ANTIOXIDANT EFFECTS OF PHYTOCHEMICALS IN TEXAS RED WINE IN BREAST CANCER CELLS

A Senior Scholars Thesis

by

ALEXANDRA ELIZABETH HAGOOD

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Nutritional Science

ANTIOXIDANT EFFECTS OF PHYTOCHEMICALS IN TEXAS RED WINE ON BREAST CANCER CELLS

A Senior Scholars Thesis

by

ALEXANDRA ELIZABETH HAGOOD

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by:

Research Advisor: Susanne Talcott
Associate Dean for Undergraduate Research: Robert C. Webb

April 2009

Major: Nutritional Science

ABSTRACT

Antioxidant Effects of Phytochemicals in Texas Red Wine on Breast Cancer Cells.
(April 2009)

Alexandra Elizabeth Hagood
Department of Nutrition and Food Science
Texas A&M University

Research Advisor: Dr. Susanne Talcott
Department of Food and Nutritional Science

The anti-cancer effects of wine have been greatly studied, mainly on *Vitis Vinifera* grapes. There is a lack of information about the effects of Texas wines in particular, and it was the goal of this study to investigate two of these wines (one Syrah and one Port). Antioxidant capacity of the wines was determined using an ORAC assay. Effects on cell proliferation and reactive oxygen species were tested on invasive estrogen-receptor negative breast cancer cells (MDA-MB-231). These cells were treated with extract concentrations from 12.5-200 µg GAE/mL diluted in DMSO (up to ~0.2% volume in medium). When treated with 50 µg GAE/mL Syrah wine extract, cell proliferation was decreased to 59% of the control, and at 200 µg GAE/mL cell proliferation was decreased to 41%. When treated with 50 µg GAE/mL Port wine extract, cell proliferation decreased to 54% at and to an impressive 18% at 200 µg GAE/mL. When treated with Syrah wine extract, the generation of reactive oxygen species decreased in a dose-dependant manner from 71.8% at 12.5 µg GAE/mL to 27.2% of the control at 200 µg GAE/mL and still 67% at 12.5 µg GAE/mL. The Port wine extract was less effective at

low concentrations (decrease in ROS to 85.8% at 12.5 µg GAE/mL). At higher concentrations, results were similar to that of the Syrah wine extract. Results were conflicting as the Syrah had a greater effect on the inhibition of generating reactive oxygen species while the Port had a greater effect of cell growth inhibition. This study will lead to further, more detailed studies identifying isolated polyphenolics from wines causing the beneficial effect, the mechanisms behind their effects and possibly, how these compounds could be utilized to prevent or slow the progression of this invasive cancer.

DEDICATION

This thesis is dedicated to my wonderful family and my soon-to-be-family fiancé. You have all supported and encouraged me throughout this endeavor. Thank you for listening to me when days in the lab were frustrating, and for joining in my excitement when my experiments worked! You have no idea how you have kept me motivated and working hard while still enjoying my last year in college. Special thanks are extended to my mother for practically planning my entire wedding while I was tied up in College Station with this project and other school work. You are the most loving and creative person I know and May 31, 2009 will be beautiful because of all the hard work you've put into it.

ACKNOWLEDGMENTS

I would like to thank first and foremost Dr. Susanne Talcott for accepting me into her lab as an elective student and then as an undergraduate scholar. Throughout the year she always kept me on track and ensured every step of my project was executed to the highest quality. Her commitment to assisting with my project and other undergraduates on their way to graduate school was profound, even while becoming a mom again!

I also need to extend infinite thanks to Armando del Follo for his unending patience. Working on his Ph.D., writing papers and running multiple projects concurrently, he would often drop what he was doing to double, triple and quadruple check my calculations and techniques and answer any and all of my questions. This project would never have been possible without him.

Dr. Steve Talcott and all of the graduate and Ph.D. students in both of the Dr. Talcotts' labs were indispensable resources during my project. Thanks must be given to Giuliana Noratto, Gabriele Angel, Michelle Bertoldi, Emily Townsley, Salvador Bertucci, Lius Maldonato, Kaylee Shepherd, Chris Duncan, Jorge Cardona, and Lisbeth Pacheco for answering my infinite questions, showing me new techniques, analyzing results, carrying materials back and forth between rooms for me when I was on crutches, and always switching the conversation to English when I entered a room!

A large part of this project is due to Abida Hasan and Kimberly Krenek who kept me motivated while also completing their own projects.

NOMENCLATURE

AOX	Antioxidant
DMSO	Dimethylsulfoxide
FBS	Fetal Bovine Serum
GAE	Gallic Acid Equivalents
MDA-MB-231	Estrogen Receptor Negative Late Stage Breast Cancer Cell Line
ORAC	Oxygen Radical Absorbance Capacity Assay
PBS	Phosphate Buffer Solution
ppm	Parts per Million
ROS	Reactive Oxygen Species
TTX	Treatment

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Cancer is the second leading cause of death in America, second to heart disease, as of 2005 (1). A trend was noticed, in that the occurrence of cancer was much lower in those who ate larger quantities of fruit and vegetables in their diet (2). Further investigation pointed to phytochemicals in many of these fruits and vegetables having antioxidant properties and that they help to prevent and/or slow the progression of many types of cancer cells. Several in vitro and in vivo studies have demonstrated the antioxidant and anti-inflammatory effect of phytochemicals relevant to several types of cancer (3-12).

This project proposes to study two Texas-specific winegrape varieties and the anti-cancer effects of their polyphenolics by measuring basic antioxidant, cell death and anti-inflammatory biomarkers in breast cancer cells. Before discussing the specifics of this project, it will be useful to give a general background of breast cancer, its risk factors and development, and why it is thought these phytochemicals from winegrapes may play a role in the future of cancer prevention and treatment.

This thesis follows the style of Molecular and Cellular Biochemistry.

Breast cancer

Among women in the United States, breast cancer is the most common form of cancer and is second only to lung cancer as the leading cause of cancer death. It has been estimated that 182,460 women will be diagnosed with invasive breast cancer and 40,480 will die of it in 2008 (13). Of those with invasive breast cancer, 5-10% of cases are known to be due to one of the most proven risk factors: heredity (12).

Unfortunately, heredity, like other known factors including genetics, age, race, menarche, breast tissue density, having had no children, personal (13) and family medical history (13, 14), late parity (or age of first full term pregnancy) (2, 14) and education (14) are unalterable. However, other risk factors such as using birth control pills (13), forgoing breast feeding, low levels of physical activity, obesity (13, 14), poor diet (2, 14) and alcohol consumption (13-15) are lifestyle behaviors that can easily be adjusted to reduce risk. Additionally, treatments such as post-menopausal hormone therapy, radiation and DES (diethylstilbestrol) are risk factors. It should be noted that while breast cancer occurs primarily in women, men can develop it as well (13).

