Elucidating Antiproliferative Mechanisms of Grapeseed, Guava, and Juniper Berry Extracts

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Abstract

Plant extracts are an untapped source of medicinal potential. Even today they are used as standalone treatments and applied alongside conventional therapies. The focus of our laboratory is to identify plant extracts exhibiting antiproliferative activity in vitro, to determine which chemicals are responsible for this activity, and to elucidate mechanism(s) by which growth is slowed/inhibited by plant extracts. Specifically, we exposed five cell lines/strains to twenty-two plant extracts and measured cell proliferation. Extracts from Vinca, Juniper Berry, Guava, Grapeseed, and Yew slowed the growth of all five lines/strains in a dose dependent fashion. We are working to understand the mechanism of antiproliferation by measuring induction of apoptosis, effects on microtubule assembly, and wound healing.

Introduction

A wide variety of plants possess medicinal compounds (Anantharaju et al 2016). Some plants, such as Pacific yew, have yielded highly effective chemotherapeutic drugs (taxanes) and others, such as willow, contain chemicals to relieve pain (Anampa et al 2015; Kelly, et al). The potential medicinal activities of many plants remain untested. Work done by previous BIO310 students, assayed the antiproliferative activity of a wide variety of plant extracts.

Building on their data, we worked with RAW 264.7 cells, a mouse monocyte macrophage cell line, established from an ascites of a tumor induced by intraperitoneal injection of Abselon Leukemia Virus (A-MuLV). We used two assays to measure apoptosis (programmed cell death): formation of an apoptotic ladder as visualized by agarose gel electrophoresis and disruption of active mitochondria as occurs in the early stages of apoptosis using a JC-1 assay. We detected evidence of apoptosis induced by grapeseed, guava, and *Vinca* extracts but not by oregano, juniper berry, or yew extracts.

Materials and Methods

Cell Lines. HeLa cells, MDA MB 231 cells and RAW 264.7 cells were a gift from Sarah Smith (Medical College of Wisconsin, Milwaukee, WI). HepG2 cells were obtained from Robbin Eppinga (Dordt College, IA). HFF-S2 cells were established from a fresh foreskin. HeLa cells and RAW 264.7 cells were maintained in 90% DMEM (Sigma-Aldrich, St. Louis, MO) with 5% FBS (Gibco, Grand Island, NY) and 5% DCS (Gibco, Grand Island, NY). HepG2 cells, HFF-S2 cells, and MDA MB 231 cells, were maintained in 90% MEM (Gibco, Grand Island, NY) with 10% FBS (Gibco, Grand Island, NY).

Extracts and Chemicals. We made extracts from *Vinca* (CaribbeanGarden, Philadelphia, PA), Japanese yew (Northwestern College, Orange City, IA), ginger root and leaves (City Market, Kansas City, MO), fresh mistletoe (ALLO Books, Amazon), aronia (Chokeberry) powder (Aronia Unlimited Inc., Sioux Falls, SD), aronia juice (Akron Apple and Aronia, Inc., Akron, IA), aronia berries (Don Vaas, Orange City, IA), dandelion root (Orange City, IA), Juniper berries (LLB Company, Los Angeles, CA), guava (Royal King), grapeseed (Naravis, Winter Springs, FL), Turmeric root (Chinatown Food Market, Kansas City, MO) by washing plant material with deionized water, drying in a 40°C oven, crushing using mortar and pestle, macerating in 80% ethanol, and evaporating the ethanol in a flow hood. We re-suspended remaining residue in DMSO (Sigma Aldrich, St. Louis, MO) and sterilized by filtration through a 0.22 micron filter (Millipore corp., Carringtwohill, Ireland). All extracts were stored at -20°C.

CyQuant Assay. We plated cells at $1x10^4$/well in 96 well plates and treated with extracts or chemicals 24 hours later. Cells within each experiment were treated with equivalent amounts of DMSO and its concentration never exceeded 0.2%. After a 24-hour treatment, we used the CyQuant Cell Proliferation Assay Kit (ThermoFisher, Waltham, MA). An excitation wavelength of 485 nm and emission of 530 nm were accomplished using a Promega GLOMAX plate reader (Madison, WI). Mean background (wells containing no cells) was subtracted from each sample.

DNA Analysis. Cultures were 80% confluent when extracts were added. Cells were treated with extract at 2 ul/ml (extract or DMSO control). After 48 hours, the media was removed and cells were treated with lysis buffer (10mM Tris, 1mM EDTA, 1% SDS) and scraped with a rubber policeman into a tube. After chloroform extraction (24:1 chloroform:isoamyl alcohol) the upper layer was removed and precipitated by bringing the salt concentration to 0.3 M sodium acetate and adding an equal volume cold isopropanol. Tubes were centrifuged, drained, and the pellets were allowed to air dry before resuspending in dH_2O. Samples were run on a 1% agarose gel in TAE and visualized with a Chemidoc (BioRad, Hercules, CA).

