Antineoplastic Effects of Rhodiola Crenulata on B16-F10 Melanoma

Maxine Dudek

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Antineoplastic Effects of *Rhodiola crenulata* Treatment on B16-F10 Melanoma

A Thesis Presented
by
MAXINE C. DUDEK

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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Molecular and Cellular Biology
Antineoplastic Effects of *Rhodiola crenulata* Treatment on B16-F10 Melanoma

A Thesis Presented

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Not knowing what to expect in the world of scientific research, I was both nervous and excited when I began pursuing this endeavor. Throughout my time at the Pioneer Valley Life Science Institute, I have been so fortunate to have had incredible advisors, mentors and friends. First, I would like to acknowledge Dr. Richard B. Arenas who first allowed me to immerse myself into the lab atmosphere. Next, I would like to acknowledge my advisor, Dr. Sallie S. Schneider. I have never known a more patient, kind and considerate person. Since the first day I entered lab, Dr. Schneider has ignited my curiosity for scientific research and nurtured me from the nervous undergraduate I once was into the researcher I am today. I truly believe the success of Dr. Schneider’s lab can be attributed to her genuine desire to help both her students and employees succeed, and her unrelenting patience and guidance. In my many lab stumbles and downfalls, Dr. Schneider never once was critical, and instead was only encouraging and supportive.

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Next, I would like to acknowledge my colleague, Lotfi M. Bassa. Lotfi showed me the value of hard work and dedication. He also pushed me to understand the mechanics behind laboratory protocols so that I would further understand how the experiment worked. Not only did Lotfi spend hours explaining techniques such as RT-PCR and Western Blots to me, but he also took time to advise me in my classes and academic work. I know that Lotfi’s desire to push others while making sure that they learn and understand during the process will make him an excellent professor.

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ABSTRACT

ANTINEOPLASTIC EFFECTS OF *RHODIOLA CRENULATA* TREATMENT ON B16-F10 MELANOMA

MAY 2015

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Melanoma remains an aggressive form of skin cancer with limited treatment options. Novel methods to treat primary tumors and prevent metastatic disease can lead to improved survival for those diagnosed with melanoma. Through this work, we have evaluated the antineoplastic effects of *Rhodiola crenulata* (*R. crenulata*) root extracts on B16-F10 melanoma both *in vitro* and *in vivo*. In this study, we observed that *R. crenulata* treatment resulted in an increased cell death as well as a reduced cell growth, proliferation and migration *in vitro*. Additionally, we observed that *R. crenulata* decreased the expression of integrin β1 and vimentin, and increased expression of E-cadherin upon *in vitro* treatment. Further, we observed in a topical *R. crenulata* based cream therapy, a more radial growth pattern of tumors as well as a reduced mitotic activity and increased tumor necrosis. Markedly, we observed that mice supplemented with *R. crenulata* orally in their drinking water also displayed reduced establishment of metastatic foci in a disseminated model of melanoma. Collectively, these findings reveal that *R. crenulata* exhibits striking anti-tumorigenic and anti-metastatic properties, and
that this extract may increase survival and harbor potential novel adjuvant therapy for the
treatment of melanoma.
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CHAPTER I
INTRODUCTION

1.1. Melanoma: A Brief Background

Each year, more than 76,000 people in the United States are diagnosed with melanoma, the most aggressive form of skin cancer [1]. Associated with UV exposure, melanoma arises from the malignant transformation of melanocytes. Melanocytes specialize in melanogenesis or the production of melanin, a pigment that protects the skin by absorbing and scattering harmful solar radiation [2-3]. Encapsulated in melanosomes, melanin is transferred by microtubules to the matrix of the cytoplasm in cells located in the outermost layer of the epidermis called keratinocytes, through calcium-dependent adhesion type-1 transmembrane protein (cadherin) interactions [4]. Once released in the keratinocyte’s matrix, melanin accumulates in the supernuclear region of the cytoplasm where it acts as a barrier against environmental damages, such as pathogens, heat, UV radiation and water loss [5].

There are five distinct stages of melanoma development that include: the common acquired nevus (benign nevus), dysplastic nevus, radial growth phase (RGP) primary melanoma, vertical growth phase (VGP) primary melanoma, and disseminated disease [5-6]. Both the benign nevus, and the dysplastic nevus arise from a malfunction in the melanin unit that leads to an increased keratinocyte to melanocyte ratio. If left untreated, both the benign nevus and the dysplastic nevus may begin to grow in situ. As melanoma undergoes more vertical growth, cancerous cells are more likely to invade the lower
dermis and penetrate through the deeper subcutaneous tissue and basement membrane, thus resulting in an increased risk of metastatic spread [5;7]. Unfortunately, the five-year survival rate for patients diagnosed with metastatic melanoma is less than 16.1%. [1].

1.2. Epithelial-mesenchymal transition transforms melanocytes into highly migratory and invasive melanoma cells

One process responsible for transforming melanocytes into malignant cells is unregulated epithelial-mesenchymal transition (EMT). During embryonic development, EMT allows cells to migrate to their designated locations by reorganizing the cytoskeleton and altering cells’ affinity for the extracellular matrix (ECM) [8]. In normal melanocytes, SNAIL2 and ZEB2 transcription factors active MITF-dependent melanocyte differentiation programs. Differentiated melanocytes utilize tyrosinase to convert tyrosine into dopa and subsequently to dopaquinone, the melanin precursor. Unregulated EMT triggers the activation of different transcription factors like TWIST1 and ZEB1 [9]. These factors along with others work together to disrupt MITF-dependent differentiation and subsequent melanin production. Ultimately this instigates the conversion of melanocytes into cancerous cells [10].

Not only is EMT responsible for the neoplastic transformation of melanocytes, but it is also responsible for increasing a malignant cell’s ability to migrate and metastasize [11-12]. As melanocytes morph into melanoma cells, E-cadherin expression is lost and vimentin becomes heavily over-expressed by mesenchymal cells [13]. E-cadherin plays a critical role in maintaining the structural integrity of the skin as well as melanocyte-
keratinocyte interaction [14] while vimentin, a mesenchymal filament protein, is involved in wound healing, angiogenesis and cancer growth [15-16]. Studies reveal that primary melanomas over-expressing vimentin and under-expressing E-cadherin tend to have a more metastatic incidence [13;17]. Therapy targeting vimentin over-expression and E-cadherin re-expression results in the significant reduction of tumor growth and invasiveness \textit{in vivo} [18-20]. Thus, vimentin and E-cadherin serve as useful biomarkers for classifying melanoma subtypes [21].

