatment down-regulated the protein level of cyclin B1 and cdc2 in MT2 and Su9T01 cells as shown

in Fig. 5A. p53 plays a critical role in the regulation of the cell cycle and apoptosis. p21^{Waf1/Cip1} is the principal mediator of cell cycle arrest and is also involved in many important cellular processes including cell cycle progression, apoptosis, and transcriptional regulation through both p53-dependent and p53-independent pathways (Abbas & Dutta, 2009). Treatment of the HTLV-1-infected T-cells MT2 resulted in a significant increase in p53 and p21^{Waf1/Cip1} protein levels (Fig. 5A). In contrast, p53 and p21^{Waf1/Cip1} were undetected in the ATLL-derived cells Su9T01. These results suggest that the p53-dependent pathway is involved in OPA-F7-induced growth inhibition of the HTLV-1-infected T-cells MT2, whereas the p53-independent pathway is involved in the inhibition of the ATLL-derived cells Su9T01.

4. Discussion

Proanthocyanidins, naturally occurring phytochemicals, induces cell cycle arrest at the G₀/G₁ and/or G₂/M checkpoint and/or apoptosis, resulting in inhibition of cell growth in various cancer cells (Nandakumar, Singh, & Katiyar, 2008). In the present study, our data revealed that an OPA fraction from BBL apparently inhibited the growth of HTLV-1-associated cell lines (Fig. 3). Chemical characteristics of proanthocyanidins, such as the galloylation of catechins and the degree of polymerization, are related to their bioactive capacity. For example, the antiproliferative capacity of a proanthocyanidins fraction toward human colorectal adenocarcinoma HT29 cells is enhanced by both polymerization and galloylation (Lizarraga, et al., 2007). As shown in Table 1, the most effective fraction (F7) contained OPA with the highest mean degree of polymerization and

the highest total activity per **proanthocyanidins**. Thiol degradation products of an OPA fraction from BBL contained catechin, epicatechin, and epicatechin-4-hydroxyethylthio ether (Inoue, et al., 2011), although it did not contain galloylated catechins such as epigallocatechin and epicatechin gallate. Therefore, proanthocyanidin polymerization should play a more important role than galloylation in the antiproliferative effect of the OPA fraction from BBL. We further showed that the cell growth inhibitory effect was due to an induction in G₂/M cell cycle arrest together with induction of apoptosis in MT2 and Su9T01 cells (Fig. 4). A previous report shows that proanthocyanidins from various plants mainly cause G₁ cell cycle arrest (Nandakumar, Singh, & Katiyar, 2008). For example, proanthocyanidins from grape seed, cranberry, apple, cocoa, and green tea leaves arrest the cell cycle at G₁ in breast carcinoma cells, esophageal adenocarcinoma cells, non-small cell lung cancer cells, epidermoid carcinoma cells, and head and neck squamous cell carcinoma cells (Agarwal, Sharma, Zhao, & Agarwal, 2000; Kozikowski, Tüchtemel, Böttcher, & Romanczyk, 2003; Kresty, Howell, & Baird, 2008; Kuo, Hsu, Lin, & Lin, 2005; Meeran & Katiyar, 2007; Pierini, et al., 2008; Prasad & Katiyar, 2012; Singh, Sharma, & Katiyar, 2011; Sun & Hai Liu, 2006). In contrast, proanthocyanidins **originated from** cocoa, grape seed and pine bark cause G₂/M arrest of the cell cycle in colon carcinoma cells and pancreatic cancer cells (Carnésecchi, et al., 2002; Lizarraga, et al., 2007; Prasad, Vaid, & Katiyar, 2012). Interestingly, studies of **proanthocyanidins** from grape seed by Katiyar *et al.* show that proanthocyanidins arrest the cell cycle at G₁ in non-small cell lung cancer cells, epidermoid carcinoma cells, and head and neck squamous cell carcinoma cells (Meeran & Katiyar, 2007; Prasad & Katiyar, 2012; Singh, Sharma, & Katiyar, 2011), whereas it arrested it at G₂/M in pancreatic cancer cells (Prasad, Vaid, & Katiyar, 2012). It is evident

from these results that whether a cell is arrested at G₁ or G₂/M by proanthocyanidins depends on the cell type. Although the OPA fraction from BBL induced G₂/M cell cycle arrest in HTLV-1-associated cell lines, the fraction might induce G₁ cell cycle arrest in other types of cancer cells. It also remains controversial what cellular environment influences proanthocyanidins-induced cell cycle arrest at G₁ or G₂/M.

