The dissertation of Deepti Dabas was reviewed and approved* by the following:

Gregory R. Ziegler  
Professor of Food Science  
Dissertation Co-Adviser  
Co-chair Committee

Joshua D. Lambert  
Assistant Professor of Food Science  
Dissertation Co-Adviser  
Co-chair Committee

Ryan J. Elias  
Assistant Professor of Food Science

Steven Weinreb  
Professor of Chemistry

John Floros  
Head of the Department of Food Science

*Signatures are on file in the Graduate School
ABSTRACT

There is an increasing consumer demand for and scientific interest in new functional food ingredients. Avocado is a commercially important crop and studies have shown that the pulp may have benefits to cardiovascular health, dermatological health and possibly anti-cancer activity. Despite being important historically, the avocado seed is considered a waste product and does not find uses currently. New research is beginning to show its potentially useful functional properties. We report here that avocado (Persea americana) seed when crushed with water develops an orange color in a time-dependent manner. Heat treatment of the seed prevented color development, whereas addition of exogenous polyphenol oxidase (PPO) restored color development. The orange color intensified as the pH was adjusted from 2.0 to 11.0 in solution, and these changes were only partially reversible in the presence of oxygen but completely reversible when the pH was adjusted and readjusted in the absence of oxygen. The color was found to be stable in solution form at -18 °C for two months at pH 7.5.

The colored avocado seed extract (CASE) was found to have radical scavenging activity, by electron paramagnetic resonance spectroscopy (EC$_{50}$ = 42.1 μg/mL) and antioxidant activity in oil-in-water emulsions. The oxygen radical absorbance capacity (ORAC) value of CASE was 2012.1 ± 300 TE/mg.

CASE inhibited the growth of human cancer cell lines (lung, colon, breast, and prostate) (IC$_{50}$= 19.1-132.2 μg/mL) in vitro. LNCaP prostate cancer cells were the most sensitive to the effects and further studies showed that CASE inhibited cell cycle
progression in the G0/G1 phase by decreasing expression of cyclin D1 and cyclin E2. Further it induced apoptosis as indicated by cleavage of caspase 3, cleavage of poly (ADP–ribose) polymerase (PARP) and externalization of phosphatidylserine. It also reduced the nuclear translocation of NF-κB, a transcription factor which promotes cell survival and replication.

In lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages, CASE reduced the formation of pro-inflammatory cytokines (IL-6, IL-1β and TNFα), and NO, a potent inflammatory mediator. Reduced NO production correlated with reduced iNOS expression. CASE reduced the formation of Prostaglandin E2, a pro-inflammatory mediator and inhibited Phospholipase A2 (IC_{50} = 36 µg/mL) by a non-competitive mechanism. CASE reduced LPS-induced nuclear translocation of NF-κB, transcription factor with pro-inflammatory actions.

Overall, these results suggest that the avocado seed may be a potential source of natural colorant which could impart additional functions including radical scavenging, cancer preventive activity and anti-inflammatory activities. However these effects need to be further tested in vivo.
TABLE OF CONTENTS

LIST OF TABLES ................................................................................................................. viii
LIST OF FIGURES ................................................................................................................ ix
ACKNOWLEDGEMENTS ........................................................................................................ xiii

Chapter 1 LITERATURE REVIEW AND OBJECTIVES ....................................................... 1

Abstract ................................................................................................................................. 1
1.1. Colorants ......................................................................................................................... 2
  1.1.1. Synthetic Colorants ................................................................................................. 2
  1.1.2. Natural Colorants .................................................................................................... 3
1.2. Avocado .......................................................................................................................... 6
  1.2.1. Avocado pulp .............................................................................................................. 8
  1.2.2. Functional properties of avocado pulp .................................................................... 8
    1.2.2.1. Dermatological uses ......................................................................................... 9
    1.2.2.2. Cardiovascular effects ...................................................................................... 9
    1.2.2.3. Anti-cancer effects .......................................................................................... 11
    1.2.2.4. Treatment of osteoarthritis ............................................................................ 12
    1.2.2.5. Anti-inflammatory effects ............................................................................. 14
    1.2.2.6. Antimicrobial effects ..................................................................................... 17
  1.2.3. Avocado seed ............................................................................................................ 17
  1.2.4. Antioxidant activity of avocado seed ..................................................................... 21
  1.2.5. Functional properties of avocado seed ................................................................... 22
    1.2.5.1. Anti-cancer effects ......................................................................................... 23
    1.2.5.2. Anti-inflammatory effects ............................................................................. 24
    1.2.5.3. Anti-diabetic effects ...................................................................................... 24
    1.2.5.4. Anti-hypertensive effects .............................................................................. 25
    1.2.5.5. Dermatological uses ...................................................................................... 25
    1.2.5.6. Antimicrobial activity ..................................................................................... 28
    1.2.5.7. Insecticidal effect ........................................................................................... 28
  1.2.6. Safety of avocado seed extract .............................................................................. 29
1.3. Polyphenol oxidase ....................................................................................................... 29
1.4. Purpose and objectives ................................................................................................. 32
1.5. References .................................................................................................................... 34

Chapter 2 A COLORED AVOCADO SEED EXTRACT AS A POTENTIAL NATURAL
COLORANT ............................................................................................................................ 43

Abstract ................................................................................................................................. 43
2.1. Introduction ..................................................................................................................... 44
2.2. Material and Methods ................................................................................................. 45
  2.2.1. Reagents .................................................................................................................. 45
Abstract .................................................................................................................. 75

3.1. Introduction ........................................................................................................ 76

3.2. Material and methods .......................................................................................... 77
  3.2.1. Reagents ......................................................................................................... 77
  3.2.2. Cell culture ..................................................................................................... 78
  3.2.3. Preparation of CASE ..................................................................................... 78
  3.2.4. Radical scavenging and antioxidant activity ................................................. 79
  3.2.5. Oxygen Radical Absorbance Capacity (ORAC) Assay ................................ 80
  3.2.6. Growth inhibitory effects ............................................................................. 81
  3.2.7. H$_2$O$_2$ production ..................................................................................... 81
  3.2.8. Cell cycle analysis ......................................................................................... 81
  3.2.9. Induction of apoptosis - Externalization of phosphatidylserine .................... 82
  3.2.10. Quantification of intracellular reactive oxygen species (ROS) .................... 82
  3.2.11. Western blot analysis .................................................................................. 83
   3.2.11.1. Preparation of whole cell lysate ............................................................... 83
   3.2.11.2. Preparation of nuclear lysate .................................................................. 83
   3.2.11.3. Immuno blots ....................................................................................... 84
  3.2.12. Data Analysis ............................................................................................. 84

3.3. Results .................................................................................................................. 84
  3.3.1. Antioxidant activity ...................................................................................... 84
  3.3.2. Growth inhibitory effects ............................................................................. 86

Chapter 3 ANTIOXIDANT AND ANTI-CARCINOGENIC EFFECTS OF A COLORED
AVOCADO SEED EXTRACT .................................................................................. 75

2.2.2. Preparation of colored avocado seed extract (CASE) ..................................... 46
2.2.3. Kinetics of color formation ........................................................................... 46
2.2.4. Role of polyphenol oxidase (PPO) in color formation ................................... 47
2.2.5. Role of peroxidase in color formation ............................................................ 48
2.2.6. Phenolic content of the colored extract ......................................................... 48
2.2.7. Effect of pH on color of the extract ............................................................... 49
2.2.8. Color stability ............................................................................................... 50
2.2.9. Chemical characterization of CASE ............................................................... 51
2.2.10. Using CASE in commercial products ......................................................... 52
2.2.11. Statistical analysis ....................................................................................... 53