1.3. The Role of Integrins and id genes in Mediating Melanoma Growth Patterns and tumorigenesis

The progression of melanoma from a RGP to VGP is a critical step in disease development and outcome for those with this malady. Melanoma lesions that display radial growth patterns are typically less aggressive and more chemo-sensitive than those displaying more vertical growth patterns. A number of items contribute to melanoma’s ability to progress from RGP to VGP. One key factor are integrins, a family of transmembrane glycoprotein receptors involved in determining cell shape and migration. As melanoma transitions from RGP to VGP certain integrins like \( \alpha 5\beta 1 \) are upregulated [22-23]. This upregulation is responsible for altering a cancer cell’s affinity to the extracellular matrix, promoting proteolytic enzymes that degrade the basement membrane, and promoting several pro-survival mechanisms to prevent apoptosis [24].

Like integrins, inhibitor of DNA binding (Id) proteins are responsible for coordinating biological processes like migration and invasion as well other cellular functions such as
cell-fate determination, proliferation, cell cycle regulation and angiogenesis [25]. In cancer cells, over-expression of ID genes contributes to an increased tumorigenesis, invasiveness, and migratory behavior [26-27]. Cancer studies have revealed that therapies targeting ID genes have potential in reducing metastatic spread [28].

1.4. Significance

At present, early detection of melanoma is the most important means to improve disease survival. Depending on the stage at presentation, a multi-disciplinary approach to therapy is required and treatment options often include a combination of surgical resection, radiation, chemotherapy, and immunotherapy. Despite aggressive therapies, treatment options remain limited and outcomes of patients with this disease, especially those with disseminated disease, remain poor. A study reviewing the data of patients with Stage I and Stage II melanoma revealed that patients with a previous history of cutaneous melanoma have a 25-fold increased incidence of developing a second melanoma [29]. Given this, investigations into novel treatment options are necessary for patients with this aggressive disease.

1.5. Rhodiola crenulata

One promising option for the treatment of melanoma is the extract derived from *Rhodiola crenulata* (*R. crenulata*) roots. *R. crenulata* is a small perennial plant cultivated in barren soils and high altitudes in the tundra regions of Siberia and the highlands of Tibet. Traditionally, it has been prized by the people in regions where it grew for its energy-enhancing ability. During the 20th century, scientists began to investigate its adaptogenic
properties and discovered its ability to exert antioxidant effects, support DNA repair, alleviate altitude and menopausal symptoms, and improve abilities such as physical exercise, memory, sexual function, learning, and work capacity [30]. Additional investigations revealed that *Rhodiola* increases cellular energy production, improves oxygen utilization and alleviates depression, fatigue and anxiety through increasing the production of serotonin, norepinephrine and dopamine [30-33]. Recent studies have demonstrated the therapeutic potential of *R. crenulata* in a variety of cancers including bladder cancer, breast cancer and glioblastoma [34-37]. *Rhodiola* contains many active ingredients such as salidroside, catechol, ferulic acid, salicylic acid, quercetin, 4-hydroxybenzoic acid, caffeic acid, gallic acid and 3,4-dihydroxybenzoic acid [38]. Unfortunately, determining the active ingredient responsible for *Rhodiola*’s activity remains challenging, as many active components are believed to work in concert in exerting *R. crenulata*’s many effects. Today, one may supplement their diet with *Rhodiola* by purchasing it in powdered, encapsulated or loose-tea form. Recommended doses range from individual to individual; however, it is suggested that one begin with 100mg *Rhodiola* ½ hour before breakfast on an empty stomach. If this dose is tolerated, an additional 100mg may be taken ½ hour before lunch. Most people do not need to exceed more than 400mg per day [30].

1.6. Rationale and Objectives

Given the anti-tumorigenic properties observed with the use of *Rhodiola* plant extracts, both *in vitro* and *in vivo*, this study aimed to evaluate the therapeutic potential of *R. crenulata*’s hydroalcoholic extract on melanoma. In this study, we set out to evaluate *R.
**crenulata**'s effects on proliferation, migration transcription and expression of key transcripts and proteins involved in tumor progression. Further, we wanted to evaluate *R. crenulata*’s effects on melanoma tumor growth and metastatic establishment *in vivo.*
CHAPTER II
MATERIALS AND METHODS

2.1. *R. crenulata* Preparation

*R. crenulata* root extract was obtained in powder form from Barrington Chemical Corporation (Harrison, NY). For cell culture experiments and oral *R. crenulata* administration, *R. crenulata* powder was dissolved in a 10% ethanol solution in distilled water and was filter sterilized. Cream-based *R. crenulata* for topical use was prepared by the addition of 5% or 10% *R. crenulata* by weight to a 10% DMSO-Eucerine™ based cream.

2.2. Cell Culture

B16-F10 murine melanoma cell culture line was utilized for this research (ATCC). Cells were cultivated at 37°C under 5% CO$_2$ and maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 100 U/mL Penicillin and 100µg/mL Streptomycin (Gibco®, Grand Island, NY).

2.3. Evaluation of Viability and Proliferation

A CellTiter96® Aqueous One Solution MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] containing an electron coupling reagent (phenazine ethosulfate; PES) (Promega, Madison, WI) assay was performed according to the manufacturer’s instructions in order to test for cell growth and proliferation. A total of $5 \times 10^3$ B16-F10
cells were seeded per well on a 96-well microtiter plate and treated with 200µg/mL R. creunulata or ethanol vehicle control in quintuplicate. After incubation for 24, 48, and 72 hours the plate was spun at 2,000g for 1 min and treated media was decanted and replaced with 100µl 1X PBS and 20µl of MTS reagent per well. The plate was then incubated at 37°C in a humidified, 5% CO2 atmosphere for 3 hours. The absorbance of each well was recorded at 490nm using an Enspire® multimode automated plate reader (PerkinElmer, Waltham, MA). The experiment was performed in sextuplicate.

