# THE EFFECTS OF SELECT PLANT EXTRACTS ON CANINE NEOPLASTIC CELL GROWTH AND SIGNALING

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# ABSTRACT

The inclusion of fruits and vegetables in the diet has been suspected to prevent cancer and the use of natural compounds, or nutraceuticals, to treat the disease has recently been entertained. While there is ongoing research in the use of nutraceuticals to treat a variety of human cancers, little work has been completed in our companion animals, specifically dogs. A screening of feed ingredients deemed safe and reliable by the Association of American Feed Control Officials was performed. After examination of cellular proliferation, apoptosis by flow cytometry, and a multitude of signaling pathways by western immunoblotting, turmeric root and rosemary leaf extracts were determined to be potent inhibitors of neoplastic cell growth. These extracts worked synergistically to induce apoptosis *in vitro* in canine mastocytoma, mammary carcinoma, and osteosarcoma. These results need further *in vivo* and clinical examination, as a dietary supplement to cancer treatment could prove efficacious.

# BIOGRAPHICAL SKETCH

Corri Levine completed her Bachelors of Science degree at Cornell University's College of Agriculture and Life Sciences in May, 2011. Her degree was awarded with a major in animal science and a minor in biological sciences with a concentration in microbiology. During her undergraduate career she completed research in the lab of Dr. Joseph Wakshlag studying muscle metabolism in calves, adiposity of Hus1 genetically modified mice, and the effects of weight loss on serum adipokines in dogs. In addition she worked at Cornell's Center for Materials Research performing electron microscopy on elephant umbilical cords infected with Elephant Endotheliotropic Herpesvirus. After graduation she interned at Terem Emergency Health Center in Jerusalem, Israel collecting data related to treatment of cellulitis. From September 2011 until June 2013 she worked in the laboratory of Dr. Steven Goldman at the University of Rochester studying the use of human fetal cells and induced pluripotent stem cells to generate myelin in a mouse model of congenital demyelination. In June 2013, Corri returned to Cornell University's School of Veterinary Medicine to perform research with Dr. Joseph Wakshlag. She began her Master's training and coursework in August 2014 in the Comparative Biomedical Sciences program concentrating in Cellular and Molecular Medicine and Pharmacology. Her work focuses on the use of naturally derived compounds to inhibit the growth of canine cancer cell lines.

This thesis is dedicated to my husband, Benjamin Perry, who has supported me through the late night and early morning trips into the laboratory so I could complete this research.

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# LIST OF ABBREVIATIONS

TE – Turmeric extract (87% curcuminoids)

RE – Rosemary extract (70% carnosic acid)

C2 – Canine mastocytoma cell line

CMT-12 – Canine mammary adenocarcinoma cell line

D17 – Canine osteosarcoma cell line

CDF – Canine dermal fibroblast primary cell line

IC – Inhibitory Concentration

SAPK/JNK – Stress-activated protein kinases/Jun amino-terminal kinases

# Chapter 1 - Introduction

## BACKGROUND

Cancer in humans is a leading cause of death in the United States[1] and worldwide,[2] and this holds true in dogs.[3] The disease onset can begin early or late in life and results in unregulated cell growth which can ultimately interfere with normal tissue and organ function. While therapies have continued to evolve, one area that has been underexplored until recently is the use of natural plant compounds. Plants and plant-derived compounds have been used for centuries around the world to treat a variety of ailments. For example, topical creams containing gel from the *Aloe vera* plant are used to soothe skin irritations and the compound salicin from white willow bark has been purified and chemical modified to make the common anti-inflammatory drug, aspirin.[4,5] In addition, several studies have examined the link between diets high in specific nutrients and the possible prevention of common diseases from heart disease to cancer.[6,7]

The term "nutraceutical" was developed in 1989 when Dr. Stephen DeFelice joined the words 'nutrition' and 'pharmaceutical' in order to describe "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease."[8] This definition is quite broad and the use of the term in marketing is currently unregulated, leading to a widespread use of over-the-counter supplements to treat various ailments with no medical supervision. This is both a problem for the medical community and an underutilized resource for epidemiologists. Many cancer patients begin using vitamins or supplements after diagnosis, and continue use throughout prescribed chemotherapy or radiation treatment.[9] In many cases, these patients do not report supplement use to their prescribing clinician. This trend has migrated into the veterinary world with owners administering supplements to their

companion animals.[10] The possibility for negative side effects or ineffective treatments is a concern to many oncologists considering the lack of studies examining the dual use of chemotherapeutics and nutraceuticals. Certain compounds can interfere with metabolism resulting in increased absorption of drugs and more extreme side effects or quicker metabolism resulting in lower plasma levels and decreased efficacy of the treatment.[11,12] The possibility for unwanted side effects or antagonism between prescribed treatments and dietary supplements highlights the need for increased research into the mechanisms and pathways affected by these naturally derived compounds.

The term nutraceutical can encompass a variety of different compounds. For my research, I have limited this definition to include plant derived extracts including, but not limited to, those derived from the leaves, root, skin, fruit, and flower. These whole extracts contain specific compounds of interest along with other, minor components; in contrast, single compounds of interest can be purified from these plants. The notion of using whole extracts, including the minor components, supposes that these compounds work synergistically and may in fact be more potent than the compound of interest alone.[13,14] Polyphenols, compounds made up of several aromatic rings, are the major compounds responsible for possible health benefits and can be divided into major subclasses, the most abundant being 1) phenolic acids such as curcuminoids and capsaicin found in the common spices curry and black pepper, respectively, 2) flavonoids such as those found in green tea, and 3) stilbenoids such as resveratrol found in grapes and wine, and 4) terpenes such as limonene found in citrus fruits and the major compounds found in the rosemary leaf, carnosic acid and carnosol.[15] An initial screening of 14 extracts derived from plant material was carried out by **Onco***design* S.A. (Dijon, France) using the ATP-Lite assay. The results from this screen yielded five extracts with the potential to be used as anti-neoplastic

agents. The five extracts came from green tea leaves, rosemary leaves, turmeric root, black pepper, and pomegranate skin. An overview of the activities of these compounds of interest are outlined in the following paragraphs.

*Curcuma longa*, or turmeric root, and its curcuminoids components have been extensively studied as a nutraceutical to treat a variety of ailments, with a record of nearly 10,000 publications on the topic and research devoted to delivery methods and increasing bioavailability.[16] The allure to using curcuminoids as a treatment modality lies in the ability of these compounds to target a variety of pathways from inflammation to apoptosis. In addition, several clinical trials in humans have been initiated and have shown limited to no toxicity or side effects with treatment.[17] The limiting factor in the administration of curcumin to patients is bioavailability due to poor solubility and rapid metabolism. A large focus of curcumin research has been focused on the synthesis of curcumin analogs and nanoparticle formulations to increase bioavailability.[18] The use of bioavailability enhancers, including other natural products[19] and total turmeric extract,[20] has shown promise in increasing cellular absorption of curcuminoids. With the complex targeting capabilities of curcumin, investigations into the potential use in the treatment of cancer in companion animals is warranted.

Phenolic acids from the plant *Rosmarinus officinalis*, commonly known as rosemary, have been used in food and cosmetic products as a potent antioxidant in order to prolong shelf-life and as an antimicrobial agent. More recently, the antioxidant effects of rosemary have been studied as potential anticancer agents, although other mechanisms of action have been found including cell cycle arrest and inhibition of P-glycoprotein (P-gp), also known as Multidrug Resistant protein 1 (MDR1).[21] The variety of cellular targets have been contributed to two major components, carnosic acid and carnosol, and to a lesser extent, rosmanol.[22] Rosemary extract as a

whole has been deemed safe for consumption and as an additive to foods making it a particularly good candidate for testing as a nutritional supplement for cancer treatments.[23]

Piperine, the main compound of interest in *Piper nigrum*, literally, black pepper, has been shown to inhibit cytochrome P450 drug metabolism, thereby allowing increased absorption and tissue distribution of co-administered compounds for improved efficacy.[24,25] Several studies have examined the anticancer potential of piperine *in vitro,* focusing on the antiproliferative and anti-metastatic effects including inhibition of protein kinase B (AKT) phosphorylation, mitogen-activated protein kinases (MAPK), and matrix metalloproteinases (MMP), but these activities were found using high concentrations of piperine (>25 μM) that may not be physiologically achievable. [26,27] While it seems as though the majority of administered piperine can be absorbed in the gut, very little of the compound reaches other tissues and instead remains in the stomach and small intestine.[28,29] While piperine may not show *in vivo* antitumor effects, its use as a bioavailability and absorption enhancer make research into any cytotoxic effects and interactions with chemotherapeutics necessary.

Extracts from the fruit of *Punica granatum*, or the pomegranate plant, contain various bioactive polyphenols, the most abundant of which are punicosides (or punicalagins). Punicosides have been found to exhibit strong antioxidant activity due to the major byproduct, ellagic acid.[30] Ellagic acid is rapidly absorbed and further metabolized by colonic bacteria by glucuronidation to urolithin A or urolithin B, and it is these metabolites that are found in the plasma.[31] Cell culture experiments show the primary action of ellagic acid is the inhibition of nuclear factor kappa-light chain enhancer of activated B cells (Nf-κB).[32] Nf-κB inhibition not only leads to decreased cell proliferation, but also a pronounced decrease in inflammation. Cell cycle arrest has also been observed in S phase in colon carcinoma due to down regulation of

cyclins A and B and the upregulation of cyclin E.[33] The necessary concentrations to be effective *in vitro* are variable and many of the concentrations tested are well beyond potential physiological levels.[34,35] In other studies using more physiological concentrations, a positive *in vivo* effect has been seen using a xenograft rodent model.[36] The rapid metabolism and elimination of ellagic acid results in a circulating blood half-life of less than two hours.[37,38] It is therefore necessary to perform *in vivo* pharmacokinetic studies before administering pomegranate juice or its purified constituents as a therapy in order to determine the bioavailability of ellagic acid and if the breakdown of the parental compounds are necessary to increase the half-life and distribution.

The health benefits of green tea extracts have been extensively studied as it contains four major polyphenols. The most abundant of these is the flavanol (-)-epigallocatechin-3-gallate (EGCG), constituting anywhere from 10 to 50% of the total flavanol content depending on the processing of the tea leaves.[34] In addition to its abundancy, EGCG seems to be the most potent of the flavanols, diminishing cell proliferation in multiple neoplastic epithelial cell lines and round cell tumor cells, with similar actions related to inhibition of Nf-κB, MAPK, and Phosphatidylinositide3-Kinase/AKT signaling.[39] EGCG can also induce apoptosis in multiple neoplastic cell types including, lung, skin, colon, pancreas and prostate cell lines due in part to p53 stabilization and direct binding to the BH3 domain of B-cell lymphoma 2 (Bcl-2) mitochondrial protein inhibiting its anti-apoptotic functions.[40,41,42] The bioavailability of EGCG is relatively low, with several studies in humans indicating a peak plasma concentration of around 1 μM[43] and a half-life less than three hours.[44] In canines, these values are higher, with 20% of the administered dose absorbed and a half-life of around eight hours,[45] even at nutritionally relevant doses.[46] It should be noted though, that

high doses of purified EGCG may be toxic to canines, causing hepatic failure.[47] In addition, the bioavailability and tissue distribution varies between species and individuals.[48,49]

## OBJECTIVES

The six extracts described were tested *in vitro* for their antineoplastic ability and the leading compounds were examined for possible mechanisms of action. I performed a secondary evaluation of antiproliferative capabilities of each extract individually and in combination. In order to characterize mechanisms of action of the leading compounds I performed a series of western blots to examine common signaling pathways and markers of apoptosis. In addition, I performed flow cytometry to observe apoptosis, changes in cell cycle, generation or reduction of reactive oxygen species and superoxide radicals, changes in cellular accumulation and efflux of compounds, and changes in mitochondrial permeability. The results of my thesis research have been used to advise Royal Canin in the development of a preliminary diet awaiting clinical trials in dogs.

Based on the knowledge gaps identified and described above, the objectives of my thesis research were therefore to (1) screen a variety of plant extracts for antineoplastic activity, (2) test leading compounds in combination to evaluate potential synergistic effects, (3) test leading compounds and combinations with the chemotherapeutic drug most commonly used for each cell type, and (4) determine mechanisms by which the leading compounds inhibited neoplastic cell growth.

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# Chapter 2 - Effects and synergy of feed ingredients on canine neoplastic cell proliferation

## ABSTRACT

Adjunctive use of nutraceuticals in human cancer has shown promise, but little work has been done in canine neoplasia. Previous human research has shown that polyphenols and carotenoids can target multiple pathways *in vitro* and *in vivo*. These compounds could synergize or antagonize with currently used chemotherapies, either increasing or decreasing the effectiveness of these drugs. Considering the routine and well controlled feeding practices of most dogs, the use of nutraceuticals incorporated into pet food is attractive, pending proof that the extracts are able to improve remission rates. The aim of this study was to examine five feed ingredients for antiproliferative effects, as well as the interaction with toceranib phosphate and doxorubicin hydrochloride, when treating canine neoplastic cell lines *in vitro*. Screening using MTT proliferation assays showed that green tea, turmeric, and rosemary extracts were the most effective. Turmeric extract was the most potent and exhibited synergy with a rosemary extract at concentrations from 1 to 25 μg $mL^{-1}$. This combination had an additive or synergistic effect with chemotherapeutic agents at selected concentrations within each cell line. No significant effects on cell viability were observed when the combination therapy was used with normal primary canine fibroblast cells. The use of turmeric and rosemary extracts in combination may be beneficial as a supplementary method of treatment of canine neoplasias. Further studies into the pharmacokinetics and mechanisms of action of these extracts should be examined.

