Journal of Animal and Veterinary Advances 14 (7): 197-204, 2015
ISSN: 1680-5593

Elderberry Extracts Suppress Melanoma Growth *In vitro*

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Abstract: Melanoma is the deadliest form of skin cancer and is gradually increasing globally amongst the elderly population. Current treatments for melanoma are invasive have adverse effects on important immunological cells and may cause immunosuppression of the patient. This study demonstrates that extracts separated from elderberry from the European black elder (*Sambucus nigra*) significantly decrease proliferation of a murine melanoma (B16-F10) and human neuroblastoma (SH-SY5Y) cell line. Extracts separated from the European black elder (*Sambucus nigra*) significantly decrease proliferation of a murine melanoma (B16-F10) and human neuroblastoma (SH-SY5Y) cell line. A transformed, non-cancerous cell line (CHO-K1) was not significantly inhibited. Elderberry fractions were investigated for their effects on murine spleen cells. Elderberry fractions were less toxic to elderly mouse spleen cells compared to young mouse spleen cells. The same elderberry fractions that killed melanoma cells did not affect spleen cell proliferation responses to the same extent. Elderberry extracts are capable of suppressing melanoma tumor growth without inhibiting important immunological factors such asIL-2 secretion. Elderberry may have use in diet-based strategies that combat incidence of cancer and other diseases that become more prevalent with aging.

Key words: Immunosenescence, elderberry, tumor, immune, anthocyanin

INTRODUCTION

Over the past 20 years, anticancer researchers have been studying minimally invasive cancer treatments to address the severity of the many cancer treatment side effects. A proposed treatment is utilization of dark-pigmented berry extracts that are able to suppress cancer cell growth (Katsube *et al*., 2003). The anticancer potential of dark-pigmented berries comes from the phytochemical properties of their phenolic components (Seeram, 2008). Several phenolic berry extracts show promise as a preventative strategy against human cancer cell growth, angiogenesis, metastasis and free radical scavenging (Zafra-Stone *et al*., 2007; Matchett *et al*., 2005). The antioxidant activity of phenolic berry extracts is widely accepted (Wu *et al*. 2004) and the body of evidence supporting the direct tumor killing ability of extracts is growing (Seeram, 2008).

Elderberry, the berry product from the European black elder (*Sambucus nigra*) may have a number of health-benefiting components. Elderberry has been used previously in traditional medicine for the treatment of viral infections and recent studies confirm the benefits of elderberry as an antiviral (Kong, 2009; Roschek *et al*., 2009; Roxas and Jurenka, 2007). Elderberry may also be used as a modulator of the immune system by causing increased production of pro-inflammatory cytokines IL-1b, TNF-α, IL-6 and IL-8 thereby stimulating a number of immune effector cells (Barak *et al*., 2001). Polyphenols, called anthocyanins have been shown to be incorporated into vascular endothelial cells and exhibit significant oxidative protection against a number of oxidative stressors, thereby maintaining cellular integrity and preventing DNA mutation (Youdim *et al*., 2000). Dark-pigmented berry anthocyanins in general have been shown to also modulate a variety of cellular mechanisms capable of inducing apoptosis of cancer cells *in vitro* (Jing *et al*., 2008).

The immune system plays a significant role in detecting and suppressing tumor cell growth (Dunn *et al*. 2002). T helper lymphocytes play a significant role in stimulating the differentiation of cytotoxic T lymphocytes capable of directly killing cancer cells by secretion of Interleukin-2 (IL-2). Many of the drugs used in chemotherapy cancer treatment, effectively suppress the proliferation of important adaptive immunity effector T lymphocytes (Zitvogel *et al*., 2008).

It was our goal to begin to separate and identify the individual components of elderberry capable of suppressing the growth of a murine melanoma cell line (B16-F10) and to examine the ability of these components to modulate immune function. Proper identification of

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melanoma-suppressing, immune-inducing elderberry fractions may lead to diet-based strategies for natural prevention and suppression of melanoma.

MATERIALS AND METHODS

Cell lines: The murine melanoma line, B16-F10 and Chinese hamster ovary cell line (CHO-K1; transformed, non-cancerous cells taken from a Chinese hamster ovary) were obtained from ATCC (Manassas, VA; #CRL-6475) (Manassas, VA; #CCL-61). SH-SY5Y neuroblastoma cells were generously provided by Dr. Robert Ross (Fordham University, Department of Neurobiology, NY). SH-SY5Y cells lack caspase-8 and are therefore resistant to apoptosis induction through caspase-8 pathways. All cell lines were maintained in RPMI-1640 media supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin (Penstrep-Amp), incubated in 75 cm^3 flasks at 35.7°C and 7.5% CO_2 and were sub-cultured every 2-4 days based on confluence.