The development of breast cancer begins in the cells of the breast tissue: cells that line the ducts of the mammary gland or cells in the lobes of the breast are damaged and over many stages develop into a malignant tumor (13). Hormones play a role in breast cancer risk and some hypothesize that alcohol (not distinguishing between beer, liquor and wine) intake increases amounts of certain circulating sex hormones and affects a

person's risk (13, 15). Most early-stage breast cancers are estrogen-receptor (ER)-positive and respond to endocrine treatment with antiestrogens. However, later-stage breast cancers are ER-negative and more aggressive, requiring treatment with cytotoxic drugs. Since antiestrogens do not affect these cases, another property of the cancer must be exploited: reactive oxygen species (ROS). ROS are small molecules or ions with a free electron and can be oxygen ions or free radicals, which are produced by the mitochondria during energy metabolism (8). It has been learned that breast cancer initially begins with ROS-induced damage to a cell's DNA (4, 11, 12) by causing lesions on the DNA that change a normal breast cell into a malignant one. Healthy human cells are equipped with repair mechanisms to rapidly mend any DNA damage. One study induced oxidative damage in different lines of breast cancer cells with hydrogen peroxide (H_2O_2) and showed that, despite a repair period, the repair mechanisms were ineffective against these lesions. Cells from the line of invasive ductal breast carcinomas were more susceptible to oxidative damage and, also unable to repair it (12). The accumulation of unrepaired damage over time will eventually lead to cancer. Research is now focused on slowing the progression of this damage to decelerate or possibly reverse present cancer, and keeping the damage from accumulating, thus preventing cancer from occurring.

Beneficial properties of wine

Herbal preparations have been extensively used as traditional medicines by most cultures for thousands of years. Various diseases have been treated with crude or refines extracts

from many natural sources, and the current widespread use of many nutraceuticals and herbal extracts stems from the long history of their therapeutic applications (16). Individual natural products from various sources have proven to be highly effective in the treatment of many diseases and some of the earliest medicines including aspirin, morphine, quinine, digitoxin and pilocarpine were derived from plants (16, 17).

Much research has been conducted in the isolation of phytochemicals from grapes and red and white wine and to determine the mechanisms behind the desirable effects they have on cancer cells. Studies have shown that compounds in grapes and wine are health-promoting and disease-preventing (16) due to their antioxidant and anti-cancer properties proven both in vitro and in vivo (11, 18). They may even decrease prevalence of coronary heart disease (14). Compounds that have been studied and proven to have helpful effects are phytochemicals; as they are disease preventing chemicals from a plant, antioxidant in function, and polyphenolic in structure due to the multiple phenol rings. The bitterness and astringency of wines can be attributed to these polyphenolics (9). It follows then, that red wine has a polyphenolic concentration that is six to seven times higher than that of white wine (11). Also, wine grapes have a higher concentration than table grapes (9). The polyphenol composition in a grape is dependant on the color, year, growth site, the degree of maturation and part of the grape. The seeds, which are normally waste from the wine making process, actually contain 5-8% polyphenolics including procyanidins and flavonoids such as gallic acid and catechin (9). Other polyphenols in wine are phenolic acids and trihydroxy-stilbenes (resveratrol) (11).

These antioxidants in wine are absorbed in the upper gastrointestinal tract after it is ingested, and tend to be found in greater concentrations in the liver, heart and kidney (11). One study showed that pure polyphenolics are more effective than in wine (11) but even antioxidants in wine extracts, which still contain non-polyphenolic compounds, protect against aging, disease and decay. These polyphenolics have four to five times the antioxidant power of Vitamins C and E (9), and it is their structure that gives them these properties. The structure of phytochemicals contains multiple phenol rings and hydroxyl (-OH groups) which allow for electron transfer and reduction, as seen below in Figure 1 (4, 9).

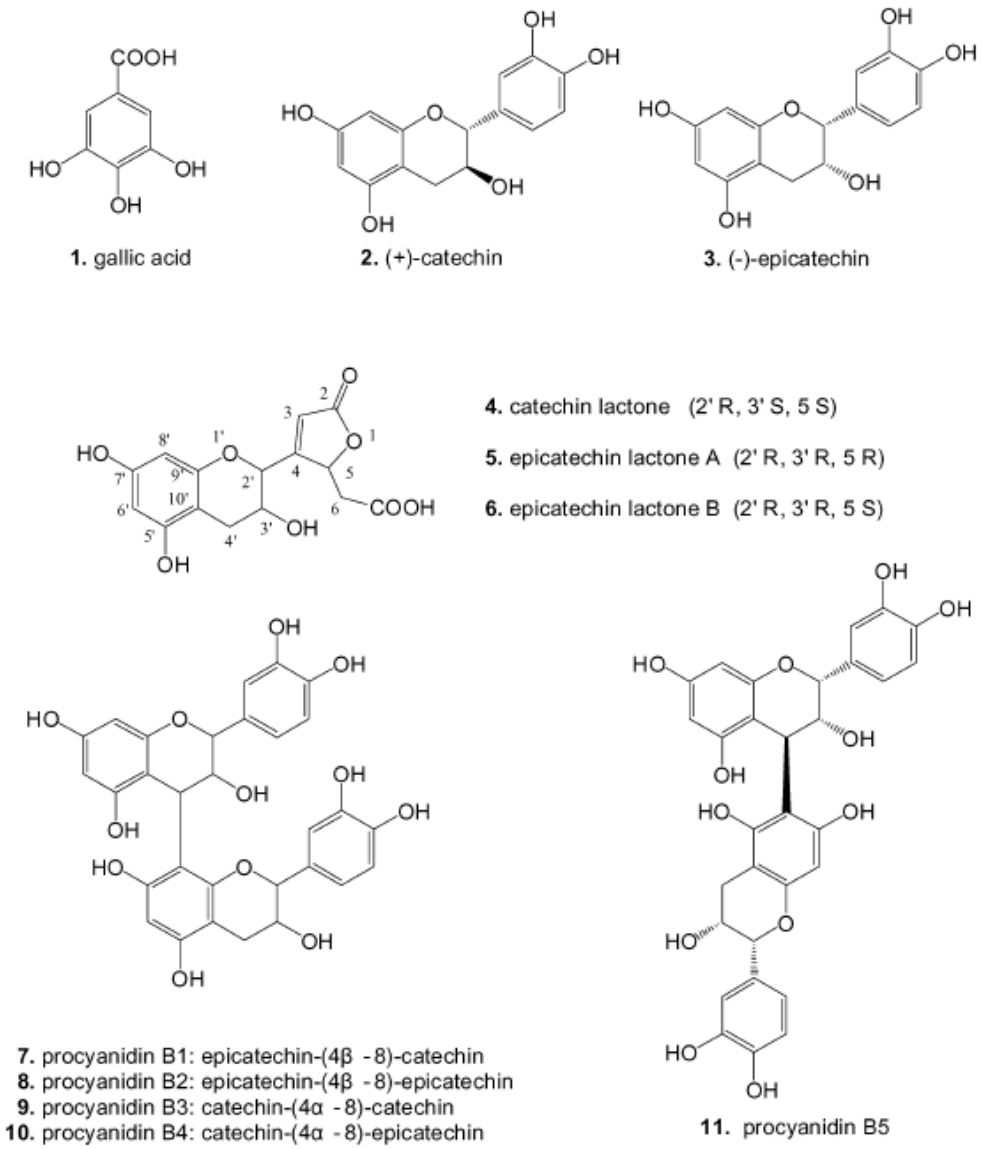


Fig. 1 Chemical Structure of 11 Individual Polyphenols (4).

