Fall 11-16-2017

Synergistic Effects of d-Tocotrienol and Xanthorrhizol in Prostate Cancer Cells

Chappell Madhani

ACCEPTANCE

This thesis, *Synergistic Effects of d-δ-Tocotrienol and Xanthorrhizol on Prostate Cancer Cells*, by Chappell Rebecca Madhani was prepared under the direction of the Master's Thesis Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the degree Master of Science in the Byrdine F. Lewis School of Nursing and Health Professions, Georgia State University. The Master's Thesis Advisory Committee, as representatives of the faculty, certify that this thesis has met all standards of excellence and scholarship as determined by the faculty.

Huanbiao Mo, Ph.D.
Committee Chair

Desiree Wanders, Ph.D.
Committee Member

Weiming Xia, Ph.D.
Committee Member

10/31/2017
Date

AUTHOR'S STATEMENT

In presenting this thesis as a partial fulfillment of the requirements for the advanced degree from Georgia State University, I agree that the library of Georgia State University shall make it available for inspection and circulation in accordance with its regulations governing materials of this type. I agree that permission to quote, to copy from, or to publish this thesis may be granted by the professor under whose direction it was written, by the Byrdine F. Lewis School of Nursing and Health Professions director of graduate studies and research, or by me. Such quoting, copying, or publishing must be solely for scholarly purposes and will not involve potential financial gain. It is understood that any copying from or publication of this thesis which involves potential financial gain will not be allowed without my written permission.

Chappell R. Madhavi

Signature of Author

NOTICE TO BORROWERS

All theses deposited in the Georgia State University library must be used in accordance with the stipulations prescribed by the author in the preceding statement. The author of this thesis is:

Chappell Rebecca Madhani
3071 Lenox Road, Unit 13
Atlanta, GA 30324

The director of this thesis is:

Huanbiao Mo, PhD
Chair and Professor
Department of Nutrition
Byrdine F. Lewis College of Nursing and Health Professions
Georgia State University
Atlanta, Georgia 30302

VITA

Chappell Rebecca Madhani

ADDRESS: 3071 Lenox Road, Unit 13
Atlanta, GA 30324

EDUCATION:

M.S.	2017	Georgia State University Nutrition
B.S.	2012	Kennesaw State University Anthropology

PROFESSIONAL EXPERIENCE:

- Senior Server, Trainer — 2013-2014
  Bistro Niko, Atlanta, GA
- Laboratory and Field Technician - Intern — 2012
  Edwards Pitman Environmental, Inc., Smyrna, GA
- Senior Server, Trainer — 2010-2013
  Outback Steakhouse, Roswell, GA
- Office Assistant — 2009-2010
  River City Legal Group, Chattanooga, TN

PROFESSIONAL SOCIETIES AND ORGANIZATIONS:

- Academy of Nutrition and Dietetics — 2014-present
- Georgia Dietetic Association — 2014-present
- Greater Atlanta Dietetic Association — 2014-present
- Nutrition Student Network, Georgia State University — 2014-2015

AWARDS AND PUBLICATIONS:

- Madhani, R., Todd, J., Mo, H. (2016) 2016 Dietary guidelines for Americans. 1:16 21. Publisher: China National Cereals, Oils & Foodstuffs Corporation Co. Ltd.
- Dean's List – Georgia State University — 2014
- Dean's List – Kennesaw State University — 2011

ABSTRACT

SYNERGISTIC EFFECTS OF DELTA- δ-TOCOTRIENOL AND XANTHORRHIZOL IN PROSTATE CANCER CELLS

by
C. Rebecca Madhani

Background: Approximately one in seven American men will be diagnosed with prostate cancer during their lifetime. The mevalonate pathway produces essential intermediates for the post-translational prenylation and dolichylation of growth-associated proteins including Ras, nuclear lamins and growth factor receptors. Dysregulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the mevalonate pathway, in prostate cancer cells supports tumor growth and therefore can be targeted for prostate cancer prevention and therapy. Previous studies have shown that isoprenoids including tocotrienols and xanthorrhizol suppress the growth of prostate cancer cells with concomitant downregulation of HMG CoA reductase.

Objective: To determine the synergistic effects of *d*-δ-tocotrienol and xanthorrhizol on growth of human DU-145 prostate carcinoma cells.

Methods: DU-145 cells were incubated with *d*-δ-tocotrienol and xanthorrhizol, individually and in blends, for 72 hours before viable cells were quantified by CellTiter 96® Aqueous One Solution. The impacts of these compounds, individually and in combination, on cell cycle distribution and the

expression of cell cycle related proteins in DU-145 cells were evaluated by flow cytometry and Western-blot in follow-up studies.

Statistical analysis: All experiments were repeated 3 times. One-way analysis of variance (ANOVA) was performed to assess the differences between groups using Prism ® 4.0 software (GraphPad Software Inc., San Diego, CA). Differences in means was assessed using Tukey's test. Levels of significance are indicated as $P < 0.05$.

Results: Blends of *d*-δ-tocotrienol and xanthorrhizol showed greater inhibition of cell growth than those of individual compounds. Current results did not show a significant change in cell cycle distribution or down regulation of Cdk4 and cyclin D1 with the combination of compounds. The level of procaspase-3 for apoptosis initiation was also not altered.

Conclusion: Further studies are warranted to confirm these initial findings and understand the mechanisms underlying the combined effect of *d*-δ-tocotrienol and xanthorrhizol on cell proliferation.

SYNERGISTIC EFFECTS OF DELTA- δ-TOCOTRIENOL AND XANTHORRHIZOL IN PROSTATE CANCER CELLS

by
C. Rebecca Madhani

A Thesis

Presented in Partial Fulfillment of Requirements for the Degree of

Master of Science in Health Sciences

The Byrdine F. Lewis College of Nursing and Health Professions

Department of Nutrition

Georgia State University

Atlanta, Georgia

2017

ACKNOWLEDGMENTS

I am deeply grateful to Huanbiao Mo, Ph.D. for sharing his lab and research, and for allowing me to flourish as a student under his direction. I would also like to thank Dr. Mo for introducing me to the fascinating world of tocotrienols, xanthorrhizol, and nutritional cancer therapy *in vitro*. This opportunity has intensified my interest in research and appreciation for time and work that is required for research. I would like to thank Desiree Wanders, Ph.D. and Weiming Xia, Ph.D. for taking time to serve on my committee and for offering their expertise.

