Understanding the Antiproliferative Activity of Plant Extracts

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Abstract

Many plants possess medicinal properties. Some, such as the Pacific yew, have yielded chemotherapeutic drugs (taxanes). Scientists report that other extracts such as the leaves of Calendula officinalis (marigold), Vinca rosea (periwinkle), Viscum cruciatum (mistletoe), and Rosmarinus officinalis (rosemary) have anti-tumor activity. In most cases, the chemical components responsible for antiproliferative activity have not been identified and it is unclear if any individual components are as effective in isolation as they are in the context of the whole extract. Furthermore, in most cases, there are no data indicating whether these extracts have synergistic effects or cause negative reactions when used with other drugs. We are using HeLa (adenocarcinoma), RAW 264.7 (leukemia), HepG2 (hepatoma), MDA-MB-231 (adenocarcinoma), and human foreskin fibroblasts (HFF, non-tumorigenic) to test the antiproliferative activity of several plant extracts. We identified five extracts, grapeseed, guava, yew, juniper berry, and Vinca, that slow the growth of all five cell lines in a dose-dependent manner. We are using a variety of methods to understand the mechanism by which these extracts are blocking cell growth.

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).

RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Promega, Madison, WI) was performed according to kit instructions. Cells were plated, treated 24 hours later, and plates were read every four hours after 20 hours of treatment.

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Discussion

We confirmed antiproliferative activity in extracts from grapeseed, juniper berries, and guava using a CyQuant assay. We were unable to confirm antiproliferative activity in old or new *Yew* extracts and are working to understand these results.

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 used a RealTime-Glo Annexin V Apoptosis and Necrosis Assay to confirm our previous apoptosis results and to see if we also have secondary necrotic death induced by our extracts. We have some evidence of apoptosis with this assay, although we think we will need to alter our time course to be sure. We do not see evidence of secondary necrosis induced by grapeseed, *Yew*, juniper berry, or guava.

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

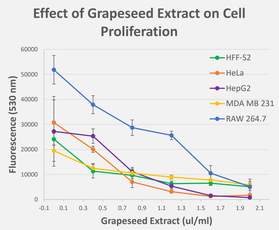

Figure 1. Extract from Grapeseed slows the proliferation of five cell lines/strains *in vitro*. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates $p<1.4x10^{-5}$ for all cell types.

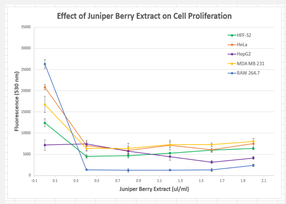

Figure 2. Extract from Juniper Berries slows the proliferation of all tested cell lines/strains except HepG2 (p=0.35). Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p<.003 for all affected cell types.

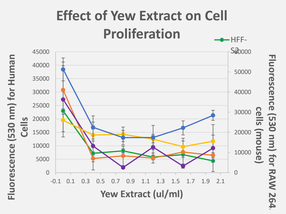

Figure 3. Extract from *Yew* needles slows the proliferation of all five cell lines/strains. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p<.00011 for all cell types.

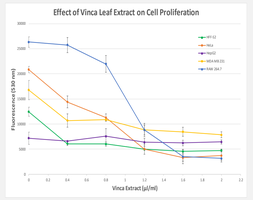

Figure 4. Extract from *Vinca* leaves slows the proliferation of all cell lines/strains tested except HepG2 (p=0.08). Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates $p<4x10^{-7}$ for all affected cell types.

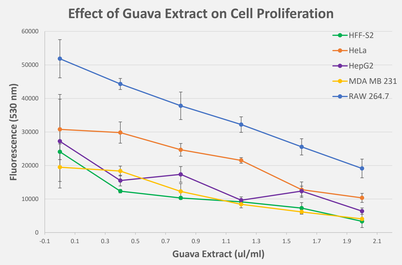

Figure 5. Extract from Guava slows the proliferation of all five cell lines/strains. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p<0.002 for all cell types.