2.3. Results .................................................................................................................. 53
  2.3.1. Kinetics of color formation ........................................................................... 53
  2.3.2. Role of PPO in color development ............................................................... 55
  2.3.3. Phenolic content .......................................................................................... 57
  2.3.4. Effect of pH on color .................................................................................... 58
  2.3.5. Stability of the colored extract .................................................................... 61
  2.3.6. Chemical characterization of CASE ............................................................. 63
  2.3.7. Effect of addition of color to the products .................................................... 63

2.4. Discussion ............................................................................................................. 66

2.5. Conclusion ............................................................................................................. 72
LIST OF TABLES

Table 1. Kinetic analysis of inhibition of secreted PLA₂ by CASE.............................111
LIST OF FIGURES

Figure 1.1. Structure of (a) Persin (b) Persenone A (c) Persenone B …………………..16

Figure 1.2. Structure of phenolic substrates present in the seed (a) 3-Hydroxy-tyrosol-1’-O-β-D-glucoside (b) Tyrosol (c) 3’-Hydroxy Tyrosol (d) Chlorogenic acid (e) Quinic acid (f) Epicatechin and (g) Catechin …………………………………………………………………………………20

Figure 1.3. The structure of the phloridzin and the colored pigment produced as a result of PPO activity…………………………………………………………………………………………………………………………..31

Figure 2.1. Kinetics of color formation in avocado seed paste. (a) A visual change in color of the paste was observed with the time of incubation. Representative results of three independent experiments. (b) Spectrophotometric analysis at 35 min showed a λmax at 480 nm. (c) Absorption at 480 nm (Mean ±SD of three replicates) and (d) ΔE increased with the time of incubation (mean of two independent experiments) ………………………………..54

Figure 2.2. Role of polyphenol oxidase and peroxidase in the development of color in avocado seed paste. (a) Role of polyphenol oxidase: The absorbance at 480 nm increased in heat-inactivated avocado paste as increasing amount of exogenous PPO was added. No color formation was observed in samples co-incubated with different doses of PPO and 58 μM tropolone (marked ‘T’ in Figure). (b) Role of peroxidase: With the addition of increasing amounts of peroxidase, only a minor increase in absorbance at 480 nm compared to negative control was observed. No effect of co-incubation with tropolone was observed. Mean ±SD of three independent experiments …………………………………………56

Figure 2.3. Comparison of the phenolic content of the colored and the uncolored avocado seed extract. Higher total phenolic concentrations were observed in the uncolored extract. This difference was statistically significant (p < 0.05). Mean ±SEM of three independent experiments ………………………………………………………………………………………………………………………………………57

Figure 2.4. Effect of pH on the color of the avocado seed extract. (a) Absorption spectra at different pH values show a λmax at 480 nm at pH 7.5, 9.0 and 11.0; at pH 11.0 an additional λmax at 440 nm was observed. Representative results of three independent experiments (b) Absorption at 480 nm and ΔE values were seen to increase as a function of pH. Mean ±SD of three replicates ………………………………………………………………………………………58

Figure 2.5. Effect of pH change and the presence or absence of air on the rehydrated freeze dried extract color (a) When the pH was increased from 7.5 to 11.0, in the presence of oxygen, the absorption intensity increased and two more absorbance maxima emerged at pH 11.0. When pH was decreased back to 7.5, only partial restoration of color character was observed. When pH was increased to 11.0 under a nitrogen atmosphere, an increase was in near UV range was observed. On reducing the pH back to 7.5, complete reversal of absorbance intensities was observed. Representative results of three independent experiments. (b) When pH was reduced from 7.5 to 2.0 and adjusted back to
similar effects were observed under both air and nitrogen atmospheres. Representative results of three independent experiments

**Figure 2.6.** Effect of storage on color of the rehydrated freeze dried extract. (a) Absorbance Intensity at 480 nm and (b) ΔE were determined during storage for 60 days at pH 7.5 at -18 °C (■), 4 °C (●), 24 °C (▲) and 40 °C (▼). Mean ±SD of three replicates

**Figure 2.7.** Effect on color after addition of CASE to (a) muffin (b) apple juice (c) grapefruit juice and (d) milk

**Figure 2.8.** Effect of addition of CASE on color of white angel food cake muffins. (a) ΔE value on the surface (b) ΔE values on the side (c) ΔE values inside were measured by cutting the muffins and measuring L, a and b values. (d) Chroma value on the surface (e) Chroma value on the side (f) Chroma values inside. Mean ± SD of two independent experiments

**Figure 2.9.** Effect of addition of CASE on the color of grapefruit juice (a) ΔE value (b) Chroma value. Mean ± SD of two independent experiments

**Figure 2.10.** Effect of addition of CASE on the color of apple juice (a) ΔE value (b) Chroma value. Mean ± SD of two independent experiments

**Figure 2.11.** Effect of addition of CASE on color of milk. (a) ΔE value (b) Chroma value. Mean ± SD of two independent experiments

**Figure 3.1.** Antioxidant activity of CASE. (a) The antioxidant activity of CASE was compared using EPR. Tempol absorbance intensity was determined after incubation for 120 min at 37°C. The Tempol absorbance intensities were normalized to the intensity of control incubated with 120 µM Trolox. Results are mean ±SEM of three independent experiments. (b) Lipid hydroperoxides in oil-in-water emulsion containing CASE at various concentrations were used to assess antioxidant activity in a model food system during storage at 37 °C. Symbols represent negative control (■), 5 µg/mL (●), 15 µg/mL (▲), 50 µg/mL (▼), 100 µg/mL (▲), 250 µg/mL (▷) and 500 µg/mL (◇). Results are represented as mean ±SD

**Figure 3.2.** Cell survival in selected cancer cell lines after treatment with CASE. The percentage survival was calculated with respect to control after incubation for 48 h. Results are mean ±SEM of three independent experiments

**Figure 3.3.** Cell survival in LNCaP cancer cell line after treatment with CASE. The percentage survival was calculated with respect to control after incubation for 12 h (■) and 24 h (●). Results are mean ±SEM of three independent experiments
Figure 3.4. Effect of CASE on the progression of cell cycle in LNCaP cells. Cells were treated with only media, IC$_{50}$ or IC$_{60}$ for 12 h. Each value represents mean ±SD of two independent experiments. .................................................................88

Figure 3.5. Expression of cell cycle related proteins as affected by treatment with CASE at IC$_{50}$ or IC$_{60}$ values for 12 h. Results are representative of three independent experiments. .................................................................89

Figure 3.6. Expression of cleaved caspase 3, cleaved PARP and γH2AX as affected by treatment for 12 h with CASE. Results are representative of three independent experiments. .................................................................90

Figure 3.7. Apoptosis as determined using staining with annexin-FITC/PI. Cells were treated with CASE for 12 h, stained with annexin and PI and analyzed using flow cytometry. Mean ± SD of two independent experiments. .................................................................91

Figure 3.8. Expression of NF-κB in the nuclear extracts as affected by treatment for 12 h with CASE. A reduction in the expression of all the three proteins was observed. Results are representative of three independent experiments. .................................................................92

Figure 4.1. Effect of CASE on the production of inflammatory mediators in RAW 264.7 cells stimulated with LPS. Cytokine levels were measured in the supernatant of cells treated with CASE. Mean ± SD of three independent experiments. ‘NC’ indicates non-LPS stimulated control.................................................................................................109

Figure 4.2. Effect of CASE on the protein expression of iNOS in LPS-stimulated RAW 264.7 cells. Cells were treated with CASE for 24 h. Figure is representative of three independent experiments. .................................................................110

Figure 4.3. The effect of CASE on the production of PGE$_2$ in RAW 264.7 cells stimulated with LPS for 24 h. Mean ± SD of three independent experiments. (b) The effect of CASE on the activity of pure secreted PLA$_2$. Symbols represent mean ± SD (n=5) (c) Kinetic analysis of CASE inhibition of secreted PLA$_2$. Concentrations of CASE used were (●) nil, (■) 36 µg/mL and (▲) 72 µg/mL. Inhibitory kinetics were determined by using Michelis-Menton analysis .................................................................111

Figure 4.4. Effect of CASE on nuclear translocation of NF-κB in LPS-stimulated RAW 264.7 cells. Nuclear and total levels NF-κB of were determined by western blot. β-actin and Histone H3 were used as protein loading controls for total and nuclear protein respectively. Representative results of two independent experiments .................................................................112
Figure A.1. Chromatogram of the fraction 15 MeOH:85 EtOAc-5. The conditions have been described in the section 2.2.9. The chromatogram was obtained at two wavelengths - 280 nm (blue line) and at 440 nm (pink line). The intensity at 440 nm was intensified 10 times for better visibility.