Thank you to Rafaela Feresin, Ph.D. for sharing her western blot procedure and best practices. I also appreciated her effort to organize and streamline our lab for a more efficient work space. Thank you to Dr. Xiangming Ji, Ph.D. for sharing his expertise on western blot and for suggesting the best companies from which to order antibodies. A big thank you to Manal Elfakhani and Sophie Yount for guiding me through laboratory methods and procedures. I would also like to thank both for helping me maintain the DU-145 cells and procedures during my rotations outside of the lab. Their efforts were integral to the completion of this project. Thank you to Darren Chan for sharing his progress and troubleshooting with me. I would also like to thank him for discovering and researching FCS Express for analysis of our cell cycle data. Finally, a big thank you to Shaligram Sharma for always taking the time to answer my questions and for providing advice on western blot and data analysis.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

ABBREVIATIONS

DU-145	human prostate cancer cells
d-δ	delta
T	tocotrienol
X	xanthorrhizol
Cdk4	cyclin dependent kinase 4
E2F	E2 transcription factor
RB	retinoblastoma
P53	tumor protein p53
Ink4a	A family of cyclin-dependent kinase inhibitors
FPP	farnesyl pyrophosphate
GGPP	geranylgeranyl pyrophosphate
GTPase	hydrolase enzyme
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
TRF	Tocotrienol rich fraction
DNA	Deoxyribonucleic acid
mL	milliliter
µL	microliter
µM	micromolar
µg	microgram

x g	times gravity
CO_2	carbon dioxide
^{o}C	degrees Celsius
cm^2	centimeters squared
RPMI	Roswell Park Memorial Institute
FBS	Fetal Bovine Serum
1x HBSS	1x Hank's Balanced Salt Solution
1x PBS	1x Phosphate Buffer Solution
V	volts
PVDF	Immun-Blot Polyvinylidene difluoride
PBST	Phosphate Buffered Saline containing 0.1% Tween-20 solution
ANOVA	One-way analysis of variance
SEM	Standard error of the mean

CHAPTER I

SYNERGISTIC EFFECTS OF DELTA- δ-TOCOTRIENOL AND XANTHORRHIZOL IN PROSTATE CANCER CELLS

Introduction

Approximately one in seven American men will be diagnosed with prostate cancer during their lifetime. In 2017, the Centers for Disease Control and Prevention reported 161,360 North American men diagnosed with prostate cancer and 26,730 deaths from prostate cancer. (1) Prostate cancer is currently treated with methods such as chemotherapy, radiation, and surgery. Radiation and surgery may cause side effects such as infections, impotence, or bladder and bowel problems. (2) Chemotherapy is accompanied by disconcerting symptoms including loss of appetite, mouth sores, fatigue, nausea, vomiting, diarrhea, and hair loss. In addition, common chemotherapy agents including Docetaxel, Mitoxantrone, and Estramustine may put patients at risk for peripheral neuropathy, leukemia, and blood clots, respectively. (3) Therefore, alternative therapies are necessary for the treatment of prostate cancer.

Two dietary factors that could potentially serve as therapeutic agents for prostate cancer are *d*-δ-tocotrienol and xanthorrhizol. *d*-δ-Tocotrienol, a minor form of vitamin E, has been found to decrease cancer cell proliferation by inhibiting HMG-CoA reductase in the mevalonate pathway, thereby preventing prenylation or dolichylation of growth-related proteins. (4) In addition, *d*-δ-tocotrienol has been associated with

cell cycle arrest in the G1 phases with a concomitant inhibition of Cdk4 and caspase-3 activation and apoptosis in melanoma cells. (5) Xanthorrhizol is a bisabolane-type sesquiterpene that has also exhibited anti-cancer effects by inducing cell cycle arrest through decreased expression of Cyclin D1 and Cdk4, and apoptosis by activation of procaspases 3 & 9 in colon cancer cells. (6)

Hypothesis and Specific Aims

Hypothesis: *d*-δ-Tocotrienol and xanthorrhizol synergistically suppress the proliferation of DU-145 prostate carcinoma cells by inducing cell cycle arrest and apoptosis.

Specific aim 1: To determine the effects of *d*-δ-tocotrienol and xanthorrhizol, individually and in combination, on prostate cancer cell proliferation.

Specific aim 2: To determine the effects of *d*-δ-tocotrienol and xanthorrhizol, individually and in combination, on cell cycle progression and cell cycle-related proteins of prostate cancer cells.

Specific aim 3: To determine the effects of *d*-δ-tocotrienol and xanthorrhizol, individually and in combination, on an apoptosis-related protein, procaspase-3, of prostate cancer cells.

CHAPTER II

Literature Review

Metabolic Regulation and Cancer Therapies

The RB pathway plays a vital role in cell proliferation by activating and suppressing growth related factors, mediating entry of cells into the cell cycle. The RB pathway consists of five protein families including D-type cyclins, CDKN, (Ink4a), E2F-transcription factors, cyclin-dependent protein kinases (e.g. Cdk4, Cdk6) and RB-family pocket of proteins. (7) Cdk4 and Cdk6 are catalytic components that bind to D-cyclins to form active kinase complexes and initiate cell progression through the cell cycle. (8) Ink4a proteins inhibit the activity of Cdk4 and Cdk6 by competing with cyclin D1 to suppress creation of the active cyclin-D1/Cdk4/6 kinase complex, which is activated during normal cell cycle entry and proliferation. (9) Other cellular targets of the D-cyclin/Cdk4/6 complexes are RB-proteins, which control chromatin structures and E2F-transcription factor activities. (8)

The E2-factor is a family of transcription factors that are downstream effectors of the RB pathway. E2F play important roles in entry of the S phase of the cell cycle (10) and p53-dependent and p53-independent apoptosis. (11) RB-pocket proteins can be directed to E2F-regulated promoters, thereby inhibiting transcription by suppressing E2F transactivation. The D-cyclin/Cdk4/6 complex can phosphorylate RB-pocket proteins and disrupt RB-E2F interaction, prompting activation of E2F-regulated gene expression. In cancer cells, the RB-pathway is modified to promote deregulated tumor cell proliferation

by failing to suppress E2F activity. Consequently, the D-cyclin/Cdk4/6 kinase complex stimulates tumor cell proliferation, however Ink4a proteins operate as tumor suppressors by inhibiting D-cyclins. (7)

Apoptosis is a regulated cellular suicide that is necessary to remove damaged cells. An accumulation of evidence has shown mitochondria to be the major organelles involved in apoptosis, through processes including DNA damage, growth factor down regulation, and chemotherapeutic drugs. (12) Apoptosis is promoted through increased E2F activity, which enhances the expression of pro-apoptotic genes such as caspases. Caspases are cysteine proteases that are activated in apoptotic cells. Caspases 2, 8, and 9 are initiator caspases that cleave downstream effectors caspases 3, 6, and 7. Effector caspases execute apoptosis by cleaving cellular proteins. (13)

The mevalonate pathway plays important roles in cell proliferation and cell death. Intermediates of the mevalonate pathway, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are required for the prenylation, membrane attachment, and biological activities of many growth-related proteins and dolichylation of growth factor receptors. Among the growth-associated proteins that are prenylated are the nuclear lamins and the small GTPases, including the Ras protein, that are involved in signaling regulating cell division. (14)

The importance of mevalonate pathway intermediates in cell proliferation and cell death renders the pathway a useful target for prostate cancer therapy. One approach is to suppress the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, of the pathway. (15) In eukaryotic cells, HMG CoA reductase is regulated at multiple levels, including sterol-mediated transcriptional downregulation and non-sterol-

mediated posttranscriptional downregulation; the latter includes ubiquitination and proteasome-mediated degradation of HMG CoA reductase. (7) One class of HMG CoA reductase inhibitors is statins. Statins are competitive inhibitors of HMG CoA reductase and are widely prescribed for their hypocholesterolemic effect. (14)

Tocotrienol and Cancer Inhibition

Tumor cell proliferation is suppressed by down regulation of HMG-CoA reductase, which is the committed step for the biosynthesis of sterols such as cholesterol through the mevalonate pathway. In contrast, tocotrienol has been shown to down regulate HMG CoA reductase, thereby inhibiting tumor cell proliferation. (14)