Cellular responses in the HTLV-1-infected T-cells MT2 and ATLL-derived cells Su9T01 by OPA-F7 differed in the expression of some apoptosis- and cell cycle-related proteins. Moreover, OPA-F7 inhibited the cell growth of Su9T01 cells at low concentrations (7.5 µg/ml) compared with treatment of MT2 cells at 45 µg/ml. By western blot analysis, the protein expression of p53 and p21^{Cip1/Waf1} were increased by OPA-F7 treatment of MT2 cells, whereas the proteins could not be detected in Su9T01 cells. Induction of the CDK inhibitor p21^{Cip1/Waf1} is mediated by both p53-dependent and -independent mechanisms of cellular stress and causes cell cycle arrest at G₁ or G₂/M resulting in a damage response and cell senescence (Roninson, 2002). Besides cell cycle arrest, p21^{Cip1/Waf1} plays essential roles in damage response by regulating fundamental processes like apoptosis and transcription (Cazzalini, Scovassi, Savio, Stivala, & Prosperi, 2010). A recent study reported that the expression of p21^{Cip1/Waf1} is very low in ATLL-derived cell lines, although its expression is high in HTLV-1-infected cell lines (Watanabe, et al., 2010). ATLL-derived cell lines have a significantly decreased rate of growth after UV irradiation, although the rate of growth of HTLV-1-infected cell lines is not changed (Watanabe, et al., 2010). In the colon carcinoma cells HCT116, ionizing radiation treatment clearly induces death of p21^{Cip1/Waf1} deficient HCT116 cells, whereas the treatment only slightly induces the death of wild-type HCT116 cells (Sohn, Essmann, Schulze-Osthoff, & Jänicke, 2006). These findings indicate that low

expression of p21^{Cip1/Waf1} may increase in response to cellular stress. Therefore, Su9T01 cells without p21^{Cip1/Waf1} expression may be more strongly inhibited by treatment with OPA-F7 in comparison to MT2 cells which express higher levels of p21^{Cip1/Waf1}. As another factor, HTLV-1 Tax, the virus-encoded regulatory protein, may also be able to explain the strong growth inhibitory effect of OPA-F7 in Su9T01 cells. Tax has been shown to play a critical role in cellular transformation by interfering with genome instability, cell cycle and apoptosis (Matsuoka & Jeang, 2007). Moreover, Tax enhances expression of various antiapoptotic proteins, including c-Flip, Bcl-xL, Bfl-1, and Hiap-1 (Silbermann & Grassmann, 2007). Although primary ATLL cells and ATLL-derived cell lines do not express significant levels of Tax, HTLV-1-infected cell lines highly express Tax (Watanabe, et al., 2010). Due to Tax-mediated antiapoptotic effects in HTLV-1-infected cell lines MT2, Su9T01 cells without Tax expression may be more strongly inhibited by treatment with OPA-F7 in comparison to MT2 cells which express higher levels of Tax.

In response to various cellular stress including DNA damage, ribonucleotide depletion, and abnormal proliferative signals, p53 activates transcription of target genes with functions in cell cycle control, DNA repair, and apoptosis (Nakamura, 2004). Treatment of MT2 cells with OPA-F7 clearly increased p53, cleaved caspase-9, caspase-3, and PARP, and decreased survivin expression. Survivin directly blocks the processing and activation of effector caspases 3 and 7 (Shin, et al., 2001). Moreover, DNA damage induces the p53-survivin signaling pathway and down-regulates survivin expression, resulting in cell cycle arrest and apoptosis (Zhou, et al., 2002). Therefore, the antiapoptotic protein survivin was down-regulated by OPA-F7 treatment of MT2 cells, suggesting that survivin also plays a role in OPA-F7-induced apoptosis. In addition to apoptosis induction, treatment of

OPA-F7 also induced cell cycle arrest at G₂/M. The role of p53 in G₂/M cell cycle arrest in response to DNA damage is multiple targets that can generally be considered to influence either the cell cycle (i.e., cyclin-B, cdc2) or the mitotic machinery (i.e., topoisomerase II) (Brown, Boswell, Raj, & Lee, 2007). Interestingly, cocoa-derived **proanthocyanidins** significantly reduces topoisomerase II activity and proliferation in cancer cell lines (Lanoue, Green, Kwik-Urbe, & Keen, 2010). Therefore, the growth inhibitory effect induced by OPA-F7 may cause by interference with topoisomerase II.