Adherent cancer cells were released from 75 cm^3 culture flasks with 1.5 mL 1x trypsin-EDTA prior to use in assays. Trypsin was neutralized using RPMI-1640 media supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penstrep-Amp and the resulting cells in RPMI/10% FBS/1% Penstrep-Amp were collected prior to counting.

Preparation of spleen cells: C57BL/6 male mice (5 months old and 18 months old) (Purdue University IACUC approval #1111000244) were sacrificed by cervical dislocation and spleens were removed and prepared as previously described (Atre and Blumenthal, 1998). The spleen cells were counted and placed into 96 well plates in triplicate at a final concentration of 1×10^6 cells per well. All but the control (resting) wells received 0.125 μg of the T cell mitogen concanavalin A (Con A). At 48 h, tritiated thymidine (H^3-Thy) was added to each well at a final concentration of 0.38 μCi per well. At 72 h, the cells were harvested and H^3-Thy levels determined.

Extraction and pooling of elderberry components: Gravity column chromatography was used to separate the components of 13% standardized *Sambucus nigra* elderberry powder, generously provided by Artemis International, Inc. (Fort Wayne, IN). The protocol used for gravity column chromatography was an adaptation of the protocol first published by Strack and Mansell (1975) using 25 g Polyvinylpyrrolidone (PVP) as the stationary phase in a 250 mL glass chromatography column. The 2 g standardized elderberry powder was dissolved in 4 mL 0.01 N hydrochloric acid (HCl) and was added to the column on top of the stationary phase. Varying concentrations of sterile water-methanol mobile phaseswith a consistent amount of 0.01 N HCl were passed through the column in order of decreasing polarity. The first mobile phase solvent was 100% (150 mL) sterile deionized water added to 15 mL 0.01 N HCl for a total volume of 165 mL. The second mobile phase solvent was 90% (135 mL) sterile deionized water, 10% (15 mL) MeOH and 15 mL 0.01 N HCl. Mobile phases were added to the column in order of decreasing polarity in 165 mL increments with the final mobile phase consisting of 100% MeOH and 15 mL 0.01 N HCl. Samples eluted off of the column were collected in 14.5 mL increments.

The bulk of the solvent was evaporated from each sample using a Buchi rotary evaporator. The remainder of solvent was evaporated dry by vacuum centrifugation and samples were rehydrated with 0.5 mL Phosphate Buffered Saline (PBS). Samples were stored at -18°C until use.

Individual column samples were pre-screened in a proliferation assay (described below) to measure cell inhibition. This data was used to pool fractions with similar suppressive activityand these pooled fractions were used in assays described herein.

Cell proliferation determination by thymidine uptake assay: The transformedcells were diluted to 5×10^5 cell/mL. In a MICROTEST™ 96-well tissue culture plate, each well was filled with 100 μL cell dilution and 100 μL additional RPMI/10% FBS/1% Penstrep-Amp, bringing the final cell concentration of each well to 5×10^4 cells/200 μL. The plate was placed in the CO_2 incubator for 24 h. At 24 h, crude elderberry and pooled Elderberry fraction treatments were added to each well. At 48 h, tritiated thymidine (Moravek Biochemicals, Brea, CA) was added to each well at a final concentration of 0.38 μCi per well. At 72 h, the cells were harvested and H^3-Thy levels determined.

Cell harvesting and counting: The 5 min prior to harvesting cancerous and transformed cells, 2 μL 1x trypsin-EDTA (0.25%) was added to each well to release the cells. Harvesting of cells was accomplished by washing the cells 25-30 times with PBS using a Brandel cell harvester (Model #M-24) and precipitating them onto Whatman filter paper disks with 10% Trichloroacetic Acid (TCA). The disks were allowed to dry, placed into 6 mL volume scintillation vials with 3 mL Ecolume scintillation cocktail and were counted with a Beckman Coulter LS 6500 multi-purpose scintillation counter. Each assay was performed a minimum of 3 times.