In addition to the statins mentioned above, tocotrienols are another class of HMG CoA suppressors. Tocotrienols, a minor form of vitamin E, include four chemical forms (α-, β-, γ-, and δ-tocotrienol). Tocotrienols vary by the number and position of methyl groups in the polar head of the molecules. Tocotrienol is derived from dietary sources including palm oil, red annatto seeds, grape seed oil, flaxseed oil, buckthorn berry, rye, oat, and barley. Tocotrienol studies began in the early 1990's and focused on regulation of cholesterol production via post-transcriptional suppression of HMG CoA reductase. (16) Tocotrienols have been found to mimic sterols by promoting ubiquitination and degradation of HMG-CoA reductase. (4) Over the past two decades, tocotrienol has been found to suppress the proliferation of tumor cells originated from various tissues, including colon, breast, prostate, skin, liver, lung, lymph gland, cervix, and nerve. (17)

Har et al. examined the effects of tocotrienol in BNL CL.2 normal liver cells and BNL 1ME A.7R.1 liver cancer cells. Tocotrienols appeared to have a greater effects in

liver cancer cells compared to normal liver cells in all experiments. Tocotrienol significantly reduced cell viability of liver cancer cells in a dose-dependent manner. Elevated caspase-3 activity was found in liver cancer cells in a time dependent manner following 9, 12, and 24 hour treatments of tocotrienol. Finally, 24-hour tocotrienol treatment induced DNA fragmentation in liver cancer cells compared to untreated cells. These data suggest that tocotrienol decreases viability in 1ME A.7R.1 liver cancer cells through apoptotic activity of caspase-3. (18)

Fernandes et al. looked at the effect of *d*-δ-tocotrienol in A2058 and A375 human melanoma cells. *d*-δ-Tocotrienol caused a dose-dependent inhibition of cell proliferation in both A2058 and A375 cell lines. Cell cycle analysis demonstrated significant increases in the percentage of cells in the G_1 phase for both cell lines after treatment with *d*-δ-tocotrienol. *d*-δ-Tocotrienol (24 μM) raised the percentage of A2058 cells in the G1 phase from 46% to 63% ($P < 0.05$). Concomitantly, S% decreased from 35% to 20% ($P < 0.05$). G1 arrest was also seen in A375 cells treated with 33 μM *d*-δ-tocotrienol. G_1 arrest was congruent with a significant decrease in expression of Cdk4, a vital catalyst in the transition from G1 to S phase, for both cell lines. (5)

A concentration-dependent cleavage of procaspase-3 was initiated by *d*-δ-tocotrienol in A2058 cells using western blot analysis. Cleavage of procaspase-3 is shown through lighter bands in western blot analysis. These data suggest that *d*-δ-tocotrienol induces cell cycle arrest in A2058 and A375 cells and initiates caspase-3 activation and apoptosis in A-2058 human melanoma cells. (5)

Srivastava and Gupta found significant growth inhibition of DU-145 human prostate cancer cells induced by tocotrienol-rich fraction (TRF). TRF composition was

14.77% α-tocotrienol, 33.97% γ-tocotrienol, 26.11% *d*-δ-tocotrienol, 18.10% α-tocopherol, and 7.05% of other tocotrienol-like compounds. DU-145 cell growth decreased by 17.9%, 20.1%, 46.5%, 78.3%, and 79.8%, after treatment with 5, 10, 20, 40, and 80 μg/ml of TRF, respectively. In addition, TRF caused a dose-dependent increase of the percentage of DU-145 cells in the G0/G1 phase, compared to the control. A concomitant decrease in the percentage of cells in the S phase of cell cycle was shown, compared to the control. These data suggest that TRF caused a dose-dependent decrease in cell growth and increase in cell cycle arrest at the G0/G1 phase in DU-145 cells. (19)

Xanthorrhizol and Cancer Inhibition

Sesquiterpenes are a class of isoprenoids that have been shown to suppress the mevalonate pathway and inhibit the biosynthesis of essential intermediates required for the function of growth related proteins such as Cdk4. Consequently, sesquiterpene-mediated downregulation of HMG CoA reductase induces cell cycle arrest and initiates apoptosis. (14)

Xanthorrhizol is a sesquiterpene derived from *Curcuma xanthorrhiza rhizome*, also known as Javanese turmeric. Xanthorrhizol is native to Indonesia and has exhibited a variety of health benefits, including anti-inflammatory, antimicrobial, antioxidant, hyperglycemic, antihypertensive, antiplatelet, nephroprotective, estrogenic, and anticancer properties. It has been speculated that xanthorrhizol suppresses carcinogenesis by delivering cytotoxic effects from its phenol group. Xanthorrhizol's anti-cancer effects have been associated with its contribution to apoptosis and cell cycle arrest. (6)

Cheah et al. found a concentration-dependent growth inhibition in MDAMB-231 human breast cancer cells, caused by 48 hour treatments of xanthorrhizol. Apoptotic levels of MDAMB-231 cells was measured using an apoptotic index after 24 hours of treatment with xanthorrhizol. A significant increase ($p<0.005$) of apoptosis was found, in a dose-dependent manner. Finally, caspase activity of MDAMB-231 cells after xanthorrhizol treatment was also assessed, which found a significant increase ($p<0.005$) in caspase-3 activity compared to control cells. These data suggest that xanthorrhizol induced growth inhibition and apoptosis by activation of caspase-3 in MDAMB-231 human breast cancer cells. (20)

Kang et al. found that 24-hour treatments of xanthorrhizol inhibited growth and increased percentage of HCT-116 human colon cancer cells at the G0/G1 phase of the cell cycle, compared to control. Percentage of HCT-116 cells in the S phase also decreased compared to the control. Concomitantly cyclin D1 and Cdk4 were downregulated in a time- and dose-dependent manner. As previously mentioned, cyclin D1 and Cdk4 form the active kinase complex for transition of cells from the G1 to S phases of cell cycle. This suggests cell cycle arrest of the G0/G1 phase induced by xanthorrhizol by down regulation of cyclin D1 and Cdk4. (21)

Kang et al. also found evidence of apoptosis through DNA fragmentation, activation of procaspases 3 & 9, release of cytochrome c, and cleavage of poly-(ADP-ribose) polymerase. DNA fragmentation was determined using an agarose gel electrophoresis with 24 and 48 hour treatments of xanthorrhizol. Apoptosis was detected in a time- and dose-dependent manner. Apoptosis was also detected using western blot analysis. Cleaved caspase-3 was increased in a dose-dependent manner after 24 and 48

hour xanthorrhizol treatments. Additionally, expression of procaspase-3 was decreased in a time- and dose-dependent manner. (21)

Handayani et al. examined the effects of xanthorrhizol in HepG2 human liver cancer cells. IC_{50} values were used to conduct cytotoxicity assays; increasing concentrations of xanthorrhizol was found to inhibit HepG2 cell growth in a dose dependent manner. Apoptotic levels of HepG2 cells was measured using an apoptotic index after 24, 48, and 72 hours of treatment with xanthorrhizol. The percentage of apoptotic cells was increased to more than 70% in all treatment groups compared to the less than 5% in the control. Western blot analysis of apoptosis-related proteins after 24, 48, and 72 hour treatments of xanthorrhizol showed a time-dependent decrease in procaspase-3 and increased expression of caspase-3. (22)

In summary, tocotrienol has shown anti-proliferative effects in BNL 1ME A.7R.1 liver (18), A2058 and A375 melanoma (5), and DU-145 prostate (19) cancer cells. Tocotrienol induced apoptotic events in BNL 1ME A.7R.1 liver cells (18) and A2058 melanoma cells (5). Tocotrienol also induced cell cycle arrest in DU-145 prostate cancer cells (19) and A2058 and A375 melanoma cells (5). Xanthorrhizol has shown anti-proliferative effects in MDAMB-231 breast (20), HCT-116 colon (21), and HepG2 liver (18) cancer cells, but not prostate cancer. Xanthorrhizol has exhibited apoptotic events in MDAMB-231 breast (20) and HepG2 liver (18) cancer cells, and both apoptosis and cell cycle arrest in HCT-116 colon cancer cells (21). No studies to date have looked at the synergistic effects of tocotrienol and xanthorrhizol in prostate cancer cells.