The structure of these compounds allows them to act as radical scavengers by breaking radical chain reactions and putting an end to their detrimental effects (9, 11). An example of this was demonstrated in a study done by Murias, et al. Hydroxyl groups were added to resveratrol and the new compound (3,3',4,4',5,5'-hexahydroxystilbene aka:

M8) interacted directly with the mitochondria-the organelle which produces free radicals. This new compound, M8, deactivated the cells' defense against antioxidants (8). Besides ridding the cell of radicals and allowing the antioxidants to work, polyphenols such as ellagic acid and quercetin can act synergistically and induce cell-death or apoptosis thus killing the cancer cells (17). Each of these compounds and their mechanisms are undergoing much research to be fully understood.

Previous studies

Many studies have been performed investigating properties of wine and its specific compounds. One potent antioxidant found in grapes and wine that has been widely researched is resveratrol. One study examined the extracts from the skin and seed of a particular grape, the muscadine grape. It was seen that resveratrol inhibited prostate cancer cells partly because of its antioxidant capacity but, as it turns out, there is not a large amount of resveratrol in the skin compared to the seed. Somewhat unexpectedly, cell growth was decreased by both. The resveratrol in the seed stopped cells during their cycle, confirming many studies done before this, such as the previously discussed Murias study. What was unique was the discovery that a compound in the skin extract disrupted Akt activity, causing apoptosis and cell death (5). In 1999, a study showed that resveratrol was the cause of decreased growth, viability and the expression of anti-apoptotic compounds in leukemia cells. These cells, which were not strong survivors, eventually self destructed because of the antioxidant. It was also the reason behind inhibition at every step in a multi-step carcinogenesis (10). While studies have shown

the positive effects of a compound, it is important to rule out any negative side effects. Rats were tested with high enough doses to correspond to 1000 times what an average ~154 lb person would consume. While this is not the case of every compound, there were no harmful effects due to elevated resveratrol (6).

Another well studied phytochemical is procyanidin. It has been seen to decrease both inflammation and cancer cell growth (9). Mouse spleen cells were treated with procyanidin B4, catechin and gallic acid and had protection from H_2O_2 at low doses. Once the level of protection peaked, damage seen to the cells increased with the concentration of catechin (4). This dose-dependence was also seen in a study done in 2000. Polyphenolics from red wine lead to decreased H_2O_2 susceptibility, cell proliferation and tumor arrest both in vitro and in vivo initially. Higher concentrations, as previously seen, did cause a stimulatory effect. Although this is counter-productive, a human would need to chronically ingest red wine for any bioeffective outcomes so the risk of ingesting too much of these antioxidants from wine is relatively small (11).

Most studies show the cancer fighting aspects of red wine compounds. In contrast, phenolic compounds piceatonnol (PIC) and myricetin (MYR) that appear in high quantities in wine were shown to have estrogen-like activity. They increased cell growth in estrogen-dependant breast cancer cells. Unlike other studies, this one concluded that drinking red wine could actually be a risk factor in post-menopausal women (7).

As is often the case, results are incomplete and sometimes contradictory. One area that is lacking is the study of Texas wines, specifically. Based on the unique climate and plant-disease conditions in the state of Texas, only specific grape varieties (*Vitis vinifera*) can be grown. It is important to identify the compounds and their antioxidant properties so these wines may be compared to more popular and more researched varieties.

CHAPTER II

METHODS

Phytochemical extracts

Barrel Port Reserve wine was provided by Messina Hof in Bryan, TX. Syrah wine was provided by Kiepersol Estates in Tyler, TX. Solid phase extraction was used to extract wine polyphenolics. The wines were diluted 1:5 in nano-pure water then filtered through a C-18 Sep-pak filter. This filter removed water, sugar and other unwanted compounds while binding the polyphenolics. Acidified methanol was used to elute the wine extract from the filter into a separate flask. Once the entire amount of wine had been filtered in this way (350mL), the methanol was evaporated using a Büchi rotavapor. Approximately 15 mL of the wine/methanol mixture were poured into a small flask and rotated in 35°C water while being rotated. A pump was attached to the rotavapor to create a vacuum, thus lowering methanol's boiling point and allowing it to evaporate. Each 15 mL amount of sample was on the rotavapor for 15 minutes to avoid decomposing the compounds. Some methanol remained. The extract was concentrated in a SpeedVac at 43°C for 1.5 hours and stored at -80°C.

Polyphenolic concentration

The concentration of polyphenolics in each vial of extract, aka: total soluble phenolics, was determined by Folin-Ciocalteu's reagent assay. The extract was suspended in 5µL

DMSO, then further by a factor of 10 with nano-pure water. Dilutions were made with gallic acid to make a standard curve with which to compare the extract sample. Gallic acid concentrations of 1000, 800, 600, 500, 400, 300, 200 and 100 ppm were utilized. 0.50 mL Folin's reagent was added to 50µL of each sample (extract and gallic acid), in a 5mL tube, mixed and left for 3 minutes. 0.50mL Sodium carbonate was then added, mixed and solutions were left for 7 minutes. After such time, 3.95mL nano-pure water was added and mixed, and then the samples were kept out of light for one hour. 100µL of each sample was added to a well in a 96 well plate and absorbance was measured in a plate reader. Final concentrations were expressed as gallic acid equivalents (GAE). These concentrations were used to dilute treatments for assays.

Cell culture

Non-hormone dependant breast cancer cell line MDA-MB-231 was cultured in Dulbecco's Modified Eagle Medium-High Glucose (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (100,000 U/L penicillin and 100 mg/L streptomycin). The cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C. MDA-MB-231 cells can be seen in Figure 2.