2.4. Florescent Viability/Cytotoxicity Assay
A Live/Dead assay was performed according to the manufacturer’s instructions to evaluate the cytotoxic effects of R. creunulata. A total of 7.5 X 10³ B16-F10 cells were seeded on each well of a Lab-Tek® Chamber Slide and treated with vehicle control or 200µg/mL R. creunulata. The chamber slide was cultivated at 37°C in a humidified, 5% CO2 atmosphere. Following 72 hours of treatment, media was decanted and replaced with 250µl 1X PBS containing 2µM Calcein and 4µM Ethidium homodimer-1. After a 3 hour incubation period, images were captured at 20X magnification with a Nikon Eclipse TE2000-U inverted microscope using MetaVue™ software (Universal Imaging Corporation, Downington, PA).

2.5. Clonogenecity Assay
A total of 200 B16-F10 cells were seeded onto 60 mm tissue culture plates. After allowing the cells to adhere for 24 hours, plates were treated with 100µg/mL R. creunulata or vehicle control for one hour followed by exposure to 5Gy radiation or sham treatment.
The media was replaced with the appropriate treatment every 4 days. By day 11, colonies were observed. Plates were then washed with 1X PBS, fixed in methanol and stained with 2% crystal violet in a 50% methanol solution for 4 minutes. Colonies were defined as a cluster of five or more cells. The number of colonies was than quantified and compared between treatment groups. The experiment was performed in triplicate.

2.6. RNA Isolation and Real-Time PCR

B16-F10 cells were treated with 200µg/mL *R. crenulata* or vehicle. After 24 hours, total RNA was extracted from cells using an acid-phenol extraction procedure [39] according to the manufacturer’s instructions (Trizol, Invitrogen, Carlsbad, CA). Relative levels of mRNA were determined by quantitative real-time Polymerase Chain Reaction (PCR) using the Stratagene Mx3005P real time PCR system (Agilent Technologies, La Jolla, CA). All values were normalized to the amplification of GAPDH. The assays were performed using the 1-step 2X Brilliant SYBR Green QRT-PCR Master Mix Kit (Agilent Technologies) containing 200nM forward primer, 200nM reverse primer, and 100ng total mRNA. The conditions for target mRNA amplification were performed as follows: 1 cycle of 50°C for 30 min; 1 cycle for 95°C for 10 min; 35 cycles each 95°C for 30x, 55°C for 1 min and 72°C for 30s.

2.7. Migration assays

Migration and growth was first evaluated using a scratch wound assay. B16-F10 cells were plated on 30mm dishes and allowed to reach 100% confluency. The tip of a 10µL pipette was used to produce a scratch wound down the center of each plate. Immediately
following scratch-wound formation, control plates were treated with 100µg/mL \textit{R. crenulata} or ethanol vehicle control and cell growth was monitored over 48 hours. Images of the plates were captured daily with Nikon Eclipse TE2000-U using Metaview\textsuperscript{TM} software (Universal Imaging Corporation).

Additional migration analysis was performed utilizing transwell migration chambers. B16-F10 cells were pre-treated with 100µg/mL \textit{R. crenulata} or vehicle control. After 24 hours, 1x10\textsuperscript{5} pre-treated cells were seeded onto a BD BioCoat\textsuperscript{TM} Matrigel migration chamber (8µm pore) plate (BD Biosciences, San Jose, CA) and treated with serum-free DMEM. Matrigel migration chambers were placed in a 24-well plate so that they hovered above complete DMEM in order to create a gradient for cell migration. After 24 hours of incubation, migration chamber membranes were then fixed for 10 min with 10\% formalin, stained with 2\% crystal violet for 20 min, and rinsed three times with distilled water. Non-migrating cells were removed from the non-migrating surface of the membrane with a cotton-tipped swab moistened with 1X PBS. The membrane was then mounted on a microscope slide with Cytoseal\textsuperscript{TM} XYL mounting medium (Richard-Allan Scientific\textsuperscript{TM}). Images were captured with an Olympus BX41 light microscope using SPOTSOFTWARE (Diagnostic Instruments, Inc., Sterling Heights, MI). The total number of migrating cells was quantified in four representative images per membrane sample at 20X power. The experiment was performed in triplicate.
2.8. Cellular Adhesion Assay

Cells (1x10^5 B16-F10 cells) were plated on fibronectin-coated plates and treated with 200µg/mL *R. crenulata* or vehicle control. Following 24 hr incubation at 37°C the plate was agitated and washed with 100µl washing buffer. Adhered cells were fixed with 50 µl 4% paraformaldehyde for 10 min, washed twice and stained with 50µl 2% crystal violet. The plate was rinsed with H₂O and allowed to dry completely overnight. The crystal violet was solubilized in 2% SDS and the absorbance of each well was recorded at 550nm using an Enspire® multimode automated plate reader (PerkinElmer, Waltham, MA). The experiment was performed in sextuplicate.

2.9. Melanogenesis Assay

100 or 1000 cells were seeded on a 96-well plate and treated with 50µg/mL, 100µg/mL, 200µg/mL *R. crenulata* or vehicle. After 96 hours, a normalized number of cells in each group was measured for absorbance at 405nm using an Enspire® multimode automated plate reader (PerkinElmer, Waltham, MA).

Melanogenesis was further analyzed by treating B16-F10 cells with 200µg/mL *R. crenulata* or vehicle. After 24 hours, a normalized number of cells were lysed for 1 hour at 60°C with 1N NaOH. After that, samples were measured for absorbance at 405nm using an Enspire® multimode automated plate reader (PerkinElmer, Waltham, MA).
2.10. Western Analysis