Modulation of IL-2 by pooled elderberry fractions: A Quantikine® ELISA (Enzyme-Linked Immunosorbant Assay) kit for mouse IL-2 was obtained from R&D Systems (Minneapolis, MN). A C57BL/6J male mouse (10 months old) was sacrificed and a single cell suspension of spleen cells was obtained and diluted to 10×10^6 cells/mL. In 12-well costar® microtiter plates, 250 μL RPMI/10% FBS/1% Penstrep-Amp and 250 μL of spleen cell dilution was added to each well, bringing the final cell concentration of each well to 2.5×10^6 cells/500 μL supplemented media. Excluding the negative control well and well designated for ConA only (+0.3125 μg ConA), each well received an addition of 25 μL pooled elderberry fraction and 0.3125 μg ConA. The 12-well microtiter plates were incubated for 24 h in a CO_2 incubator (7.5% CO_2). Following incubation, the contents of each well were gently centrifuged for 5 min and the supernatant was removed for use in the assay. The assay was performed by the manufacturer's instructions and treatments were assayed in duplicate. A Packard SpectraCount™ was used to determine the Optical Density (OD).

RESULTS AND DISCUSSION

Melanoma B16-F10 cell assays: Counts Per Minute (CPM) measurements from the scintillation counter were analyzed and the percent proliferation of elderberry treated cells was assessed based on the growth of control cells at 100%. All 39 pooled fractions decreased B16-F10 cell proliferation by 40% or more (Fig. 1). The assay was repeated 3 times and the average of the trials revealed that the tumor-suppressive ability of the pooled fractions was dose dependent. The 10 mg mL^{-1} crude elderberry treatment significantly suppressed B16-F10 cell proliferation by 51.9% and the 1 mg mL^{-1} crude elderberry treatment decreased B16-F10 cell proliferation by 19.1%, although this was not significantly different from the control.

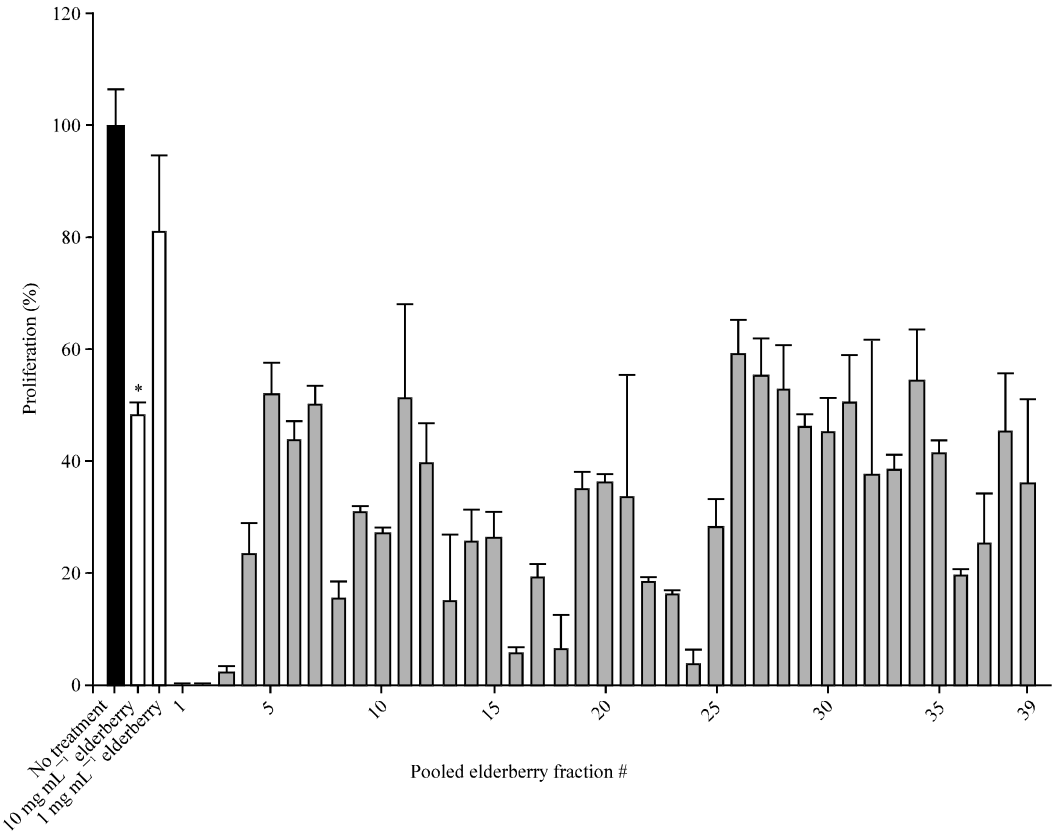

Fig. 1: B16-F10 cell suppression by 10 μL treatments of pooled elderberry fractions. Asterisk represent a significant proliferative difference vs. control cell growth ($p<0.05$) (n = 3) and all of the fractions exhibited significant inhibitory activity with fractions 16, 18 and 24 showing >95% inhibitory activity. As can be seen, 10 mg mL^{-1} of unfractionated elderberry demonstrated significant inhibition while 1 mg mL^{-1} did not significantly inhibit B16 melanoma cell proliferation as compared to control cells