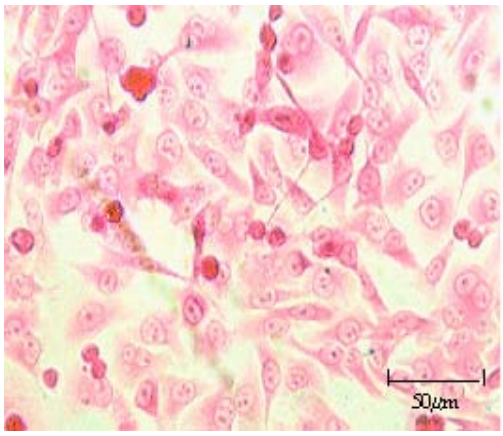


Fig. 2 MDA-MB-231 Breast Cancer Cells (19)

ORAC

The antioxidant capacity of the Syrah and Port wines were determined with an ORAC assay as described by Cao (19). The wine was diluted 200, 150 and 100x with ORAC buffer and compared to a standard curve made with different concentrations of Trolox-a water soluble form of Vitamin E, a known antioxidant (200, 100, 50, 25, 12.5ppm). 50µL samples of each dilution were added to wells in a black, clear bottom 96-well plate. 100 µL fluorescin dilution (18 µL fluorescin in 50mL ORAC buffer) was added to the samples. 330mg ?, a radical, was dissolved in 5mL ORAC buffer to make AARH solution just before 50 µL was added to the wells. Fluorescence was read at 37°C in a plate reader every 5 minutes for two hours.

Cell proliferation

To study the effect of the wine extracts on the growth of MDA-MB-231 breast cancer cells, 15,000 cells were first seeded into each well of a 24-well plate. To seed the cells, or attach them to the plate to prepare for the assay, medium was removed from the plate and the cells were washed with 10 mL buffer PBS to remove any debris and dead cells. 2mL trypsin were added to the dish and incubated for 4 minutes to detach the cells. 10 mL FBS was then added to the dish and the plates' contents were placed in a falcon tube and centrifuged for 2 minutes at 1000rpm. After the FBS was removed a pellet of cells remained at the bottom of the tube. These were re-suspended in 5 mL of DMEM containing 2.5% FBS and counted. Once a solution of the desired concentrations of cells was prepared (30,000 cells/well), 0.5mL was added to each well and incubated for 24 hours. Plates were treated with different concentrations of wine extract (200, 150, 100, 75 and 50 mg GAE/mL) and incubated for 72 hours. The control "treatment" contained only medium with 0.2% DMSO (as did the other treatments) so to offset any effect it may have caused to the cells. The layout of the plate was as in Figure 3.

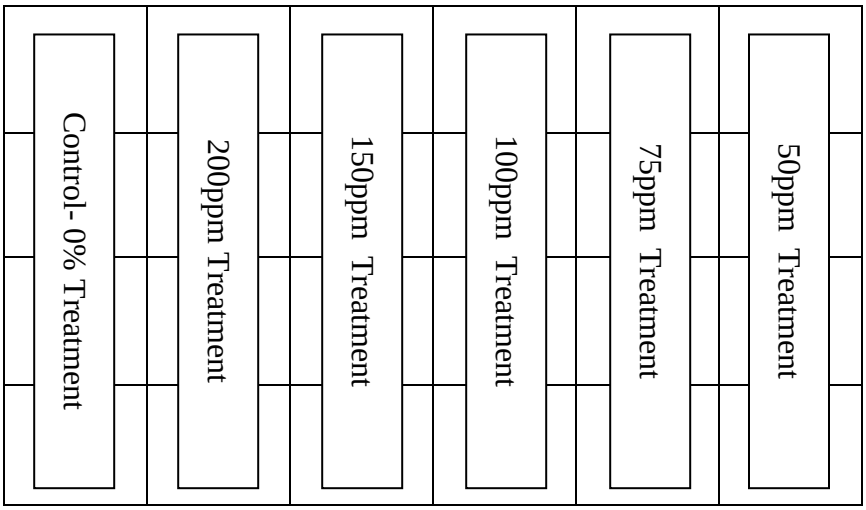


Fig. 3 Cell Proliferation Plate Layout

After the 72 hour incubation time, cells were detached with 0.25mL of trypsin. Instead of medium, trypsination was arrested with PBS containing 10% FBS. Contents of each well were transferred into a cuvette containing 20mL isotonic solution. Remaining cells were removed with a cell scraper. After scraping, buffer was added to each well to re-suspend the newly detached cells. All contents of each well were recovered and transferred into the well's corresponding cuvette. The number of cells, and thus cell growth, was determined using a cell counter (Beckman Coulter, Fullerton, CA).

Generation of reactive oxygen species

Inhibition of ROS generation was measured with the ROS assay. 10,000 MDA-MB-231 breast cancer cells were seeded in each well of a 96 well plate and incubated for 24 hours. Cells were treated with different concentrations (0, 12.5, 25, 50, 75, 100, 150,

200 µg GAE/mL) of extract and incubated for 24 hours. The plate layout is shown in Figure 4.

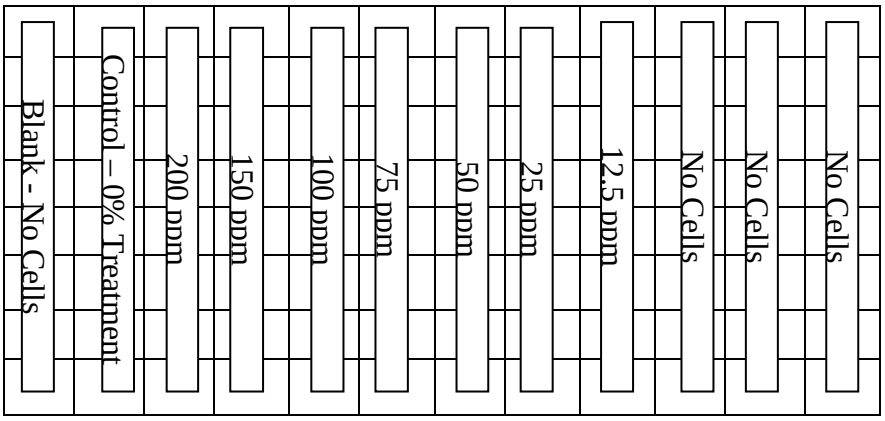


Fig. 4 ROS Plate Layout

Oxidative stress was induced with 200µM hydrogen peroxide for 2 hours. Cells were washed with PBS and incubated with 10mM complete name (DCFH-DA) for 15 minutes. Intensity of fluorescence was read at 37°C after 15 minutes in a microplate reader (485 nm excitation and 538 nm).

CHAPTER III

RESULTS

Polyphenolic concentration

Results from the Folin-Ciocalteu's assay are shown in Table 1.