B16-F10 cells were treated with 200µg/mL \textit{R. crenulata} or vehicle control. Following treatment for 72 hours, cells were lysed in RIPA buffer containing phosphatase and protease inhibitors. Lysates were collected and protein concentration was quantified using a BCA™ Protein Assay kit (Pierce, Rockford, IL). For each sample, 40µg/mL of protein was run at 180V for one hour on a 10% SDS PAGE polyacrylamide gel electrophoresis and transferred onto a Whatman® Schleicher & Schuell® Westran S PVDF protein sequencing membrane for 90 minutes at 100V at 4°C (Sigma Aldrich®, St. Louis, MO). The membrane was blocked at room temperature for one hour using a 5% milk TBS-T solution and was incubated at 4°C in primary antibody [rabbit anti-Vimentin #3932 (Cell Signaling)] diluted 1:1000 in 5% BSA TBS-T solution overnight. Following this, the membrane was incubated with secondary antibody [anti-rabbit IgG HRP-linked Antibody #7074 (Cell Signaling, Danvers, MA)] (1:2000 in 5% milk TBS-T) for two hours. Labeled protein bands were detected using HyGLO™ Quick Spray chemiluminescent detection reagent (Denville Scientific Inc., South Plainfield, NJ). This protocol was repeated for β-Actin as a normalizer for Vimentin [(Primary: mouse anti-β-Actin #47778 (Santa Cruz Biotechnology, Dallas, TX); Secondary: goat anti-mouse IgM-GRP #2064 (Santa Cruz Biotechnology)]; β1 [(Primary: rabbit anti-β1 #4706 (Cell Signaling, Danvers, MA); Secondary: anti-rabbit IgG HRP-linked Antibody #7074 (Cell Signaling, Danvers, MA)] and β-Actin as a normalizer for β1 [(Primary: rabbit anti-β-Actin #ab8227 (abcam, Cambridge, MA)].
2.11. Fluorescent Immunocytochemistry

1x10^5 B16F10 cells were plated onto glass coverslips and coated with Attachment Factor 1X (Gibco®) following the manufacturer’s protocol in a 24 well plate. Cells were treated with 200µg/mL *R. crenulata* or vehicle for 72h. Next, the media was decanted and cells were fixed with 4% paraformaldehyde at 4°C. Cells were permeabilized with 0.5% TritonX-100 and washed 3 times with 1X PBS:Glycine (130mM NaCl, 7mM Na2HPO4, 3.5mM NaH2PO4, 100mM Glycine). Cells were blocked with 1X IF Buffer (130mM NaCl, 7mM Na2HPO4, 3.5mM NaH2PO4, 7mM NaN3, 0.1% BSA, 0.2% TritonX-100, 0.05% Tween-20) plus 10% goat serum for 1-2h. The cells were blocked with secondary blocking buffer consisting of 1X IF buffer, 10% goat serum, and 20µg/mL goat anti-mouse F(ab’)2 for 30-45 min. Mouse anti-E-cadherin (BD Transduction Laboratories™, Franklin Lakes, NJ) was diluted 1:50 in secondary blocking buffer O/N. Secondary antibody Alexa Flour-568 goat anti-mouse (Molecular Probes, Invitrogen) was diluted 1:500 in 10% goat serum IF buffer and incubated for 45-60min. coverslips were removed from the wells and mounted onto microscope slides with Vecta-shield Mounding Medium for Fluorescence with DAPI (Vector Laboratories Inc., Burlingame, CA). Images were captures with a Zeiss 200M inverted microscope at the same exposure and time using Axiovision software 4.8.2.


All mouse work was completed in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*. Thirty eight-week-old virgin c57bl/6 female
mice were housed individually in plastic cages and were permitted free access to food and water. A total of $1 \times 10^6$ B16-F10 cells were implanted subcutaneously above the scapular fat pad. Twenty-four hours following tumor implantation, daily topical *R. crenulata* treatment was initiated. Treatment groups included: 10 mice treated with 5% *R. crenulata* cream, 10 mice treated with 10% *R. crenulata* cream, and 10 mice treated with DMSO control cream. Mice were evaluated daily and tumor volume measurements were obtained once tumors became palpable. Tumor volume was calculated by $(\text{tumor length}^2 \times \text{tumor width})/2$, in which the tumor length was the smaller of the two measured values. Mice were euthanized if they appeared unhealthy, exhibited greater than 15% weight loss, or when tumor volume exceeded $1.5 \text{ cm}^3$. Upon euthanasia, necropsy was performed and tissues were preserved in 10% formalin or flash frozen.

Excised tumors were paraffin embedded and stained with hematoxylin and eosin (H+E). Images were captured with an Olympus BX41 light microscope using SPOTSOFTWARE (Diagnostic Instruments, Inc., Sterling Heights, MI). The total number of mitotic cells was quantified in three representative images at 40X power per tumor sample obtained from each mouse in the DMSO and 10% *R. crenulata* treated groups.

2.13. Animal Studies (enteral treatment of subcutaneous tumor growth)
Twenty eight-week-old virgin c57bl/6 female mice were housed in plastic cages. Mice were pre-treated for one day with *R. crenulata* (100mg/kg in their water) or vehicle supplemented water. A total of $1 \times 10^6$ B16-F10 cells were implanted subcutaneously above the scapular fat pad. Mice were evaluated daily and water was replaced with
appropriate treatment every 48 hours. Tumor volume measurements were obtained once tumors became palpable. Tumor volume was calculated by \((\text{tumor length}^2 \times \text{tumor width})/2\), in which the tumor length was the smaller of the two measured values. Mice were euthanized if they appeared unhealthy or exhibited more than 15% weight loss. Upon euthanasia, necropsy was performed and tissues were preserved in 10% formalin or flash frozen.

2.14. Animal studies (Dissemination Model)

Sixteen eight-week-old virgin c57bl/6 female mice were housed in plastic cages. Mice were pre-treated for three days with \(R. \text{ crenulata}\) (100mg/kg in their water) or vehicle supplemented water. A total of \(1 \times 10^5\) B16-F10 cells were injected via lateral tail vein to model a disseminated disease state. Mice were evaluated daily and water was replaced with appropriate treatment every 48 hours. Mice were euthanized if they appeared unhealthy or exhibited more than 15% weight loss. All surviving mice were euthanized 30 days following tumor injection. At the time of euthanasia, lungs were preserved in fekete’s solution, while remaining organs were preserved in 10% formalin. Lungs were evaluated for gross establishment of metastatic foci and photos were obtained. Formalin fixed paraffin embedded lungs, were further analyzed histologically via H+E staining. Representative images of H+E stained lung sections were taken using an Olympus BX41 light microscope using SPOTSOFTWARE (Diagnostic Instruments, Inc., Sterling Heights, MI).
2.15. Statistical Analysis

All results were analyzed using either a two-tailed student’s t-test, one-way analysis of variance (ANOVA), or a two-ANOVA with a Bonferroni’s correction. Statistical outliers were identified utilizing a rout test. All statistical analysis was performed with GraphPad Prism Software (Prism, GraphPad Software, Inc., San Diego, CA).
CHAPTER III  
IN VITRO RESULTS

3.1. *R. crenulata* induces morphological changes and decreases proliferation and survival in B16-F10 cells