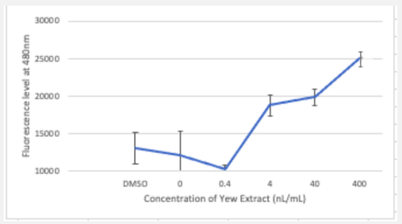

Figure 6. Fresh (2023) extract from Yew appears to stimulate the proliferation of Hela cells. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent three replicates +/- SD.

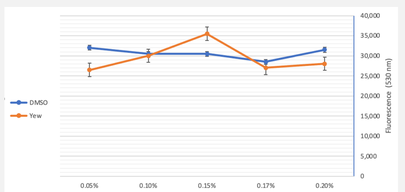

Figure 7 Fresh (2023) extract from Yew appears to have no effect on the proliferation of Hela cells. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent three replicates +/- SD.

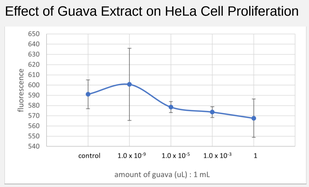

Figure 8. Extract from guava slows the proliferation of Hela cells. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent three replicates +/- SD.

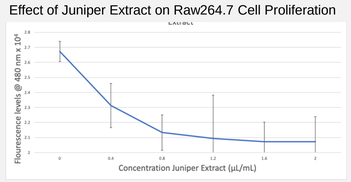

Figure 9. Extract from juniper berries slows the proliferation of RAW 264.7 cells. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent three replicates +/- SD.

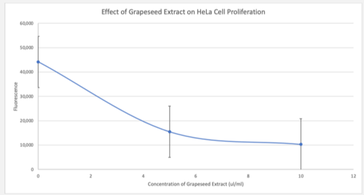

Figure 10. Extract from grapeseed slows the proliferation of HeLa cells. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent three replicates +/- SD.

Extracts from Many Plants Have No Effect on Cell Proliferation

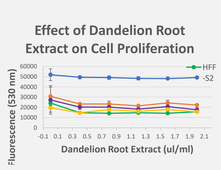

Figure 11. Extract from Dandelion root has no effect on cell proliferation in any tested cell lines/strains. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p>0.05 for all tested cell types.

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 Leaves

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

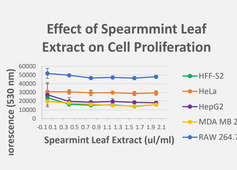

Figure 12. Extract from Spearmint leaves has no effect on cell proliferation in any tested cell lines/strains. Fluorescence (excitation 485 nm, emission 530 nm). Data points represent at least four replicates +/- SD. ANOVA analysis indicates p>0.05 for all tested cell types.

Extracts from Grapeseed and Guava Induce Apoptosis

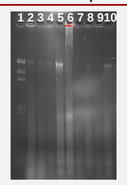

Figure 13. DNA isolated from cells treated with plant extracts. Lane 2-4 DMSO control. Lanes 5-8 Oregano extract. Lanes 8-10 Juniper Berry extract. None of these treatments show a distinct apoptotic ladder. Lane 1: λ/HindIII ladder.

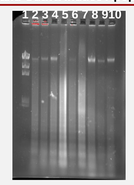

Figure 14. DNA isolated from cells treated with plant extracts. Lanes 2-4 grapeseed extract. Lanes 5-7 guava extract. Lanes 8-10 camptothecin. Guava extract-treated cells show a relatively distinct apoptotic ladder. Cells treated with grapeseed extract and camptothecin show an apoptotic ladder that is less distinct. Lane 1: λ/HindIII ladder.

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

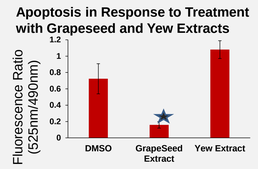

Figure 15. RAW 264.7 cells showed disruption of active mitochondria as occurs in early stages of apoptosis when treated with grapeseed extract but not yew extract. *Indicates statistically different from DMSO control p<0.0002.