Table 1 Polyphenolic Concentration of Wine Extracts

Extract Vial	Syrah 1	Syrah 2	Port 1	Port 2	Port 3
Equation of Best Fit Line	y=1289.2x-172.86	y=1155.5x-177.49	y=1186.6x-167.63	y=1214.6x-134.1	y=121.17x-1048.6
R^2	0.9975	0.9984	0.9926	0.9951	0.9833
Average µg GAE/mL	6347.522	6109.462	4339.762	4663.173	9956.891

ORAC

The antioxidant capacities of the wines were determined with an ORAC assay. The standard curve made with different concentrations of Trolox is shown in Figure 5.

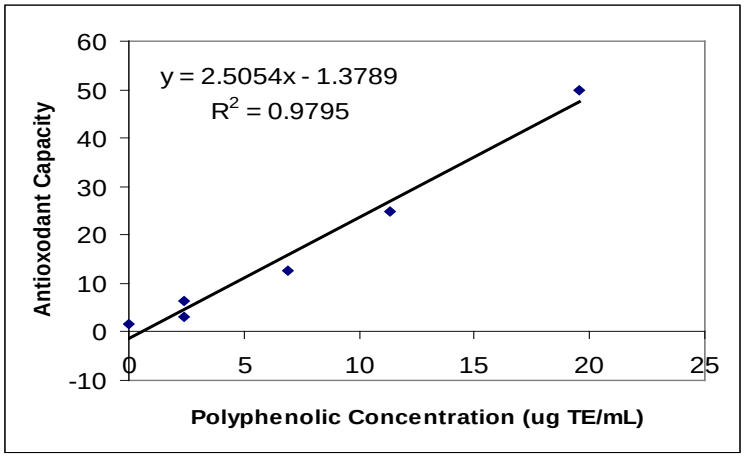


Fig. 5 ORAC Standard Curve with Trolox

Values for the wine dilutions were calculated in the equation of the standard curve and can be seen in Table 2.

Table 2 ORAC Results-Antioxidant Capacity for Syrah and Port

	Y=2.5054x-1.3789	
Dilution Factor	Average Polyphenolic Concentration (µM TE/g)	Antioxidant Capacity
Syrah		
200x	33.28	81.99422
150x	19.53	47.5605
100x	9.67	22.8367
Port		
200x	43.93	108.675
150x	24.15	59.1307
100x	9.82	23.225

These values show that the less concentrated dilutions are relatively equal in antioxidant capacity, 22.8 and 22.2uM TE/g respectively. At a dilution of 150x, AOX began to differ slightly: 47.6 uM TE/g for the Syrah vs. 59.1 uM TE/g for the Port. At 200x, the wines were significantly different: 81.9 uM TE/g for the Syrah and 108.68 uM TE/g for the Port. The Syrah antioxidant capacity is 80.5% of the Port when diluted 200x.

Cell proliferation

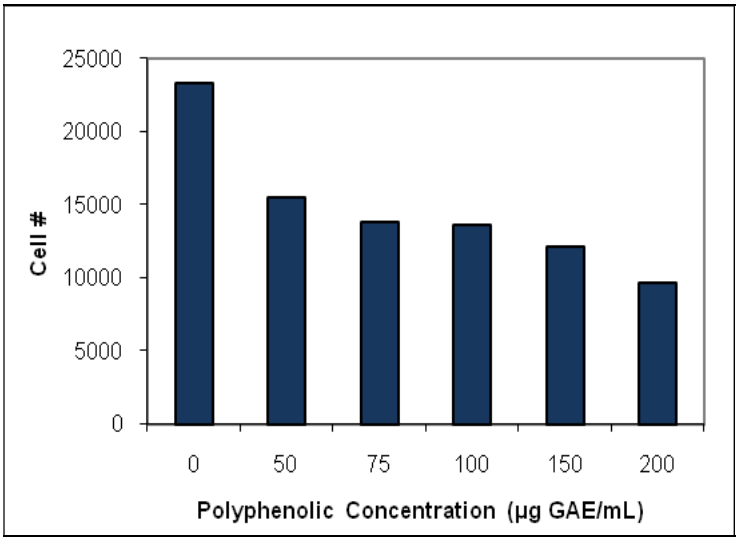
Contents of each well were transferred into a cuvette and read twice in the cell counter. Results from the Syrah are shown in Table 3.

Table 3 Cell Proliferation Readings-Syrah

TTX [conc]	0	200	150	100	75	50
Row 1	900, 920	410, 408	530, 386	600, 628	582, 548	628, 566
Row 2	**976**, 999	441, 488	569, 544	567, 556	567, 543	745, 724
Row 3	1078, 952	335, 387	434, 473	569, 588	613, 643	654, 647
Row 4	***662, 714***	385, 365	566, 540	522, 501	568, ***480***	613, 579

Averages were taken of each concentration and multiplied by the dilution factor of 402. These values are the actual number of cells in each well after 72 hours of incubation. These values are seen in Figure 4 with error bars too small to be seen. The percentage of cells in each well as compared to the control is seen in Figure 6.

A



B

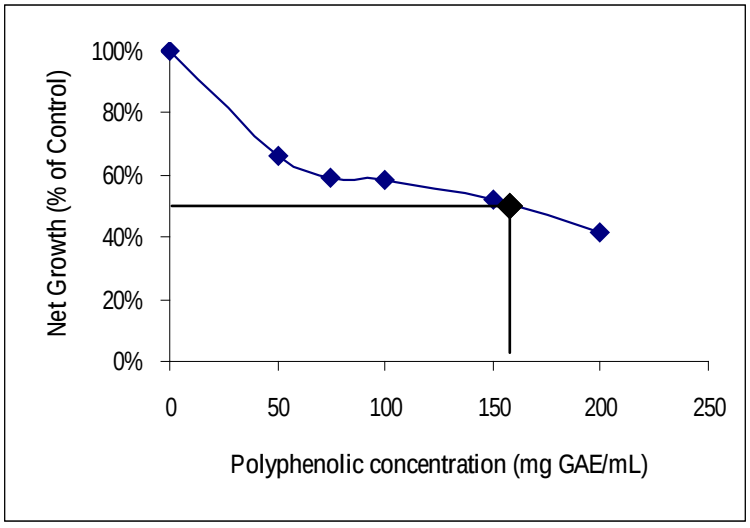


Fig. 6 Cell Proliferation Syrah Results. **A** Polyphenolic Concentration vs. Cell # **B** Polyphenolic Concentration vs. % Cell Proliferation

At the lowest concentration, 50 µg GAE/mL, the Syrah wine extract decreased cell proliferation to 59% of the control. Proliferation continued to decrease when higher concentrations were used. At 200 µg GAE/mL, cell proliferation was decreased to 41%. The IC50 (concentration at which proliferation is half of the control) is shown to be ~160 µg GAE/mL.

Results from the Port are shown in Table 4 and Figure 7.