MitoPT JC-1 Assay (ImmunoChemistry Technologies, Bloomington, MN). We treated 90% confluent RAW264.7 plates with concentrations of extract that produced an ED_{50} four hours before beginning the assay. We followed the assay protocol and measured our results using the Promega GloMax Plate Reader with a 490 nm excitation 510-570 nm emission probe and then a 525 nm excitation and 580-640 nm emission probe (Madison, WI).

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Extracts from Grapeseed, Juniper Berries, Yew, *Vinca*, and Guava Slow Proliferation of Cells

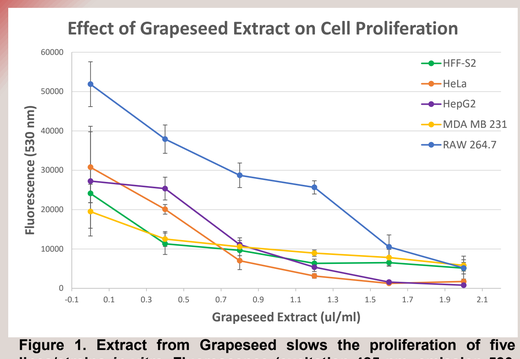


Figure 1. Extract from Grapeseed slows the proliferation of five cell lines/strains *in vitro*. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates $p<1.4x10^{-5}$ for all cell types.

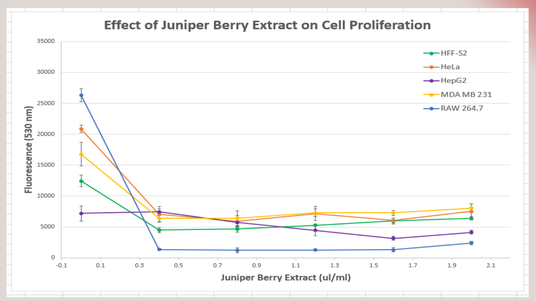


Figure 2. Extract from Juniper Berries slows the proliferation of all tested cell lines/strains except HepG2 (p=0.35). Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p<.003 for all affected cell types.

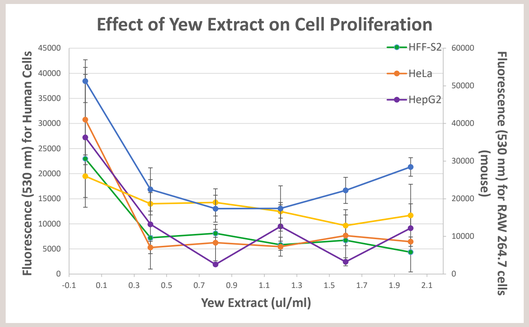


Figure 3. Extract from *Yew* needles slows the proliferation of all five cell lines/strains. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p<.00011 for all cell types.

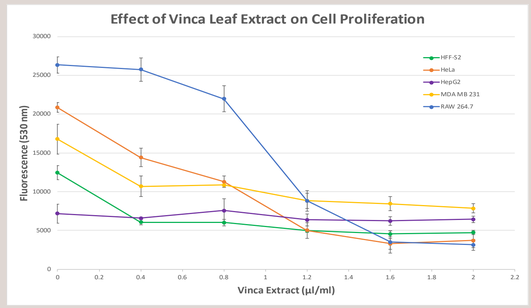


Figure 4. Extract from *Vinca* leaves slows the proliferation of all cell lines/strains tested except HepG2 (p=0.08). Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates $p<4x10^{-7}$ for all affected cell types.

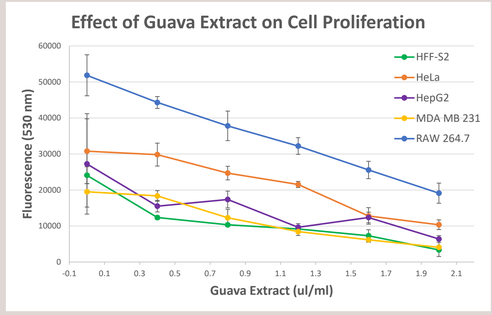


Figure 5. Extract from Guava slows the proliferation of all five cell lines/strains. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p<0.002 for all cell types.

Extracts from Many Plants Have No Effect on Cell Proliferation

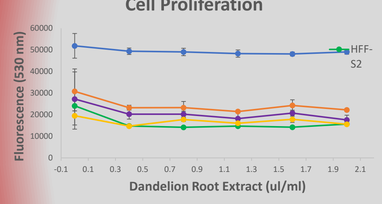


Figure 6. Extract from Dandelion root has no effect on cell proliferation in any tested cell lines/strains. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p>0.05 for all tested cell types.

Additional Extracts Tested With No Significant Effect on Proliferation
Basil Leaves
Impatiens Leaves
Lavender Leaves
Marigold Leaves
Chocolate Mint Leaves
Oregano Leaves
Sage Leaves
Mistletoe Leaves
Ginger Root
Rosemary

Table 1 . Additional extracts were tested but no significant effect on cell proliferation was detected (data not shown).