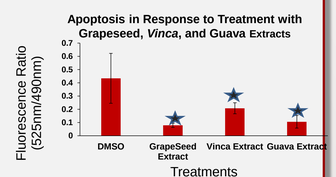

Figure 16. RAW 264.7 cells showed disruption of active mitochondria as occurs in early stages of apoptosis when treated with grapeseed, *Vinca*, and guava extracts. *Indicates statistically different from DMSO control p<0.007.

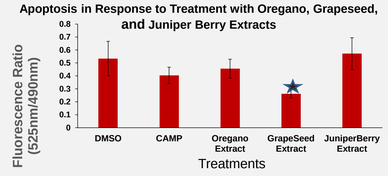

Figure 17. RAW 264.7 cells showed disruption of active mitochondria as occurs in early stages of apoptosis when treated with grapeseed extract but not oregano or juniper berry extracts. *Indicates statistically different from DMSO control p<0.0003.

Extracts from Grapeseed, Guava, Juniper berries, *and Yew* Induce Apoptotic but Not Necrotic Death

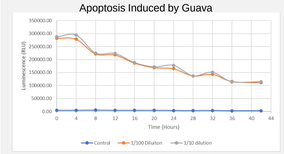

Figure 18. HeLa cells showed luminescence patterns typical of late apoptosis when treated with guava extract. Data points represent three replicates.

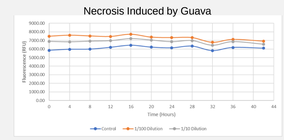

Figure 19. HeLa cells did not show fluorescence patterns typical of secondary necrosis when treated with guava extract. Data points represent three replicates.

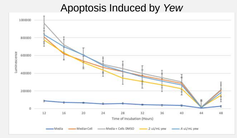

Figure 20. HeLa cells showed luminescence patterns typical of late apoptosis when treated with *Yew* extract. Data points represent three replicates.

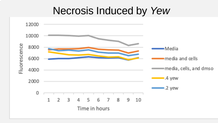

Figure 21. HeLa cells did not show fluorescence patterns typical of secondary necrosis when treated with *Yew* extract. Data points represent three replicates.

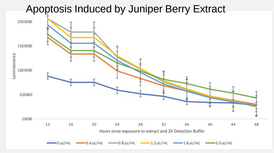

Figure 22. RAW 264.7 cells showed luminescence patterns typical of late apoptosis when treated with *juniper berry* extract. Data points represent three replicates.

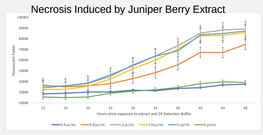

Figure 23. RAW 264.7 cells did not show fluorescence patterns typical of secondary necrosis when treated with juniper berry extract. Data points represent three replicates.

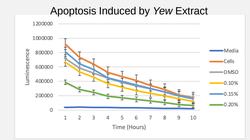

Figure 24. HeLa cells showed luminescence patterns typical of late apoptosis when treated with *Yew* extract. Data points represent three replicates.

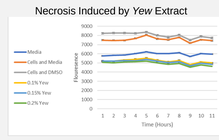

Figure 25. HeLa cells did not show fluorescence patterns typical of secondary necrosis when treated with *Yew* extract. Data points represent three replicates.

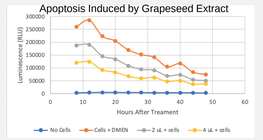

Figure 26. HeLa cells showed luminescence patterns typical of late apoptosis when treated with grapeseed extract. Data points represent three replicates.

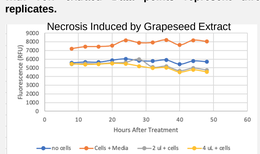

Figure 27. HeLa cells did not show fluorescence patterns typical of secondary necrosis when treated with grapeseed extract. Data points represent three replicates.