Figure A.2. Chromatogram of the isolated colored peak from the fraction 15 MeOH:85 EtOAc-5. The conditions have been described in the section 2.2.9. The chromatogram was obtained at two wavelengths - 280 nm (blue line) and at 440 nm (pink line). The intensity at 440 nm was intensified 10 times for better visibility.

Figure A.3. Proton NMR spectra of isolated colored peak from the fraction 15 MeOH:85 EtOAc-5.

Figure A.4. Carbon NMR spectra of isolated colored peak from the fraction 15 MeOH:85 EtOAc-5.

Figure A.5. Infrared spectra of isolated colored peak from the fraction 15 MeOH:85 EtOAc-5.

Figure B.1. Antioxidant activity of the uncolored extract was determined using EPR. Tempol absorbance intensity was determined after incubation for 120 min at 37 °C. The Tempol absorbance intensities were normalized to the intensity of control incubated with 120 μM Trolox. Mean ± SEM of three independent experiments.

Figure B.2. Viability of selected cancer cell lines after treatment with the uncolored avocado seed extract. The percentage survival was calculated with respect to control after incubation for 48 h. Cell lines are represented as: (○) LNCaP, (●) MCF7, (▲) HT29 and (■) H1299. Results are mean ±SEM of three independent experiments.
ACKNOWLEDGEMENTS

I would like to express my deepest appreciation for my co-adviser Dr. Joshua D. Lambert for his guidance, help and valuable discussions throughout my research. I would like to thank my co-adviser Dr. Gregory R. Ziegler for his support and helpful suggestions during the course of my research. I would also like to acknowledge the help of my committee members Dr. Ryan J. Elias and Dr. Steven M. Weinreb for being on my committee and for valuable discussions and suggestions.

I would like to thank Dr. Devin Peterson (University of Minnesota) for help with mass spectrometry experiments. I would like to thank the Food Science Department and the Pennsylvania State University for financial support.

I would like to acknowledge the unending support from my family and friends. I would also like to thank fellow graduate students especially my lab mates for their support and help with experiments during my research.
Chapter 1

REVIEW OF LITERATURE AND OBJECTIVES

Abstract

Avocado (Persea americana, Lauraceae) is a commercially important crop which is grown in the United States and also imported in large quantities. It has been reported to have cardiovascular benefits and some current studies, mainly of the pulp, have demonstrated anti-cancer and anti-inflammatory effects. For example, avocado unsaponifiables are used to treat osteoarthritis and also have dermatological applications. The avocado seed has a rich ethno pharmacological history and has been used traditionally for treatment of a number of indications. It has high phenolic content and high antioxidant activity. Recent studies have demonstrated the anti-cancer, anti-diabetic, anti-inflammatory, blood pressure reducing, anti-microbial, insecticidal and dermatological activities of seed preparations. The acute and sub-chronic safety of orally-administered seed extract was evaluated in the rat and it was found to be safe even at high doses. With avocado being produced and transported across countries and consumed in large quantities, there is a need to find suitable applications for seed which is regarded as a waste product. Its use as a natural colorant which may have additional health beneficial properties represents a possible application.
1.1. Colorants

Color plays a key role in determining the expectations and perceptions of consumers with respect to food (Delgado-Vargas 2000). It is one of the most obvious characteristics of a food and, if not appealing, negatively impacts consumer acceptance. Colors have a strong impact on people’s emotions, for example, yellow color is recognized as cheerful and red as exciting (Delgado-Vargas 2000). Colorants have been used for centuries to improve the appearance of food, cosmetics and clothing and colorants were given due importance in the Food and Drug Act of 1906, the first major comprehensive legislation on food additives (Delgado-Vargas and Paredes-Lopez 2003).

1.1.1. Synthetic colorants

During the 19th century, inorganic colored compounds like copper sulfate were used to color foods despite being toxic in nature. With the industrial production of large amounts of organic synthetic substitutes, colorants derived from petroleum and coal tar took precedence. These were used without proper safety evaluations sometimes resulting in health problems, intoxications and even death as a result of consumption. The 1938 Food Drug and Cosmetic act (FD&C) specified a list of colorants allowed in foods and presumably held them to a higher standard of purity than those available for other industrial uses. This act also made it mandatory to require Food and Drug Administration (FDA) certification for using organic colorants (Delgado-Vargas 2000). The 1960 color additive amendment to the FD&C specified two groups of colorants- certified color additives and color additives exempt from certification. Also, the manufacturers had to submit each batch of certifiable colors to the FDA for approval (Delgado-Vargas 2000;
Delgado-Vargas and Paredes-Lopez 2003). Subsequently the number of synthetic dyes were limited to seven FD&C colorants and two colorants for restricted use (Socaciu 2008).

Synthetic colorants are easy to produce, stable, less expensive and have better coloring properties than natural colorants (Pintea 2008). Usually these colors are blended to get the desired color and the required stability under conditions of processing and storage. The behavior of synthetic colors under different conditions like pH, light, acid, alkali and heat is well-characterized and hence individual colors or different blends can be suitably selected if information on the food product is available (Francis 1999). Synthetic colorants belong to concern level III and require the strictest safety evaluations. Although artificial colorants have a long history of use, consumers have increasingly begun to consider synthetic colorants undesirable (Socaciu 2008).

1.1.2. Natural colorants

By definition, a natural pigment is one that is synthesized by and accumulated and/or excreted by living cells. It may also be synthesized by cells under stress or by dying cells (Hendry and Houghton 1992). However it may also be ‘nature-identical’ and may be synthesized chemically, for example β carotene and canthaxanthin. Historically, natural pigments (e.g. saffron, turmeric, paprika and petals of various flowers) were used to color food products. Currently, 26 natural colorants including anthocyanins, curcumin, carminic acid, lycopene, betanin, paprika and saffron are permitted for use as exempt colorants in the United States.
Since colors have been categorized as ‘additives’ by the FDA, even natural colors are considered “added colors” but unlike synthetic colorants, they do provide clean-label alternative to the food product. Consumers look for products colored with food-based agents rather than synthetic coloring agents and additionally expect health effects from food. For example, anthocyanins are a class of colors which may provide additional health effects (Duangmal and others 2008). Technical advances in natural colors have allowed for better shades, stability and cost. Some products such as cheddar cheese and poultry products are acceptable only when they are pigmented with natural colorants (Delgado-Vargas and Paredes-Lopez 2003). Consequently, there has been decreased interest in the development of new synthetic colorants and increased effort to discover new natural alternatives (Socaciu 2008).

Of the total market of $1.7 billion, natural colors make up some $0.65 billion, and the colors market is forecast to reach $1.8 billion by 2014. The market for natural color is expected to increase by 6.5 % per year while total growth in the color sector is 4 % indicating negative growth rate for synthetic colorants (Trim 2006).

1.2. Avocado

The avocado (Persea americana Mill.) is a member of the commercially important plant family, Lauraceae, which also includes Cinnamonum, Camphor and Aniba which are used for aromatic oils, and Persea, Chlorocardium, Eusideroxylan and Laurus - used for timber although avocado has other uses as well (Chanderbali and others 2008). Lauraceae, along with Annonaceae, Magnoliaceae and Proteaceae, ranks among the oldest recorded flowering plants and has arisen from woody magnolia forebears. The
genus *Persea* is of African Gondwanaland origin, and its ancestral species migrated to Asia and via Europe to North America and via Antarctica to South America probably by the Paleocene. The avocado originated in the tropical regions of the New World but is now widely cultivated around the world. The archaeological record reveals it to be one of the oldest food plants of Mexico (8000 BC) (Heiser 1979).