Caspase 8 is activated by DRs including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors (DR4 and DR5) and CD95. The OPA fraction from BBL significantly increased cleaved/active caspase 8 in MT2 and Su9T01 cells (Fig. 5A). The fraction also increased DR5 protein in the membrane fraction of these cells (Fig. 5B), suggesting that the DRs will trigger signaling pathways in the treated cells. TRAIL interacts with the death domain-containing receptors DR4 and DR5, resulting in apoptosis induction (Srivastava, 2001). TRAIL selectively induces apoptosis in tumor cells without damaging normal tissues (Srivastava, 2001). Therefore, TRAIL is one of the most promising therapeutic agents against different types of cancer. However, some tumor cells remain resistant to TRAIL-induced apoptosis (Zhang & Fang, 2005). Recent studies have reported that DR can be up-regulated by some natural phytochemicals including quercetin (Psahoulia, Drosopoulos, Doubravska, Andera, & Pintzas, 2007), flavonolignan silibinin (Kauntz, Bousserouel, Gossé, & Raul, 2012), and apple **procyanidins** (Maldonado, Bousserouel, Gossé, Lobstein, & Raul, 2010; Maldonado-Celis, Bousserouel, Gossé, Lobstein, & Raul, 2009), with a resulting decrease in TRAIL-induced resistance to apoptosis. ATLL is also highly resistant to TRAIL-mediated cell death. Rocaglamide, a

naturally occurring herbal compound, reduces TRAIL resistance in HTLV-1-associated cells (Bleumink, et al., 2011). Wogonin and the structurally related natural flavones apigenin and chrisin also reduce TRAIL resistance in HTLV-1-associated cell lines by up-regulation of DR5 (Ding, et al., 2012). Therefore, OPA-F7 from BBL may also limit TRAIL resistance in HTLV-1-associated cell lines.

5. Conclusions

This is the first study to show the cell growth inhibitory effect of an OPA fraction from BBL on HTLV-1-associated cell lines. The fraction inhibited the growth of these cell lines by inducing apoptosis and G₂/M cell cycle arrest. OPA fraction-induced apoptosis of HTLV-1-associated cell lines requires the death receptor-mediated caspase-dependent pathway of cell death and treatment of the fraction resulted in arrest at G₂/M by down-regulation of cyclin B1 and cdc2. The OPA fraction from BBL may have the potential to serve as a source of novel compounds for reducing the risk of developing ATLL. However, the growth inhibitory effect induced by the OPA fraction has not yet been verified *in vivo*. Although proanthocyanidins from BBL are high-molecular weight oligomers, orally administered proanthocyanidins must be absorbed from the digestive tract to provide beneficial effects. Recent studies provide evidence that orally administered apple proanthocyanidins (consisting of dimers to pentamers) can be rapidly absorbed and reach both the plasma and liver tissue (García-Ramírez, et al., 2006; Ou & Gu, 2013; Shoji, et al., 2006). Moreover, orally administered proanthocyanidins from grape seed inhibit the growth of tumor cells in mouse tumor models (Prasad, Vaid, & Katiyar, 2012; Singh, Sharma, &

Katiyar, 2011). Recent *in vivo* studies of ATLL have shown that food and plant constituents such as curcumin, soy-isoflavones, fucoidan, fucoxanthin, and *Bidens pilosa* inhibit the growth of tumor cells in immunodeficient mice transplanted with HTLV-1-associated cell lines (Haneji, et al., 2005; Ishikawa, et al., 2008; Nakama, Ishikawa, Nakachi, & Mori, 2011; Tomita, et al., 2006; Yamasaki, et al., 2007). Further studies of the OPA fraction from BBL on HTLV-1-associated cell lines are needed to evaluate the growth inhibitory effect *in vivo* and the molecular mechanism responsible for the effect *in vitro*.