# INTRODUCTION

Neoplasia is the cause of death in 20-30% of dogs in the United States, and the incidence and mortality increases to 50% in dogs over the age of ten.[1,2,3] Risk factors for developing cancer vary based on age, breed, sex, environmental exposures, and nutrition.[4] The incidence and, in some cases, prevalence of specific tumor types are different between dogs and humans, with lymphoma, mast cell disease, osteosarcoma, and mammary neoplasia being particularly prevalent in dogs.[5] Current treatments rely on the use of surgical intervention, chemotherapeutics, and/or radiation therapy. The efficacy of such treatments varies greatly and relies heavily on the stage of cancer progression when treatment is provided, genetic predispositions, and tolerance to the chemotherapy and/or radiation treatments.

Most chemotherapeutic treatments are limited and target DNA replication (cell cycle), cellular metabolism, or a single regulatory pathway. In the case of high grade and refractory tumors, multiple drug combinations are often used in order to achieve remission. As the number of treatment modalities increase there can be compounding side effects that are not always well tolerated by patients.[6]

The use of natural compounds, or nutraceuticals, in combination with traditional chemotherapy might prove useful in treating various cancers, and 40% of pet owners admit to using nutritional supplements as a complementary therapy.[7] Natural products have been used in the initial design of chemotherapeutic agents, with well over half of all compounds synthesized or screened being more potent derivations of natural products.[8] The effects of these compounds, whether natural or synthetically made, act through a variety of mechanisms that may contribute to the antiproliferative, antimutagenic, and/or pro-apoptotic properties.[9,10] In fact, a single compound might be able to induce apoptosis through several different pathways.[11] Much of the

natural product research performed has been on human or rodent cell lines, particularly of epithelial origin, with little research being done on canine cell lines.[12] More research is necessary to determine the specific effects of these natural products in canine cells and in combination with chemotherapeutic agents commonly used in veterinary medicine, as there is a paucity of information.[13,14,15]

Plant extracts consist of various polyphenols, terpines, stilbenoids and carotenoids.[16] Several epidemiological studies in humans have found benefits to the inclusion of polyphenol and carotenoid rich fruits and vegetables in the diet.[17,18] A case-control study has been completed in dogs, and the results indicated an inverse relationship between the consumption of vegetables and the risk of developing transitional cell carcinoma of the urinary bladder in Scottish Terriers.[19] The potential use of certain polyphenols and carotenoids as a treatment option is ongoing in pre-clinical trials with treatment of human epithelial carcinomas on the horizon.[20,21,22] However, it is necessary to investigate the potential effectiveness in canine cancers which represent other cellular lineages with hematopoietic, mesenchymal and epithelial neoplasias all being prevalent. In veterinary patients, there have been few intervention studies to examine nutraceutical dietary additions, with two studies examining possible treatment of lymphoma in dogs.[23,24] Considering that the typical daily feeding patterns of companion dogs are consistent (once or twice per day), the potential for incorporation of natural anti-carcinogenic ingredients is feasible, so long as the added herbal, fruit, or vegetable extract is considered generally reliable and safe by the Association of American Feed Control Officials (AAFCO).[25]

The objective of this study was to examine the antiproliferative effects of five commonly used natural food extracts chosen from an initial cytotoxic screening, which could be utilized in commercial dog food, on canine mastocytoma, mammary carcinoma, and osteosarcoma cell

lines. Extracts were selected based on current literature and only those in which the effective concentration was deemed physiologically relevant based on findings in humans and rodents were used for further evaluation. After defining efficacy of the leading extracts we set forth to determine if combinations of extracts could work synergistically. In addition, we sought to determine if these extracts had an additive effect when used in conjunction with the chemotherapeutic drugs most often utilized in the treatment of each neoplastic disease represented in the cell culture systems.

# METHODS

## Substances

Natural extracts were received directly from the manufacturer and the content of each compound of interest based on the manufacturers' purity analysis was verified by a secondary laboratory using High Performance Liquid Chromatography (Table 2.1). Extracts were dissolved at 20 mg $mL^{-1}$ in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to make fresh solutions before every experiment. Chemotherapeutic agents used were toceranib phosphate (Palladia™, Zoetis Animal Health, Florham Park, NJ) and doxorubicin hydrochloride (Sigma Aldrich, St Louis, MO); fresh dilutions were made from stock before each experiment.

**Table 2.1 – Characteristics of natural extracts**

[a] Purity value represents the percent of the main compound of interest in each extract as determined by manufacturer.

| Common Name | Latin Name | Part used | Compound of interest | Purity[a] | Manufacturer | Product Name | Product Number | Batch/Lot Number |
|---|---|---|---|---|---|---|---|---|
| Black Pepper | *Piper nigrum* | Fruit | Piperine | 95.02% | Sabinsa | VetPerine | FP-0215-06 | C130329 |
| Green Tea | *Camellia sinensis* | Leaf | EGCG | 45.76% | Naturex | Green tea extract | EA140362 | A043/008/A13 |
| Pomegranate | *Punica granatum* | Skin | Punicalagins | 35.60% | Polinat | Pomegranate extract [40% punicosides] | P40P | P40P13-2102 |
| Rosemary | *Rosmarinus officinalis* | Leaf | Carnosic acid | 66.90% | Vitiva | Rosemary extract INOLENS70 | 302036 | LAB.13-036001 |
| Turmeric | *Curcuma longa* | Root | Curcuminoids | 87.59% | Naturex | Turmeric extract | DA251471 | A060/006/D13 |

Cell culture

Three canine neoplastic established cell lines were used, representing hematopoietic, epithelial, and mesenchymal tumor types: mastocytoma C2 (University of California, San Fransisco, USA), mammary gland adenocarcinoma CMT-12 (Auburn University, Alabama, USA), and osteosarcoma D17 (#CCL-183; ATCC, Manassas, VA, USA). The cell lines were grown on tissue culture-treated plates (Laboratory Product Sales [LPS], Rochester, NY, USA) with appropriate medium containing 10% heat inactivated fetal bovine serum (HI-FBS; Invitrogen, Carlsbad, CA, USA) and 1% antibiotic-antimycotic (Invitrogen). Cell lines were grown at 37°C and 5% $CO_2$ for all experiments and passage of cells, unless otherwise noted. Canine primary dermal fibroblasts (CDF; Applied Biological Materials [ABM], Richmond, BC, Canada) were used to investigate effects on normal cells and were propagated and maintained on PriCoat T25 flasks (ABM) in Prigrow II medium (ABM) containing 10% HI-FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

MTT proliferation assay

Cells were plated at a density of $4 \times 10^3$ cells per well on 96-well tissue culture-treated flat bottom plates (LPS) and incubated overnight in complete medium. Cells were treated the following day with DMSO vehicle control or extract using a twofold serial dilution for eight final concentrations ranging from 0.4 to 100 µg $mL^{-1}$ for 48 h representing physiological and supra-physiological concentrations to assess all extracts for potential effectiveness at reducing cellular proliferation. A twofold serial dilution of chemotherapy was also tested ranging from 1.7 to 100 nM toceranib phosphate for the C2 cell line, and 0.03 to 2 µM doxorubicin hydrochloride for the CMT-12 and D17 cell lines. To quantify cellular proliferation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye; Alfa Aesar, Ward Hill, MA, USA) assays were

performed by adding 30 µL of MTT dye (5 mg $mL^{-1}$ in phosphate-buffered saline solution) to each well and incubating at 37°C for 1 h. Media were then aspirated and the cells were solubilized in 200 µL of isopropanol. The optical density of each well was analyzed on a spectrophotometric plate reader (Epoch; Biotek, Winooski, VT, USA) at a wavelength of 570 nm as previously described.[26] Single extract experiments were assayed in duplicate in four independent experiments.

Synergy between extracts was examined using combinations of two extracts at six concentrations: 0.8, 1.7, 3.1, 6.3, 12.5, or 25 µg $mL^{-1}$ as a representation of potentially high end physiological concentrations. All pairwise combinations of extract concentrations were tested. The percent proliferating cells of control for each treatment was pooled from all experiments and is reported as mean ± standard error of the mean.

Interaction with toceranib phosphate/doxorubicin hydrochloride using proliferation assay

Cells were plated at a density of 4 x $10^3$ cells per well on 96-well tissue culture-treated plates (LPS) and incubated overnight in complete medium. Cells were treated the following day with 50 µL of a combination of TE (0.8, 1.7, 3.1, or 6.3 µg $mL^{-1}$), RE (0.8, 1.7, 3.1, or 6.3 µg $mL^{-1}$), and chemotherapeutic drug. The C2 cell line was treated with toceranib phosphate (3.1, 6.3, 12.5, 25, 50, 100 nM) and the CMT-12 and D17 cell lines were treated with doxorubicin hydrochloride (0.03, 0.06, 0.13, 0.25, 0.5, 1, 2 µM). DMSO was used as a vehicle control for all treatments. Cells were treated for 48 h before performing MTT assays. Wells treated with DMSO were considered to represent 100% proliferating cells. All combinations were tested in duplicate in two independent experiments.

Trypan blue exclusion assay of cell viability

The trypan blue exclusion assay was performed on CDF cells due to the slow rate of proliferation and low metabolic activity of these normal canine cells, precluding productive MTT assays. The effects of extract treatments were compared to the results obtained on the C2, CMT-12, and D17 cell lines. For the CDF cells, Applied Cell Extracellular Matrix (ABM) was applied overnight to 24-well tissue culture-treated plates (LPS). For all cell lines, cells were plated at a density of $5 \times 10^3$ cells per well and incubated until 60% confluent before treatment with DMSO vehicle control, 6.3 µg $mL^{-1}$ TE, 6.3 µg $mL^{-1}$ RE, or a combination of 3.1 µg $mL^{-1}$ each of TE and RE. After 48 h of treatment, cells were collected and centrifuged at 1,900g for 10 min. With the exception of the C2 cell line, cells were detached with 0.05% Trypsin/EDTA. The cell pellet was resuspended in 0.1% trypan blue (Sigma) in phosphate-buffered saline solution and 1% FBS, loaded on a hemocytometer, and visualized on an inverted microscope. Cells which stained blue were considered non-viable. All treatments were performed in triplicate and the percent of viable cells was averaged. All values were standardized to the vehicle control treatment which was considered to represent 100% viable cells.

Data management and calculations

Raw data from proliferation assays (optical density of each well) were normalized to the vehicle alone treatment for individual assays, considered to represent 100% proliferating cells (single or combined treatment). The percent proliferating cells was then averaged across each replicate. The 50% inhibitory concentration ($IC_{50}$) for each extract was then calculated across experiments by Probit analysis.

The compound interactions were calculated by multiple drug effect analysis using CalcuSyn software (v.2.11; Biosoft, Cambridge, GB, United Kingdom) which employs the median equation principle according to the methodology described by Chou and Talalay[27] to determine a combination index (CI) value by the formula:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1(D)_2}{(D_x)_1(D_x)_2}$$

Where $(D)_1$ and $(D)_2$ are the doses of both compounds in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of each compound alone at x percent of inhibition. CI values $\leq 0.9$ indicate synergism, a CI value $> 0.9$ and $< 1.1$ indicates an additive effect, and CI values $\geq 1.1$ indicate antagonism.

Statistical analysis

All statistical analysis on the outcome of percent proliferating cells as measured by MTT assay was performed using JMP Pro (v. 11.2.1; SAS Institute Inc., Cary, NC, USA). The residuals of the statistical model were evaluated for normality and found to be not normally distributed. Therefore, non-parametric Kruskal-Wallis test was used to compare differences in percent proliferating cells for every treatment concentration used within each cell line across experiments. Comparisons between each treatment group and vehicle control group were carried out using the Steel method adjusting alpha risk for multiple comparisons.

For the outcome of percent viability determined by the trypan blue exclusion assay, residuals of the statistical model were found to be normally distributed, and therefore analyzed using analysis of variance with Dunnett's method for comparison to vehicle control, controlling for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

# RESULTS

## Single treatment versus dual combination treatment on three types of cancer cell lines

Antiproliferative effects were examined using the MTT assay to determine the potency of the extracts. All three cell lines were most sensitive to TE, with an $IC_{50}$ below 13 µg $mL^{-1}$ and a significant decrease in cell proliferation at concentrations of at least 0.8 µg $mL^{-1}$ in the C2 cell line, and at concentrations of 6.3 µg $mL^{-1}$ and higher in both the CMT-12 and D17 cell lines ($p < 0.0001$). RE also had an effect with $IC_{50}$ less than 14 µg $mL^{-1}$ in all three cell lines, and a significant decrease in cell proliferation at a concentration of 6.3 µg $mL^{-1}$ and above in the C2 cell line ($p = 0.0203$) and at 12.5 µg $mL^{-1}$ and above in the CMT-12 and D17 cell lines ($p < 0.0001$). Low concentrations of RE caused a minor increase in proliferation when used alone: 9% increase with 0.8 µg $mL^{-1}$ in C2; an average of 11% increase with concentrations of 3.1 µg $mL^{-1}$ and below in CMT-12; average of 12% increase with concentrations of 3.1 µg $mL^{-1}$ and below in D17 ($p < 0.05$). Green tea, black pepper, and pomegranate [40% punicosides] extracts required a concentration greater than 20 µg $mL^{-1}$ to reduce cell proliferation (Table 2.2).