The avocado is a large drupe (a fleshy fruit containing a single seed) and has the highest oil content of all fruits, with the possible exception of the olive. It is also possibly the most nutritious of all fruits (Chanderbali and others 2008). The avocado seed weight is around 16% of the total fruit weight (Ramos-Jerz 2007). Fruit color at maturity is green, black, purple, or reddish, and skin varies in thickness and texture depending on the variety (Yasir and others 2010). The avocado’s status as a food varies with the region where it is consumed and the degree of familiarity with which it is regarded by the local populace. The fruit is a traditional staple in Guatemala and nearby countries. It is also an ingredient of widely consumed, elaborately prepared traditional foods in Mexico such as guacamole, which is composed of pureed avocado pulp and other vegetables. The avocado’s high nutritional value and the potential usefulness of unsaturated oils in promoting health of heart and circulatory system are expected to attract the interest of populations among which this fruit is a newly offered item (Knight 2002).

There are eight well-defined geographical ecotypes of *Persea americana*, of which three (so called horticultural races) comprise the commercial avocado crop. These horticultural races are the Mexican (*P. americana* var. *drymifolia*), the West Indian (*P. americana* var. *americana*) and the Guatemalan (*P. nubigena* var. *nubigena* and *P. nubigena* var. *guatemalensis*) (Heiser 1979). Current commercial varieties are hybrids of
the races. For example the Hass variety belongs to the Guatemalan-Mexican hybrid group (Chanderbali and others 2008). Common varieties in commerce include Hass, Bacon, Feurte, Gwen, Lamb Hass, Pinkerton, Reed and Zutano (Ding and others 2007). The Hass variety is commonly grown in Mexico and California (USDA 2006). The fruits of Hass variety are pear to ovoid shaped and the skin is tough, leathery and dark purple or black when ripe; the seed is smaller than other common varieties (Morton and Dowling 1987). Harvesting time begins in mid-March in California. Total U.S. avocado production during the 2007/08 season was 170,000 tons (USDA 2008). The total world production in 2007/08 was 1.4 million tons with Mexico, being the largest producer, producing 1.1 million tons (USDA 2008). The European Union and North America are the largest importers of avocados (Chanderbali and others 2008).

1.2.1. Avocado pulp

Avocado pulp contains up to 30% oil and its consumption is believed to improve lipid profile of people with normal to high blood cholesterol levels (Kate and Lucky 2009). Avocado pulp is rich in oleic acid which is negatively associated with cardiovascular disease (Plaza and others 2009). The chemical composition of pulp of Hass variety was evaluated as: moisture, 77.3%; fat, 15.8%; protein, 1.83% and ash 1.01%. In addition to high content of monounsaturated fats, the pulp contains several bioactive phytochemicals including carotenoids, B vitamins, vitamins C and E, D manno-heptulose, β - sitosterol, persenone A and B. These constituents have demonstrated antifungal, antitumor and antioxidant activity in some studies (Lu and others 2005). The concentration of carotenoids in pulp was analyzed by Lu and others (2009) and lutein was
the most abundant carotenoid (213-340 μg/100 g). Other carotenoids were identified as: zeaxanthin, 8-18 μg/100 g; β-cryptoxanthin, 21-38 μg/100 g and α-carotene, 19-28 μg/100 g. The effect of month of harvest (January, April, July and September) from four different locations across California on the fat content and the carotenoids of the pulp was studied. As the season progressed, an increase in fat content and carotenoid concentrations was observed (Lu and others 2009). Fatty acids, sterols and antioxidant activity were studied in avocado pulp slices packaged in plastic bags under nitrogen, air or vacuum, stored at 8 °C. Oleic acid was the major fatty acid (57% of total content) and β sitosterol was the major sterol (89% of total sterol content). During refrigerated storage for 13 days, the fatty acid content was reduced whereas antioxidant capacity increased, with the maximum effect observed in vacuum samples. However, changes in the carotenoids were not studied, which may be the major contributors to the antioxidant activity (Plaza and others 2009).

Some of the major phenolic compounds in the pulp were identified using chromatography– hydroxybenzoic acids were quantified at 280 nm, as gallic acid equivalents (GAE), hydrocinnamic acid at 320 nm as chlorogenic acid equivalents and flavonols at 365 nm as rutin equivalents. The pulp contained 2.2 mg/100 g catechins, 34.6 mg/100 g hydroxybenzoic acids, 111.3 mg/100 g hydrocinnamic acids and 73.4 mg/100 g procyanidins. No flavonol equivalents were detected in the pulp. The total phenolic content of ethyl acetate, acetone or methanolic extracts of pulp were evaluated with the acetone extract giving the maximum amount of phenolics -100 mg GAE/100 g, followed by the extracts of methanol and ethyl acetate (Rodríguez-Carpena and others 2011).
1.2.2. Functional properties of avocado pulp

Avocado pulp is considered a health promoting food and as a result, there is increasing consumption and research (Lu and others 2009). Traditionally avocado pulp has been consumed for diverse health related reasons including wound healing and hair growth (Ding and others 2007). In West African countries, avocado pulp is widely used as baby food. It is also used as an aphrodisiac (Duke and Vasquez 1994).

Avocado pulp has been found to have in vitro growth inhibitory effects against cancer. Lutein, other carotenoids, and tocopherols are proposed as the active compounds (Lu and others 2009). Oil extracted from the pulp is being increasingly used in cosmetics, particularly the unsaponifiable fraction, because of its skin improvement properties (Koulbanis and others 1982). The unsaponifiable fraction is also used to treat osteoarthritis. Acetogenins extracted from the pulp have insecticidal, anti-cancer and anti-inflammatory properties which are explained in detail below.

1.2.2.1. Dermatological uses

Avocado oil specifically unsaponifiables (fraction of avocado oil which after hydrolysis does not produce soap) have been successfully tested for restoration of physiological equilibrium including water retention in the skin (Rosenblat and others 2010). Various formulations for improving suppleness of skin containing 20-40% avocado unsaponifiables as one of the ingredients, were generated (Koulbanis and others 1982). Avocado/soybean unsaponifiables (ASU), in the ratio 1:2 (2-10 µg/mL) were applied to human cultured foreskin fibroblasts in presence of an apoptosis inducing agent, phorbol myristyl acetate. Co-treatment resulted in an inhibition of apoptosis by the ASU.
Preparations for topical application and oral application in the form of capsule were patented (Segal and others 2002).

Paoletti and others (2010) demonstrated the up regulation of Human β- defensin (HBD) in skin keratinocytes as a result of treatment with AV119 (patented C7 sugar alcohols extracted from avocado pulp). HBD is an endogenous peptide produced by stimulated keratinocytes which has direct anti-microbial activity against a broad spectrum of pathogens. Activation of protein kinase C occurred which led to activator protein-1 (AP-1) and HBD gene activation (Paoletti and others 2010).

Treatment of keratinocytes (1 μg/mL for 60 min) with polyhydroxylated fatty alcohols (PFA) isolated from pulp and seed, prior to exposure to ultra violet (UV) B radiation, resulted in decreased generation of pro-inflammatory mediators - Interleukin-6 (Il-6) and Prostaglandin E2 (PGE2), and reduced formation of cyclobutane pyrimidine dimers. In human skin explants, treatment with the PFA significantly reduced UV-induced cellular damage. The major constituents were identified as 1-acetoxy-2,4-dihydroxy-heptadec-16-ene and 1-acetoxy-2,4-dihydroxy-heptadec-16-yne (Rosenblat and others 2010).