Acknowledgment

This work was supported in part by Grant-in-Aid for Scientific Research of Priority Area from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a research fund from the Miyazaki Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, JST. Part of this work was conducted at Miyazaki Prefectural Industrial Support Foundation, Miyazaki, Japan. We thank Dr. Michiyuki Maeda (Kyoto University) and Dr. Naomichi Arima (Kagoshima University) for providing HTLV-1-associated cell lines. We also thank Dr. Masao Yamasaki (Miyazaki University) for his expertise in flow cytometric analysis.

473 **References**

- 474 Abbas, T., & Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities.
475 *Nature Reviews Cancer*, 9(6), 400-414.
- 476 Agarwal, C., Sharma, Y., Zhao, J., & Agarwal, R. (2000). A polyphenolic fraction from
477 grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468
478 cells by inhibiting mitogen-activated protein kinases activation and inducing G1
479 arrest and differentiation. *Clinical Cancer Research*, 6(7), 2921-2930.
- 480 Arima, N., Molitor, J. A., Smith, M. R., Kim, J. H., Daitoku, Y., & Greene, W. C. (1991).
481 Human T-cell leukemia virus type I Tax induces expression of the Rel-related
482 family of kappa B enhancer-binding proteins: evidence for a pretranslational
483 component of regulation. *Journal of Virology*, 65(12), 6892-6899.
- 484 Bleumink, M., Köhler, R., Giaisi, M., Proksch, P., Krammer, P. H., & Li-Weber, M. (2011).
485 Rocaglamide breaks TRAIL resistance in HTLV-1-associated adult T-cell
486 leukemia/lymphoma by translational suppression of c-FLIP expression. *Cell Death*
487 *and Differentiation*, 18(2), 362-370.
- 488 Brown, L., Boswell, S., Raj, L., & Lee, S. W. (2007). Transcriptional targets of p53 that
489 regulate cellular proliferation. *Critical Reviews in Eukaryotic Gene Expression*,
490 17(1), 73-85.
- 491 Carnésecchi, S., Schneider, Y., Lazarus, S. A., Coehlo, D., Gossé, F., & Raul, F. (2002).
492 Flavanols and procyanidins of cocoa and chocolate inhibit growth and polyamine
493 biosynthesis of human colonic cancer cells. *Cancer Letters*, 175(2), 147-155.
- 494 Cazzalini, O., Scovassi, A. I., Savio, M., Stivala, L. A., & Prosperi, E. (2010). Multiple
495 roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response.
496 *Mutation Research*, 704(1-3), 12-20.
- 497 Ding, J., Polier, G., Köhler, R., Giaisi, M., Krammer, P. H., & Li-Weber, M. (2012).
498 Wogonin and related natural flavones overcome tumor necrosis factor-related
499 apoptosis-inducing ligand (TRAIL) protein resistance of tumors by down-regulation
500 of c-FLIP protein and up-regulation of TRAIL receptor 2 expression. *Journal of*
501 *Biological Chemistry*, 287(1), 641-649.
- 502 Folin, O., & Denis, W. (1915). A colorimetric method for the determination of phenols
503 (and phenol derivatives) in urine. *Journal of Biological Chemistry*, 22, 305-308.
- 504 García-Ramírez, B., Fernandez-Larrea, J., Salvadó, M. J., Ardèvol, A., Arola, L., & Bladé,
505 C. (2006). Tetramethylated dimeric procyanidins are detected in rat plasma and
506 liver early after oral administration of synthetic oligomeric procyanidins. *Journal of*
507 *Agricultural and Food Chemistry*, 54(7), 2543-2551.
- 508 Haneji, K., Matsuda, T., Tomita, M., Kawakami, H., Ohshiro, K., Uchihara, J. N., Masuda,
509 M., Takasu, N., Tanaka, Y., Ohta, T., & Mori, N. (2005). Fucoidan extracted from
510 Cladosiphon okamuranus Tokida induces apoptosis of human T-cell leukemia virus
511 type 1-infected T-cell lines and primary adult T-cell leukemia cells. *Nutrition and*
512 *Cancer*, 52(2), 189-201.
- 513 Inoue, N., Nagao, K., Nomura, S., Shirouchi, B., Inafuku, M., Hirabaru, H., Nakahara, N.,
514 Nishizono, S., Tanaka, T., & Yanagita, T. (2011). Effect of *Vaccinium ashei* reade