**Table 2.2 – $IC_{50}$ of extracts and chemotherapy determined by MTT assays**

Values were determined by averaging duplicate wells in four independent experiments and using Probit analysis.
[a]C2 treated with toceranib phosphate, CMT-12 and D17 treated with doxorubicin hydrochloride.

| | Green tea extract | Pomegranate extract [40% punicosides] | Rosemary extract [70% Carnosic Acid] | Turmeric extract | Black pepper extract | Chemotherapy[a] |
|---|---|---|---|---|---|---|
| | $IC_{50}$ (µg $mL^{-1}$) | $IC_{50}$ (µg $mL^{-1}$) | $IC_{50}$ (µg $mL^{-1}$) | $IC_{50}$ (µg $mL^{-1}$) | $IC_{50}$ (µg $mL^{-1}$) | $IC_{50}$ |
| C2 | **11.5** | **48.5** | **11.9** | **4.8** | **21.8** | **12.5 nM (6.2 ng $mL^{-1}$)** |
| CMT-12 | **20.4** | **40.9** | **13.0** | **9.1** | **34.5** | **0.3 µM (163.1 ng $mL^{-1}$)** |
| D17 | **47.8** | **93.8** | **13.6** | **12.3** | **36.5** | **0.5 µM (271.8 ng $mL^{-1}$)** |

Turmeric extract (TE) and rosemary extract [70% carnosic acid] (RE) combination

The most effective and consistently synergistic combination was between TE and RE. No other dual extract treatments were found to have considerable or consistent synergy at any combination of extract across all three cancer cell lines (data not shown for 4 negative combinations). The combination of TE and RE resulted in a decrease in the concentrations of each extract needed to reach an $IC_{50}$ in all three cell lines suggesting a synergistic combination (Figure 2.1A-C). The $IC_{50}$ required in combination (determined using equal concentrations of extracts) was 2.9 μg $mL^{-1}$ of each extract in C2, 4.9 μg $mL^{-1}$ of each extract in CMT-12, and 7.5 μg $mL^{-1}$ of each extract in D17. Analysis using Calcusyn software resulted in CI values below 0.9, indicating synergy, in treatment concentrations at or below 12.5 μg $mL^{-1}$ of each extract in all three cell lines (Table 2.3A-C). With treatment using this combination, a significant decrease in cell proliferation compared to vehicle control was first observed at a concentration of 1.7 μg $mL^{-1}$ TE + 1.7 μg $mL^{-1}$ RE in the C2 ($IC_{28}$, $p = 0.0001$) and CMT-12 ($IC_{13}$, $p = 0.0019$) cell lines, and at a concentration of 3.1 μg $mL^{-1}$ TE + 3.1 μg $mL^{-1}$ RE in the D17 cell line ($IC_{11}$, $p = 0.0073$). Once a concentration of 6.3 μg $mL^{-1}$ TE + 6.3 μg $mL^{-1}$ RE was reached in the C2 cell line ($p < 0.0001$) and a concentration of 12.5 μg $mL^{-1}$ TE + 12.5 μg $mL^{-1}$ RE in the CMT-12 ($p = 0.0052$) and D17 ($p = 0.0041$) cell lines, further increases in inhibition were not detected with higher concentrations when compared to DMSO treatment alone.

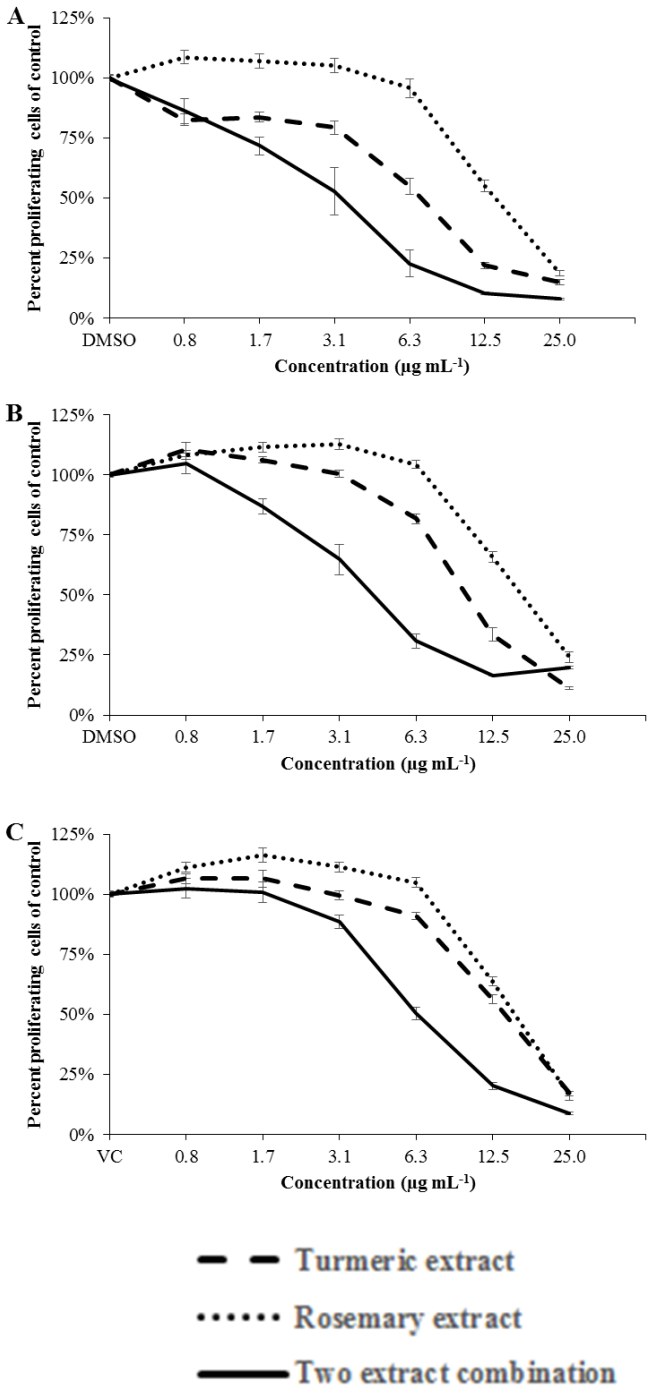


**Figure 2.1 – Synergistic combinations of TE and RE**

Percent proliferating cells of control is represented as mean ± SEM and was determined by MTT assays on C2 (A), CMT-12 (B), and D17 (C) cell lines. TE and RE were used individually (dashed lines) or in combination at equal concentrations (solid line) ranging from 0.8 to 25 µg $mL^{-1}$. Lowest concentration that induced a significant ($p < 0.05$) decrease in percent proliferation compared to DMSO control indicated by + (TE alone), # (RE alone), and ^ (dual extract combination).

**Table 2.3 – Combination index for two extract treatment**

Combination Index (CI) values for C2 (A), CMT-12 (B), and D17 (C) cell lines treated with TE and RE in combination at concentrations of 0.8, 1.7, 3.1, 6.3, 12.5, and 25.0 μg $mL^{-1}$. CI values ≤ 0.9 indicate synergism (bold values), a CI value > 0.9 and < 1.1 indicates an additive effect, and CI values ≥ 1.1 indicate antagonism (italicized values). NP = no calculation possible due to antagonism with single extract alone (RE).

**A**

| Rosemary extract [70% Carnosic Acid] (μg $mL^{-1}$) \ Turmeric extract (μg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 | 12.5 | 25.0 |
|---|---|---|---|---|---|---|
| 0.8 | *2.551* | *1.786* | *1.307* | **0.362** | **0.128** | **0.216** |
| 1.7 | *2.168* | 0.959 | **0.573** | **0.197** | **0.105** | **0.225** |
| 3.1 | *1.339* | **0.678** | **0.373** | **0.152** | **0.118** | **0.208** |
| 6.3 | **0.775** | **0.53** | **0.226** | **0.182** | **0.147** | **0.174** |
| 12.5 | **0.175** | **0.174** | **0.164** | **0.142** | **0.204** | **0.221** |
| 25.0 | **0.213** | **0.306** | **0.473** | **0.328** | **0.423** | **0.316** |

**B**

| Rosemary extract [70% Carnosic Acid] (μg $mL^{-1}$) \ Turmeric extract (μg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 | 12.5 | 25.0 |
|---|---|---|---|---|---|---|
| 0.8 | NP | *1.182* | **0.738** | 1.005 | **0.471** | **0.742** |
| 1.7 | **0.611** | **0.682** | **0.655** | **0.793** | **0.355** | **0.703** |
| 3.1 | **0.877** | **0.771** | **0.766** | **0.655** | **0.406** | **0.671** |
| 6.3 | 0.971 | **0.859** | **0.789** | **0.647** | **0.524** | **0.775** |
| 12.5 | **0.629** | **0.603** | **0.523** | **0.528** | **0.687** | 1.048 |
| 25.0 | **0.853** | 0.944 | **0.871** | **0.869** | **0.882** | *1.601* |

**C**

| Rosemary extract [70% Carnosic Acid] (μg $mL^{-1}$) \ Turmeric extract (μg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 | 12.5 | 25.0 |
|---|---|---|---|---|---|---|
| 0.8 | NP | NP | NP | **0.866** | **0.617** | **0.531** |
| 1.7 | NP | NP | NP | 0.909 | **0.59** | **0.527** |
| 3.1 | NP | NP | **0.69** | **0.665** | **0.644** | **0.573** |
| 6.3 | **0.619** | **0.584** | **0.547** | **0.522** | **0.434** | **0.561** |
| 12.5 | **0.746** | **0.655** | **0.597** | **0.505** | **0.583** | **0.664** |
| 25.0 | **0.491** | **0.526** | **0.558** | **0.666** | **0.61** | **0.79** |

**Natural extracts and chemotherapy interaction on growth inhibition of cancer cell lines**

The CI values generated for the C2 cell line with dual extract treatment in the presence of toceranib phosphate at the $IC_{25}$ (Table 2.4A) and $IC_{50}$ (Table 2.4B) showed that when both extracts were added at 0.8 or 1.7 μg $mL^{-1}$ there was a mild antagonistic to additive effect, while at 3.1 μg $mL^{-1}$ of both extracts there was a definitive additive effect. When either extract was added at 6.3 μg $mL^{-1}$ there was a definitive synergistic effect with toceranib phosphate regardless of the amount of the other extract. At the $IC_{75}$ (Table 2.4C) of toceranib phosphate, synergy was only seen when both extracts were used at 6.3 μg $mL^{-1}$, all other combinations produced an additive or mildly antagonistic effect. When the CMT-12 cell line was treated with an $IC_{25}$ of doxorubicin hydrochloride, synergy or an additive effect could be seen when both extracts were used at a concentration of 1.7 μg $mL^{-1}$ or higher and antagonism was seen when either extract was used at a concentration of 0.8 μg $mL^{-1}$ (Table 2.4D). When doxorubicin hydrochloride was used at the $IC_{50}$ (Table 2.4E) or $IC_{75}$ (Table 2.4F), if either extract was used at 3.1 μg $mL^{-1}$ or lower there was modest antagonism. When extracts were combined at concentrations of 3.1 μg $mL^{-1}$ or higher there was an additive or synergistic effect. The D17 cell line showed a modest additive or synergistic effect at any combination of extracts when used with any IC of doxorubicin (Table 2.4G-I) in general, with the weakest effect at the $IC_{50}$ with possible mild antagonism at 1.7 and 3.1 μg $mL^{-1}$ (Table 2.4H).

**Table 2.4 – Combinatorial effects of TE/RE and chemotherapy on tumor cell proliferation**

Combination Index (CI) values on C2 (A-C), CMT-12 (D-F), and D17 (G-I) cell lines treated with TE and RE in combination at concentrations of 0.8, 1.7, 3.1, and 6.3 µg $mL^{-1}$ in the presence of chemotherapeutic agents. Toceranib phosphate used for C2 cell line at (A) $IC_{25}$ of 6.3 nM, (B) $IC_{50}$ of 12.5 nM, and (C) $IC_{75}$ of 25 nM; doxorubicin hydrochloride was used for the CMT-12 cell line at (D) $IC_{25}$ of 0.1 µM, (E) $IC_{50}$ of 0.3 µM, and (F) $IC_{75}$ of 1 µM; doxorubicin hydrochloride was used for the D17 cell line at (G) $IC_{25}$ of 0.3 µM, (H) $IC_{50}$ of 0.5 µM, and (I) $IC_{75}$ of 2 µM. CI values ≤ 0.9 indicate synergism (bold values), a CI value > 0.9 and < 1.1 indicates an additive effect, and CI values ≥ 1.1 indicate antagonism (italicized value).