1.2.2.2. Cardiovascular effects

Avocado pulp has a high content of monounsaturated fatty acids (MUFA), primarily oleic acid, and may improve the fatty acid composition of blood (Colette 2003). A number of studies have been carried out in humans to study these effects.
Substitution of 30 g of mixed dietary oil with 200 g/d avocado pulp increased blood levels of oleic acid and decreased blood levels of myristic acid in 61 volunteers after 6 weeks (Pieterse and others 2005).

In a randomized controlled study, the effects of a diet rich in avocado MUFAs were examined in 37 adult subjects with mild hypercholesterolemia (with or without hypertriglyceridemia) and 30 healthy subjects after 7 d; significant decreases in TC (total cholesterol) and LDL-C (low density lipoprotein cholesterol) concentrations in healthy and hypercholesterolemic subjects were observed. In subjects with hypertriglyceridemia, a significant reduction in TG (triglycerides) concentration was also observed (López Ledesma R 1996).

Effects of a diet rich in MUFA using avocado as the major source in the diet (30% of the total calories were consumed as fat and 75% of the total fat was from the avocado) were studied in patients with phenotype II or phenotype IV dyslipidemia for 4 wk. Levels of TC and LDL-C decreased significantly in patients with phenotype II dyslipidemia. A significant increase in HDL-C (high density lipoprotein cholesterol) was also seen in patients with phenotype II and IV dyslipidemia (Carranza 1995).

In a randomized cross-over study, patients with non–insulin-dependent diabetes mellitus (NIDDM) were treated with isocaloric diets supplemented with avocados or high carbohydrate diet. A higher reduction (20%) in plasma triglyceride level was reported after consuming diet containing avocado as compared to diet with high carbohydrate content (8% reduction) (Lerman-Garber and others 1994). In another similar study, 15 women with NIDDM consumed a diet containing avocado or a high carbohydrate diet for 3 wk and lipid markers were measured before and after the study. Diet containing
avocado showed a higher reduction in total cholesterol and in LDL-C and in apolipoprotein-B. LDL-C and apolipoprotein-B did not significantly change as a result of the high carbohydrate diet (Colquhoun and others 1992).

1.2.2.3. Anti-cancer effects

The anti-carcinogenic effects of avocado pulp have been investigated. Inhibition of PC3 and LNCaP prostate cancer cell lines was observed after treatment with an acetone extract of the pulp of Hass variety (IC$_{50}$ = 295.0 and 97.6 µg/mL respectively). PC3 cells were dose dependently arrested in G$_2$/M phase after 72 h treatment. Increased expression of the cyclin dependent kinase (CDK) inhibitors, – p21 and p27, was observed (Lu and others 2005). A chloroform extract of avocado pulp selectively inhibited growth of an oral epithelial cancer cell line (IC$_{50}$ = 14 µg/mL) but not normal human oral epithelial cell lines (Ding and others 2007). This selective inhibition was associated with generation of reactive oxygen species (ROS) and resultant apoptosis in the cancer cells (Ding and others 2009).

The chloroform extract and two acetogenins isolated from pulp- [(2S,4S)-2,4-dihydroxyheptadec-16-ynyl acetate] (compound I) and [(2S,4S)-2,4-dihydroxyheptadec-16-ynyl acetate] (compound II) were tested for their role in targeting epidermal growth factor receptor (EGFR) /Ras /Raf /mitogen activated protein kinase (MEK)/extracellular-signal-regulated kinases (ERK1/2) pathway and thus inhibiting cancer. Treatment of oral cancer cells, 83-01-82 CA/MEK$_{CA}$ with chloroform extract of avocado pulp for 48 and 72 h down-regulated levels of EGFR, phosphorylated c-Raf and ERK1/2 in a concentration dependent manner. While compound I acted by inhibiting the activation of c-Raf and
ERK1/2, compound II acted by blocking EGF induced EGFR phosphorylation (D'Ambrosio and others 2011).

Persin, [(+)-(Z,Z)-1-acetyloxy)-2-hydroxy-12,15-heneicosa-dien-4-one] is synthesized in idioblast oil cells present in pulp and leaves and is known to act as a natural insecticide and fungicide (Rodriguez Saona and Trumble 2000). When tested in MDA-MB-231, MCF-7 and T-47D breast cancer cell lines, persin induced G₂/M cell cycle arrest and acted as microtubule stabilizing agent. Also, persin was shown to cause caspase-dependent apoptosis. Interest in persin is tempered by the fact that it has been shown to induce necrosis and apoptosis in mammary gland of lactating mice when administered by gavage as a single dose of 100 mg/kg (Butt and others 2006). This result suggests non-specific mammary gland toxicity.

1.2.2.4. Treatment of osteoarthritis

Osteoarthritis is characterized by failure of the repair process of damaged cartilage due to biomechanical and biochemical changes in the joint. Cartilage being non-vascularised has a restricted supply of nutrients and oxygen to the chondrocytes, which are cells responsible for maintenance of extracellular matrix. Initially, chondrocytes form clusters in damaged area subsequently resulting in increased synthesis of tissue destructive proteinases and increased apoptotic death of chondrocytes resulting in a matrix that is unable to withstand normal mechanical stresses. At a later stage, the bone and synovial tissue are also affected– leading to synovial inflammation and joint swelling, these processes further cause imbalance of cartilage matrix degradation and repair. The disease most commonly affects older people and is characterized by pain and
discomfort especially during physically demanding activities. Drugs and surgery are commonly used to manage the disease (Bijlsma and others 2011).

ASU is a mixture of one part avocado oil unsaponifiable to two parts soybean oil unsaponifiable. It forms an unconventional treatment for osteroarthritis and was first studied in Europe. It has been approved as a prescription drug in France and introduced as food supplement in Denmark (Angermann 2005).

In an animal study carried out in outbred dogs, ASU treatment for 3 months at a dose of 300 mg ASU/day or 300 mg ASU/3 days increased the formation of transforming growth factor (TGF-β) in joint fluid compared to control. There was no significant difference in the two treatments. TGF-β stimulates the production of extracellular matrix components including type II collagen and proteoglycan by chondrocytes (Altinel and others 2007). In horses with experimentally induced osteoarthritis, treatment with ASU extracts (300 mg/day for 70 d) did not have an effect on signs of pain or lameness, but did significantly reduce the severity of articular cartilage erosion and synovial hemorrhage, and significantly increased the articular cartilage glycosaminoglycan synthesis, compared to control (Kawcak and others 2007). In another clinical study, 164 patients with osteoarthritis were randomly divided into control and treatment groups (ASU at 300 mg/d) for 6 months. Pain was reduced to a greater extent in the ASU group and non steroidal anti inflammatory drug (NSAID) use was also reduced (Maheu and others 1998). Three other clinical studies using human patients have been reviewed, out of which two observed improvement in osteoarthritis with the use of ASU (Ernst 2003).

In another animal study, the in vivo effects of treatment with ASU on the development of osteoarthritic structural changes in the anterior cruciate ligament dog
model (osteoarthritis was induced by anterior cruciate ligament transection of the knee) were explored. The animals received either placebo or ASU (10 mg/kg per day) for 8 wk. The size of macroscopic lesions on the tibial plateaus was decreased in dogs treated with the ASU. Histologically, the severity of cartilage lesions on both tibial plateaus and femoral condyles, and cellular infiltration into the synovium were significantly decreased. Treatment with ASU also reduced loss of subchondral bone volume. ASU significantly reduced the level of inducible nitric oxide synthase (iNOS) and matrix metalloproteinase-13 (MMP-13), both of which are key mediators in the structural changes that take place in cartilage during the progression of osteoarthritis (Boileau C. 2009).

A treatment containing a combination of ASU and green tea for the treatment of arthritis has also been patented. The study demonstrated the in vivo effects of combination of catechins and ASU for varying period of time for treatment of arthritis in human subjects (Henderson and others 2006).