- leaf extracts on lipid metabolism in obese OLETF rats. *Bioscience, Biotechnology and Biochemistry*, 75(12), 2304-2308.
- Ishikawa, C., Tafuku, S., Kadekaru, T., Sawada, S., Tomita, M., Okudaira, T., Nakazato, T., Toda, T., Uchihara, J. N., Taira, N., Ohshiro, K., Yasumoto, T., Ohta, T., & Mori, N. (2008). Anti-adult T-cell leukemia effects of brown algae fucoxanthin and its deacetylated product, fucoxanthinol. *International Journal of Cancer*, 123(11), 2702-2712.
- Iwanaga, M., Watanabe, T., & Yamaguchi, K. (2012). Adult T-cell leukemia: a review of epidemiological evidence. *Frontiers in Microbiology*, 3, 322.
- Kai, H., Akamatsu, E., Torii, E., Kodama, H., Yukizaki, C., Sakakibara, Y., Suiko, M., Morishita, K., Kataoka, H., & Matsuno, K. (2011). Inhibition of proliferation by agricultural plant extracts in seven human adult T-cell leukaemia (ATL)-related cell lines. *Journal of Natural Medicines*, 65(3-4), 651-655.
- Kauntz, H., Bousserouel, S., Gossé, F., & Raul, F. (2012). The flavonolignan silibinin potentiates TRAIL-induced apoptosis in human colon adenocarcinoma and in derived TRAIL-resistant metastatic cells. *Apoptosis*, 17(8), 797-809.
- Kozikowski, A. P., Tückmantel, W., Böttcher, G., & Romanczyk, L. J. (2003). Studies in polyphenol chemistry and bioactivity. 4.(1) Synthesis of trimeric, tetrameric, pentameric, and higher oligomeric epicatechin-derived procyanidins having all-4beta,8-interflavan connectivity and their inhibition of cancer cell growth through cell cycle arrest. *Journal of Organic Chemistry*, 68(5), 1641-1658.
- Kresty, L. A., Howell, A. B., & Baird, M. (2008). Cranberry proanthocyanidins induce apoptosis and inhibit acid-induced proliferation of human esophageal adenocarcinoma cells. *Journal of Agricultural and Food Chemistry*, 56(3), 676-680.
- Kuo, P. L., Hsu, Y. L., Lin, T. C., & Lin, C. C. (2005). The antiproliferative activity of prodelphinidin B-2 3'-O-gallate from green tea leaf is through cell cycle arrest and Fas-mediated apoptotic pathway in A549 cells. *Food and Chemical Toxicology*, 43(2), 315-323.
- Langner, E., & Rzeski, W. (2012). Dietary derived compounds in cancer chemoprevention. *Wspolczesna Onkologia*, 16(5), 394-400.
- Lanoue, L., Green, K. K., Kwik-Urbe, C., & Keen, C. L. (2010). Dietary factors and the risk for acute infant leukemia: evaluating the effects of cocoa-derived flavanols on DNA topoisomerase activity. *Experimental Biology and Medicine*, 235(1), 77-89.
- Li, C., Feng, J., Huang, W. Y., & An, X. T. (2013). *Composition of polyphenols and antioxidant activity of rabbiteye blueberry (Vaccinium ashei) in Nanjing. Journal of Agricultural and Food Chemistry*, 61(3), 523-531.
- Lizarraga, D., Lozano, C., Briedé, J. J., van Delft, J. H., Touriño, S., Centelles, J. J., Torres, J. L., & Cascante, M. (2007). The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS Journal*, 274(18), 4802-4811.
- Maeda, M., Shimizu, A., Ikuta, K., Okamoto, H., Kashihara, M., Uchiyama, T., Honjo, T., & Yodoi, J. (1985). Origin of human T-lymphotrophic virus I-positive T cell lines in adult T cell leukemia. Analysis of T cell receptor gene rearrangement. *Journal of Experimental Medicine*, 162(6), 2169-2174.