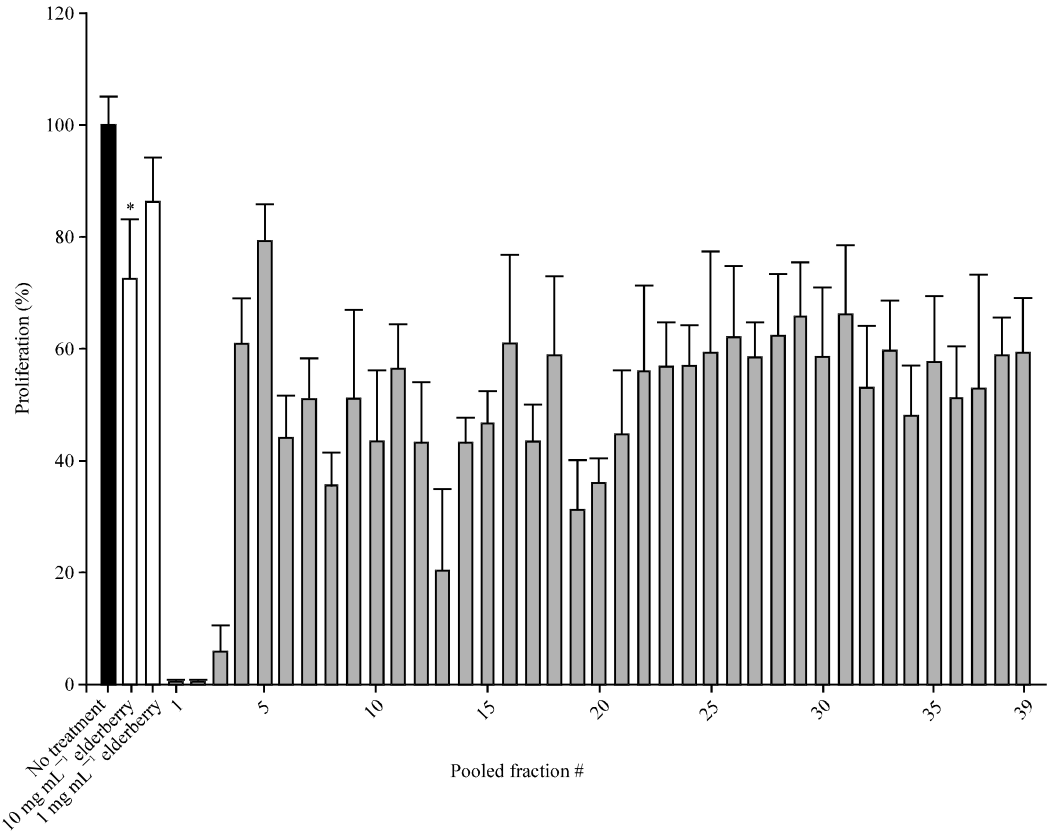

Fig. 2: SH-SY5Y cell suppression by 10 μL treatments of pooled elderberry fractions. Asterisk represents a significant proliferative difference vs. control cell growth ($p<0.05$) (n = 3) and all of the fractions exhibited significant inhibitory activity. The percent inhibition of the elderberry fractions was not as great as shown against the B16 melanoma cells with the greatest amount of inhibition being about 80% in fraction #13. As can be seen, 10 mg mL^{-1} of unfractionated elderberry demonstrated significant inhibition while 1 mg mL^{-1} did not significantly inhibit SH-WY5Y cell proliferation as compared to control cells

SH-SY5Y cell assays: All 39 pooled elderberry fractions decreased SH-SY5Y cell proliferation by 20% or more compared to control, untreated cells (Fig. 2). The assay was repeated 3 times and the average of the trials revealed that the tumor-suppressive ability of the pooled fractions was significantly different than the control. The 10 mg mL^{-1} crude, unfractionated elderberry samples significantly suppressed SH-SY5Y cell proliferation by 27.3% and the 1 mg mL^{-1} crude elderberry treatment decreased SH-SY5Y cell proliferation by 13.7%, although not significantly different from the control. The amount of cell suppression caused by both the 10 mg mL^{-1} and the 1 mg mL^{-1} concentrations was generally much less in the SH-SY5Y cells than in the melanoma cells.