B16-F10 cells are a well-studied mouse cell line used to model melanoma. These cells are typically adherent, flat, and have multiple dendritic projections. We treated these cells with increasing doses of *R. crenulata* and observed significant effects at 200µg/mL (Fig. 1B). Phenotypically, the B16-F10 cells treated with *R. crenulata* exhibited reduced dendritic projections, became rounder and appeared less adherent (Fig. 1B). To assess *R. crenulata*’s effect on growth and proliferation, we performed an MTS assay using 200µg/mL *R. crenulata* over time. At 24 hours, a trend towards decreased proliferation was noted in groups treated with 200µg/mL *R. crenulata* (Fig. 1A) compared to vehicle control. However, by 48 hours and 72 hrs the decrease in proliferation was significant [(p<.01) and (p<.0001) respectively]. To evaluate the cytotoxic effects of *R. crenulata* on B16-F10 cells, a live dead assay was performed. An increase in the number of dead cells was observed in samples treated with *R. crenulata* (Fig. 1B), especially evident upon administration of 200µg/mL *R. crenulata*.

In other cell types, we have found that treatment with *R. crenulata* sensitizes cancer cells to radiation treatments [37]. Thus, we were interested in whether treatment with *R. crenulata* could enhance the sensitivity of B16-F10 cells to radiation induced cell death. To evaluate treatment efficacy via colony formation and survival, a clonogenecity assay
was performed. In agreement with the MTS and the live dead assay, *R. crenulata* significantly reduced the number (p<.001 and p<.0001 respectively) compared to vehicle control (Fig. 1C-D). However, radiation sensitivity did not appear to be affected by the presence of *R. crenulata* in this cell line.

3.2. *R. crenulata* increases melanogenesis in B16-F10 Melanoma Cells

As melanoma develops, the production of melanin by melanocytes may depreciate. In order to evaluate *R. crenulata*’s effects on melanogenesis, we treated B16-F10 cells with 50µg/mL, 100µg/mL or 200µg/mL *R. crenulata*. After 96 hours, we observed a dose-dependent increase in melanin content (p<.05, Fig. 2). This trend was also observed in treated B16-F10 cells that were lysed open prior to being analyzed (data not shown).

3.3. *R. crenulata* inhibits migration of B16-F10 melanoma cells

As noted above, the metastatic ability of B16-F10 cells is of paramount importance to the survival of melanoma patients. To test whether *R. crenulata* could affect migration of B16-F10 cells, a scratch wound assay was performed. Plates treated with vehicle control exhibited complete wound closure by 48 hours while plates treated with 100µg/mL *R. crenulata* maintained distinct wound boundaries with minimal growth at 48 hours (Fig. 3A). We confirmed these results using a transwell migration assay. We observed a significant reduction in the number of migrating cells in wells treated with 100µg/mL *R. crenulata* for 24 hours compared to vehicle treated wells (p<.0001, Fig. 3B-C).
3.4. *R. crenulata* decreases expression of ID genes, Integrin β1 and Vimentin in B16-F10 Cells

A decrease in migration could be caused by a number of different mechanisms. We had noted in our early experiments a slight rounding and lifting of the B16-F10 cells in response to *R. crenulata* treatment. To evaluate *R. crenulata*'s effects on cellular adhesion, we tested the ability of *R. crenulata* or vehicle treated cells to adhere to a fibronectin-coated plate. A significant decrease in adhesion was noted following 24 hours of 200µg/mL *R. crenulata* treatment (p<.0001, Fig 4A). Because fibronectin interacts with certain integrins, and integrins have been shown to be important for migration, we examined integrins β1 protein levels by Western Analysis. Upon 72 hours of 200µg/mL *R. crenulata* treatment, expression of β1 was observed to be significantly reduced (p=.0362, Fig. 4B).

Similar to integrins, ID genes play an important role in migration and invasion. We evaluated ID gene expression levels by qRT-PCR. Following 24 hours of 200µg/mL *R. crenulata* treatment we observed an overall decrease in the expression levels of IDs 1-4 with a significant reduction in the expression levels of both ID-2 and ID-3 (p<.05, Fig. 4C).