- Maldonado, M. E., Bousserouel, S., Gossé, F., Lobstein, A., & Raul, F. (2010). Implication of NF- κ B and p53 in the expression of TRAIL-death receptors and apoptosis by apple procyanidins in human metastatic SW620 cells. *Biomedica*, 30(4), 577-586.
- Maldonado-Celis, M. E., Bousserouel, S., Gossé, F., Lobstein, A., & Raul, F. (2009). Apple procyanidins activate apoptotic signaling pathway in human colon adenocarcinoma cells by a lipid-raft independent mechanism. *Biochemical and Biophysical Research Communications*, 388(2), 372-376.
- Matsuo, Y., Fujita, Y., Ohnishi, S., Tanaka, T., Hirabaru, H., Kai, T., Sakaida, H., Nishizono, S., & Kouno, I. (2010). Chemical constituents of the leaves of rabbiteye blueberry (*Vaccinium ashei*) and characterisation of polymeric proanthocyanidins containing phenylpropanoid units and A-type linkages. *Food Chemistry*, 121(4), 1073-1079.
- Matsuoka, M., & Jeang, K. T. (2007). Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nature Reviews Cancer*, 7(4), 270-280.
- Meeran, S. M., & Katiyar, S. K. (2007). Grape seed proanthocyanidins promote apoptosis in human epidermoid carcinoma A431 cells through alterations in Cdk1-Cdk-cyclin cascade, and caspase-3 activation via loss of mitochondrial membrane potential. *Experimental Dermatology*, 16(5), 405-415.
- Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K., & Hinuma, Y. (1981). Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature*, 294(5843), 770-771.
- Naczek, M., Grant, S., Zadernowski, R., & Barre, E. (2006). Protein precipitating capacity of phenolics of wild blueberry leaves and fruits. *Food Chemistry*, 96(4), 640-647.
- Nagahama, K., Eto, N., Yamamori, K., Nishiyama, K., Sakakibara, Y., Iwata, T., Uchida, A., Yoshihara, I., & Suiko, M. (2011). Efficient approach for simultaneous estimation of multiple health-promoting effects of foods. *Journal of Agricultural and Food Chemistry*, 59(16), 8575-8588.
- Nakama, S., Ishikawa, C., Nakachi, S., & Mori, N. (2011). Anti-adult T-cell leukemia effects of *Bidens pilosa*. *International Journal of Oncology*, 38(4), 1163-1173.
- Nakamura, Y. (2004). Isolation of p53-target genes and their functional analysis. *Cancer Science*, 95(1), 7-11.
- Nandakumar, V., Singh, T., & Katiyar, S. K. (2008). Multi-targeted prevention and therapy of cancer by proanthocyanidins. *Cancer Letters*, 269(2), 378-387.
- Ou, K., & Gu, L. (2013). Absorption and metabolism of proanthocyanidins. *Journal of Functional Foods*. (in press)
- Pierini, R., Kroon, P. A., Guyot, S., Ivory, K., Johnson, I. T., & Belshaw, N. J. (2008). Procyanidin effects on oesophageal adenocarcinoma cells strongly depend on flavan-3-ol degree of polymerization. *Molecular Nutrition and Food Research*, 52(12), 1399-1407.
- Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., & Gallo, R. C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proceedings of the National Academy of Sciences of the United States of America*, 77(12), 7415-7419.

- Pop, C., & Salvesen, G. S. (2009). Human caspases: activation, specificity, and regulation. *Journal of Biological Chemistry*, 284(33), 21777-21781.
- Prasad, R., & Katiyar, S. K. (2012). Bioactive phytochemical proanthocyanidins inhibit growth of head and neck squamous cell carcinoma cells by targeting multiple signaling molecules. *PLoS One*, 7(9), e46404.
- Prasad, R., Vaid, M., & Katiyar, S. K. (2012). Grape proanthocyanidin inhibit pancreatic cancer cell growth in vitro and in vivo through induction of apoptosis and by targeting the PI3K/Akt pathway. *PLoS One*, 7(8), e43064.
- Psahoulia, F. H., Drosopoulos, K. G., Doubravska, L., Andera, L., & Pintzas, A. (2007). Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. *Molecular Cancer Therapeutics*, 6(9), 2591-2599.
- Roninson, I. B. (2002). Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. *Cancer Letters*, 179(1), 1-14.
- Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., & Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO Journal*, 17(6), 1675-1687.
- Shin, S., Sung, B. J., Cho, Y. S., Kim, H. J., Ha, N. C., Hwang, J. I., Chung, C. W., Jung, Y. K., & Oh, B. H. (2001). An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. *Biochemistry*, 40(4), 1117-1123.
- Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., Kanda, T., Ohtake, Y., & Goda, Y. (2006). Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, 54(3), 884-892.
- Silbermann, K., & Grassmann, R. (2007). Human T cell leukemia virus type 1 Tax-induced signals in cell survival, proliferation, and transformation. *Signal Transduction*, 7(1), 34-52.
- Singh, T., Sharma, S. D., & Katiyar, S. K. (2011). Grape proanthocyanidins induce apoptosis by loss of mitochondrial membrane potential of human non-small cell lung cancer cells in vitro and in vivo. *PLoS One*, 6(11), e27444.
- Sohn, D., Essmann, F., Schulze-Osthoff, K., & Jänicke, R. U. (2006). p21 blocks irradiation-induced apoptosis downstream of mitochondria by inhibition of cyclin-dependent kinase-mediated caspase-9 activation. *Cancer Research*, 66(23), 11254-11262.
- Srivastava, R. K. (2001). TRAIL/Apo-2L: mechanisms and clinical applications in cancer. *Neoplasia*, 3(6), 535-546.
- Sun, J., & Hai Liu, R. (2006). Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. *Cancer Letters*, 241(1), 124-134.
- Takami, Y., Uto, H., Takeshita, M., Kai, H., Akamatsu, E., Moriuchi, A., Hasegawa, S., Oketani, M., Ido, A., Kataoka, H., & Tsubouchi, H. (2010). Proanthocyanidin derived from the leaves of *Vaccinium virgatum* suppresses platelet-derived growth factor-induced proliferation of the human hepatic stellate cell line LI90. *Hepatology Research*, 40(4), 337-345.