**A**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | *1.239* | *1.409* | *1.186* | **0.753** |
| 1.7 | *1.418* | *1.478* | 1.049 | **0.766** |
| 3.1 | *1.378* | *1.252* | 0.941 | **0.775** |
| 6.3 | 0.946 | **0.842** | **0.736** | **0.707** |

**B**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | *1.1* | *1.158* | **0.886** | **0.669** |
| 1.7 | *1.424* | *1.444* | 1.044 | **0.855** |
| 3.1 | *1.527* | *1.223* | 1.051 | **0.887** |
| 6.3 | 1.018 | **0.887** | **0.808** | **0.729** |

**C**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | *1.104* | *1.182* | *1.11* | 1.016 |
| 1.7 | 1.062 | 1.044 | 0.922 | 1.005 |
| 3.1 | *1.585* | *1.357* | *1.278* | *1.346* |
| 6.3 | 1.038 | 1.049 | 0.91 | **0.899** |

**D**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | *1.625* | *1.307* | *1.445* | 1.088 |
| 1.7 | *1.622* | *1.272* | 1.002 | **0.895** |
| 3.1 | *1.46* | **0.89** | **0.836** | **0.809** |
| 6.3 | *1.259* | **0.846** | **0.748** | **0.717** |

**E**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | *2.093* | *1.493* | *1.793* | *1.187* |
| 1.7 | *1.654* | *1.241* | *1.256* | 0.928 |
| 3.1 | *1.632* | *1.127* | 1.017 | **0.791** |
| 6.3 | *1.462* | 0.918 | **0.894** | **0.718** |

**F**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | *2.111* | *1.764* | *1.38* | 1.048 |
| 1.7 | *1.594* | *1.586* | *1.234* | **0.87** |
| 3.1 | *1.456* | *1.262* | 0.91 | **0.767** |
| 6.3 | *1.339* | *1.333* | **0.866** | 0.913 |

**G**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | **0.629** | **0.73** | **0.829** | 0.993 |
| 1.7 | **0.685** | **0.743** | **0.84** | 0.923 |
| 3.1 | 1.032 | 1.035 | 1.028 | 1.053 |
| 6.3 | 1.073 | 1.034 | 1.003 | **0.853** |

**H**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | *1.154* | *1.134* | *1.31* | *1.158* |
| 1.7 | *1.132* | *1.189* | *1.451* | 0.992 |
| 3.1 | *1.224* | *1.136* | *1.377* | **0.899** |
| 6.3 | 1.083 | 1.081 | *1.17* | **0.69** |

**I**

| Rosemary extract (µg $mL^{-1}$) \ Turmeric extract (µg $mL^{-1}$) | 0.8 | 1.7 | 3.1 | 6.3 |
|---|---|---|---|---|
| 0.8 | **0.361** | **0.399** | **0.421** | **0.554** |
| 1.7 | **0.589** | **0.63** | **0.591** | **0.588** |
| 3.1 | **0.599** | **0.639** | **0.603** | **0.647** |
| 6.3 | **0.792** | **0.775** | **0.842** | **0.772** |

**Cytotoxic activity of TE and RE against cancer cell lines without affecting normal cells**

The trypan blue exclusion assay (Figure 2.2) showed that individual extracts at 6.3 µg $mL^{-1}$ or a combination of 3.1 µg $mL^{-1}$ TE and 3.1 µg $mL^{-1}$ RE did not induce a significant decrease in cell viability in the control primary cells, CDF. In comparison, the three cancer cell lines were also assayed using the same conditions. These concentrations did not induce cytotoxicity on the D17 cell line, while the C2 and CMT-12 cell lines had a significant decrease in cell viability when treated with 6.3 µg $mL^{-1}$ TE alone (29% and 36%, respectively $p < 0.01$) or with the combination of 3.1 µg $mL^{-1}$ each extract (26% and 51%, respectively $p < 0.01$).

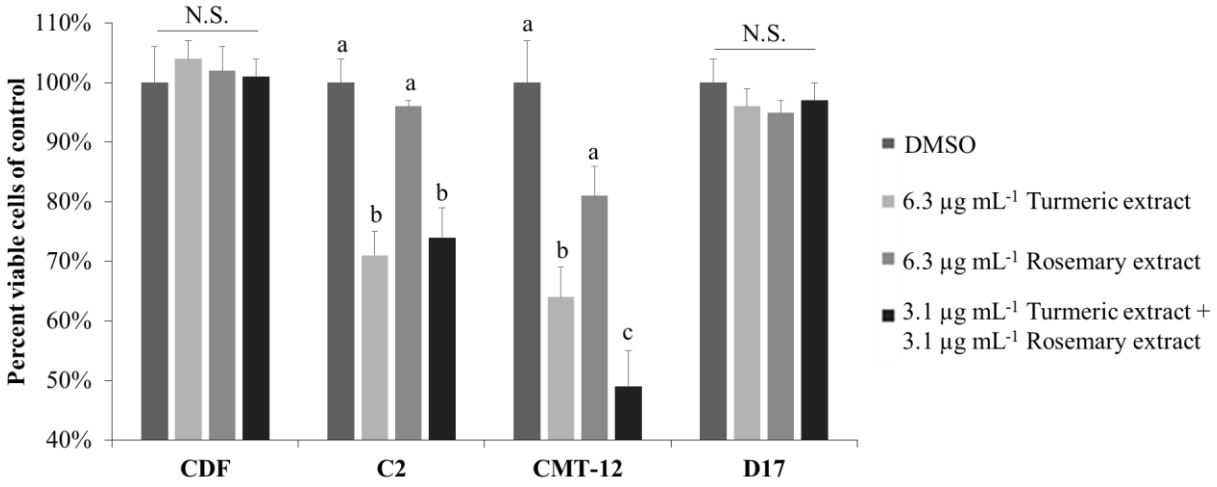


**Figure 2.2 – Cytotoxic activity of TE, RE, and combination on tumor versus normal cells**

Percent viable cells determined by trypan blue exclusion assay after 48 h treatment is represented as mean ± SEM in comparison with DMSO vehicle treatment. Within each cell line, means with different letters are significantly different ($p < 0.05$). NS = Not significant.

# DISCUSSION

The use of bioactive polyphenol and/or carotenoids from feed ingredients is well studied in human cancer cells, and there may be efficacy in the utilization of many of these bioactive components in treating canine cancers.[28,29] In the current study we surveyed five extracts which were selected based on scientific literature and an initial screen of feed ingredients, choosing antiproliferative compounds that could hinder cell proliferation at 25 μg $mL^{-1}$, based on a relatively robust assumption regarding peak absorption kinetics of around 20 μM or less. Our combination experiments were designed to focus on concentrations that may have some utility *in vivo* focusing on concentrations of less than 10 μg $mL^{-1}$. Our results indicated that TE was the most potent at inhibiting proliferation at low microgram concentrations. To further confirm the single extracts and potential interaction of TE and RE observed in the MTT assay, trypan blue exclusion assays were also performed to assess viable versus non-viable cells. The modest differences in terms of inhibitory effects when comparing the trypan blue and MTT assays may be a reflection of membrane permeability and cytotoxicity being measured with the trypan blue assay versus metabolic cellular activity being measured with the MTT assay. This is particularly evident in the D17 cell line which was less sensitive than the C2 or CMT-12 cell lines, showing diminished activity on MTT metabolic/proliferative assays and no increase in cell death in the trypan blue assay. This suggests a diminished cell proliferation response rather than cell death response in the D17 cell line. The response of C2 and CMT-12 cell lines showed that the combination effect of TE with RE at concentrations of 1.7 – 12.5 μg $mL^{-1}$ each extract was superior in diminishing cell growth as well as increasing the number of non-viable cells than treatment with TE alone, suggesting synergy in this treatment strategy.

Of the two plant extracts that were most effective, TE, which contains 87% curcuminoids, showed the most potent anti-proliferative effects. These effects could be seen at a concentration below 15 μg $mL^{-1}$ in all cell lines examined. The high potency of curcumin may be related to its binding affinity for at least 30 different proteins.[30] The antiproliferative effects have been examined in cell culture models of nearly every type of neoplastic condition including leukemia, breast, prostate, bladder, melanocytic, skin, ovarian, hepatic and uterine cancer cells.[31] In most cell-based models, low micromolar concentrations of curcumin affect various intracellular pathways ranging from transcriptional activation to induction of apoptosis to halting of the cell cycle.[32] These include: transcriptional activators AP-1, Nf-κB, β-catenin, STAT-3, hypoxia induced factor-1, and notch-1; receptor signaling cascades EGF, HER2, VEGF, PDGF, IGF, and FGF; all three major MAP Kinases ERK1/2, p38, and JNK, and protein kinase C. Intrinsic mitochondrial apoptosis induction by changes in mitochondrial membrane permeability via Bcl-2 and Bax family member proteins are also documented.[33] The wide array of molecular targets has led to over 100 clinical trials in humans to study the use of curcumin to treat various pathologies from obesity related diseases to neurological diseases to various neoplastic conditions.[34] Although curcumin and turmeric extracts are effective *in vitro*, the bioavailability and absorption of curcuminoids is somewhat limited. Typically, less than 10% of curcumin is absorbed, even with aggressive treatment regimens, leading to high nanomolar serum concentrations in rodents and humans.[35,36] The curcumin that is absorbed is quickly glycosylated, sulfated, or hydroxylated, and it is unclear if these metabolites are as effective as the unconjugated curcumin.[37,38] Several approaches to increase bioavailability are being examined including the use of curcumin analogs,[39] liposomal curcumin,[40] curcumin nanoparticles,[41] and adjuvant therapy[32] with bioavailability enhancers such as piperine from black pepper extract.[42] These modifications have led to increased blood curcumin concentrations and a half-life of nearly 10 hours.

Recently, there have been several studies looking into using whole turmeric extract mixtures instead of pure curcumin alone. Specifically, extract formulations including turmerones has led to increased solubility, absorption, and bioavailability.[43] Generally, treatment with curcumin has exhibited no adverse side effects even at high doses and because of this, a maximum tolerated dose has not been established.[44] Only two canine studies have been completed, with low dosing regimens of 4 mg $kg^{-1}$ given orally twice a day showing no side effects after two months of treatment.[45,46]

Rosemary extract rich in carnosic acid (RE) also generated interesting results considering the $IC_{50}$ for cellular proliferation is likely within physiological ranges (plasma concentration maximum 42.52 mg $L^{-1}$) as seen in a rodent model after intragastric administration.[47] Even more intriguing was the synergistic effect of RE with TE. *Rosmarinus officinalis* contains several phenolic compounds including carnosic acid, carnosol, and rosmarinic acid.[48] In our study, as well as others, carnosic acid and carnosol were more potent in decreasing cellular proliferation than rosmarinic acid in various types of cancer cell lines at concentrations below 20 µM.[49,50] Carnosic acid and carnosol have been shown to have several mechanisms of action including cell cycle arrest, induction of apoptosis, free radical scavenging, inhibition of metastatic markers, and inhibition of P-glycoprotein mediated drug efflux.[51,52,53] Intracellular pathways affected include inhibition of PI3-Kinase/AKT/Nf-κB signaling,[54] downregulation of cyclins A and B,[55] induction of apoptosis by decreases in Bcl-2,[56] and inhibition of all three major MAP Kinases ERK1/2, p38, and JNK.[57] In rodent studies, the use of a topical[58] or oral[59] rosemary extract has been well tolerated and effective. Toxicity studies in rats have shown that up to 3 g $kg^{-1}$ of rosemary oil is acceptable[60,61] and biologically relevant levels of around 10 µM can be reached through dietary administration,[62] however canine studies are lacking.

We found synergy between TE and RE, which agrees with previous *in vitro* studies using the same combination.[63,64] While RE alone was only effective at concentrations above 6.3 µg $mL^{-1}$ in all

three cancer cell lines, its use with TE significantly decreased the concentrations needed to reduce cell proliferation. In all three tumor cell lines, these extracts worked synergistically at concentrations between 1 – 10 μg $mL^{-1}$ of each extract. When used in combination, extrapolation of our data accounting for the percentage of the compound of interest (curcumin and carnosic acid) suggest that the $IC_{50}$ is 6.8 μM curcumin and 7.6 μM carnosic acid for C2, 12 μM curcumin and 13 μM carnosic acid for CMT-12, and 18 μM curcumin and 20 μM carnosic acid for D17. Neither of the extracts, when used alone or in combination, showed effects on cell viability in the normal canine dermal fibroblasts, suggesting the effects on normal cell death or proliferation is minimal.

When the C2 cell line was incubated with the TE/RE combination in the presence of toceranib phosphate, a synergistic or additive effect was seen when either extract was used at 6.3 μg $mL^{-1}$, or when TE was used at 3.1 μg $mL^{-1}$ or higher. When the CMT-12 cell line was treated with the TE/RE combination in the presence of doxorubicin hydrochloride, there was a modest antagonistic when extracts were used at lower concentrations (below 3.1 μg $mL^{-1}$ of each), but a synergistic or additive effect could be seen with a higher concentration of 6.3 μg $mL^{-1}$ of both extracts. The D17 cell line showed considerable additive and synergistic effects with all extract combinations at the $IC_{25}$ and $IC_{75}$ of doxorubicin hydrochloride in general. Mild antagonism was seen when extracts were used at 3.1 μg $mL^{-1}$ or lower in combination at the $IC_{50}$, but this was diminished or absent when either extract reached a concentration of 6.3 μg $mL^{-1}$. The mild antagonism of these extracts with doxorubicin hydrochloride could be attributed to their antioxidant properties. Doxorubicin has been known to increase reactive oxygen species, and the presence of endogenous free radical scavengers, such as glutathione, can dampen the effects of the drug.[65] Considering these findings, further testing of TE and RE with other chemotherapeutic agents to ensure similar synergy, additive, or antagonistic effects is warranted. Furthermore, considering the

lack of basic pharmacokinetics with oral TE and RE, studies in canines are needed to examine whether these feed ingredients would have any utility. Studies to examine the effect these compounds may have with chemotherapeutic agents *in vivo* is also necessary as synergy may allow for a decrease in the administered dose.

Other extracts examined in the MTT assay were piperine from black pepper, pomegranate extract, and green tea extract. Effective $IC_{50}$ for these extracts across the cell lines were typically above 25 μg $mL^{-1}$ which would be considered supraphysiological. This takes into account that most animals cannot reach concentrations greater than approximately 10 – 20 μM of any specific bioactive component from these extracts when used at relatively large doses for any significant period of time. That said, these compounds were also tested for synergistic, antagonistic, or additive effects and were not observed to increase TE or RE effectiveness (data not shown) and were discounted for further examination with commonly used chemotherapeutic agents.

## CONCLUSIONS

This study of commonly used feed ingredients showed that a combination of TE and RE diminished the growth of cancer cells. This synergistic effect was observed at 10 μg $mL^{-1}$ and below indicating a potential for physiological effects, however *in vivo* pharmacokinetic and efficacy studies are needed. Although we are unsure of the bioactive molecules inducing these effects, the high concentrations of curcuminoids and carnosic acid are likely involved. The antiproliferative effects of chosen chemotherapeutic agents were not hindered when these extracts were used in combination at concentrations of 3.1 and 6.3 μg $mL^{-1}$. Further testing of other chemotherapeutic agents with these specific extracts is warranted to ensure no distinct

antagonism is evident. In addition, further examination of the potential apoptotic effects and cellular pathways affected by these extracts individually and in combination may be fruitful in determining the similarities and differences of their effects between cell lines.