CHAPTER III

Methods and Procedures

Materials

Human DU-145 prostate carcinoma cells were received as a gift from the Biology Department at Georgia State University Biology and were used in all procedures. All supplies needed for cell culturing were purchased from Fisher Scientific, Inc. (Pittsburgh, PA) and Thermo Scientific (Waltham, MA). Xanthorrhizol and *d*-δ-tocotrienol were received as a gift from American River Nutrition, Inc. (Hadley, MA).

Cell Culture

DU-145 cells were cultured in Roswell Park Memorial Institute (RPMI-1460) media and supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, and 0.8% gentamicin in 25 cm^2 flasks. Cells were then incubated for 48 hours at 37^oC in a humidified atmosphere of 5% CO_2. At 48 hours, cells were subcultured into a 175 cm^2 flask, grown to 80 - 90% confluence, and then prepared according to experimental procedure. Nine treatments were used for each experiment, as follows: control (C), *d*-δ-tocotrienol 5 µM (T5), *d*-δ-tocotrienol 10 µM (T10), xanthorrhizol 16.25 µM (X16.25), xanthorrhizol 32.5 µM (X32.5), *d*-δ-tocotrienol 5 µM & xanthorrhizol 16.25 µM (T5/X16.25), *d*-δ-tocotrienol 5 µM & xanthorrhizol 32.5 µM (T5/X32.5), *d*-δ-tocotrienol 10 µM & xanthorrhizol 16.25 µM (T10/X16.25), *d*-δ-tocotrienol 10 µM & xanthorrhizol 32.5 µM (T10/X32.5). Each treatment was dissolved in ethyl alcohol, with the control containing ethyl alcohol only.

Cell Proliferation

Cell titer was performed to quantify the effects of *d*-δ-tocotrienol, xanthorrhizol, and a combination of both on DU-145 cell proliferation. Human prostate cancer (DU-145) cells were cultured as previously described and seeded in 96-well plates at 1,500 cells per well. Cells were treated at 0.1% volume for 72 hours with respective treatments of xanthorrhizol and *d*-δ-tocotrienol. At 72 hours, complete RPMI media was aspirated from wells; cells were then washed with 1x Hank's Balanced Salt Solution (1x HBSS), and refreshed with 100 µL serum-free RPMI media and 20 µL Cell Titer 96® Aqueous One Solution. Cells were then incubated for 2 hours at 37°C, 5% CO_2. At 2 hours, the plate was measured using a Biotek, Synergy HT plate reader; results were analyzed using the Gen5^{TM} software from Biotek® Instruments, Inc. (Winooski, VT, USA).

Cell Cycle Analysis

Xanthorrhizol and *d*-δ-tocotrienol were tested for their effects on cell cycle distribution in DU-145 cells. Cells were seeded in 25 cm^2 flasks (Becton Dickinson Labware, Franklin Lakes, NJ) at $1.5x10^6$ cells per flask with 3 mL RPMI medium per flask and incubated for 24 hours. At 24 hours, medium was aspirated and replaced with fresh medium containing respective treatments of xanthorrhizol and *d*-δ-tocotrienol. After 24 hours of incubation, cells were harvested by trypsinization and pelleted by centrifugation at 13,000 x g for 5 minutes. Cell pellets were then fixed in 1 mL of 70% ethanol at -20 degrees Celsius overnight. In preparation for analysis, cells were washed twice with a 1x Phosphate Buffer Solution (1x PBS) and centrifuged at 5 minutes after each wash. Cells were re-suspended in 500 µL of 1x PBS containing RNase A (Sigma-Aldrich) and incubated at 37 degrees Celsius without light for 30 minutes. One hundred

µL of a PBS-TritonX100-propidium iodide solution was added to each sample and held in the dark, at room temperature for 15 minutes. Stained cell samples were divided into 200 µL aliquots and analyzed for DNA content using a BD LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA). The distribution of cells in the G1, S, and G2 phases of the cell cycle was determined using FCS Express software (De Novo Software, Glendale, CA).

Western Blot Analysis

Western-blot was performed to examine the proteins Cdk4, Cyclin D-1, and Procaspase-3. Protein concentration of DU-145 cells treated with respective treatments of xanthorrhizol and *d*-δ-tocotrienol was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Treatment samples containing 10 µg of protein was mixed with 4x Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) at a 1:1 ratio (v/v) and β-Mercaptoethanol before loading into a 12% SDS-polyacrylamide gel. Gels were then loaded onto a Mini PROTEAN Tetra electrophoresis unit (Bio-Rad Laboratories) and run at 100 volts (V) for 10 minutes, and 150 V for 1.5 hours. Proteins were transferred from the gel onto an Immun-Blot Polyvinylidene difluoride **(**PVDF) membrane (Bio-Rad Laboratories) with a Trans Blot© Turbo™ Transfer System (Bio-Rad Laboratories) at 25 V and 1 amp (A) for 30 minutes. Polyvinylidene difluoride membranes were then be incubated in blocking solution (5% non-fat dry milk in 1x PBS) and shaken at room temperature for 20 minutes. After blocking, membranes were rinsed with Phosphate Buffered Saline containing 0.1% Tween-20 solution (PBST), twice for five minutes and once for ten minutes. Membranes

were then incubated with a 1:1000 dilution of PBST and monoclonal antibodies to beta-actin at 4 degrees Celsius on a rocker overnight.

After overnight incubation, membranes were washed with PBST twice for five minutes and once for ten minutes and incubated with a 1:1000 dilution of PBST and secondary antibody (horseradish peroxidase linked; Cell Signaling Technology) for one hour. After a third wash period, membranes were exposed to a Super Signal West Pico Chemiluminescence Kit (Pierce) and then photographed with an Image Quant LAS 4000 system (GE Healthcare Life Sciences, Pittsburgh, PA).

Membranes were incubated again with blocking solution for twenty minutes, washed three times, and incubated with primary antibodies of interest at 4 degrees Celsius on a rocker overnight. Antibodies included cyclin-dependent kinase-4 (Cdk4) (Santa Cruz Biotechnology, Santa Cruz, CA) and procaspase-3 (PC-3) (Santa Cruz Biotechnology) with a 1:500 PBST solution, and Cyclin-D1 (Cell Signaling Technology, Danvers, MA) with a 1:1000 PBST solution overnight. Membranes were washed again three times and incubated with PBST and respective secondary antibodies in a 1:1000 dilution (Cdk4: mouse; Procaspase-3: mouse; Cyclin-D1: rabbit; horseradish peroxidase linked) for one hour at room temperature. Finally, membranes were washed three times, treated with the Super Signal Chemiluminescence solution, and imaged as described above.