- 649 Takeshita, M., Ishida, Y., Akamatsu, E., Ohmori, Y., Sudoh, M., Uto, H., Tsubouchi, H., &
650 Kataoka, H. (2009). Proanthocyanidin from blueberry leaves suppresses expression
651 of subgenomic hepatitis C virus RNA. *Journal of Biological Chemistry*, 284(32),
652 21165-21176.
- 653 Tomita, M., Kawakami, H., Uchihara, J. N., Okudaira, T., Masuda, M., Takasu, N.,
654 Matsuda, T., Ohta, T., Tanaka, Y., Ohshiro, K., & Mori, N. (2006). Curcumin
655 (diferuloylmethane) inhibits constitutive active NF-kappaB, leading to suppression
656 of cell growth of human T-cell leukemia virus type I-infected T-cell lines and
657 primary adult T-cell leukemia cells. *International Journal of Cancer*, 118(3),
658 765-772.
- 659 Watanabe, M., Nakahata, S., Hamasaki, M., Saito, Y., Kawano, Y., Hidaka, T., Yamashita,
660 K., Umeki, K., Taki, T., Taniwaki, M., Okayama, A., & Morishita, K. (2010).
661 Downregulation of CDKN1A in adult T-cell leukemia/lymphoma despite
662 overexpression of CDKN1A in human T-lymphotropic virus 1-infected cell lines.
663 *Journal of Virology*, 84(14), 6966-6977.
- 664 Yamasaki, M., Fujita, S., Ishiyama, E., Mukai, A., Madhyastha, H., Sakakibara, Y., Suiko,
665 M., Hatakeyama, K., Nemoto, T., Morishita, K., Kataoka, H., Tsubouchi, H., &
666 Nishiyama, K. (2007). Soy-derived isoflavones inhibit the growth of adult T-cell
667 leukemia cells in vitro and in vivo. *Cancer Science*, 98(11), 1740-1746.
- 668 Zhang, L., & Fang, B. (2005). Mechanisms of resistance to TRAIL-induced apoptosis in
669 cancer. *Cancer Gene Therapy*, 12(3), 228-237.
- 670 Zhou, M., Gu, L., Li, F., Zhu, Y., Woods, W. G., & Findley, H. W. (2002). DNA damage
671 induces a novel p53-survivin signaling pathway regulating cell cycle and apoptosis
672 in acute lymphoblastic leukemia cells. *Journal of Pharmacology and Experimental*
673 *Therapeutics*, 303(1), 124-131.

Figure Captions

Fig. 1. Separation of the polyphenols from BBL-HWE using a Sephadex LH-20 column chromatography.

Fig. 2. Cell growth inhibitory effects of BBL-HWE in HTLV-1-associated cell lines. Cells were treated in the presence or absence of BBL-HWE and cell growth was measured by the WST-8 assay at 72 h. The relative growth of the cultured cell is presented as the mean $\pm$ SE (n=3). The data obtained were statistically analyzed using one way ANOVA followed by Dunnett's test for individual comparison of groups with HTLV-1-negative leukemic Molt4 T cells. * p <0.05, ** p <0.01, and *** p <0.001.