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# Chapter 3 - Cellular effects of a turmeric root and rosemary leaf extract on canine neoplastic cell lines

## ABSTRACT

Adjunctive use of nutraceuticals in human cancer has shown promise, but little work has been done in canine neoplasia. We previously identified two plant extracts, turmeric (TE) and rosemary (RE), which work synergistically to reduce neoplastic cell growth. We set forth to determine the mechanism of action of these extracts individually and in combination. Three canine neoplastic cell lines were used: C2 (mastocytoma), CMT-12 (mammary gland carcinoma), and D17 (osteosarcoma). Cells were treated with 6.3 µg/mL extract individually, or 3.1 µg/mL each extract in combination. We assessed apoptosis, antioxidant effects, cellular accumulation of curcumin, inhibition of drug efflux, and perturbation of signaling pathways. The combination treatment resulted in caspase-3/7 cleavage and apoptosis in all cell lines, beyond the effects of TE alone, and CMT-12 cells were the most susceptible. Both extracts had a significant antioxidant effect and induced increases in activated c-jun N-terminal kinase (JNK). We found that the presence of RE increased cellular accumulation of curcumin. TE and RE interact synergistically to induce apoptosis in canine neoplastic cell lines. The effects of RE increasing cellular accumulation of curcumin may be part of the underlying mechanisms.

# INTRODUCTION

The use of natural remedies, or nutraceuticals, in the treatment of cancer and a variety of other diseases has increased dramatically in recent years. The use of these products has been around for centuries, but investigations into the mechanisms of action have only just begun and appear to be highly varied in cell culture systems. The effective compounds of interest have been determined and purified from a variety of plants and used in treating various diseases including cancer.[1] The benefit of using these plant extracts to treat cancer is in ability of a single extract to affect several different pathways at once. While purified or synthetically constructed compounds often prove efficacious, the presence of minor compounds in an extract may allow for a more potent effect by increasing cellular absorption and/or binding to other targets.[2]

The effects of these purified compounds have been examined *in vitro* in a variety of human neoplasias including cell lines derived from tumors of the colon, skin, and breast tissue,[3] but only a few studies have looked at the effects in canine cancer cells lines.[4,5,6] The major types of cancer found in the dog differ from humans with lymphoma, mast cell disease, osteosarcoma, and mammary neoplasia being the most frequently seen in veterinary practices.[7]

We previously identified two extracts, turmeric extract (TE) and rosemary extract (RE), which were shown to be cytotoxic and reduce proliferation in a synergistic manner in canine mastocytoma, mammary carcinoma, and osteosarcoma cell lines.[8] The use of TE and its major compound of interest, curcumin, has been extensively studied to treat a variety of diseases and ailments, perhaps due to its ability to bind and interact with a variety of proteins.[9] Unfortunately, the use of TE *in vivo* has been limited by its poor bioavailability and efforts are underway to increase the absorption and bioavailability of the curcuminoids found in this extract.[10] This obstacle may be overcome through the use of combination treatments with other extracts that

improve bioavailability or hinder additional pathways. In our previous study, RE worked in a synergistic manner to decrease cellular proliferation. Carnosic acid, the compound of interest in RE, is capable of targeting a variety of signaling pathways, many of which overlap with those targeted by curcumin. The effects of these two compounds in combination have been examined in acute myeloid leukemia cells[11] and breast cancer cells,[12] showing synergy in anti-proliferative effects and increased pro-apoptotic signaling.

The objective of this study was to determine the effects on canine cancer cell death and possible mechanisms by which TE and RE exert antiproliferative and cytotoxic effects individually and in combination on canine mastocytoma, mammary carcinoma, and osteosarcoma cell lines. We set forth to examine markers of apoptosis, antioxidant capabilities, and screened for changes in the activation of common signaling pathways after treatment.

## METHODS

### Extracts

Turmeric extract (TE; Naturex, Avignon, France) and rosemary extract (RE; Vitiva, Markovcih, Slovenia) were solubilized in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 20 mg $mL^{-1}$. Fresh extract solutions were prepared and used for every experiment.

### Cell culture

Three canine neoplastic established cell lines, representing hematopoietic, epithelial, and mesenchymal tumor types were used for all experiments; mastocytoma C2 (Dr. Warren Gold, University of California, San Francisco, USA), mammary gland carcinoma CMT-12 (Dr. R. Curtis Bird, Auburn University, Alabama, USA), and osteosarcoma D17 (#CCL-183; ATCC,

Manassas, VA, USA). Cell lines were grown on tissue culture-treated plates (Laboratory Product Sales [LPS], Rochester, NY, USA) at 37°C and 5% $CO_2$ for all experiments and passage of cells, unless otherwise noted. Cell lines were cultured in complete medium as previously described.[8] All culture reagents were purchased from Invitrogen, Carlsbad, CA, USA, unless otherwise indicated.

Caspase 3/7 Activation Assay

Cells were plated at a density of 4 x $10^3$ cells per well on white walled 96-well tissue culture-treated plates (ThermoFisher Scientific, Waltham, MA, USA) and incubated overnight in complete medium. Cells were treated the following day with DMSO vehicle control, 6.3 µg $mL^{-1}$ extract alone or 3.1 µg $mL^{-1}$ each extract in combination for 36 h. Chemotherapeutic drugs at a 50% inhibitory concentration ($IC_{50}$) were used as a positive control; 12.5 nM toceranib phosphate (Palladia™, Zoetis Animal Health, Florham Park, NJ) was used for the C2 cell line, and 0.3 or 0.5 µM doxorubicin hydrochloride (Sigma Aldrich, St Louis, MO) was used for the CMT-12 and D17 cell lines, respectively. Background fluorescence and luminescence was measured in wells containing treatments but no cells. Caspase 3/7 activation was measured using the ApoLive-Glo™ Multiplex Assay (Promega, Madison, WI, USA) following manufacturer's instructions. Briefly, after 36 h of treatment, viability reagent was added to the wells and incubated at 37°C for 30 m and fluorescence was measured at $400_{Ex}/505_{Em}$. Next, Caspase-Glo 3/7 Reagent was added to all wells, incubated for 30 m at room temperature, and luminescence was measured. Fluorescence and luminescence was measured using SpectraMax M3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Flow Cytometry

Cells were plated on 60 mm tissue culture-treated plates (LPS) and incubated in complete medium until 60% confluent. Cells were then treated with medium, DMSO vehicle control, extract alone or extracts in combination. Cells were treated for 12 h (reactive oxygen species generation), 24 h (curcumin accumulation), or 48 h (Apoptosis/Necrosis, Cell Cycle). All flow cytometric analysis was performed on BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

**Examination of Apoptosis and Necrosis**

Apoptosis and necrosis after 48 h treatment was measured using Annexin-V and 7-AAD staining. Briefly, cells were detached with Accumax (Innovative Cell Technologies, San Diego, CA, USA), collected and centrifuged for 10 m at 500 rcf at 4°C. The pellet was washed once with PBS before resuspension in Annexin Binding Buffer (ABB; 10mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) at a density of 1 x $10^6$ cell $mL^{-1}$. Annexin-V and 7-AAD conjugates were added to the cell suspensions and incubated for 15 m at room temperature. After the incubation, ABB was added to the cell suspension and kept on ice until analysis.

**Reactive Oxygen Species**

Reactive oxygen species were measured using Dihydrorhodamine 123 (DHR123; Invitrogen, Carlsbad, CA, USA) according to literature.[13] Briefly, cells were detached using Accumax (Innovative Cell Technologies), collected and centrifuged for 10 m at 500 rcf at 4°C. The pellet was washed once with Phosphate Buffered Saline (PBS) before resuspension in 1 mL of stain (30 μM DHR123 in DMEM). The cell suspension was then incubated at 37°C for 30 m, pelleted, and resuspended in 1 mL DMEM and filtered before analysis.

**Cellular Accumulation of Curcumin**

The cellular accumulation of curcumin was measured by exploiting the auto-fluorescent properties of this compound.[14] After treatment, cells were detached with Accumax (Innovative Cell Technologies), collected and centrifuged for 10 m at 500 rcf at 4°C. The cell pellet was washed once with PBS before resuspension in DMEM, and filtered before analysis.

**Competitive Inhibition of Efflux Pumps**

Activity of MDR1/P-glycoprotein was measured by examining the amount of Rhodamine 123 present within CMT-12 cells after incubation with selected treatments. The assay was performed per manufacturer's instructions (ECM910; Invitrogen). Briefly, cells were detached using Accumax (Innovative Cell Technologies), collected and centrifuged for 5 m at 200 rcf. Cells were resuspended at 1 x $10^6$ cells $mL^{-1}$ in cold Rhodamine 123 buffer for 30 m on ice in order to allow for cellular uptake of the dye. After incubation, cell suspensions were centrifuged for 5 m at 200 rcf and washed twice with cold efflux buffer. 2.5 x $10^5$ cells were used for each treatment. Treatments included: 4°C negative control, 22 nM Vinblastine positive control, DMSO vehicle control, 6.3 µg $mL^{-1}$ TE, or 6.3 µg $mL^{-1}$ RE. With the exception of the 4°C treatment, all tubes were transferred to a 37°C water bath and incubated for 1 h. After the incubation period, 5 mL cold efflux buffer was added to each tube and placed on ice. Cell suspensions were collected by centrifugation at 4°C for 5 m at 200 rcf and washed once in cold efflux buffer. Cell pellets were resuspended in 0.5 mL cold propidium iodide buffer and kept on ice until analysis by flow cytometry using FL-1 for Rhodamine 123 and FL-3 for propidium iodide. Samples maintained at 4°C retain the dye due to inactivity of the pumps at this temperature. In the positive control samples, vinblastine acts as a competitive inhibitor, blocking the export of the dyes by MDR1/P-gp. Longer incubations (up to 3 h) with treatments were

tested and no significant difference was found. The C2 and D17 cell lines were tested but no major efflux of the dye was seen (Figure 3.1).

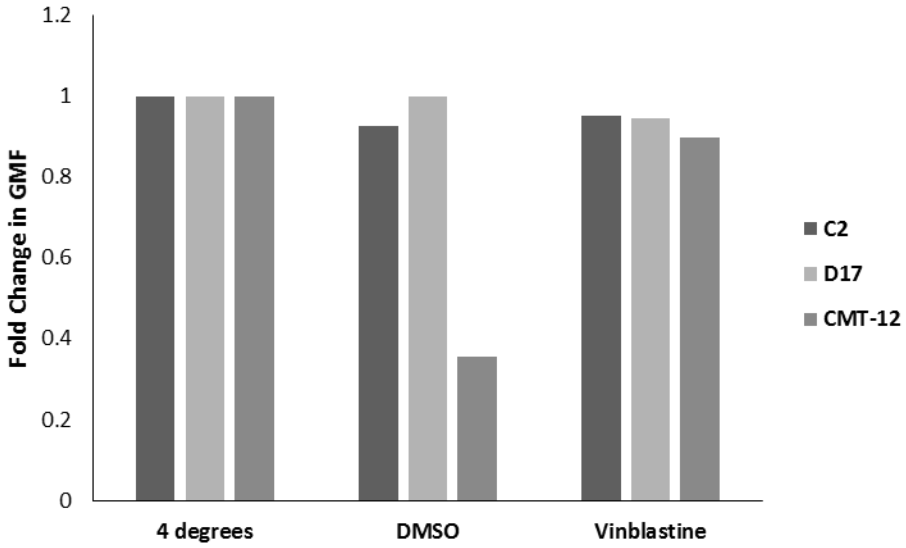


**Figure 3.1 – MDR1/P-gp activity**

MDR1/P-gp activity was assessed across the three cell lines (C2, CMT-12, and D17) by measuring the change in geometric mean fluorescence (GMF) after incubation with DMSO vehicle control or the competitive inhibitor vinblastine. Only the CMT-12 cell line showed an efflux of the dye, Rhodamine 123, after incubation with DMSO, as would be expected.

**Examination of Cell Cycle Dynamics**

Cell cycle dynamics were analyzed after 24 h (data not shown) and 48 h treatment using propidium iodide staining. Briefly, cells were detached with Accumax (Innovative Cell Technologies), collected in tubes coated with 2% fetal bovine serum (FBS) in PBS and centrifuged for 5 m at 300 rcf at 4°C. The pellet was washed twice with 1% FBS in PBS, filtered, and resuspended in 70% cold ethanol for overnight fixation. The following day, samples were centrifuged for 10 m at 500 rcf at 4°C, resuspended in cold PBS and filtered. Samples were centrifuged again for 5 m at 300 rcf at 4°C and resuspended in DNA staining solution [2% propidium iodide (Sigma Aldrich), 0.1% Triton X-100 (Sigma Aldrich), in PBS]. Samples were

incubated for 30 m at room temperature and analyzed using FL-2. Only C2 and D17 cell lines were analyzed due to the presence of doublets with CMT-12 cells, resulting in an artificial accumulation in the G2/M phase.