Statistical Analysis

All experiments were repeated 3 times. One-way analysis of variance (ANOVA) was performed to assess the differences between groups using Prism® 4.0 software (GraphPad Software Inc., San Diego, CA). Differences in means was assessed using Tukey's test. Values are mean ± standard error of the mean (SEM). Levels of significance are indicated as $P < 0.05$.

CHAPTER IV

Results

Cell proliferation of DU-145 cells was first analyzed using the predefined treatment groups. Figure 1 shows the cytotoxic effect of *d*-δ-tocotrienol and xanthorrhizol on DU-145 human prostate cancer cells. Proliferation of DU-145 cells treated with 5 μM of *d*-δ-tocotrienol (T5) and 10 μM of *d*-δ-tocotrienol (T10) and 32.5 μM of xanthorrhizol (X32.5) was 56 ± 37%, 67 ± 7%, and 43 ± 7%, respectively, of that of untreated cells. Xanthorrhizol treatment reduced cell proliferation to a level significantly different from that of untreated cells ($P < 0.05$). A combination of 5 μmol/L of *d*-δ-tocotrienol and 32.5 μmol/L of xanthorrhizol (T5/X32.5) decreased cell proliferation to 17 ± 7% of that of untreated cells. The 83% inhibition of proliferation achieved by the combination is greater than the 44% (*d*-δ-tocotrienol) and 57% (xanthorrhizol) inhibitions induced by the two agents individually ($P < 0.05$). Similarly, the 81% inhibition of proliferation achieved by the combination of 10 μmol/L of *d*-δ-tocotrienol and 32.5 μmol/L of xanthorrhizol (T10/X32.5) is greater than the 33% (*d*-δ-tocotrienol) and 57% (xanthorrhizol) inhibitions ($P < 0.05$).

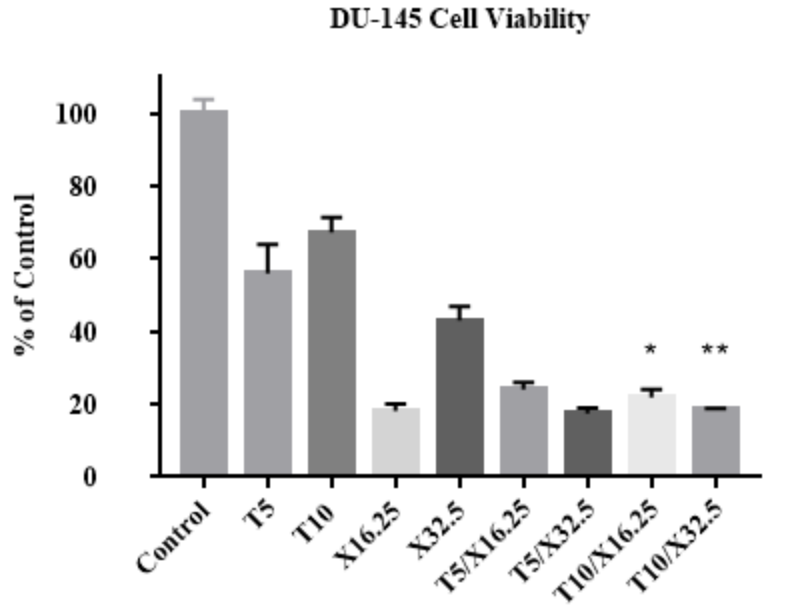


Figure 1. d-δ-Tocotrienol and Xanthorrhizol Treatments (μM)

Figure 1. Suppression of DU-145 prostate carcinoma cell proliferation induced by *d*-δ-tocotrienol and xanthorrhizol. Cells were plated at a density of 1.5 x 103 cells/well in 96-well plates with complete RPMI media. After 24 hours of incubation, wells were divided into respective treatment groups (6 replicates/group) and incubated for an additional 72 hours. Viable cell count was then determined using a CellTiter 96® Aqueous One Solution. Data are based upon 3 assays. Vertical bars represent percentage of viable cells according to respective treatments. Asterisks * and ** represent a significant difference between T10 and groups T10/X16.25 ($P < 0.01$), and T10/X32.5 ($P < 0.001$), respectively. Values are mean ± SEM, n = 18.

Cell cycle distribution of DU-145 cells was then evaluated (Fig. 2) to determine whether inhibition of cell proliferation induced by *d*-δ-tocotrienol and xanthorrhizol was attributed, at least in part, to cell cycle arrest. Percentage of cells in the G1 phase for 0

hour (when the treatment started) and control groups at 24 h was 44 ± 2% and 53 ± 4%, respectively. Cells treated with 5 μM of *d*-δ-tocotrienol (T5), 10 μM of *d*-δ-tocotrienol (T10), 32.5 μM of xanthorrhizol (X32.5), 5 μM of *d*-δ-tocotrienol and 32.5 μM of xanthorrhizol (T5/X32.5), and 10 μM of *d*-δ-tocotrienol and 32.5 μM of xanthorrhizol (T10/X32.5) exhibited G1 percentages of 46 ± 6%, 46 ± 3%, 46 ± 5%, 47 ± 7%, and 49 ± 12%, respectively. The percentage of cells in the S phase for 0 hour and control groups was 32 ± 6% and 28 ± 3%, respectively. Cells treated with T5, T10, X32.5, T5/X32.5, and T10/X32.5 exhibited S phase percentages of 36 ± 9%, 37 ± 5%, 44 ± 10%, 34 ± 12%, and 35 ± 11%, respectively. The percentage of cells in the G2 phase for 0 hour and control groups was 24 ± 4% and 19 ± 6%, respectively. Cells treated with T5, T10, X32.5, T5/X32.5, and T10/X32.5 exhibited G2 phase percentages of 19 ± 11%, 18 ± 6%, 9 ± 9%, 19 ± 11%, and 16 ± 6%, respectively. No significant differences in G1% were observed with any of the treatments. X32.5 significantly reduced the G2% as compared to untreated cells ($P < 0.05$), as noted by ***. Asterisks * and ** for G2 phase denote significant differences between groups X16 and X32, and X32 and T5/X16, respectively.

Figure 2. Cell Cycle Analysis

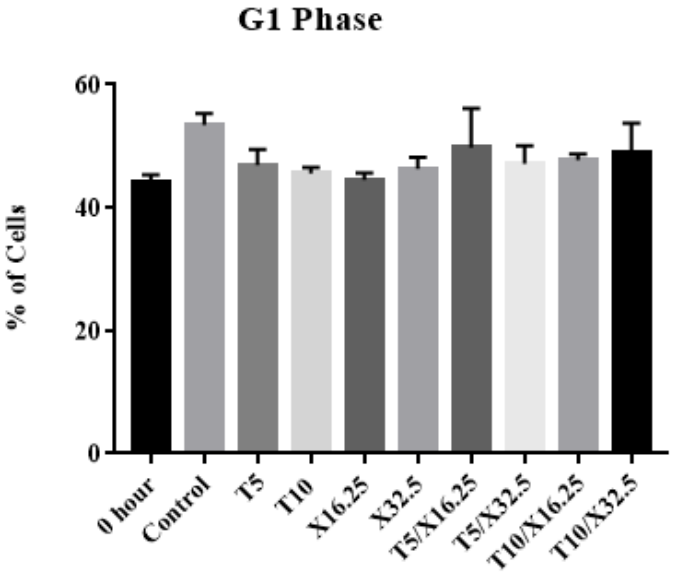


Figure 2a.