CHO-K1 cell assays: The 25 of the 39 pooled fractions decreased CHO-K1 cell proliferation by a statistically significant percent (Fig. 3). Neither the 10 mg mL^{-1} nor 1 mg mL^{-1} crude, unfractionated elderberry sample significantly decreased CHO-K1 cell proliferation significantly compared to the control cells whereas the 10 mg mL^{-1} crude, unfractionated elderberry did significantly suppress both B16-F10 and SH-SY5Y cells. In general, pooled elderberry fractions that were exceptionally inhibitory in the B16-F10 and SH-SY5Y cell proliferation assays also decreased cell proliferation of CHO-K1 cells.

Young and old mouse comparative spleen cell proliferation assay with pooled elderberry fractions: Young mice have a much greater proliferation response to ConA than older mice. Addition of crude elderberry treatments (10 and 1 mg mL^{-1}) to spleen cells isolated from both young and old mice in the presence of ConA, demonstrated that all proliferation responses were less than untreated control levels. Addition of

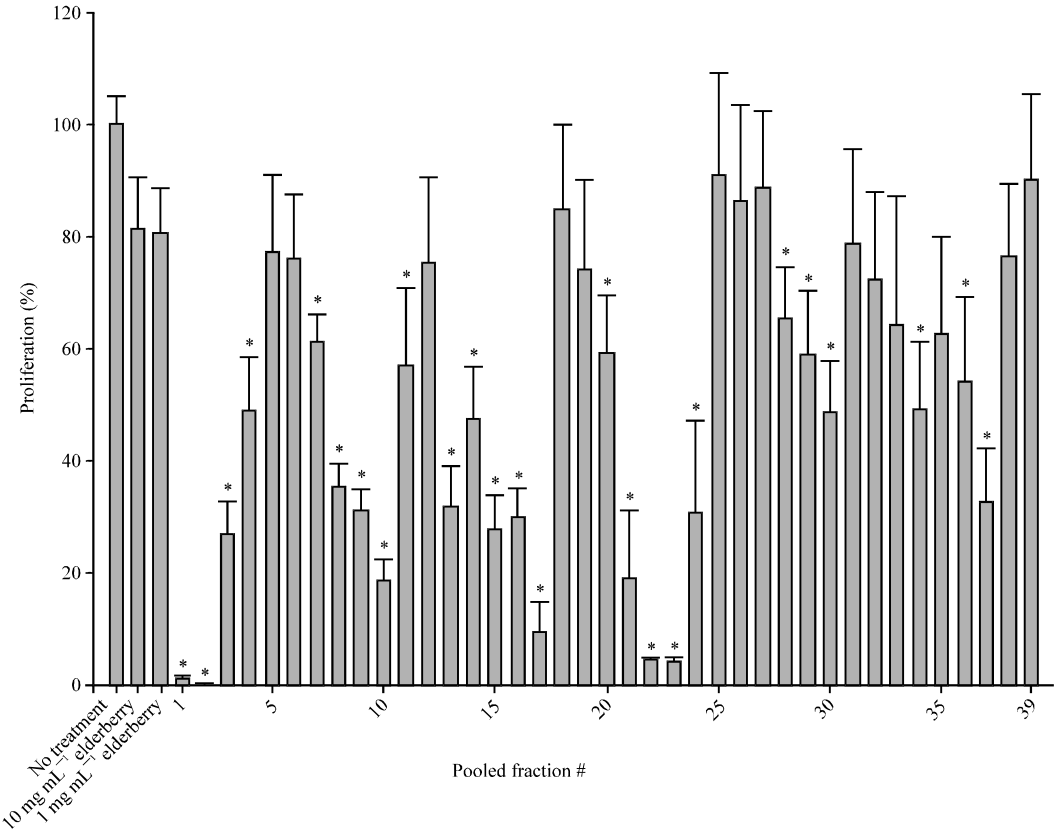

Fig. 3: CHO-K1 cell suppression by 10 μL treatments of pooled elderberry fractions. Asterisks represent a significant proliferative difference vs. control cell growth ($p<0.05$) (n = 3). The amount of inhibition of cell proliferation was highly variable with fractions #17, 22 and 23 giving greater than 90% inhibition

pooled elderberry fractions to senescent spleen cells demonstrated less inhibition of proliferation when compared to younger spleen cells (Fig. 4). While pooled elderberry fractions did reduce spleen cell proliferation the reduction of spleen cells compared to tumor cells was not as great. Raw CPM values are given for young and old mouse spleen cells treated with 0.125 μg ConA (insert).