Table 4 Cell Proliferation Readings-Port

TTX [conc]	0	200	150	100	75	50
Row 1	108, 119	44, 46	74, 65	85, 75	92, 83	101, 81
Row 2	210, 218	27, 26	82, 91	90, 76	81, 108	73, 73
Row 3	201, 181	32, 16	80, 73	94, 75	74, ***69***	***68***, 85
Row 4	126, 103	21, 18	73, 79	106, 96	***58, 50***	97, 93

A

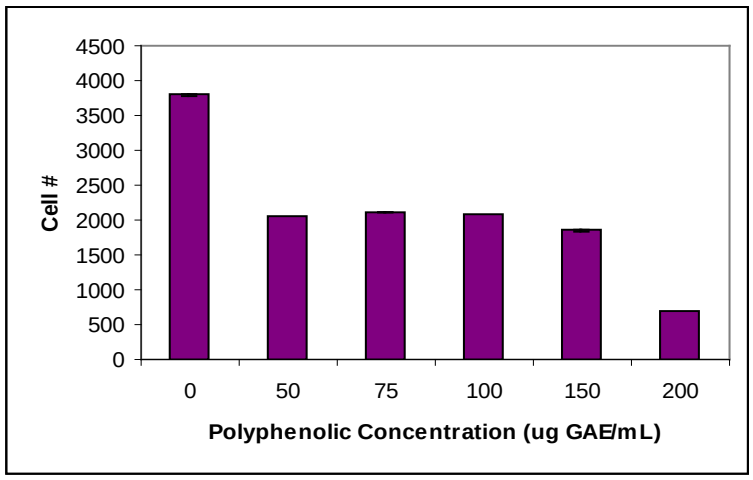


Fig. 7 Cell Proliferation Port Results. A Polyphenolic Concentration vs. Cell # B Polyphenolic Concentration vs. % Cell Proliferation

B

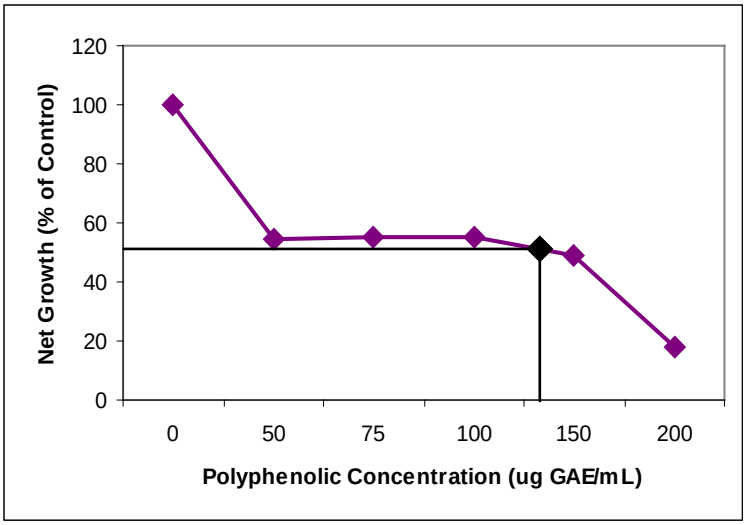


Fig . 7 Cont.

At 50 µg GAE/mL, the Port wine extract decreased cell proliferation to 54% of the control, while at the same concentration, the Syrah wine extract decreased cell proliferation by 59%. At 200 µg GAE/mL, the Port wine extract decreased cell proliferation to 18% where the same concentration of the Syrah only decreased cell growth to 41%. The IC50 for the Port was ~140 µg GAE/mL. The large difference in the two wines' IC50s would show that the Port has the greater effect of inhibiting cell growth. This is supported by looking at the percentage of the control's cell growth at 200 µg GAE/mL . 18% is significantly lower than 41%.

Generation of reactive oxygen species

Fluorescence values at 15 and 30 minutes are shown below. Bold and italicized valued were discarded as they were dissimilar enough from other values to cause an increase in standard deviation and skewed results. Results from the Syrah are shown in Table 5.

Table 5 ROS Results-Syrah. **A** Fluorescence after 15 minutes **B** Fluorescence after 30 minutes

A

15 Raw Data

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	6493	36070	14048	20605	***31724***	25002	30060	32740	30700	-	-	-
B	6050	***33572***	17015	14027	18211	22472	26648	27044	31427	-	-	-
C	6121	41351	18147	12756	19163	20069	30010	27438	34150	-	-	-
D	6039	35122	12947	12601	15072	25669	23578	35453	33290	-	-	-
E	6068	39617	15359	11820	19664	27748	27312	33130	30633	-	-	-
F	6249	40889	11979	17789	15450	25333	25096	28190	***23411***	-	-	-
G	6611	58573	16047	17422	29656	25951	25193	29551	27469	-	-	-
H	6293	52416	***22023***	***22187***	***29469***	25913	***41029***	27674	***20638***	-	-	-

B

30 Raw Data

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	9229	67710	25186	37543	58277	45065	56069	61797	58410	-	-	-
B	8660	64095	30746	24523	32838	40549	49013	51755	59828	-	-	-
C	8965	79348	33469	22536	35001	36693	57433	51689	65439	-	-	-
D	8903	67918	23196	22449	27596	49633	44819	68042	64096	-	-	-
E	8905	78397	28476	21543	36679	53727	51537	62387	58456	-	-	-
F	9226	79733	21634	33252	28451	48148	47404	54266	44796	-	-	-
G	9793	***113444***	29428	32367	56494	49871	47766	56147	51829	-	-	-
H	9326	98564	***41155***	42247	55813	47463	77865	51575	***37420***	-	-	-

Fluorescence ranged from 14048 to 58573 when concentrations ranged from 12.5-200 µg GAE/mL. These values were used to calculate the "fold" which represents the percent fluorescence of cells incubated with treatment versus the control. Results from the Syrah are shown in Figure 8.