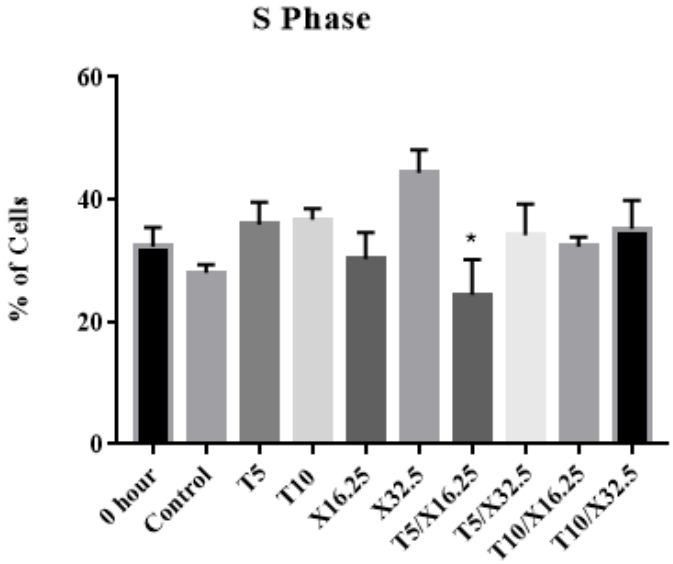


Figure 2b.

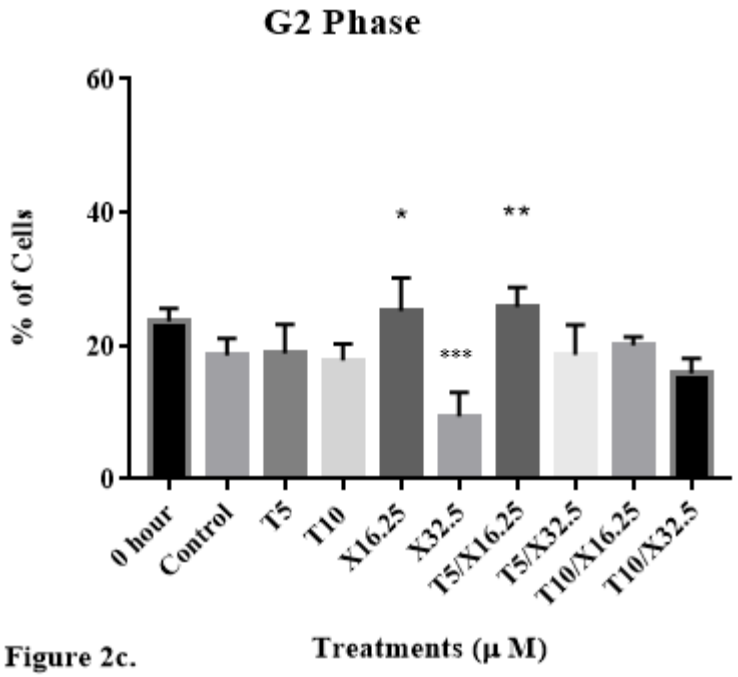


Figure 2c.

Figure 2. The impact of individual and combined effects of *d*-δ-tocotrienol and xanthorrhizol on the distribution of human DU-145 prostate cancer cells in the G1, S, and G2 phases of the cell cycle following 24 h of incubation. DU-145 cells were plated at a density of 1.5 x 10^6 cells per 25 cm^2 flask with 3 ml media and incubated for 24 hours. Cells were then treated and incubated for 24 hours. After incubation, cells were trypsinized, stained with propidium iodide, and analyzed for cell cycle distribution using flow cytometry. Brackets in the S phase denotes a significant difference between T5 and T5/X16.25; brackets in the G2 phase denotes a significant difference between X32.5 and the control. Tukey's multiple comparison test was used for repeated-measures analysis of variance. Values are mean ± SEM, n = 6.

Figures 3a and 3b illustrate the impact of individual and combined treatments of *d*-δ-tocotrienol (T) and xanthorrhizol (X) on 24-h expression of Cdk4 and cyclin D1,

respectively, two regulatory proteins of the G1/S phase transition in the cell cycle of DU-145 cells. Levels of Cdk4 were 97 ± 24%, 96 ± 39%, 76 ± 21%. 97 ± 40%, 91 ± 20% of that of control for cells treated with T5, T10, X32.5, T5/X32.5 and T10/X32.5, respectively. Levels for cyclin D1 were 128 ± 14%, 114 ± 16%, 112 ± 18%. 103 ± 32%, 155 ± 34% of that of control cells for cells treated with T5, T10, X32.5, T5/X32.5 and T10/X32.5, respectively. No significant difference was observed between any groups.

Figure 3c. illustrates the impact of *d*-δ-tocotrienol (T) and xanthorrhizol (X), individually and in combinations, on 24-h expression of procaspase-3 (PC-3), a pro-apoptotic protein. Levels for PC-3 were 118 ± 15%, 91 ± 23%, 112 ± 25%, 77 ± 4%, 107 ± 84% of that of control cells for cells treated with T5, T10, X32.5, T5/X32.5 and T10/X32.5, respectively. No significant difference was observed between any groups.

Figure 3. Western Blot Analysis

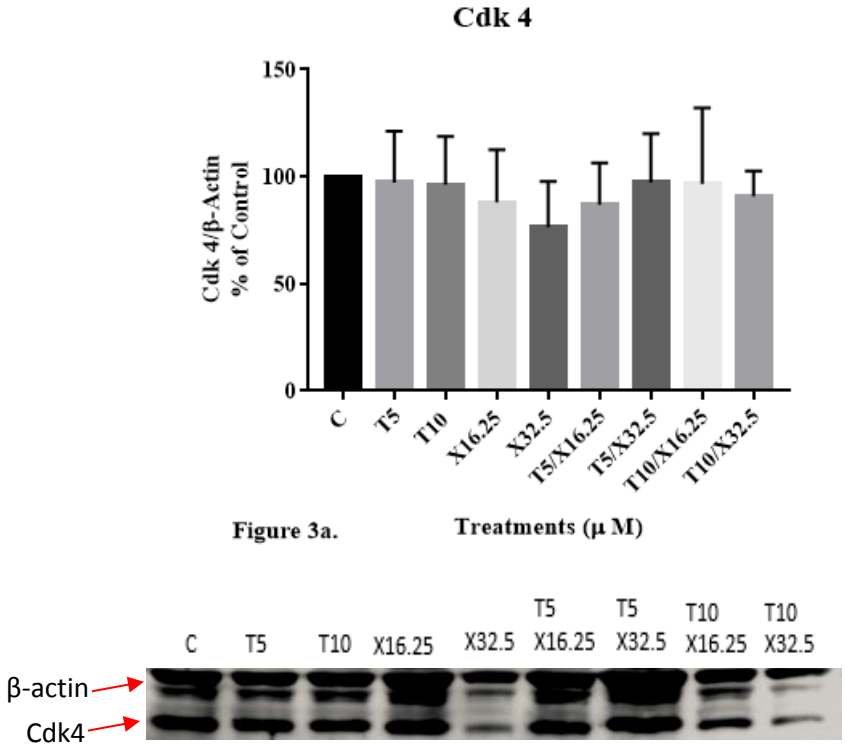


Figure 3a.