Modulation of IL-2 by pooled elderberry fractions: Neither crude elderberry nor any of the pooled fractions inhibited the secretion of IL-2 from spleen cells compared to the positive control (spleen cells with ConA only). Some of the pooled fractions increased IL-2 secretion from unstimulated spleen cells (Fig. 5).

The use of bioactive foods as naturopathic treatments for many diseases and health conditions is a growing field of interest. Phytochemicals from dark-pigmented berries have been the focus of many studies that examine natural therapeutic modulators of cardiac disease, neurological disease, viral infection and cancers. The antioxidant benefits of such berries has been well characterized. It was the objective of this research to begin to identify the active components of elderberry capable of inhibitingtumor cell proliferation *in vitro* and to determine the effect of active elderberry components on T lymphocyte proliferation.

We found that all of the elderberry components separated by column chromatography affected the growth of murine melanoma B16-F10 cells, suppressing proliferation by at least 40%. Additionally, the growth of human neuroblastoma SH-SY5Y cells was reduced by 20% or more compared to untreated controls. SH-SY5Y cells are void of caspase-8, suggesting that they cannot undergo apoptosis through the caspase-8 pathway. Previous studies have shown that polyphenols present in berries may be able to induce cancer cell death by activating caspase pathways, leading to nuclear DNA degradation and apoptosis (Yeh and Yen, 2005). It is possible that one mechanism used by elderberry polyphenols to inhibit cancer cells is through a caspase pathway which would explain why SH-SY5Y cells are not as sensitive to inhibition by pooled elderberry fraction

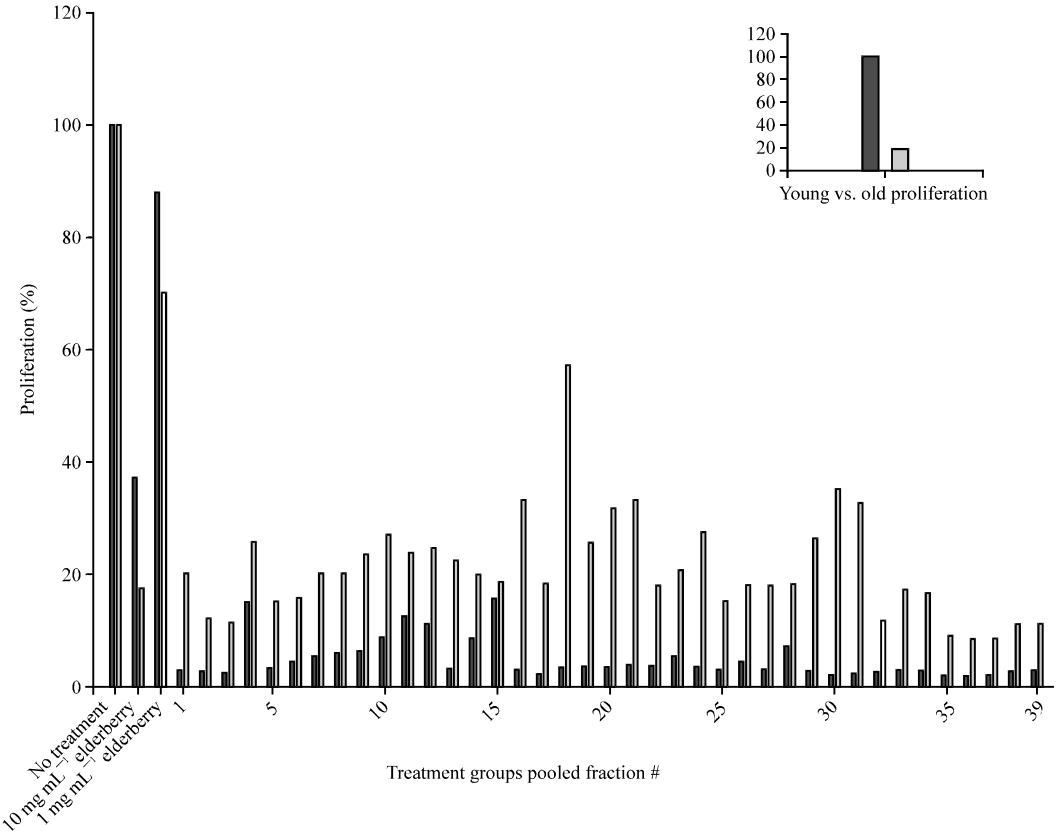

Fig. 4: Young and old mouse spleen cell growth stimulation by pooled elderberry fractions. The insert shows the relative age difference between spleen cells from young (black bars) and old mice (gray bars): the old response is only about 20% of the young spleen cell when stimulated with ConA. When normalized to 100% proliferation relative to untreated cells, the spleen cells from young mice were inhibited to a much greater extent than from older mice