A second pathway that is critical for migration and invasion of tumors involves epithelial to mesenchymal transition. In this pathway the epithelial cell E-cadherin, important for maintaining adherence junctions and melanocyte-keratinocyte interactions, is down-
regulated while vimentin, an intermediate filament expressed in mesenchymal cells, is upregulated. Western Analysis demonstrated that following 72 hours of 200μg/mL *R. crenulata* treatment, a significant reduction in the expression levels of vimentin in B16-F10 was observed (p=.0287, Fig. 4D). Immunocytochemical analyses confirm an increase in E-cadherin levels consistent with a mesenchymal to epithelial (MET) transition following 72 hours of treatment with 200μg/mL *R. crenulata*, but not observed with vehicle control (Fig. 5).
Figure 1. *R. crenulata* causes morphological changes, inhibits proliferation and decreases viability and survival in B16-F10 cells. (A) 5,000 B16-F10 cells were seeded on a 96-well plate and treated with 200µg/mL *R. crenulata* or vehicle. Cell proliferation was assessed with an MTS assay at 24, 48, and 72 hours. The experiment was performed in sextuplicate. Data was compared using a one-way ANOVA with corresponding Bonferroni t-test; bars ±SEM. Statistical significance was noted after *R. crenulata* treatment. (B) Phase contrast image representation of B16-F10 cells treated with 200µg/mL *R. crenulata* or vehicle (left panel). Image representation of a Viability/Cytotoxicity Assay (right panel): 7,500 cells were seeded on Chamber Slides and treated with 200µg/ml *R. crenulata* or vehicle. After 72 hours, viable and dead cells were labeled using Calcein and Ethidium homodimer-1 respectively. Images were captured at 20X magnification. (C) Continuous Treatment Clonogenecity Assay. 200 B16F10 cells were plated on 60mm plates and treated as described in Material and Methods. After 8 days, surviving colonies were stained with crystal violet and quantified. The experiment was performed in triplicate. Results were analyzed using a two-way ANOVA with a corresponding Bonferroni t-test; bars ±SEM. Statistical significance was observed after treatment with *R. crenulata* and after treatment with both *R. crenulata* and 5Gy irradiation. (D) Image representation of continuous clonogenecity assay. The purple dots represent surviving crystal violet stained colonies. (*p<.05, **p<0.01, ***p<0.001, ****p<0.0001).
Figure 2. *R. crenulata* treatment increases melanogenesis in B16-F10 cells. 100 or 1000 cells were seeded on a 96-well plate and treated with 50µg/mL, 100µg/mL, 200µg/mL *R. crenulata* or vehicle. 96 hours later, a normalized number of cells were measured for absorbance at 405nm. The experiment was performed in triplicate. Data was compared using a two-way ANOVA with corresponding Bonferroni t-test; bars ±SEM. Statistical significance was noted after *R. crenulata* treatment. (*p<.05).
Figure 3. *R. crenulata* decreases cell migration of B16-F10 cells. (A) Scratch Wound Healing Assay. Cells were seeded on 60mm plates and allowed to reach confluency. Using a 10µl pipette, a scratch was made in the middle of each plate. Cells were subsequently treated with 100µg/mL *R. crenulata* or vehicle. Images were captured at 15 minutes, 4 hours, 24 hours and 48 hours at 20X magnification. (B) Migration Assay. B16-F10 cells were treated with 100µg/mL *R. crenulata* or vehicle. After 24 hours, 100,000 B16-F10 cells were plated on BD BioCoat™ Matrigel migration chambers with 8µm pores. 24 hours later, migrating cells were stained with crystal violet and images were captured at 20X magnification. (C) The number of migrating cells within each representative 20X field was counted. The experiment was performed in triplicate. Data was compared using a student’s t-test; bars represent ±SEM. Statistical significance was noted after *R. crenulata* treatment. (****p<.0001).
Figure 4. *R. crenulata* decreases the expression of genes associated with proliferation, migration and tumorigenesis and increases the expression of genes involved in differentiation. (A) *R. crenulata* decreases the ability of B16-F10 cells to adhere to a fibronectin-coated plate. $1 \times 10^5$ B16-F10 cells were plated on a fibronectin-coated plate and treated for 24 hours with 200µg/mL *R. crenulata* or vehicle. Adhered cells were fixed and stained with crystal violet. The absorbance of each well was recorded at 550nm. The experiment was performed in sextuplicate. Data was compared using a student’s t-test; bars represent ±SEM. Statistical significance was noted after *R. crenulata* treatment. (****p<.0001). (B) *R. crenulata* treatment reduces the expression of β1 in B16-F10 cells. B16-F10 cells were treated with 200µg/mL *R. crenulata* or vehicle. After 72 hours, proteins were collected, quantified, and β1 expression levels were measured via Western Analysis. The experiment was performed in quadruplicate. Results were normalized to β-Actin. Blots are shown above with the corresponding graph below. Bars represent ±SEM of the relative intensity. Results significantly differed from vehicle treated cells via a student’s t-test. (*p<.05). (C) *R. crenulata* reduces gene expression of genes associated with migration, invasion, and tumorigenesis. Real-time PCR analysis was carried out for ID-1, ID-2, ID-3, and ID-4. B16-F10 cells were treated with 200µg/mL *R. crenulata* or vehicle. After 24 hours, total RNA was isolated. The results shown represent experiments performed in triplicate for *R. crenulata* and duplicate for vehicle. All results were normalized to GAPDH. Bars represent ±SEM of the fold change with respect to vehicle treated cells. Data was compared using a two-way ANOVA with a corresponding Bonferroni t-test. A significant decrease was observed in *R. crenulata* treated groups for ID-2 and ID-3. (*p<.05). (D) *R. crenulata* treatment reduces the expression of Vimentin in B16-F10 cells. B16-F10 cells were treated with 200µg/mL *R. crenulata* or vehicle. After 72 hours, proteins were collected, quantified, and Vimentin expression levels were measured via Western Analysis. The experiment was performed in quadruplicate. Results were normalized to β-Actin. Blots are shown above with corresponding graph below. Bars represent ±SEM of the relative intensity. Results significantly differed from vehicle control treated cells via a student’s t-test. (*p<.05).
Figure 5. *R. crenulata* treatment increases E-cadherin expression in B16-F10 cells. B16-F10 cells were treated with 200µg/mL *R. crenulata* or vehicle. After 72 hours, they were immunostained with E-cadherin and counterstained with DAPI. Images were captured at 20X magnification; scale bar 50µm.
CHAPTER IV  
*IN VIVO RESULTS*

4.1. Topical *R. crenulata* treatment alters patterns of tumor growth *in vivo*

Our *in vitro* results suggest that *R. crenulata* may have a potent effect on the growth and establishment of metastasis of B16-F10 *in vivo*. To evaluate the effects of *R. crenulata* application on B16-F10 tumors, we performed an *in vivo* experiment utilizing a subcutaneous syngenic melanoma tumor model in c57bl/6 mice, which we topically treated with *R. crenulata*, or vehicle in a Eucerin™ based cream (Fig. 6A). Tumors were measured daily using calipers, and for ease of measurement the pigmented region was what was measured. Interestingly, we did observe gross differences in patterns of tumor growth between treatment groups, although there was no difference in the overall size of the tumor observed. The tumors in mice treated with vehicle control tended to be raised while the tumors in the *R. crenulata* treated animals exhibited more outward growth over time (Fig. 6C). Since the widths were the measured areas, no significant difference in overall tumor size or survival was observed between the *R. crenulata* and vehicle control groups. The mice treated with *R. crenulata* survived slightly longer: 5% *R. crenulata* and 10% *R. crenulata* met requirements for euthanasia by days 15 and 20, respectively, compared to the vehicle (14 days). Further, one mouse treated topically with 5% *R. crenulata* exhibited complete regression of subcutaneous tumor and survived for over 30 days (Fig. 6B).

Given the differences in growth patterns detected upon topical *R. crenulata* treatment, we evaluated the mitotic rate within subcutaneous tumors. We observed that tumors treated
with vehicle control exhibited significantly more mitotic activity as these tumors had an average of 2.731 mitotic bodies per high power field while tumors treated with 10% *R. crenulata* had an average of 1.714 mitotic bodies per high power field (p=.0374, Fig. 6D).