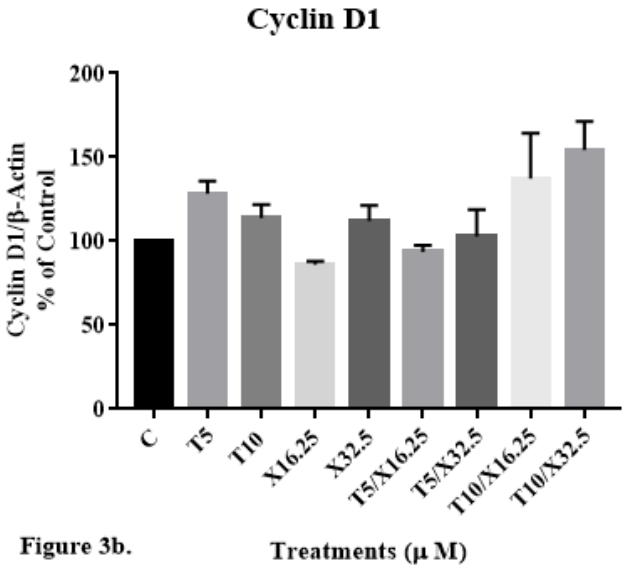
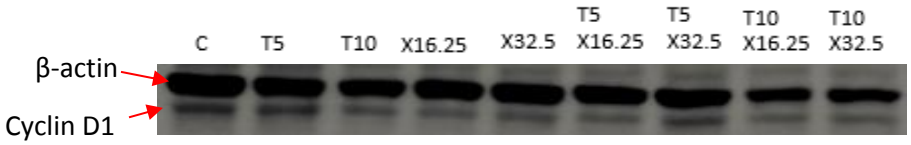


Figure 3b.



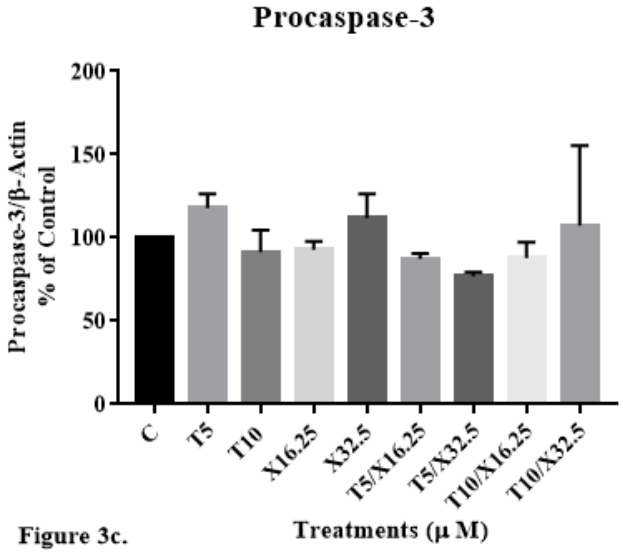


Figure 3c.

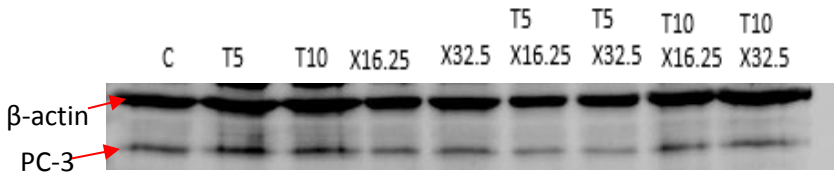


Figure 3. Representative blots showing the impact of *d*-δ-tocotrienol (T) and xanthorrhizol (X), individually and in combinations, on 24-h expression of cyclin D1 (a), Cdk4 (b), and procaspase-3 (c), in human DU145 prostate cancer cells. Western blots procedures were conducted on cell lysates and blots were detected by chemiluminescence and quantified using ImageJ software. Tukey's multiple comparisons test was used to analyze significant variance between groups. Values are mean ± standard error of the mean (SEM), n = 3.

CHAPTER V

Discussion and Conclusions

Building on previous studies showing the anti-proliferative, cell cycle arresting, and apoptotic effects of tocotrienols and xanthorrhizol in cancers of prostate, liver, breast, colon and skin, this thesis studied the synergistic effects of tocotrienol and xanthorrhizol in prostate cancer cells for the first time. Our cell proliferation data show a similar pattern, with decreased cell proliferation shown in cells treated with *d*-δ-tocotrienol and xanthorrhizol, individually and in combination. The combined treatments (T5/X32.5 and T10/X32.5) showed a significant decrease in cell viability compared to the control (no treatment). Proliferation of cells treated with *d*-δ-tocotrienol (T5, T10) was not significantly lower than the control, whereas individual treatment with xanthorrhizol (X32.5) led to cell proliferation significantly lower than that of the control. There was a significant decrease in groups T5/X32.5 and T10/X32.5 ($p < 0.001$), compared to group T10.

These data suggest that combined treatments of *d*-δ-tocotrienol and xanthorrhizol may have a more potent impact on DU-145 cell viability compared to treatments of the two compounds alone. The X16.25 group showed inconsistent results in cell proliferation. Inadvertent variations in preparation of xanthorrhizol stock solution and density of inoculated DU-145 cells may have contributed to the variations. Additional studies are needed to more accurately determine the effect of xanthorrhizol on DU-145 cell proliferation. The lack of consistent response with X16.25 treatment also precludes

the possibility of a CompuSyn analysis because a minimum of two concentrations of either compounds in a combination is required for the isobologram analysis.

Cell cycle analysis showed no significant differences between any of the treatment groups and the control in the G1, S, and G2 phases with one exception; X32.5 significantly reduced the percentage of cells in G2. Additionally, T5/X32.5 significantly increased the percentage of cells in S phase compared to X32.5. It is perplexing that X32.5 and T5/X16.25 caused opposite effect on the distribution of cells in the G2 phase. Literature has shown that tocotrienols induce G1/S arrest in human prostate (19) and melanoma (5) cancer cells and xanthorrhizol induces G1 and G2/M arrests in human colon cancer cells (21). The asynchronized cells that started in various phases of the cell cycle before treatments and the timing and length of the treatments may have caused some of the variations in these findings. Further studies are required to elucidate the effect of these compounds on cell cycle distribution in DU-145 cells.

The lack of consistent cell cycle arrest induced by the compounds may be congruent with western blot analysis finding no significant difference in Cdk4 and cyclin D1 levels between treatment groups and the control. Previous studies have shown that tocotrienols reduced Cdk4 expression in human melanoma cells. (5) Cell line-specific responses, different sources of antibodies, and technique-related variations in blot quantification may have led to the lack of detection of treatment effects. Additionally, varying treatment times may have affected cell response. Cell viability was assessed after 72 hours of treatment, whereas cell cycle analysis and western blot lysate collection was conducted after 24 hours of treatment.