1.2.2.5. Anti-inflammatory effects

Anti-inflammatory effects of ASU have been reported. PGE$_2$ and NO production were reduced by 40% and 30%, respectively in LPS-induced bovine chondrocytes by treatment with 25 μg/ml ASU for 72 h. The mRNA expression of cyclooxygenase-2 (COX-2) and iNOS were reduced by 60 and 50 % respectively. The mRNA expression of pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα) and IL-1β, was also reduced by 40 and 50%, respectively. Expression of TNFα and IL-1β were also reduced in THP-1(human macrophage) cells by 40 and 50% after treatment with ASU for 72 h (Au and others 2007).
Human articular chondrocytes were stimulated with 17 ng/mL IL-1β (to induce an osteo-arthritic condition) and then treated with ASU (10 μg/mL) or avocado or soybean unsaponifiables alone (3.3, 6.6 and 10 μg/mL respectively) for 72 h. Both the mixture and the individual unsaponifiable inhibited the formation of IL-8 and PGE$_2$ at 10 μg/mL, however only the avocado unsaponifiables and avocado-soybean unsaponifiables inhibited the formation of IL-6 at all the concentrations tested. At a concentration of 10 μg/mL, the mixture had a more pronounced effect on cytokine production than either unsaponifiables alone. The activities of collagenase and stromelysin, both of which are important in arthritic progression, were inhibited by the mixed unsaponifiables at a concentration of 10 μg/mL (Henrotin and others 1998).

The avocado unsaponifiables consisted of compounds with furyl nucleus and a aliphatic mono or polyunsaturated chain (chain length = 3-17) substitution at position 2 (almost 50% by weight), squalene (2%), long chain saturated hydrocarbons (up to 5%), polyols (up to 15%), tocopherols (trace amounts) and sterols (between 4-20%) (Henrotin and others 1998).

(2R)-(12Z,15Z)-2-hydroxy-4 oxoheneicosa-12,15-dien-1-yl acetate (persin) and two novel similarly structured compounds, persenone A and persenone B were isolated from avocado fruit (Figure 1.1). These compounds inhibited the formation of NO, induced by LPS and IFN-γ in RAW 264.7 murine macrophage cells with IC$_{50}$ = 1.2 - 3.6 μM; by comparison, IC$_{50}$ for docosahexanoic acid, a naturally occurring inhibitor of iNOS induction was 4.3 μM. The compounds were also found to be potent inhibitors of O$_2^-$ generation by TPA differentiated HL-60 cells (Kim and others 2000). In another study, 20 μM persenone A completely suppressed both iNOS and COX-2 protein
expression in RAW 264.7 cells. Persenone A when applied on mouse skin, before application of TPA significantly suppressed H$_2$O$_2$ production (Kim 2000).

Figure 1.1. Structure of (a) Persin (b) Persenone A (c) Persenone B

Transforming growth factor (TGF-β) is involved in restoration of inflamed articular cartilage (caused by various agents including metalloproteinases and various cytokines). An increased expression is also beneficial to bone remodeling and thus management of osteoporosis. Plasminogen activator inhibitor 1 (PAI-1) inhibits the activity of matrix metalloproteinases. Expression of TGF-β and PAI-1 after treatment of chondrocytes with unsaponifiables from avocado and soybean (1:2) at a concentration of
10 µg/mL, was increased therefore unsaponifiables were considered useful for treatment of osteoarthritis and arthritis (Boumediene and others 2008).

1.2.2.6. Antimicrobial effects

The antimicrobial activity of an acetone:water (70:30, v/v) extract of avocado pulp against a battery of bacteria was evaluated using the agar disk diffusion method. The zone of inhibition induced by 2 mg extract against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli* was 41.5%, 33.9%, 33.0% and 44.6% of that of positive control (10 µg of chloramphenicol) (Rodríguez-Carpena and others 2011). Castillo-Juarez and others (2009) demonstrated the antibacterial activity of a methanolic avocado extract against *Helicobacter pylori* (Israel Castillo-Juárez 2009).

1.2.3. Avocado seed

The proximate composition of the seeds of two varieties of avocado, Hass and Feurte, were determined as follows: moisture content, 54.1% and 50.2%; ash content, 1.24% and 1.34%; protein, 2.38% and 2.45%; sugars, 3.5% and 2.2%; starch, 27.5% and 29.6%; and fat 0.80% and 1.03%, respectively (Weatherby and Sorber 1931). Seeds of both varieties had lower fat content than the corresponding pulp. In another study, the chemical composition of seed of Hass variety was evaluated as: moisture, 55.7%; fat, 1.39%; protein, 2.19%; and ash, 0.7% (Ramos-Jerz 2007).

The lipid and fatty acid composition of the pulp and seed of the Feurte, Bacon and Hass varieties was investigated. Total lipid content of the seeds of the Feurte, Bacon and Hass were 1.1, 1.6 and 1.1 %, respectively. The majority of lipids in the pulp were neutral...
lipids (95-96.8%), with a smaller amount of glycolipids (2.5-3.21%) and phospholipids (0.7-2.1%). By contrast in the seed, the percentage of neutral lipids was reduced (77-80%), and the percentage of other two fractions – glycolipids (12-13.2%) and phospholipids (7.4-10.9%) was higher. The neutral lipids were largely monoacylglycerols, diacylglycerols, free sterols and free fatty acids with a lesser amount of triacylglycerols in the neutral lipids of seeds than pulp (Takenaga and others 2008).

Phytochemical studies on avocado seeds have identified various classes of natural products including phytosterols, triterpenes, fatty acids, furanoic acids, abscisic acid, proanthocyanidins and other polyphenols (Leite and others 2009; Ding and others 2007). Phenolics are present at higher levels in the avocado seed than in the pulp and have reported health benefits (Tesfay and others 2010; Wang and others 2010). The phenolic content of the seeds vary with the variety, conditions of growth and stage of maturity (Tesfay and others 2010). The determination is also influenced by the method of extraction employed in the experiment. Soong and Barlow (2004) determined the phenolics in the seed as 88.2 mg/g gallic acid equivalent (GAE). Wang and others (2010) determined the phenolics for the seed of Hass variety as 51.6 mg/g GAE. However the phenolic content for the Florida varieties (varieties having bigger fruit size and lesser pulp fat content) were lower than Hass and varied from 19.2 to 40.2 mg/g GAE. The content of procyanidins in the Hass variety was found to be 47.7 mg/g. Phenolic content was found to be 137 µg/mg tannic acid in seed (Lee and others 2008). Total phenolic content of ethyl acetate, acetone and methanolic extracts of seed was evaluated using gallic acid as standard. The acetone extract contained the maximum phenolics with the content being 608.2 g GAE/100 g followed by the extracts of methanol and ethyl acetate.
(Villa-Rodríguez and others 2011). Tesfay and others (2010) determined the effect of ontogenic stage of the phenolic content in avocado seed of Hass variety. At 6 months before maturity the phenolic content was 320 µg/g GAE, but as maturity approached, the content declined to 137 µg/g GAE. It was hypothesized that the phenolics play a role as antioxidants during development and are reduced at maturity.