Knowing that *R. crenulata* exhibited cytotoxic effects on B16-F10 cells *in vitro* we attempted to evaluate these effects *in vivo*. Fixed, paraffin-embedded tumor sections were assayed by the TUNEL method to detect DNA nicks that is characteristic of apoptosis. Subjectively, there appeared to be an increased amount of positive-TUNEL reactions in *R. crenulata* treated tumors (Fig. 7); however, these results were confounded by the fact that both melanin and positive-TUNEL reactions stained brown. Distinguishing positive-TUNEL reactions and melanin pigment in the tumor cells was challenging; thus, we cannot conclude that there was an increased amount of apoptosis in *R. crenulata* treated tumors.

This same problem was encountered when we attempted to evaluate E-cadherin by immunohistochemistry (IHC). Again, although there appeared to be an increased amount of E-cadherin in tumors treated with 10% *R. crenulata* cream, these results were confounded by the fact that both E-cadherin stain and melanin pigment appears brown under the microscope (Fig. 7).
4.2. *R. crenulata* does not cause tumor regression when administered enterally

Given the differences in growth patterns observed with a topical application of *R. crenulata* we decided to test whether these results would be mimicked or amplified in mice receiving *R. crenulata* enterally rather than topically. We performed an *in vivo* experiment utilizing a subcutaneous syngenic melanoma tumor model in c57bl/6 mice which we enterally treated with *R. crenulata* or vehicle through the mice’s water. Tumors were measured daily using calipers, and for ease of measurement the pigmented region was what was measured. We did not observe a gross difference in tumor volume between treatment groups. Additionally, we did not observe a difference in tumor growth patterns. Mice drinking water supplemented with vehicle reached a tumor volume of 500 mm$^3$ in 12 days, whereas mice drinking water supplemented with *R. crenulata* reached a tumor volume of 500 mm$^3$ in 13 days. All mice drinking water supplemented with vehicle met requirements for euthanasia by day 24 while all mice drinking water supplemented with *R. crenulata* met requirements for euthanasia by day 21.

4.3. *R. crenulata* inhibits establishment of metastatic melanoma

In order to evaluate whether *R. crenulata* can prevent establishment of metastatic disease, we provided *R. crenulata* (100µg/mL) or vehicle control enterally in water to c57bl/6 animals. After three days they were injected via the lateral tail vein with B16-F10 cells and were monitored following tumor injection for 30 days (Fig. 8A). As previous studies have suggested that this cell type will metastasize to the lung, we collected the lungs for analysis. We observed a gross difference in the number of established tumor foci within the lungs of mice treated with *R. crenulata* compared to vehicle control (Fig. 8B).
Further, a significant decrease in lung weight was observed in mice treated with *R. crenulata* secondary to a reduced tumor burden (p<0.05, Fig. 8C). Histological analysis of lungs confirmed reduced establishment of tumor microscopically within the lungs upon *R. crenulata* treatment. (Fig. 8B) Pathological consultation suggested that the reduced tumor burden corresponded with reduced inflammatory involvement and reduced angiogenesis.
**Figure 6.** *R. crenulata* alters tumor growth and reduces mitotic tumor rate *in vivo*. (A) Schematic: Topical Treatment of Subcutaneous Tumor Growth. 1x10⁶ B16-F10 cells were injected subcutaneously in 8-week-old c57bl/6 mice. Injection sites were treated daily with a topical administration of 5%, 10% *R. crenulata* or vehicle cream. Every other day, tumor volumes were measured and recorded. Mice were sacrificed when tumor volume exceeded 1.5cm³ or when 15% of initial body weight was lost. (B) Survival curve for c57bl/6 mice treated topically with *R. crenulata* or vehicle cream. An upward trend in survival days for mice treated topically with 5% or 10% *R. crenulata* cream was observed. One mouse treated with 5% cream survived for over 30 days. (C) Image representation of tumors treated topically with 10% *R. crenulata* or vehicle cream. Images were captured after the mouse had been sacrificed. (D) Image representation of histological tumor specimens stained with hematoxylin and eosin. After mice had been sacrificed, tumors were excised, fixed, paraffin embedded and stained with hematoxylin and eosin. Red arrows indicate mitotic cells. Images were captured at 40X magnification. (E) The number of mitotic cells within each representative 40X field was counted. Each sample was photographed three times and the number of mitotic cells within each represented 40X field was quantified and averaged. Results were analyzed using a student t-test; bars represent ±SEM. Statistical significance was noted after topical *R. crenulata* treatment. (*p<.05).
Figure 7. Immunohistochemical Analysis of Excised Subcutaneous Tumors. Tumors excised from 10% *R. crenulata* and vehicle treated c57bl/6 mice were fixed, and paraffin embedded. Sections were stained with the indicated reactions/antibodies. Images were captured at 4X, 20X, and 40X magnification.
Figure 8. Enteral R. crenulata inhibits establishment of metastatic melanoma in c57bl/6 mice. (A) Schematic depicting the experimental metastasis model. 72 hours prior to the injection of 10,000 B16-F10 cells via the tail vein, mice were given vehicle control or 100mg/kg R. crenulata supplemented water. Supplemented water was refreshed every other day. 30 days later, mice were sacrificed and affected tissue was excised. (B) Image representation of gross excised lungs and histological tumor specimens stained with hematoxylin and eosin from mice treated enterally with 100mg/kg R. crenulata or vehicle. After 30 days of treatment, mice were sacrificed and lungs were excised, fixed and photographed. Black residues represent tumor burden. Lungs were then paraffin embedded and stained with hematoxylin and eosin. (C) Percent of Total Body Weight. At the time of sacrifice, mice’s body weight was compared to the weight of their lungs. Results were analyzed using a student t-test; bars represent ±SEM. Statistical significance was noted after enteral R. crenulata treatment. (*p<.05).
CHAPTER V
DISCUSSION AND FUTURE DIRECTIONS

Last year melanoma was responsible for the death of 9,710 Americans [1] and the incidence of this disease continues to rise annually. Melanoma is the most aggressive and deadliest form of skin cancer and currently, prevention and early detection are the best methods to reduce these mortality rates as effective treatment options, once diagnosed, remain limited. Thus, there is a necessity to unearth compounds with therapeutic potential to combat this deadly disease. *Rhodiola* plant extracts have exhibited various anti-tumorigenic properties in a variety of cancers and have been shown to promote cell death and inhibit both proliferation and angiogenesis [34-35; 40-41]. Given this, *R. crenulata* extract and the compounds contained within it, serve as a potential source of novel melanoma therapy.