Western blot screen of affected pathways

Cells were plated on 100 mm tissue culture-treated plates (LPS) and incubated overnight in complete medium until 60% confluency was reached. Cells were treated the following day with DMSO vehicle control, 6.3 µg $mL^{-1}$ extract alone, or 3.1 µg $mL^{-1}$ each extract in combination. Cells were harvested and lysed at 12 h and 24 h after treatment using Mammalian Lysis Buffer (MLB; 25 mM Tris, 100 mM NaCL, 1 mM EDTA, 1% Triton X-100, pH 7.4) and sonication, and then centrifuged for 5 m at 14,000 rcf at 4°C. The supernatant was collected and the protein concentration was determined using the Bradford assay (Coomassie-dye; ThermoFisher Scientific Pierce, Waltham, MA, USA). Samples were equilibrated to a common volume (µg $µL^{-1}$) in MLB and 5x laemmili loading buffer (300 mM Tris-HCl pH 6.8, 10% Sodium dodecyl suflfate, 50% glycerol, 12.5% β-Mercaptoethanol, 0.025% Bromophenol blue). For each protein of interest, 30 µg total protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on gels ranging from 6 to 15% based on the molecular weight of the protein of interest. The proteins were then transferred to 0.45 µm pore size polyvinylidene fluoride membrane (Immobilon-P Membrane; EMD Millipore, Billerica, MA, USA) for 1 h at 333 mA and then blocked in 5% milk in Tris-buffered saline/0.05% Tween-20 solution (TBST). Membranes were incubated overnight in primary antibody solutions at a dilution of 1:1000 in TBST on a rocking platform at 4°C. Primary antibodies included mouse anti- phosphorylated-gamma H2A.X and extracellular regulated kinase (ERK) (R&D Biosciences, Boston, MA, USA); rabbit anti- caspase 3, protein kinase B (AKT), Ser473

phosphorylated-AKT, stress-activated protein kinase/jun-amino-terminal kinase (SAPK/JNK), Thr183/Tyr185 phosphorylated-SAPK/JNK, focal adhesion kinase (FAK), Tyr397 phosphorylated-FAK, Tyr576/Tyr577 phosphorylated-FAK, Tyr925 phosphorylated-FAK, Src, Tyr416 phosphorylated-Src, Tyr527 phosphorylated-Src, mammalian target of rapamycin (mTOR), Ser2448 phosphorylated-mTOR, Janus kinase 2 (JAK2), Tyr1007/Tyr1008 phosphorylated-JAK2, Ser727 phosphorylated-signal transducer and activator of transcription 3 (STAT3), Tyr705 phosphorylated-STAT3, B-Cell CLL/Lymphoma 2 (BCL2), and BCL2-Associated X Protein (BAX) (Cell Signaling Technology, Danvers, MA, USA); mouse anti-Thr202/Tyr204 phosphorylated p44/42 MAPK (ERK1/2) and STAT3 (Cell Signaling Technology). Membranes were washed three times with TBST and incubated at room temperature for 1 h in the corresponding secondary anti-rabbit IgG or anti-mouse IgG horseradish peroxidase-conjugated antibody at a dilution of 1:2000 (Cell Signaling Technology). Membranes were washed three times with TBST and visualized with a chemi-luminescent reagent (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA, USA). Digital images were captured using an imaging system (Biospectrum 410; UVP, Upland, CA, USA). After images were collected, membranes were washed three times in TBST and incubated with a 1:10,000 dilution in TBST of the house-keeping antibody β-Actin (Sigma-Aldrich) for 1 h at room temperature. Membranes were washed, incubated with mouse secondary antibody at a dilution of 1:2000, and imaged as described.

Data management and calculations

Caspase 3/7 activation was determined as caspase activation per viable cells for each treatment. Raw data from the viability portion of the assay (individual fluorescence values of each well) were normalized to the vehicle alone treatment for each cell line, considered to

represent 100% proliferating cells. The ratio of caspase activation to viable cells is represented as fold increase over DMSO treatment alone.

For all flow cytometry experiments 10,000 events were collected per sample and then gated based on a forward-scatter/side-scatter plot. Three independent replicates were examined for each treatment and analyzed with Cell Quest software (BD Biosciences). For measurements of reactive oxygen species, an unstained control was used to determine the baseline geometric mean fluorescence (GMF) of each extract. This value was subtracted from the GMF of stained samples in order to correct for any shift due to auto-fluorescence of the extract alone. The GMF from each treatment was compared to the DMSO treated samples and represented as fold change. A media only treatment control was used but no significant difference was found between the media only and DMSO treatment, therefore all measurements are compared to DMSO treatment. For MDR1/P-gp efflux pump activity, only one replicate was completed across all cell lines. The GMF for each condition was compared to the GMF of the sample maintained at 4°C.

Statistical Analysis

All statistical analyses were performed using JMP Pro (v. 11.2.1; SAS Institute Inc., Cary, NC, USA). The residuals of the statistical model were found to be normally distributed and therefore analyzed using analysis of variance with Tukey's method for comparison between all treatments, controlling for multiple comparisons. In the case of cell cycle dynamics, Dunnett's method was used to control for multiple comparisons when studying the difference from DMSO control. Differences were considered statistically significant at $p < 0.01$.

# RESULTS

## Caspase 3/7 Activation and Apoptosis

Treatment with 6.3 µg $mL^{-1}$ TE alone resulted in a significant increase in apoptotic cells in the C2 and CMT-12 cell lines as determined by Caspase 3/7 activation (Figure 3.2) and Annexin-V staining (Figure 3.3). A treatment with 6.3 µg $mL^{-1}$ RE alone resulted in a statistically significant increase in caspase activation in all three cell lines when compared to vehicle control. When the combination of 3.1 µg $mL^{-1}$ TE + 3.1 µg $mL^{-1}$ RE was used, an increase in Annexin-V positive cells compared to vehicle control was seen in the C2 cell line, but this was not significant compared to 6.3 µg $mL^{-1}$ TE alone; however in the CMT-12 and D17 cell lines, the combination treatment induced a significantly greater percentage of apoptotic cells compared to 6.3 µg $mL^{-1}$ TE and RE alone. This was further validated with the caspase activation assay in which all three cell lines showed a significant increase in cleaved caspase 3/7 when the combination treatment was used compared to 6.3 µg $mL^{-1}$ of single extracts alone.

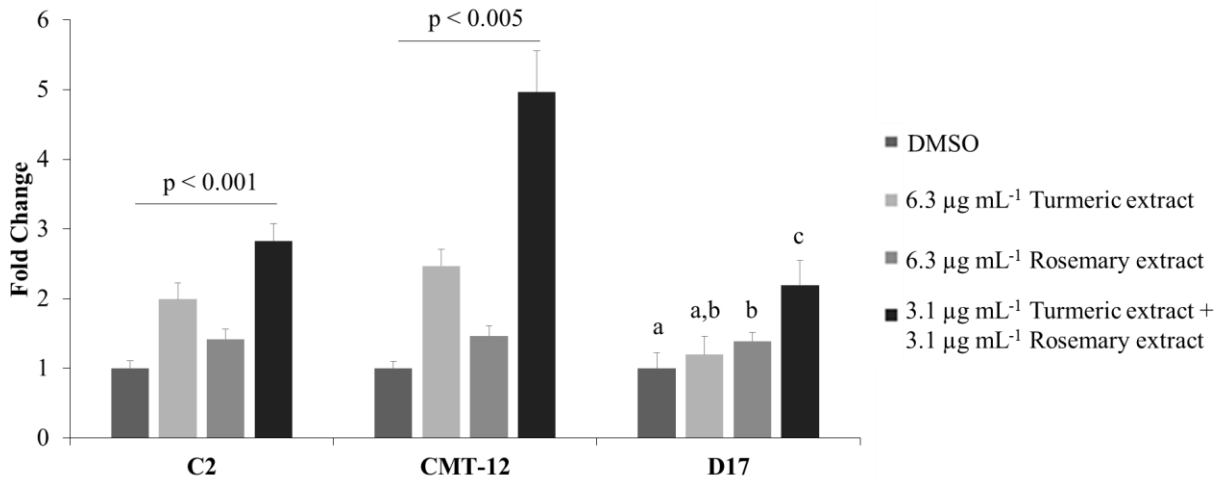


**Figure 3.2 – Caspase 3/7 activity**

Activated caspase 3/7 per viable cells after 36 h treatment is represented as mean ± SD. Values are represented as fold increase compared to DMSO vehicle control. In the C2 and CMT-12 cell lines, all treatments were statistically different; in the D17 cell line, means with different letters are significantly different ($p < 0.05$).

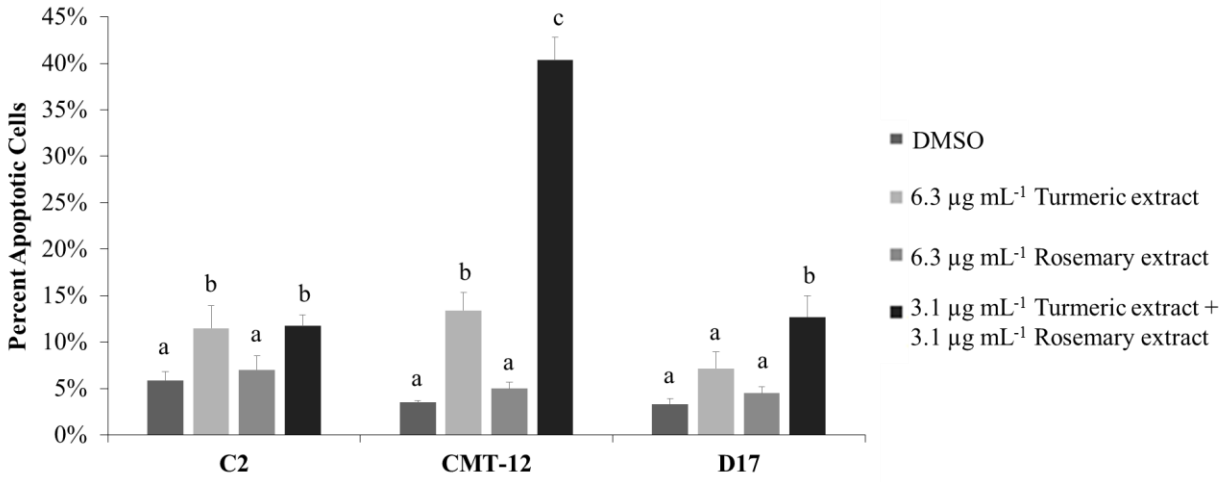


**Figure 3.3 – Induction of apoptosis by TE and RE**

Percent apoptotic cells as determined by AnnexinV staining after 48 h treatment is represented as mean ± SD. Within each cell line, means with different letters are significantly different ($p < 0.01$).

## Antioxidant Activity of Extracts

Since curcumin and carnosic acid have been implicated as antioxidants, we utilized Dihydrorhodamine123 to determine the amount of reactive oxygen species (ROS) present after treatment with each extract. TE was a stronger antioxidant than RE alone ($p < 0.05$ for C2; $p < 0.0001$ for CMT-12 and D17) and the combination treatment using half the dose (3.1 µg $mL^{-1}$ each extract) was as effective as 6.3 µg $mL^{-1}$ TE alone in all three cell lines (Figure 3.4).

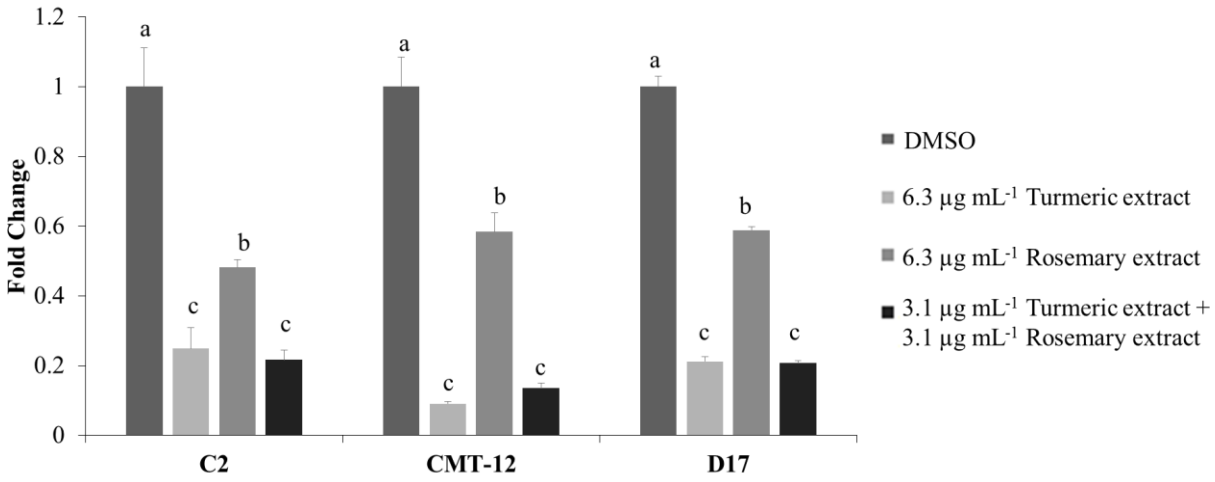


**Figure 3.4 – Reactive oxygen species**

Amount of reactive oxygen species as determined by Dihydrorhodamine123 staining after 12 h treatment is represented as mean ± SD. Values are represented as fold change compared to DMSO vehicle control. Within each cell line, means with different letters are significantly different (C2 $p < 0.05$; CMT-12 and D17 $p < 0.0001$).

## Cellular Accumulation of Curcumin

Observation from previous flow cytometry experiments showed an increase in the GMF when cells were treated with TE and excited at a wavelength of 488 nm, but no change was observed when RE was used alone. We therefore investigated the possibility that RE increases the cellular accumulation of the fluorescent compound curcumin by measuring the GMF when these compounds were used in combination. TE alone at a concentration of 3.1 µg $mL^{-1}$

significantly increased the GMF in the C2 and D17 cell lines (Figure 3.5). When cells were treated with the combination of TE + RE at a concentration of 3.1 µg $mL^{-1}$ of each extract, the GMF increased significantly ($p < 0.0001$) compared to TE alone in all three cell lines.