As one of the early events in initiating apoptosis, procaspase-3 is cleaved to a shorter but active caspase-3 that drives apoptosis. Treatments with T5/X16.25, T5/X32.5, and T10/X16.25 led to slight and non-significant decreases in the level of procaspase-3 (87%, 77%, and 88%, respectively) when compared to the control (100%). This finding suggests that caspase-3 may be activated with combined treatments but that the doses of treatments may need to be altered to show a significant effect for the initiation of apoptosis.

Data from this study showed a significant decrease in cell viability from individual and combined treatments of *d*-δ-tocotrienol and xanthorrhizol. However, we did not show significant down regulation of Cdk4 or cyclin D1 for induction of cell cycle arrest, or procaspase-3 activation for apoptosis initiation. Multiple explanations for these observations are possible. Firstly, changes in doses of *d*-δ-tocotrienol and xanthorrhizol in individual and combined treatments may increase their efficacies in downregulation of the proteins of interest. Secondly, *d*-δ-tocotrienol is a fat-soluble vitamin and may have contributed to the cellular uptake of xanthorrhizol.

Third, detached cells were not used in any of the experiments described. After plating and treatment, some cells may detach before collection. Including detached cells for analysis may contribute to a larger sample size with a higher proportion of apoptotic cells with activation of pro-caspase-3.

Fourth, using a colorimetric assay to detect caspase-3 activity in DU-145 cells may be assessed in future studies, providing a direct approach for detecting apoptosis. Other caspases may also provide insight to apoptosis as a potential mechanism of action. As previously mentioned, caspases 2, 8, and 9 are initiator caspases that cleave

downstream effectors caspases 3, 6, and 7. Effector caspases execute apoptosis by cleaving cellular proteins. Initiator and effector caspases may need to be studied simultaneously to determine if initiation and/or execution of apoptosis has occurred in DU-145 cells after individual and combined treatments of *d*-δ-tocotrienol and xanthorrhizol.

Alternatively, suppression of cell proliferation may have been caused by other signaling pathways that also regulate cell growth. For example, *d*-δ-tocotrienol has been shown to decrease viability of prostate and esophageal cancer cells, respectively, by inhibiting nuclear factor kappa B cells (NF-kB) of the Akt/NF-kB pathway. NF-kB, a pro-inflammatory transcription factor, is an important regulator in cell survival. Downregulation of NF-kB decreases inflammation, thereby decreasing the chance of certain cancers. (16)

In conclusion, combination of *d*-δ-tocotrienol and xanthorrhizol inhibited the growth of DU-145 cells with greater potencies than those of individual agents. Our limited studies did not show a significant change in cell cycle arrest or down regulation of Cdk4 and cyclin D1 in induction of cell cycle arrest, or activation of procaspase-3 for apoptosis initiation. Further studies are needed to confirm these results.

REFERENCES

1. Key Statistics for Prostate Cancer. American Cancer Society. https://www.cancer.org/cancer/prostate-cancer/about/key-statistics.html. Published 2017. Accessed October 27, 2017.

2. What Are the Symptoms of Prostate Cancer? Centers for Disease Control and Prevention. https://www.cdc.gov/cancer/prostate/basic_info/symptoms.htm. Published December 4, 2013. Accessed May 12, 2017.

3. Chemotherapy for Prostate Cancer. American Cancer Society. https://www.cancer.org/cancer/prostate-cancer/treating/chemotherapy.html. Updated March 11, 2016. Accessed October 28, 2017.

4. Song BL, DeBose-Boyd RA. Insig-dependent ubiquitination and degradation of 3-hydroxy-3-methylglutaryl coenzyme a reductase stimulated by δ- and γ-tocotrienols. *J Biol Chem.* 2006; 281(35): 2554-61.

5. Fernandes NV, Guntipalli PK, Mo H. *d*-δ-Tocotrienol-mediated cell cycle arrest and apoptosis in human melanoma cells, *Antican Res*. 2010; 30: 4937-4944.

6. Seok Fang O, Nallappan M, Yew Hoong C, et al. Xanthorrhizol: a review of its pharmacological activities and anticancer properties. *Can Cell Int.* 2015; 15: 1-15.

7. Knudsen ES, Wang JY. Targeting the RB-pathway in cancer therapy, *Clin Can Res*. 2010; 16: 1094-1099.

8. Hydbring P, Malumbres M, Sicinski P. Non-canonical functions of cell cycle cyclins and cyclin-dependent kinases. *Nat Rev Mol Cell Bio*. 2016; 17(5): 280-292.

9. Gil J, Peter G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one and one for all. *Nat Rev Mol Cell Bio*. 2016; 7(9): 667.

10. Dimova D, Dyson N. The E2F transcriptional network; old acquaintances with new faces. *Onc.* 2005; 24(17): 2810.

11. Dynlacht BD. E2F and p53 make a nice couple: converging pathways in apoptosis. *Cell Death and Diff. 2005;* 12: 313-314.

12. Pradelli L, Beneteau M, Ricci J. Mitochondrial control of caspase-dependent and independent cell death. *Cell and Mol Life Sci.* 2010; 67(10): 1589-1597.
13. Hengartner M. The biochemistry of apoptosis. *Nat.* 2000; 407(6805): 770.

14. Mo H, Elson CE. Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. *Exp Biol Med.* 2004; 229: 567-585.

15. Goldstein JL, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. *Cell*. 2006; 124: 35-46.

16. Tan, Barrie. Watson, Ronald. Preedy, Victor. *Tocotrienols: Vitamin E Beyond Tocopherols.* Boca Raton, FL: CRC Press; 2013.

17. Mo H, Elfakhani M, Shah A, et al. Mevalonate-suppressive tocotrienols for cancer chemoprevention and adjuvant therapy. Edtion ed. In: Watson RR, Preedy VR, Tan B, eds. Tocotrienols: vitamin E beyond tocopherols. Boca Raton: CRC Press, 2013: 135-49.

18. Har C, Keong C. Effects of tocotrienols on cell viability and apoptosis in normal murine liver cells (BNL CL.2) and liver cancer cells (BNL 1ME A.7R.1), in vitro. *Asia Pac of Clin Nutr.* 2005; 14(4): 374-380.

19. Srivastava JK, and Gupta S. Tocotrienol-rich fraction of palm oil induces cell cycle arrest and apoptosis selectively in human prostate cancer cells. *Biochem and Biophy Res Comm.* 2006; 346(2): 447-453.

20. Cheah YH, Nordin FJ, Sarip R, et al. Antiproliferative property and apoptotic effect of xanthorrhizol on MDAMB-231 breast cancer cells. *Antican Res*. 2008; 28: 3677–90.

21. Kang YJ, Park KK, Chung WY, Hwang JK, Lee SK. Xanthorrhizol, a natural sesquiterpenoid, induces apoptosis and growth arrest in HCT116 human colon cancer cells. *J Pharm Sci*. 2009; 111:276–84.

22. Handayani T, Sakinah S, Nallapan M, Pihie AH. Regulation of p53-, Bcl-2-, and caspase- dependent signaling pathway in xanthorrhizol-induced apoptosis of HepG2 hepatoma cells. *Antican Res*. 2007; 27: 965–7.