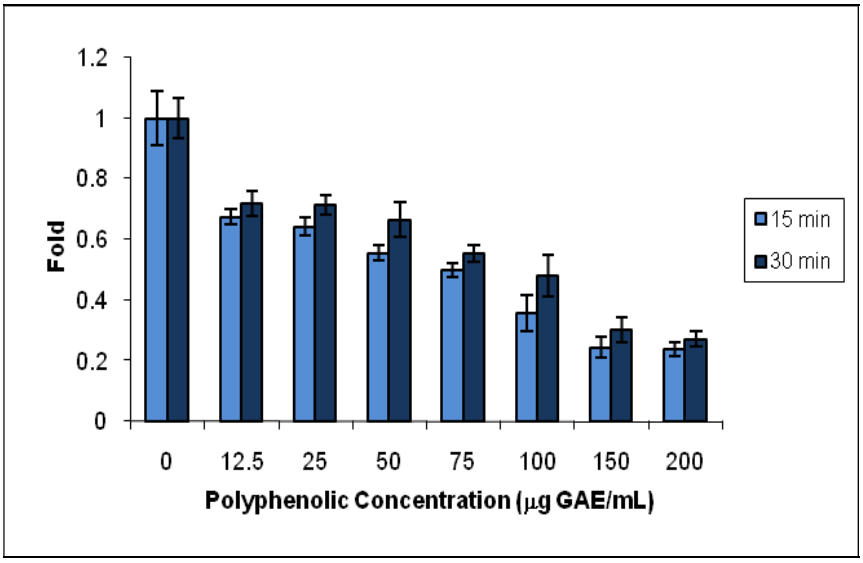


Fig. 8 ROS Syrah Results: Polyphenolic Concentration vs. Fold

Results taken at 15 minutes are most accurate and thus, the values used for analysis. At this time, the generation of Reactive Oxygen Species was decreased to 67.3% of the control when treated with 12.5 µg GAE/mL extract. ROS steadily decreased to 24.3% when treated with 150 µg GAE/mL extract and became level as cells treated with the 200 µg GAE/mL extract had decreased to 23.8%. Similar analysis was done for the Port wine. The data can be seen in Table 6 and Figure 9.

Table 6 ROS Results-Port. **A** Fluorescence after 15 minutes **B** Fluorescence after 30 minutes

A

Raw Data

	Blank	200 ppm	150 ppm	100 ppm	75 ppm	50 ppm	25 ppm	12.5 ppm	Ctl
A	6402	***18366***	***33571***	33842	***43821***	32113	***53699***	33797	43283
B	6438	17435	27315	***40841***	35475	***42432***	39225	42181	44168
C	6521	14038	25307	29038	42594	40313	47452	32386	44586
D	6542	14331	26889	31470	30839	30717	29776	37333	36198
E	6468	***12378***	24281	24330	29743	39100	29005	30140	34622
F	6430	15758	29876	24752	29042	31598	28950	30259	35926
G	6321	14559	20159	28702	26529	24341	34234	34674	35908
H	6716	16522	24056	31535	37135	30517	32540	34303	37340

B

Raw Data

	Blank	200 ppm	150 ppm	100 ppm	75 ppm	50 ppm	25 ppm	12.5 ppm	Ctl
A	8710	***31412***	***56663***	57078	***74192***	54570	***91291***	58119	76033
B	8590	29601	46009	***69480***	60698	***72011***	67435	***74132***	78115
C	8806	23330	42910	49957	***73009***	69845	***83227***	57532	78502
D	8870	23809	45082	53623	53616	53924	51191	65724	63289
E	8745	20182	40656	***40739***	51161	67625	50134	51950	60191
F	8615	26074	50891	***41620***	50140	53554	49305	52231	61916
G	8439	23147	32970	47667	***44607***	***40167***	57679	58963	60207
H	9031	26995	39200	51941	61485	***50292***	53949	56710	63200

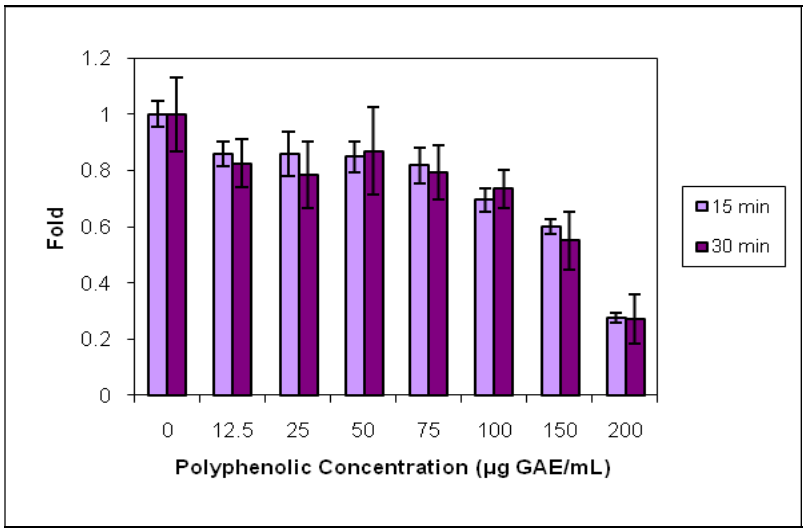


Fig. 9 ROS Port Results: Polyphenolic Concentration vs. Fold

At fifteen minutes, fluorescence ranged from 14083 to 44586 at concentrations ranging from 12.5 to 200µg GAE/mL. At 12.5 µg GAE/mL, the Port wine extract decreased fluorescence to 85.8% of the control, where at the same concentration the Syrah wine extract caused a decrease to 71.8%. There was insignificant change until steady decrease from 75-200 µg GAE/mL. At 200 µg GAE/mL there was a drastic decrease in fluorescence down to approximately 27.6% of the control where the Syrah decreased ROS generation to 27.2%. At high concentrations, as seen in the Cell Proliferation assay, the two wine extracts give similar results. At the low concentrations, however, the Syrah wine extract was more effective at inhibition the generation of reactive oxygen species.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary

It has been shown that phytochemicals found in grapes and red wines are polyphenolic compounds that have antioxidant properties. Their antioxidant properties may prevent or decrease the growth of breast cancer. Polyphenolic compounds were extracted out of two Texas red wines (Port and Syrah) and tested on estrogen-receptor negative MDA-MB-231 breast cancer cells. The effects of these extracts on cell proliferation and generation of reactive oxygen species were tested through various assays. The ORAC results showed both the Syrah and Port wines having similar antioxidant capacity at a dilution of 100x, but that when diluted further to 200x, the Syrah's antioxidant capacity was only 80.5% of that of the Port suggesting that the Port wine has a higher antioxidant capacity.

The effects of the Syrah and Port wines on cell growth were tested with a Cell Proliferation assay. The proliferation of MD-MBA-231 breast cancer cells was inhibited by treatments of the wines in concentrations ranging from 50-200 µg GAE/mL. When treated with the Syrah wine extract, cell proliferation was decreased by 33-59% with doses ranging from 50-200 µg GAE/mL. When treated with the Port wine extract, cell proliferation decreased by 45-82% with the same dosing ranges. The IC50 when treated with the Syrah wine extract was approximately 165 µg GAE/mL. The IC50 when

treated with the Port was ~140 µg GAE/mL. This shows that a smaller concentration of the Port wine extract is required for the same inhibition and that the Port wine is therefore a more potent antioxidant.

The antioxidant effects of the wine extracts were directly tested on the cells with a Reactive Oxygen Species (ROS) assay. When cells were treated with the Syrah wine extract, the generation of ROS decreased from 29-73% with concentrations ranging from 12.5-200 µg GAE/mL. When treated with the Port wine extract, the generation of ROS decreased from 14-72% with the same concentration ranges. This shows that higher concentrations of these wine extracts have the same effects, but that the Syrah is a more effective antioxidant at lower concentrations.