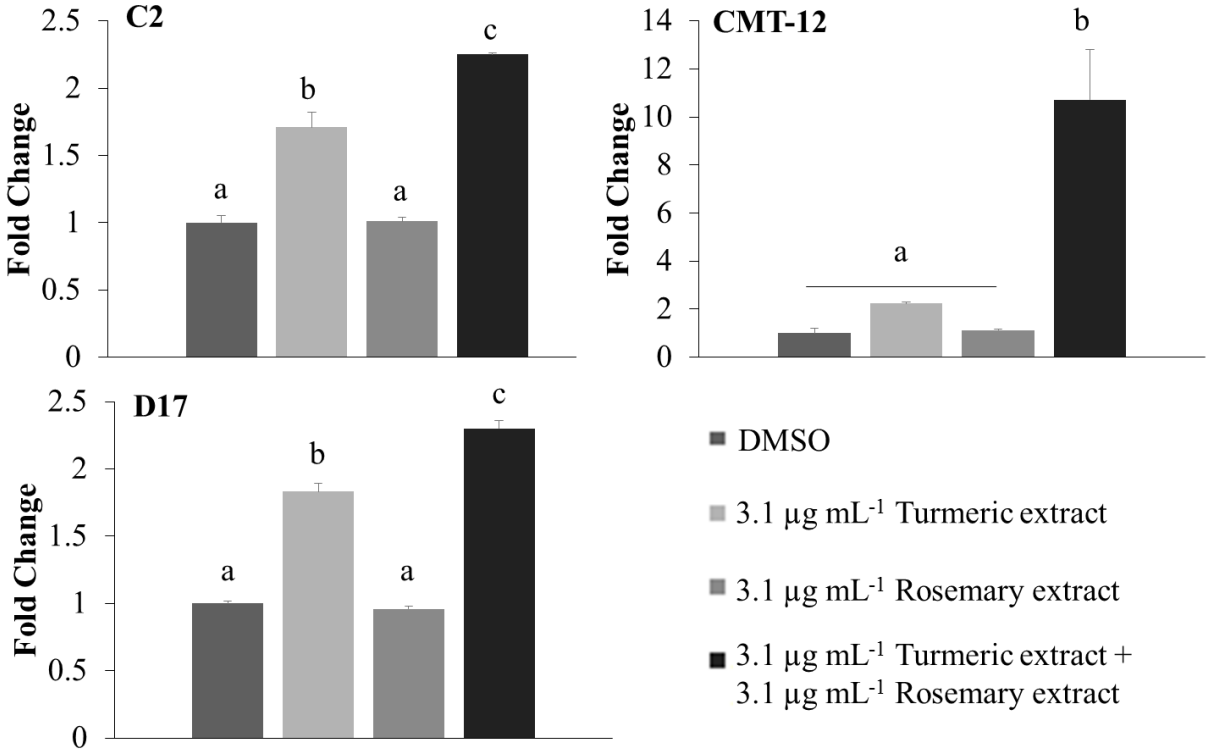


**Figure 3.5 – Cellular accumulation of curcumin**

Cellular accumulation of curcumin after 24 h treatment is represented as ± SD. Y-axis values represent the geometric mean fluorescence of all cells compared to DMSO. Within each cell line, means with different letters are significantly different ($p < 0.0001$).

## Cellular Efflux Activity

We examined if TE or RE acted as an MDR1/P-gp inhibitor using a cellular efflux assay to measure Rhodamine 123 efflux from cells. Canine mammary tumors have been shown to have increased expression of a variety of ATP-binding cassette proteins, including MDR1/P-gp, MRP-1, and BCRP;[15,16] we therefore utilized the CMT-12 cell line for this study. Treatment with RE or TE produced no observable changes from DMSO treatment indicating these extracts are not competitive inhibitors (Figure 3.6).

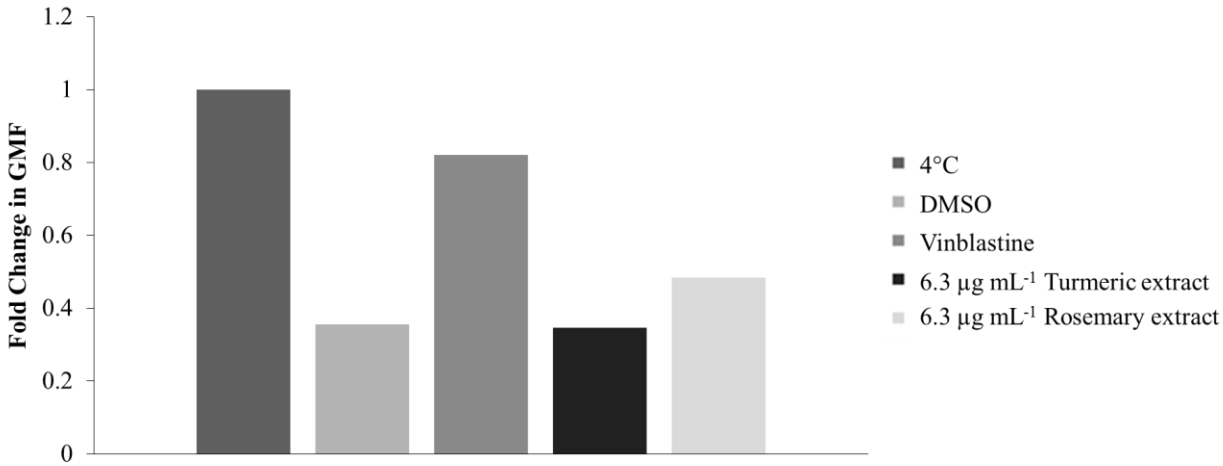


**Figure 3.6 – Activity of MDR1/P-gp in CMT-12 cells**

Cellular efflux of Rhodamine123 by MDR1/P-gp in the CMT-12 cell line in the presence of the competitive inhibitor vinblastine, TE, or RE. Y-axis values represent the geometric mean fluorescence (GMF) of all cells compared to samples kept at 4°C representing inactive MDR1/P-gp.

## Cell Cycle Dynamics

We examined whether TE and/or RE had an effect on cell cycle progression using propidium iodide staining. Cell cycle dynamics were examined after 24 h and 48 h of incubation with the treatments; no difference was seen between these two time-points therefore only data from the 48 h time point is shown (Figure 3.7). Treatment with 6.3 µg $mL^{-1}$ TE resulted in a

minor, but significant decrease in S phase in the D17 cell line. Treatment with 6.3 µg $mL^{-1}$ RE induced a significant decrease in G1/G0 phase in the D17 cell line, a reduction in S phase in both cell lines, and an increase in G2/M phase in the D17 cell line. The combination treatment using 3.1 µg $mL^{-1}$ both extracts induced a decrease in S phase in only the C2 cell line, and an increase in G2/M phase in only the D17 cell line. While these differences were significant, we did not consider the shifts severe enough to continue examining pathways related to cell cycle arrest.

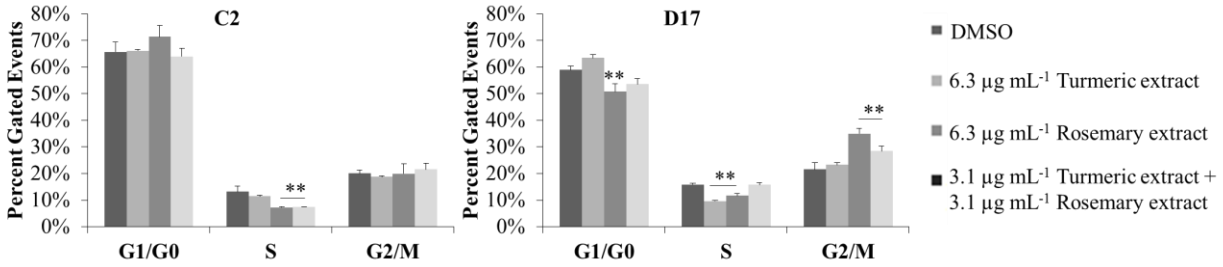


**Figure 3.7 – Cell cycle dynamics**

Percent of cells within each cell cycle phase was determined by prodium iodide DNA staining after 48 h treatment and is represented as mean ± SD. Y-axis values represent percent of gated events; x-axis values represent phases of the cell cycle. All treatments were compared to DMSO vehicle control and are represented at mean ± SD. $p < 0.01$ is represented by **.

## Cellular Pathway Analysis

After examination of several signaling pathways, no consistent trend was seen in the phosphorylation status of AKT, mTOR, JAK/STAT, FAK/Src, ERK, or phosphorylated histone γH2A.X (DNA damage repair marker). In addition, no consistent changes in the mitochondrial proteins BCL2 and BAX were observed. We did however observe changes in Thr183/Tyr185 phosphorylated-SAPK/JNK (p-SAPK/JNK; Figure 3.5). Treatment with 6.3 µg $mL^{-1}$ TE resulted in an increase in p-SAPK/JNK after 24 h in the C2 cell line, stable activation in the CMT-12 cell line, and a minor increase in the D17 cell line. Activated SAPK/JNK appeared to be transient in

the C2 and D17 cell lines after treatment with 6.3 μg $mL^{-1}$ RE, but a stable, although mild, increase was seen in the CMT-12 cell line at both 12 h and 24 h. The greatest increase in p-SAPK/JNK was seen with the combination of 3.1 μg $mL^{-1}$ each of TE and RE in the CMT-12 cell line, but this was not seen in the C2 and D17 cell lines. These results demonstrate a possible reason behind the observed susceptibility differences across the three cell lines.

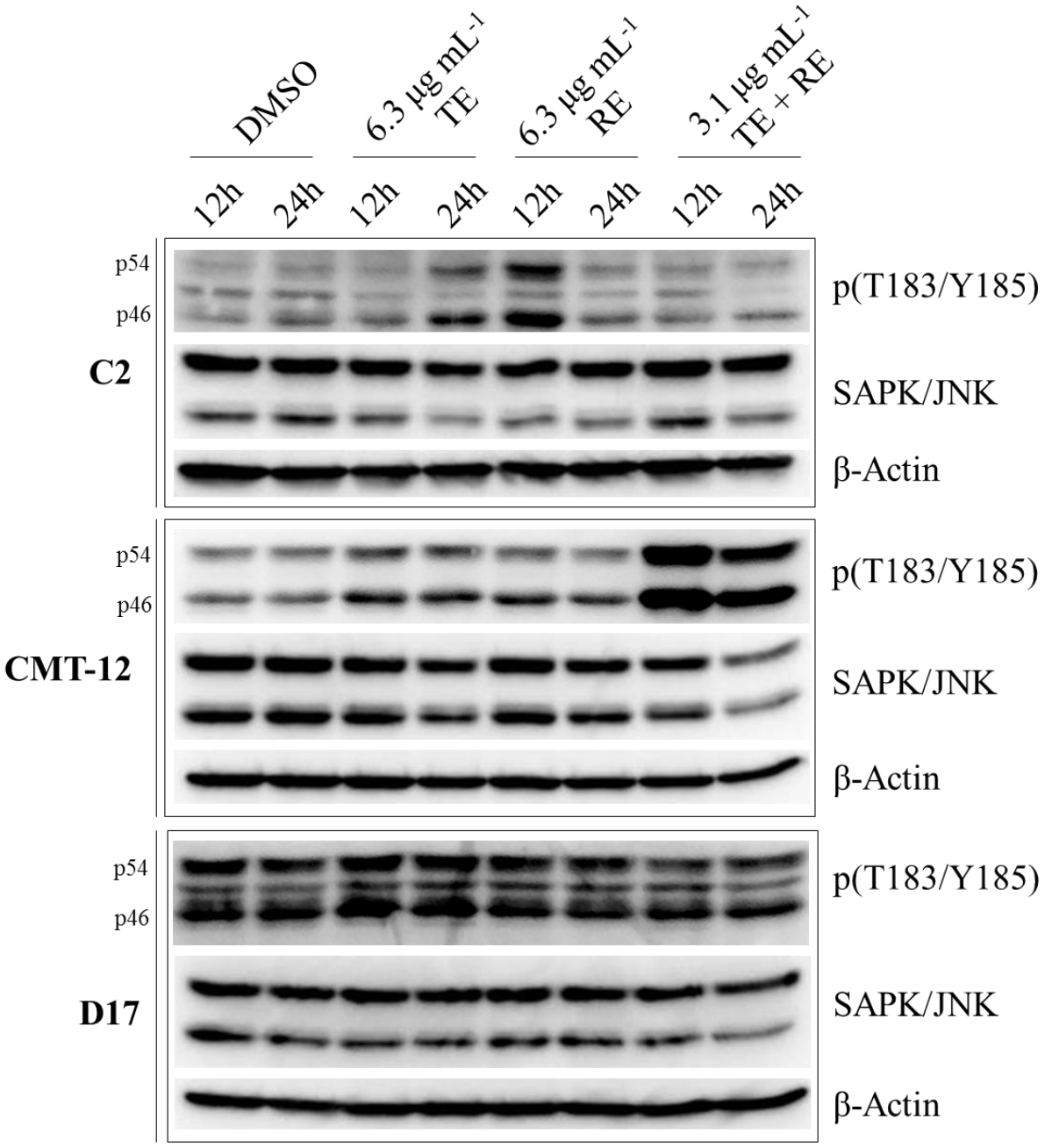


**Figure 3.8 – Pathway analysis by western blotting**

Western blot images of Thr183/Tyr185 phosphorylated-SAPK/JNK (p46/p54), total SAPK/JNK, and housekeeping protein β-Actin. Cells were harvested and lysed after 12 h or 24 h treatment with DMSO vehicle control, 6.3 µg $mL^{-1}$ Turmeric extract, 6.3 µg $mL^{-1}$ Rosemary extract, or combination of 3.1 µg $mL^{-1}$ each of Turmeric and Rosemary extracts.