In this study, we evaluated the effect of *R. crenulata* on the aggressive melanoma cell line, B16-F10. Upon treatment with *R. crenulata*, B16-F10 cells exhibited morphological changes, reduced proliferation, increased cell death and reduced colony formation compared to a vehicle control. While analyzing *R. crenulata*’s effect on B16-F10 colony formation, we observed that *R. crenulata*’s ability to reduce colony establishment was more effective than that of radiation alone. Combining both *R. crenulata* and radiation did not significantly reduce colony establishment when compared to just using *R. crenulata* treatment, suggesting that it is not affecting the frequent radio-resistance exhibited by melanoma cells. Overall, these results reveal the therapeutic potential of *R. crenulata* extracts for the treatment of melanoma *in vitro.*
Melanoma’s ability to exhibit uninhibited migration contributes to its aggressive phenotype and high mortality rate. This augmented migratory behavior is due, in part, to the ID genes. Upregulation of IDS have been associated with tumorigenesis by inhibiting cell differentiation, stimulating proliferation, enhancing invasiveness and facilitating tumor growth [42]. Previous studies report that *R. crenulata* reduces transcriptional levels of ID-1 and ID-3 in MDA-MB-231 breast cancer cell lines [34]. In this study, we observed a significant decrease in the transcriptional levels of ID-2 and ID-3 and a decreasing trend in ID-1 and ID-4 in *R. crenulata* treated B16-F10 cells. These results support and further suggest that *R. crenulata* exerts anti-migratory and anti-proliferative characteristics on B16-F10 cells.

Another pathway that contributes to melanoma’s ability to migrate and invade is epithelial-mesenchymal transition (EMT), a reversible switch implicated in the invasive properties of many carcinomas [43]. Vimentin, a mesenchymal filament protein, is heavily involved in attachment, migration, and cell signaling [44]. As it is highly expressed in mesenchymal cells, vimentin serves as an EMT marker and plays a critical role in wound healing, angiogenesis and cancer growth. Over expression of vimentin significantly increases the migratory and invasive potential in melanoma cells [15-17]. In this study, we utilized a scratch wound assay and Matrigel Migration assay to demonstrate *R. crenulata’s* ability to inhibit migration. We also showed that *R. crenulata* significantly decreased expression levels of vimentin and increased expression of melanogenesis and E-cadherin in B16-F10 cells. These results suggest that *R. crenulata*
may be inducing mesenchymal to epithelial transition (MET) and thus hints at a possible role in inducing a more differentiated state.

In agreement with a more differentiated state, we observed a flatter growth pattern of subcutaneous tumors, which is more reminiscent of a transition to radial growth phase (RGP). This is important because RGP melanomas harbor a 1-2% chance of metastasis whereas VGP melanomas harbor a metastasis rate of up to 31% [45] within 10 years of diagnosis. The transition from RGP to VGP, and subsequent increase in metastatic potential, is frequently associated with the upregulated expression of certain integrins such as \( \beta_1 \) [23; 46; 57]. Importantly, an increase in integrin \( \beta_1 \) expression of primary cutaneous malignant melanomas was observed to be associated with subsequent occult lymph node metastasis [47]. Upregulated expression of \( \alpha_5 \), an integrin subunit predominantly coupled with the integrin \( \beta_1 \) subunit, has been associated with B16-F10 metastasis via its interactions with fibronectin [48]. In this study, we observed a decrease in integrin \( \beta_1 \) in response to \( R. crenulata \) treatment \textit{in vitro} consistent with our decreased migration and decreased adhesion and in support of our \textit{in vivo} results suggesting a more differentiated tumor.

Like vertical growth, high mitotic rates in primary cutaneous melanomas are known to be associated with a more aggressive disease phenotype and lower survival rate [49-52]. Additionally, melanoma tumorigenesis is directly related to a tumor’s mitotic rate [45]. Therapeutic agents that reduce the mitotic rate in melanoma have the potential to reduce tumor growth and improve outcomes of this disease. In this study, we observed that tumors treated topically with \( R. crenulata \) had significantly less mitotic bodies than
tumors treated with vehicle control. This further suggests that *R. crenulata* can reduce the aggressive potential of melanoma tumors *in vivo*. *Rhodiola* plant species have been previously used topically to reduce melanotic lesions [53], but our study is the first one to suggest that a topical application may alter and potentially improve melanoma outcomes. The endpoints for our *in vivo* subcutaneous study was based on the size of the pigmented area; however, we had not evaluated the vertical axis in tumor volume measurements. Thus *R. crenulata* treated animals may have lasted significantly longer than the vehicle treated animals if this had been a consideration.

Our second *in vivo* study evaluated the ability of *R. crenulata* to prevent metastatic establishment. Our results revealed that upon enteral administration of *R. crenulata* in a disseminated tumor model, the resulting burden of established metastatic foci within the lung was greatly reduced both grossly and histologically. These results were impressive and exciting. Following tumor resection, an increase in the amount of circulating malignant tumor cells in the blood has been observed [54]. This, combined with the physiological results of stress during the perioperative period following tumor resection is associated with an increased recurrence rate [55]. Some studies even reveal an increased in-transit metastases risk in patients with melanoma who remove non-palpable metastases by elective lymph node dissection following tumour-positive sentinel node diagnosis [56]. We are encouraged that a treatment with the ability to reduce the establishment of malignant cells following tumor resection would have a significant preventative and therapeutic impact. Future studies will need to be performed to examine the effects of *R. crenulata* on human melanoma lines, as well as to analyze the potential
of topical treatment to prevent progression from the benign nevi to more malignant
disease.

In conclusion, *R. crenulata* exhibits promising antineoplastic properties upon treatment of
B16-F10 melanoma, both *in vitro* and *in vivo*. As therapeutic options for melanoma
remain limited, identification of novel agents with chemotherapeutic potential, such as *R.
crenulata* are instrumental in improving survival for those with this aggressive form of
cancer. The future of melanoma therapy may have a place for *R. crenulata* as an adjuvant
for current treatment methods. In this study, we show that *R. crenulata* may impede the
transition from RGP to VGP, a key progression involved in the establishment of
metastatic disease. Further, we also demonstrate that a topical administration of *R.
crenulata* is able to exert protective effects. Finally, we show exciting data for the
potential anti-metastatic activity of this extract.
REFERENCES