treatment compared to B16-F10 cells with intact caspase function. Pooled elderberry fractions suppressed the growth of both sets of cancer cells to a greater degree compared to the crude treatment.

Crude elderberry treatments added to the transformed cell line CHO-K1 did not significantly suppress cell growth. Many of the pooled fractions that significantly decreased both B16-F10 and SH-SY5Y cell proliferation did not significantly suppress CHO-K1 cell growth *in vitro*, suggesting that the pooled fractions may have a more selective killing effect on cancerous cell lines compared to transformed, non-cancerous cell lines.

When spleen cells from a young mouse and a senescent mouse were incubated with pooled fractions, the results suggest that the same concentration decreased spleen cell proliferation in senescent mice less than in young mice. These results demonstrate that pooled elderberry fractions may be able to maintain T lymphocyte proliferation in elderly individuals who are more susceptible to disease, compared to younger individuals who are more likely to have maintained functional immunity. Additionally, six of eight active pooled elderberry fractions elicited a stronger IL-2 response from unstimulated mouse spleen cells suggesting that these pooled fractions do not have adverse effects on the growth and differentiation of T lymphocytes. It is important to note that the three pooled fractions that increased IL-2 secretion (Pool 5, 18 and 26) significantly suppress the B16-F10 and SH-SY5Y cells but do not significantly inhibit CHO-K1 cell growth. Therefore, separated elderberry components may be able to elicit a "dual-edged" sword effect by directly inhibiting tumor cell growth and inducing an immune response specifically in elderly individuals who are at higher risk for cancer development.

Future directions for this study would be to chemically identify the active components in the pooled elderberry fractions, particularly those that suppress cancer cell growth and induce IL-2 secretion from murine spleen cells. We have evidence (manuscript in

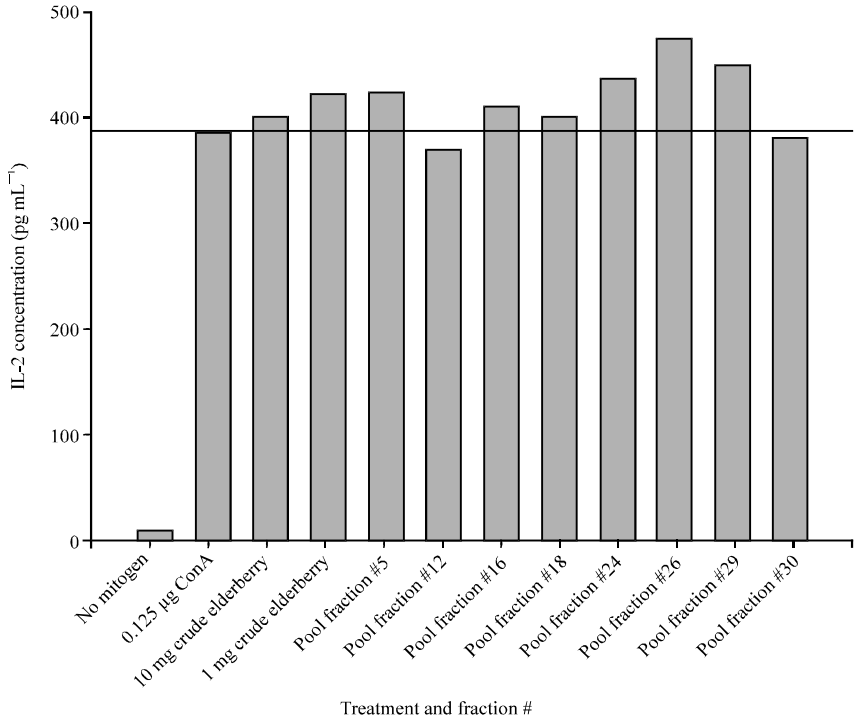

Fig. 5: Stimulation of IL-2 secretion from spleen cells isolated from young mice and treated with pooled elderberry fractions for 18 h when the culture supernatant was collected and assayed. Elderberry did not inhibit IL-2 secretion

preparation) that the different active column fractions contain characteristic levels of different anthocyanins. Also, it would be relevant to study the effect of active pooled elderberry fractions in a murine melanoma model to determine the suppressive effects of the fractions *in vivo*.