Wang and others (2010) reported the presence of catechin, epicatechin, and A- and B-type proanthocyanidins (degree of polymerization = 2-6) in the seed (Soong and Barlow 2004). Catechin, epicatechin and leucoanthocyanidins (3, 4-flavandiols) were identified as major phenolics in seed (Prabha and Patwardhan 1980). The phenolic compounds– hydroxybenzoic acids, were quantified at 280 nm as gallic acid equivalents; hydrocinnamic acid quantified at 320 nm as chlorogenic acid equivalents; and flavonols at 365 nm as rutin equivalents. The seed contained 237.8 mg/100 g catechins, 282.7 mg/100 g hydrocinnamic acid, 1.7 mg/100 g flavonols and 4.59 g/100 g procyandins. No hydroxybenzoic acid equivalents were detected in the seed (Rodriguez-Carpena and others 2011). Tyrosol derivatives, epicatechin (4β→8)-catechin, epicatechin (4β→8) epicatechin, and A-type trimers A2→(+) catechin and A2→(-) epicatechin were identified as higher molecular weight phenolics. Some of the phenolics are presented in Figure 1.2 (Ramos-Jerz 2007).
Figure 1.2. Structure of phenolic substrates present in the seed (a) 3-Hydroxy-tyrosol-1’-O-β-D-glucoside (b) Tyrosol (c) 3’-Hydroxy Tyrosol (d) Chlorogenic acid (e) Quinic acid (f) Epicatechin (g) Catechin
1.2.4. Antioxidant activity of avocado seed

The antioxidant capacity of avocado seeds has been studied by mainly electron transfer based assays. Soong and Barlow (2004) used ethanol: water (1:1, v/v) extract of the avocado seed to determine the ascorbic acid equivalent antioxidant capacity (AEAC) using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) cation radical scavenging assay and Ferric reducing antioxidant power (FRAP) assay, and determined the values to be 1160 µmol/g and 1484 µmol/g respectively. These values were 55.3 and 154.5 times greater than that of the pulp respectively. The antioxidant activity of the ethyl acetate, acetone and methanol extracts of seed using ABTS radical scavenging assay was evaluated with the acetone extract giving highest antioxidant activity – 158.3 mmol 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent/g fresh matter (Villa-Rodríguez and others 2011). Tesfay and others (2010) determined the antioxidant capacity using ABTS and FRAP assays as 2.59 mmol/g and 1.13 mmol/g, respectively. The values for the pulp were 784 µmol/g and 67.3 µmol/g, respectively. The radical scavenging capacity of 20 µg/mL methanolic extract of avocado seed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was comparable to 15 µM Trolox in the DPPH assay. Nagaraj and others (2010) prepared a methyl alcohol:water (4:1 v/v) extract of avocado seeds which was further extracted with ethyl alcohol. The resulting fraction contained alkaloids and terpenoids. These fractions were tested for their antioxidant activity using the DPPH radical assay and the terpenoids fraction had the maximum antioxidant activity (97.2 %) when normalized to 5µg/mL ascorbic acid. Antioxidant activity for methyl alcohol fraction, ethyl alcohol fraction and alkaloid fraction were 72 %, 94.6 % and 68.2 % respectively.
The antioxidant activity of the seed extract has also been evaluated using the oxygen radical absorbance capacity (ORAC) assay, a hydrogen atom transfer based reaction the seed extract was found to contain 428.8 TE/g, whereas the pulp contained only 11.6 TE/g (Wang and others 2010). Total antioxidant levels were higher in the peel and seed tissues than in the pulp. This represents a possible reason why avocados are susceptible to post-harvest mesocarp disorders. The antioxidant activity of seed was due to presence of ascorbic acid and total phenols, while the C7 sugar, D-mannoheptulose was responsible for the antioxidant activity of the pulp (Bertling and others 2007).

Antioxidant activity has also been evaluated in food systems. An acetone: water (70:30, v/v) extract from 10 g of avocado seed was added to 1 kg porcine patties, the product stored at 5 °C for 15 days and color (Lightness, L*, redness, a*, and yellowness, b*), thiobarbituric acid reactive substance (TBARS) and protein hydrazones were measured. The samples with avocado seed had better color preservation and also lower TBARS and hydrazones values on day 15 than the controls. Peel extracts added to the patties also showed similar effects (Rodríguez-Carpena and others 2011).

1.2.5. Functional properties of avocado seed

Potential medicinal properties of avocado seed have been reported in the literature. Ethno pharmacological studies of the Aztec and Maya cultures showed the use of decoctions of avocado seeds for the treatment of mycotic and parasitic infections (Kunow 2003). Seed preparations have also been reported for use against diabetes and for gastrointestinal irregularity. They have reportedly also been used to decrease muscle pain and as anti-inflammatory preparation (Ramos-Jerz 2007). The seed is considered as anti-
anemic and diuretic (Duke and Vásquez 1994). Historically, colored exudates from avocado seeds was used as indelible ink by the *Conquistadors* in the 1500s (Morton and Dowling 1987). A piece of the seed or the decoction put into tooth cavity is reported to relieve toothache. The powdered form of avocado seed is believed to cure dandruff and the seed oil is applied to skin eruptions (Morton and Dowling 1987).

Topical application of avocado seed paste has been used for treatment of arthritis. In Nigeria, it is mixed with soups and puddings because it is considered to be effective in management of hypertension (Ozolua and others 2009). Seed was also used to treat snakebite. Crushed seed was used as a contraceptive and as an abortive (Duke and Vasquez 1994). In African tradition, the ground seeds were consumed to treat whitlows and dysentery (Hatfield 2004).

### 1.2.5.1. Anti-cancer effects

Only one paper has reported the anti-carcinogenic effects of avocado seed and peels. Treatment of MDA-MB-231 breast cancer cells with the methanolic extract of avocado seed led to induction of apoptosis as measured by increased caspase-3, caspase-7 and poly (ADP ribose) polymerase (PARP) cleavage at concentrations greater than 100 µg/mL. DNA laddering was also observed. The peel extract more potently induced similar effects. This correlated with higher total polyphenols, total flavonoid and higher radical scavenging activity in the peel than in the seed (Lee and others 2008).
1.2.5.2. Anti-inflammatory effects

Arachidonic acid is the precursor to a large family of compounds called eicosanoids which includes COX-derived prostaglandins and lipoxygenase-derived leukotrienes. Eicosanoids play important physiologic and pathologic functions including as mediators of inflammation. The enzyme Phospholipase A2 (PLA2) releases arachidonic acid from membrane phospholipids from sn-2 position and thus plays an important role in inflammation (Sudhir 2005). Lipidic polyols purified from avocado seed were found to inhibit activity of secretary PLA2. The active compounds were identified as 1-acetoxy-2,4-dihydroxy-n-heptadeca-16-en (olefin A), 1,2,4-trihydroxy-n-heptadeca-16-en (olefin B), 1-acetoxy-2,4-dihydroxy-n-heptadeca-16-yn (acetylene A) and 1,2,4-trihydroxy-n-heptadeca-16-yn (acetylene B). Compared to control, inhibition of approximately 98% of enzyme activity was caused by 12.2 mM of olefin A, 7.5 mM of acetylene A, 0.17 mM olefin B or 0.08 mM of acetylene B (Etozioni 2003).

1.2.5.3. Anti-diabetic effects

Seed extracts have been shown in laboratory models to reduce blood glucose and ameliorate diabetes. Treatment of alloxan-induced diabetic rats with 450 and 900 mg/kg ethanolic seed extracts for 14 days reduced blood glucose levels by 47 -55 %. Histological study suggested a restorative effect of extract on pancreatic islets (Edem 2009). Edem, Ekanem and others found that treatment with 300 and 600 mg/Kg aqueous seed extract for 21 days reduced plasma glucose concentrations by 73 and 78 % respectively in alloxan-induced diabetic rats. The glucose levels of non-diabetic rats were also reduced by 35 - 39 %. The hypoglycemic effects are hypothesized to be due to
presence of insulin like substances in the extract, stimulation of β cells to produce more insulin, or enhanced glucose metabolism (Edem and others 2009).

1.2.5.4. Anti-hypertensive effects

An extract of avocado seed is used traditionally in Nigeria for the treatment of hypertension (Imafidon and Amaechina 2010). An aqueous seed extract from Fuerte avocados (200 - 700 mg/kg) significantly reduced the blood pressure of hypertensive rats after 4 weeks. A reduction in plasma triacylglycerol, total cholesterol and low density lipoprotein levels was also observed at doses of 500 mg/kg and higher (Imafidon and Amaechina 2010). In a similar study, 200 - 700 mg/ Kg aqueous avocado seed extract dose-dependently reduced blood pressure, cholesterol, glucose, urea and sodium levels in hypertensive rats after 5 weeks (Kate and Lucky 2009).