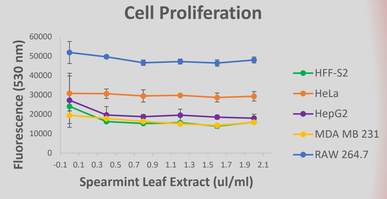
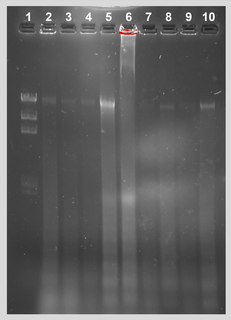


Figure 7. Extract from Spearmint leaves has no effect on cell proliferation in any tested cell lines/strains. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p>0.05 for all tested cell types.

Extracts from Grapeseed and Guava Induce Apoptosis

Figure 8. DNA isolated from cells treated with plant extracts. Lane 2-4 DMSO control. Lanes 5-8 Oregano extract. Lanes 8-10 Juniper Berry extract. None of these treatments show a distinct apoptotic ladder. Lane 1: λ/HindIII ladder.



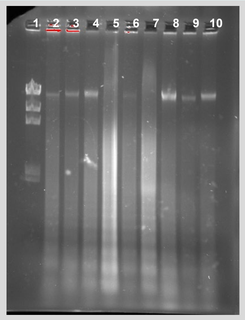


Figure 9. DNA isolated from cells treated with plant extracts. Lanes 2-4 grapeseed extract. Lanes 5-7 guava extract. Lanes 8-10 camptothecin. Guava extract-treated cells show a relatively distinct apoptotic ladder. Cells treated with grapeseed extract and camptothecin show an apoptotic ladder that is less distinct. Lane 1: λ/HindIII ladder.

Extracts from Grapeseed, Guava, and *Vinca* Induce Apoptosis

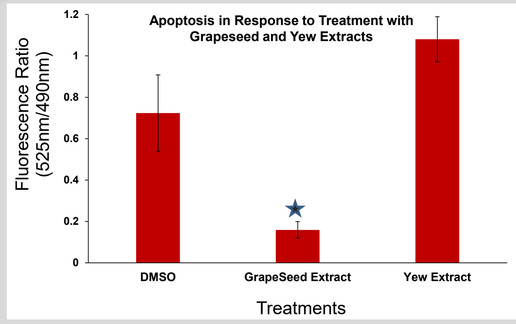


Figure 10. RAW 264.7 cells showed disruption of active mitochondria as occurs in early stages of apoptosis when treated with grapeseed extract but not yew extract. *Indicates statistically different from DMSO control p<0.0002.

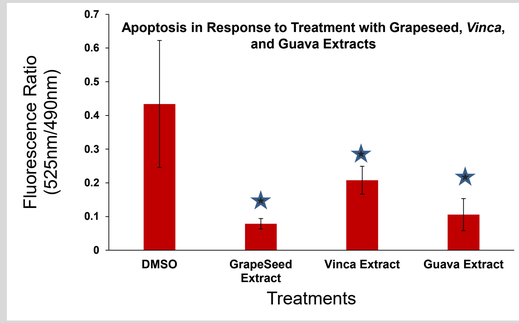


Figure 11. RAW 264.7 cells showed disruption of active mitochondria as occurs in early stages of apoptosis when treated with grapeseed, *Vinca*, and guava extracts. *Indicates statistically different from DMSO control p<0.007.

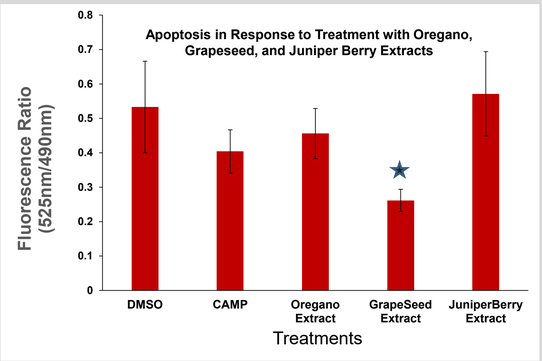


Figure 12. RAW 264.7 cells showed disruption of active mitochondria as occurs in early stages of apoptosis when treated with grapeseed extract but not oregano or juniper berry extracts. *Indicates statistically different from DMSO control p<0.0003.

Discussion

We detected antiproliferative activity in extracts from grapeseed, juniper berries, guava, *Vinca*, and Japanese yew (Figures 1-5) using a CyQuant assay. We tested many other plant extracts but were unable to detect statistically significant antiproliferative activity in them.

We saw evidence of apoptosis induced by grapeseed, *Vinca*, and guava extracts but not yew, oregano, or juniper berry extracts as indicated by detecting disruption of active mitochondria. We saw evidence of apoptosis induced by guava and grapeseed extracts in formation of an apoptotic ladder. Since the apoptotic ladder and JC-1 assay detect different events in apoptosis, we feel confident that we are seeing the induction of apoptosis by grapeseed and guava extracts.

We are continuing to assay additional extracts for the formation of an apoptotic ladder and additional cell lines using both assays.