Conclusions

The ORAC results lead to a hypothesis that at low concentrations the Port wine extract would have greater antioxidant effects on cell growth and the generation of ROS. The IC50 when the Syrah wine extract was used was ~165 µg GAE/mL where that of the Port is ~140. These values support the hypothesis that the Port wine is a more potent antioxidant.

When 12.5 µg GAE/mL treatments of wine extract were used, the generation of ROS was inhibited 14% by the Port wine extract and 29% by the Syrah wine extract. Contradictory to the results of Cell Proliferation, the inhibition of the generation of ROS

was more profound when the Syrah wine extract was used suggesting that the Syrah wine is the more potent antioxidant.

To explain this contradiction, an ORAC would be performed on the extracts used in the assays to give a more accurate representation of the antioxidant capacity of the extracts as opposed to the pure wines. It is also possible that the mechanism behind the antioxidant inhibition of cell growth is different from that of inhibition of ROS. If taken further, this study could include gene expression studies. Apoptosis biomarkers such a p53 and capsase 3 would be investigated in the hopes of suggesting a mechanism behind the wines' growth inhibition effect of MDA-MB-231 cancer cells.

This study, by no means, suggests that drinking of wine will decrease or prevent breast cancer. It is a beginning of new knowledge of the beneficial effects of Texas red wines. Further studies would determine the polyphenolic composition of each wine via HPLC. Once compounds are isolated, it can be determined exactly which compounds are the causes of specific effects by testing them individually. Synergistic and inhibitory effects of the compounds on each other can be determined by testing them once isolated. On a larger scale, clinical trials could be performed to document any effect of drinking these wines will have on women currently suffering from this breast cancer. Much more research is needed but with every new study comes new knowledge which brings the scientific community closer to preventing and treating breast cancer.

REFERENCES

1. Kung Hea: Final Data for 2005. National Vital Statistics Report, CDC 10: 1-121, 2008.

2. Sant M, Allemani C, Sieri S, Krogh V, Menard S, Tagliabue E, Nardini E, Micheli A, Crosignani P, Muti P, Berrino F: Salad vegetables dietary pattern protects against HER-2-positive breast cancer: a prospective Italian study. Int J Cancer 121: 911-4, 2007.

3. Atten MJ, Attar BM, Milson T, Holian O: Resveratrol-induced inactivation of human gastric adenocarcinoma cells through a protein kinase C-mediated mechanism. Biochem Pharmacol 62: 1423-32, 2001.

4. Fan P, Lou, H.: Effects of polyphenols from grape seeds on oxidative damage to cellular DNA. Molecular and Cellular Biochemistry 267: 67-74, 2004.

5. Hudson TS, Hartle DK, Hursting SD, Nunez NP, Wang TT, Young HA, Arany P Green JE: Inhibition of prostate cancer growth by muscadine grape skin extract and resveratrol through distinct mechanisms. Cancer Res 67: 8396-405, 2007.

6. Juan ME, Vinardell MP, Planas JM: The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful. J Nutr 132: 257-60, 2002.

7. Maggiolini M, Recchia AG, Bonofiglio D, Catalano S, Vivacqua A, Carpino A, Rago V, Rossi R, Ando S: The red wine phenolics piceatannol and myricetin act as agonists for estrogen receptor alpha in human breast cancer cells. J Mol Endocrinol 35: 269-81, 2005.

8. Murias M, Luczak MW, Niepsuj A, Krajka-Kuzniak V, Zielinska-Przyjemska M, Jagodzinski PP, Jager W, Szekeres T, Jodynis-Liebert J: Cytotoxic activity of 3,3',4,4',5,5'-hexahydroxystilbene against breast cancer cells is mediated by induction of p53 and downregulation of mitochondrial superoxide dismutase. Toxicol In Vitro 22: 1361-70, 2008.

9. Shi J, Yu, J., Pohorly, J. E., Kakuda, Y.: Polypenolics in grape seeds-biochemistry and functionality. Journal of Medicinal Food 6: 291-299, 2003.

10. Surh YJ, Hurh YJ, Kang JY, Lee E, Kong G, Lee SJ: Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. Cancer Lett 140: 1-10, 1999.

11. Damianaki A, Bakogeorgou E, Kampa M, Notas G, Hatzoglou A, Panagiotou S, Gemetzi C, Kouroumalis E, Martin PM, Castanas E: Potent inhibitory action of red wine polyphenols on human breast cancer cells. J Cell Biochem 78: 429-41, 2000.

12. Nyaga SG, Jaruga P, Lohani A, Dizdaroglu M, Evans MK: Accumulation of oxidatively induced DNA damage in human breast cancer cell lines following treatment with hydrogen peroxide. Cell Cycle 6: 1472-8, 2007.

13. Learn About Cancer. In: Learn About Breast Cancer. American Cancer Society, 2008.

14. Bessaoud F, Daures JP: Patterns of alcohol (especially wine) consumption and breast cancer risk: a case-control study among a population in Southern France. Ann Epidemiol 18: 467-75, 2008.

15. Berstad P, Ma H, Bernstein L, Ursin G: Alcohol intake, breast cancer risk among young women. Breast Cancer Res Treat 108: 113-20, 2008.

16. Mertens-Talcott SUJ-S, P.; Rios, J.; Hingorani, L.; Derendorf, H.: Absorption, metabolism, and antioxidant effects of pomegranate (Punica granatum l.) polyphenols after ingestion of a standardized extract in healthy human volunteers. J Agric Food Chem 54: 8956-61, 2006.

17. Mertens-Talcott SU, Bomser JA, Romero C, Talcott ST, Percival SS: Ellagic acid potentiates the effect of quercetin on p21waf1/cip1, p53, and MAP-kinases without affecting intracellular generation of reactive oxygen species in vitro. J Nutr 135: 609-14, 2005.

18. Lee JH, Johnson JV, Talcott ST: Identification of ellagic acid conjugates and other polyphenolics in muscadine grapes by HPLC-ESI-MS. J Agric Food Chem 53: 6003-10, 2005.

19. Haupt LM, Thompson EW, Griffiths LR, Irving MG: IS-RT-PCR assay detection of MT-MMP in a human breast cancer cell line. Biochem Mol Biol Int 39: 553-61, 1996.

CONTACT INFORMATION

Name:	Alexandra Elizabeth Hagood
Professional Address:	c/o Dr. Susanne Talcott Department of Nutrition and Food Science Texas A&M University College Station, TX 77843
Email Address:	ahagood@neo.tamu.edu
Education:	B.S. Nutritional Science, Texas A&M University, May 2009 Summa Cum Laude Undergraduate Research Scholar