Fig. 3. Cell growth inhibitory effects of OPA-F7 in HTLV-1-associated cell lines. Cells were treated in the presence or absence of OPA-F7 and cell growth was measured by the WST-8 assay at 72 h. The relative growth of the cultured cell is presented as the mean $\pm$ SE (n=3). The data obtained were statistically analyzed using one way ANOVA followed by Dunnett's test for individual comparison of groups with HTLV-1-negative leukemic Molt4 T cells. ** p <0.01, and *** p <0.001. IC₅₀ data are summarized in the corresponding table below graph.

Fig. 4. OPA-F7 induces G₂/M cell cycle arrest and apoptosis in HTLV-1-associated cell lines. (A) Cell cycle analysis of MT2 treated with OPA-F7, (B) Cell cycle analysis of

Su9T01 treated with OPA-F7. Cells were treated in the absence or presence of OPA-F7. DNA content was analyzed to indicate the stage of the cell cycle. Data represent the mean percentages of the cells at each stage of the cell cycle from three independent experiments. (C) Induction of apoptosis in the HTLV-1-infected T-cell line MT2 by OPA-F7, (D) Induction of apoptosis in the ATLL-derived cell line Su9T01 by OPA-F7. Sub-G₁ percentage represents apoptotic cells. The results are representative of three independent experiments. The data obtained from the experiment were statistically analyzed using one way ANOVA followed by Dunnett's test for individual comparison of groups with non-treated cells. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Fig. 5. Effects of OPA-F7 on the expression of cell cycle-associated and pro- and antiapoptotic proteins. MT2 and Su9T01 cells were treated with or without OPA-F7 for 24 h. (A) Amounts of cell cycle-associated proteins cyclin B1, cdc2, p21^{Waf1/Cip1}, p53, and apoptotic proteins cleaved caspase-3, -8, -9 and PARP, survivin, XIAP, Bcl-2 and Bax. (B) The cytosolic fraction (left) and membrane fraction (right) were extracted. Western blot analysis was carried out with anti-DR4 and DR5 antibodies. To assure comparable loading, the same blot was probed with anti- β -actin antibody.

Table 1. Fractionation of growth inhibition potency of BBL-HWE toward MT2 Cells.

	Weight (g)	IC ₅₀ ^a (µg/ml)	Specific activity (1/IC ₅₀)	Total phenols content ^b (mg/g)	Proanthocyanidins content ^c (mg/g)	Total activity per total phenols (mg/IC ₅₀)	Total activity per proanthocyanidins (mg/IC ₅₀)	Mean degree of polymerization
BBL-HWE ^d	57.74	39.5	2.53×10 ⁻²	NT ^e	NT	NT	NT	NT
F0	21.40	>100.0	<1.00×10 ⁻²	22.8	0.1	<0.2	<0.0	NT
F1	6.46	>100.0	<1.00×10 ⁻²	74.1	1.9	<0.8	<0.0	NT
F2	1.56	67.2	1.49×10 ⁻²	297.6	1.2	4.4	<0.0	NT
F3	1.17	>100.0	<1.00×10 ⁻²	417.9	0.8	<4.2	<0.0	NT
F4	2.69	>100.0	<1.00×10 ⁻²	445.5	2.6	<4.5	<0.0	NT
F5	3.89	>100.0	<1.00×10 ⁻²	415.1	17.0	<4.2	<0.2	NT
F6	4.71	51.2	1.95×10 ⁻²	606.4	95.6	11.8	1.9	2.4
F7	8.69	13.8	7.25×10 ⁻²	670.0	202.4	48.6	14.7	3.1

^aIC₅₀: 50% inhibitory concentration. ^b expressed in mg gallic acid equivalents per gram of dry weight. ^c expressed in mg cyaniding chloride equivalents per gram of dry weight. ^d BBL-HWE: Blueberry leaf hot water extract. ^e NT: Not tested.

Cell Line	IC ₅₀ (µg/ml)	Cell Line	IC ₅₀ (µg/ml)
MOLT-4	32.4	ED-40515	12.7
HUT-102	22.2	S1T	10.7
MT2	13.8	Su9T01	2.8