# DISCUSSION

Bioactive molecules derived directly from plants or modeled after plant compounds have become an active area of research. The majority of these studies have been focused on human and rodent cancer models and the effects of these plant extracts and select compounds vary depending on species and cell origin.[17,18] Few studies have been completed in canines and it is therefore necessary to examine the effects of these compounds before using them in veterinary practice. In the current study, we examined the molecular effects of two extracts, turmeric root and rosemary leaf extracts, previously shown to inhibit proliferation in three canine cell lines.[8] Our experiments were designed to focus on concentrations that may have utility *in vivo* and that would show any synergistic effect of the compounds. In agreement with our previous proliferation and cytotoxicity results, we found that TE was more potent than RE. TE had a greater effect on inducing apoptosis as measured by Annexin V staining and Caspase 3/7 activation, and the combination treatment using half the concentration of each extract induced a similar, if not greater, response. Across the three cell lines used we saw varying degrees of susceptibility, with the CMT-12 cell line being the most susceptible to treatment, perhaps due to an increase in intracellular curcumin as shown by flow cytometry. An increase in curcumin fluorescence was seen across the three cell lines, but the most dramatic effect was seen in the CMT-12 cell line. The presence of RE in combination with TE resulted in a 4-fold increase in GMF beyond that of TE alone. A previous study in the human breast cancer cell line, MCF-7, showed an increase in intracellular accumulation of various chemotherapeutic drugs which was attributed to competitive inhibition of P-gp by rosemary extract.[19] This inhibition was not seen in our experiments indicating that a different mechanism may be involved. The C2 and D17 cell lines did not exhibit P-gp activity as seen by a lack of change in GMF when cells were pre-

loaded with Rh123, a known substrate of this efflux pump. It is possible that this increase in fluorescence is due to a curcumin binding at the cell surface or endocytosis, though these mechanisms have not yet been examined.

Across many of our experiments we saw an additive or synergistic interaction when TE and RE were used in combination compared to either extract alone. This could be in part due to overlapping effects on various signaling pathways including NF-κB, SAPK/JNK, ERK 1/2, and membrane permeability proteins Bcl-2 and Bax.[18,20,21] Previous literature has seen a synergistic effect between these two extracts, specifically the cleavage of poly ADP-ribose polymerase (PARP) and Caspase-8, -9, and -3.[11] After screening several signaling pathways, we found a consistent increase in the phosphorylated, or active, form of SAPK/JNK. This pathway has been implicated in driving cells to apoptosis when faced with environmental stressors such as oxidative stress, inhibition of protein synthesis, changes in the cell-matrix interaction, or signaling from inflammatory cytokines.[22] Consistent with our results, studies have shown that the downstream effects of SAPK/JNK activation are both cell and context dependent; pathway activation can be either pro-apoptotic or pro-proliferative.[23] It is possible that different isoforms, generated by alternative splicing, are involved in these specific responses. In addition, these outcomes often rely on a balance between SAPK/JNK activation and the activation of the mitogen-activated protein kinase MAPK/ERK pathway, and the time and extent of activation. Early, transient activation of JNK may lead to cell survival, while sustained activation can induce apoptosis.[24] Our results showed an increase in phosphorylated SAPK/JNK after 12 h and 24 h of treatment with TE. RE induced an increase in phosphorylation after 12 h and 24 h of treatment in the CMT-12 cell line, while in the C2 cell line this increase was only seen at 12 h and returned to baseline by 24 h. The combination treatment had the greatest effect in the CMT-12 cell line,

resulting in phosphorylated SAPK/JNK at levels greater than either extract alone. Only in the CMT-12 cell line, did we see sustained activation of SAPK/JNK with the combination treatment. This sustained activation, associated with apoptosis, may be the underlying reason behind the increased susceptibility of this cell line. The transient nature of activated SAPK/JNK in the C2 and D17 cell lines lead us to believe a different pathway may be involved in the induction of apoptosis in these cell lines. This data further demonstrates the cell line and context specific effects of these extracts.

## CONCLUSIONS

The results of this study shed light on possible mechanisms by which TE and RE induce apoptosis across three canine neoplastic cell lines. The safety of these commonly used feed ingredients and continual synergy between the extracts make them good candidates for inclusion in a diet for canines diagnosed with a variety of tumor types. Our results indicate that different tumor types are likely to have a differential response to such a therapeutic intervention, with the mammary carcinoma cell line, CMT-12, being the most susceptible to treatment. This susceptibility may be due to the increased accumulation of curcumin when the combination treatment was used. In addition, SAPK/JNK signaling may play an important role in this cell line, and the sustained activation by TE could be the driver of apoptosis. The results of this study warrant further investigations into the pharmacodynamics and pharmacokinetics of these extracts in a canine model with potential for clinical trials.

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# Chapter 4 - Conclusions and future direction

Nutritional supplements have been used for medicinal purposes for centuries and more recently, purification of specific plant compounds and synthetic production to mimic these compounds has become a popular trend. Much of the research around the use of these extracts and compounds has focused on human cell lines, rodent models, and human clinical trials. The results of these studies have shown promise and could be translated to companion animals as well. This research explored the effects of several plant extracts on canine neoplastic cell lines and examined interactions between multiple extracts. Furthermore, the mechanisms behind the extracts cellular effects were investigated.

## CONCLUSIONS

The results from my experiments show evidence that specific plant extracts may provide utility in the treatment of canine cancer. My first objective was to screen an assortment of plant extracts for antineoplastic effects *in vitro*. Results from an initial proliferation screening by **Onco***design* S.A. (data not shown) and my secondary screening revealed turmeric root extract (TE) as the most potent of the extracts tested. Rosemary leaf extract (RE) was the next most effective, followed by black pepper, green tea, and pomegranate extracts. The latter three extracts were only effective at concentrations deemed above physiologically achievable levels, and the effects of green tea extract were quite variable across the three cell lines.

In order to evaluate a possible beneficial interaction between compounds, my second objective involved testing the five leading extracts in two extract combinations to determine if synergistic, additive, or antagonistic interactions occurred. If synergy occurred, these extracts could be used at lower concentrations in order to achieve the same effect, increasing the potential

to reach an effective concentration *in vivo*. I was able to demonstrate a synergistic interaction between TE and RE at a range of concentrations below 25 μg $mL^{-1}$ across all three cell lines.

While cell proliferation was hindered, I wanted to determine if these extracts were inducing cell death. I assayed for cytotoxicity and determined that TE induced cell death in both the C2 and CMT-12 cell lines. The combination treatment reduced the percent of viable cells in these cell lines as well, with a greater effect than TE or RE alone observed in the CMT-12 cell line. The D17 cell line showed a decrease in viable cells, although this was not significant, indicating these cells may require prolonged exposure to the extracts or higher concentrations in order to induce cell death rather than just altering proliferation or metabolism. I continued to examine markers of cell death, specifically apoptosis. An increase in early apoptotic cells could be seen in the C2 and CMT-12 cell when treated with TE and in all three cell lines when the combination treatment was used. Caspase activation was increased two-fold after treatment with TE in the C2 and CMT-12 cell lines. A mild but significant increase was seen when all three cell lines were treated with RE, and the combination treatment increased caspase cleavage beyond that of either extract alone in all three cell lines. These two assays further demonstrated the potency of TE and the higher susceptibility of the CMT-12 line.

My third objective was to test this combination in conjunction with two common chemotherapeutic agents: doxorubicin hydrochloride, a DNA intercalating agent, and toceranib, a receptor tyrosine kinase inhibitor. The results of these assays showed varying results based on the cell line and concentration of the chemotherapeutic agent used. A mild antagonistic effect was seen when CMT-12 cells were treated with low concentrations of extracts when doxorubicin hydrochloride was used at all concentrations tested. This held true with the C2 cells when treated with toceranib. Once extracts reached a concentration of 3.1 μg $mL^{-1}$, additive or synergistic

interactions were seen. The osteosarcoma cell line, D17, was treated with doxorubicin hydrochloride and additive or synergistic interactions were seen at both the $IC_{25}$ and $IC_{75}$. Only at the $IC_{50}$ were mildly antagonistic effects observed. The mixed results across the three cell lines suggest that caution should be taken when utilizing these natural compounds in conjunction with standard chemotherapy protocols, as the circulating concentration of these extracts may be low, resulting in unwanted antagonistic effects.

My fourth objective was to determine the mechanism of action of TE and RE. The molecular effects of curcumin have been extensively studied leading to a seemingly endless list of possible targets. In contrast, studies on the targets of RE and carnosic acid have mostly focused on the antioxidant abilities and cancer prevention. Based off the hypothesis that many plant-derived compounds act as antioxidants, I examined the ability of TE and RE to quench reactive oxygen species (ROS). RE reduced the amount of ROS by 40-50% and TE by 75-80%. When the extracts were used in combination, the strong effects of TE prevailed. The relatively short treatment time suggests that this likely a direct effect rather than an induction of endogenous antioxidant systems in cells, but differentiation would require further studies into gene expression. This experiment agrees with the hypothesis that these compounds act as antioxidants, although the benefits of antioxidants have typically been attributed to preventing cancer, not as a treatment, and further research is necessary to determine the role of ROS in cancerous cells.

One of the possible mechanisms of synergy that I studied was that RE increases the cellular accumulation of curcumin, an auto-fluorescent compound in TE. This accumulation could arise by several mechanisms: increased cellular absorption, decreased cellular efflux, or increased association with the cell membrane. I utilized the endogenous fluorescent properties of

curcumin in order to measure the mean fluorescence of cells after treatment with TE alone and in combination with RE. Across all three cell lines, the presence of RE significantly increased the mean fluorescence beyond that of TE alone. The CMT-12 cell line had the greatest overall increase in fluorescence when the combination treatment was used, perhaps revealing the reason for the increased susceptibility of these cells to the treatment. I examined the hypothesis that RE acts as an inhibitor of cellular efflux pumps, specifically MDR1/P-glycoprotein. After initial testing, the CMT-12 cell line was the only one to exhibit efflux activity and was used for all experiments. The effects of RE as either a down-regulator of efflux pump proteins or a competitive inhibitor were examined, but no significant changes were observed. The underlying cause to this increase in cellular accumulation of curcumin is still uncertain, although I hypothesize it may be related to the ability of cells to directly absorb this compound.

Finally, I set out to investigate any changes in a variety of cellular pathways. I examined several signaling pathways including the pro-proliferative PI3K/AKT/mTOR pathway, the transcriptional regulating MAPKs including ERK and SAPK/JNK, the JAK/STAT signal transduction pathway, regulation of cellular adhesion by FAK/Src, DNA damage response by phosphorylation of histone γH2A.X, and mitochondrial permeability proteins Bcl-2 and Bax. The only protein which showed a consistent change in activation status was SAPK/JNK. In all three cell lines, treatment with TE resulted in an increase in phosphorylated SAPK/JNK, RE treatment caused an increase after 12 h of treatment only in the C2 cell line, and the CMT-12 cell line had the greatest increase in phosphorylated SAPK/JNK when TE and RE were used concurrently. These results hint at a possible mechanism of action, but also demonstrate the differences between these three cell lines. This agrees with the current literature where a variety of pathways can be targeted by these extracts dependent on the cell line used.

# FUTURE DIRECTION

While the data from these studies suggest that curcumin and rosemary act synergistically to induce apoptosis, the exact mechanism by which this occurs across the three cell lines is still unclear. In addition, I was unable to determine the mechanism behind the increased cellular accumulation of curcumin when administered with RE. These gaps have led me to pursue a genome wide screening of mRNA transcription through the nuclear run-on assay, PRO-seq. This experiment will offer a myriad of data that will led us to determine if either extract up- or down-regulates specific regulatory pathways and/or is involved in histone modifications. We plan to examine the effects of each extract individually and in combination and compare these results to the effects of pure curcumin and carnosic acid.

Based on the data from this project, pharmacokinetic and pharmacodynamic studies are warranted. Given the low bioavailability and absorption of curcumin as modeled in the rodent and human, it will be necessary to examine the distribution in the dog. It will also be necessary to examine *in vivo* the hypothesis that RE enhances the cellular accumulation of curcumin. It is possible that the inclusion of RE would increase absorption in the gut therefore allowing for systemic distribution. On the other hand, this effect may only be seen at tumor sites, in which case a bioavailability enhancer such as piperine could be used, as others have previously shown its effects on increasing absorption of curcumin. Based on the exaggerated effects seen in the CMT-12 cell line, therapy consisting of supplemental turmeric and rosemary extracts could prove beneficial in patients with mammary carcinoma. While serum concentrations of these compounds may be low, this cell line showed additivity or synergism at the lowest concentrations tested. Care would need to be taken if this treatment was combined with the administration of doxorubicin hydrochloride as these low extract concentrations appeared to

have an antagonistic effect with the chemotherapeutic drug. This nutritional therapy, though, could be valuable in the case of refractory tumors or with owners who decline standard treatment protocols.

Naturally derived compounds or whole extract supplements provide a valuable resource as therapeutic agents. The ability to use these extracts as a dietary supplement provides a unique strategy and the opportunity for the potential prevention and treatment of cancers. Moreover, these compounds may work in an additive or synergistic manner with traditional therapeutic agents. Safety and efficacy for many of these extracts have been proven in humans and in rodent models of disease. Thus, it is a natural progression to use these supplements to treat cancer in our companion animals, such as dogs, that also serve as an important spontaneous animal model of human cancers. While challenges remain with regard to how such nutraceutical compounds can be used in terms of quantity and schedule of administration, this field of research continues to grow and offers a promising complement to current cancer therapies. The results of this study provide background on a variety of naturally derived extracts for use in dogs, and they highlight the potency and synergy between turmeric root and rosemary leaf extracts. I hope the exciting results provided by this work continues to advance the area of nutraceuticals for the treatment of canine cancer.