CONCLUSION

Our results are the first to describe the potential for elderberry components to induce secretion of IL-2, an important cytokine in T lymphocyte growth and differentiation. Combined with their direct tumor suppressive function and decreased toxicity in older animals, multiple extracts isolated from elderberry may be validated for use in immunotherapy and natural chemotherapy as well as support their use as a disease-preventative, naturopathic treatment for melanoma and other diseases prevalent amongst elderly individuals.

ACKNOWLEDGEMENTS

We would like to thank Dr. Robert Ross (Fordham University, NY) for supplying the SH-SY5Y neuroblastoma cell lines and Artemis International Inc. (Fort Wayne, IN) for contributing the 13% standardized elderberry powder.

REFERENCES

Atre, D. and E.J. Blumenthal, 1998. Melatonin: Immune modulation of spleen cells in young, middle-aged and senescent mice. Mechanisms Ageing Dev., 103: 255-268.

Barak, V., T. Halperin and I. Kalickman, 2001. The effect of sambucol, a black elderberry-based, natural product, on the production of human cytokines: 1. Inflammatory cytokines. Eur. Cytokine Network, 12: 290-296.

Dunn, G.P., A.T. Bruce, H. Ikeda, L.J. Old and R.D. Schreiber, 2002. Cancer immunoediting: From immunosurveillance to tumor escape. Nat. Immunol., 3: 991-998.

Jing, P., J.A. Bomser, S.J. Schwartz, J. He, B.A. Magnuson, M.M. Giusti, 2008. Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. J. Agric. Food Chem., 56: 9391-9398.

Katsube, N., K. Iwashita, T. Tsushida, K. Yamaki and M. Kobori, 2003. Induction of apoptosis in cancer cells by Bilberry (*Vaccinium myrtillus*) and the anthocyanins. J. Agric. Food Chem., 51: 68-75.

Kong, F.K., 2009. Pilot clinical study on a proprietary elderberry extract: Efficacy in addressing influenza symptoms. Online J. Pharmacol. Pharmacokinetics, 5: 32-43.

Matchett, M.D., S.L. MacKinnon, M.I. Sweeney, K.T. Gottschall-Pass and R.A. Hurta, 2005. Blueberry flavonoids inhibit matrix metalloproteinase activity in DU145 human prostate cancer cells. Biochem. Cell Biol., 83: 637-643.

Roschek, B., R.C. Fink, M.D. McMichael, D. Li and R.S. Alberte, 2009. Elderberry flavonoids bind to and prevent H1N1 infection *in vitro*. Phytochem., 70: 1255-1261.

Roxas, M. and J. Jurenka., 2007. Colds and influenza: A review of diagnosis and conventional, botanical and nutritional considerations. Altern. Med. Rev., 12: 25-48.

Seeram, N.P., 2008. Berry fruits for cancer prevention: Current status and future prospects. J. Agric. Food Chem., 56: 630-635.

Strack, D. and R.L. Mansell, 1975. Polyamide column chromatography for resolution of complex mixtures of anthocyanins. J. Chromatogr. A., 109: 325-331.

Wu, X., L. Gu, R.L. Prior and S. McKay, 2004. Characterization of anthocyanins and proanthocyanidins in some cultivars of Ribes, Aronia and Sambucus and their antioxidant capacity. J. Agric. Food Chem., 52: 7846-7856.

Yeh, C.T. and G.C. Yen, 2005. Induction of apoptosis by the Anthocyanidins through regulation of Bcl-2 gene and activation of c-Jun N-terminal kinase cascade in hepatoma cells. J. Agric. Food Chem., 53: 1740-1749.

Youdim, K.A., A. Martin and J.A. Joseph, 2000. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. Free Radical Biol. Med., 29: 51-60.

Zafra-Stone, S., T. Yasmin, M. Bagchi, A. Chatterjee, J.A. Vinson and D. Bagchi, 2007. Berry anthocyanins as novel antioxidants in human health and disease prevention. Mol. Nutr. Food Res., 51: 675-683.

Zitvogel, L., L. Apetoh, F. Ghiringhelli and G. Kroemer, 2008. Immunological aspects of cancer chemotherapy. Nat. Rev. Immunol., 8: 59-73.