Acute doses of 260 mg/Kg aqueous seed extract, when given to hypertensive rats, for 10 days, led to a reduction in mean arterial pressure (MAP) of 39.3 – 43.5 %, from baseline. Heart rate also reduced by 9.4 -19.8% (Anaka and others 2009).

1.2.5.5 Dermatological uses

Avocado seed may promote the growth of keratinocytes. Polyhydroxylated fatty alcohols derived from hexane extract of seed were applied to human keratinocytes before and after UV B irradiation and the cell viability was higher compared to control. The treatment also reduced the numbers of sunburnt cells in human skin explants, enhanced DNA repair and reduced the secretion of pro-inflammatory mediators IL-6 and PGE2. 1-acetoxy-2,4-dihydroxy-hepta-dec-16-ene and 1-acetoxy-2,4-dihydroxy-hepta-dec-16-yne
were identified as the major active constituents in the extract (Rosenblat and others 2010).

Proliferation and cell viability of keratinocytes were triggered by 10 μg/mL of the water-ethyl acetate extract and a high-speed countercurrent chromatography (HSCCC) fraction of the seed (Ramos-Jerz and others 2007).

Avocado seed unsaponifiables were patented for use in a cosmetic base containing 5 to 15 % unsaponifiable fraction of avocado seed (UFA) lipids to improve the skin by measuring skin properties in human subjects on the basis of different studies. In the first study, after six weeks of twice daily topical applications, 28 out of 40 volunteers showed at least mild or moderate improvement in reducing stretch marks and keratosis (a condition characterized by excess growth of keratin in the skin). The second study observed that with a treatment for four weeks, UFA cosmetic application was well correlated with improvement in appearance of stretch marks as opposed to no difference in the control. A third study observed that redness, hypopigmentation, hyperpigmentation and ridging were reduced significantly as a result of treatment. A fourth study observed that 33% increase in skin moisture retention and an 8% increase in skin elasticity occurred compared to the control after two weeks of UFA application. The fifth study measured a 37.5 % increase in thickness with changes happening in both epidermal and dermal layer with application for six weeks and the sixth study measured the effect on keratoses in 25-65 years aged women who applied UFA and control for five weeks after which the resolution of keratoses was observed by clinical experts, and 75% of patients showed improvement. Addition of metal chelators improved the UFA’s effect on the damaged skin (Moy 1999).
The unsaponifiable component of avocado seed oil was shown to inhibit the action of lysyl oxidase. This enzyme initiates cross links in collagen and in collagentic connective tissue including skin and overabundance of these cross links results in wrinkles, and a lack of elasticity in the skin (Werman and others 1990).

Linoleic furan, extracted from the seeds and after combining with a cosmetic carrier can be applied topically for enhancing metabolic activity in dermal and epidermal tissues. Hairless mice were used for this study with four sets of 8 female mice being treated for 14 days with 3% natural or synthetic isolated linoleic furan. After treatment, animals were sacrificed and skin used for analysis. The enhanced metabolic activity caused an increase in elasticity, protein content, DNA content and increase in thickness in the treatment group. Also, increased fibroblast population, collagen synthesis and glucose metabolism were observed. Compounds with a similar structure, having at least 9 carbons units attached to number 2 position of furan ring were also considered effective (Counts and Huber 1996). The compounds have been subject of two patents (Counts and Huber 1996; Ruiseco 1989).

Avocado seed is an important ingredient of oil based scalp treatment which has been the subject of a patent. The preparation involves soaking seeds in a mineral oil for around four weeks, combining with other ingredients and using it on the scalp. It prevents frying and falling of hair and has been tried successfully in human subjects (Ruiseco 1989).
1.2.5.6. Antimicrobial activity

The antimicrobial activity of ethanolic extract from seed extract was tested on select gram positive and gram negative bacteria and the extract was found to be effective against *Salmonella enteritidis, Citrobacter freundii, Pseudomonas aeruginosa* and *Enterobacter aerogenes* (median inhibitory concentration = 125 to 250 µg/mL) (Raymond Chia and Dykes 2010).

Fungicidal effects were observed against *Candida* spp, *Cryptococcus neoformans* and *Malassezia pachydermatis*, an organism with intense pathological activity in carnivorous animals. The minimal inhibitory concentration for the methanol extract was respectively in the range of 0.125 to 0.625 mg/mL, 0.08 to 0.156 mg/mL and 0.312 to 0.625 mg/mL (Leite and others 2009). The antimicrobial activity of crude terpenoid fractions and crude alkaloid fraction obtained using solvent extraction was assessed and both the fractions were found to inhibit the growth of *S. aureus* and *B. subtilis* (Nagaraj and others 2010).

1.2.5.7. Insecticidal effect

There is a pressing need for new effective insecticides as exotic insect species are spread and serve as vectors for various disease organisms, for example malaria and West Nile Virus in the western hemisphere (Michaelakis and others 2009). Leite and others (2009) tested the larvicidal activity and *in vitro* antifungal potential of hexane and methanol extracts of avocado seeds and found the LC$_{50}$ against *Aedes aegypti* larvae (Yellow Fever Vector) as 16.7 mg/mL and 8.9 mg/mL for hexane and methanol extract, respectively. Phytochemical analysis revealed the presence of 1,2,4-trihydroxy decane
and β-sitosterol in the hexane fraction and flavonoids, tannins, alkaloids and triterpenes in the methanol extract (Leite and others 2009).

The methanol extract of the seed showed moderate activity against mobilization of epimastigotes and trypomastigotes of Trypanosoma cruzi in vitro (minimum inhibitory concentration for 24 h was >500 μg/mL). Six 1,2,4-trihydroxyheptadecane derivatives and two 1,2,4-trihydroxynonadecane derivatives were isolated as active compounds (Abe and others 2005).

1.2.6. Safety of avocado seed extract

Crucial to its use in color applications and as a potential medicinal agent is the safety of the seed extract. An aqueous extract of avocado seed (2-10 g/kg) administered as a single dose to rats by gavage resulted in no signs of toxicity in the two subsequent weeks. On sub-chronic administration of 2.5 g/kg for 28 days, no gross or hematological changes took place (Ozolua and others 2009).

1.3. Polyphenol oxidase

The enzyme polyphenol oxidase (PPO) is of immense economic importance as it is found universally in almost all fruits and vegetables and leads to changes in color during improper post harvest storage. It has a dinuclear copper centre, which inserts oxygen in a position ortho to an existing hydroxyl group in an aromatic ring, followed by the oxidation of diphenol to the corresponding quinone (Lin and others 2010). The reactive o-quinones then undergo non-enzymatic pathways to produce brown colored products (Mustafa and others 2005). The polymerization of quinones into red, brown or
black pigments depends on conditions like the nature and amount of endogenous phenolic compounds, the presence of oxygen, reducing substances, or metallic ions, the pH and temperature, and the activity of the PPO (Dogan and others 2006).

Although often associated with undesirable browning reactions, PPO has also shown to produce attractive colors in some systems. PPO-catalyzed conversion of catechins to theaflavins in the production of black tea is responsible for the characteristic orange color of that beverage (Balentine and others 1997). Yellow-orange color production occurs as a result of oxidation of polyphenols by the PPO during processing of apples into ciders (Guyot and others 2007). Mustafa and others (2005) synthesized a yellow colored product through oxidation of ferulic acid with laccase, an enzyme functionally similar to PPO, and present in fruits and vegetables. They found that in a biphasic system containing ethyl acetate a stable yellow colored product was formed unlike in aqueous phase in which browning occurred. They attributed this color to the incomplete activity of PPO in the medium containing ethyl acetate. Jiménez-Atiénzar and others (2004) oxidized catechin at pH 7.5 using PPO and found that the absorbance maxima of the product was 430 nm which corresponded to a yellow colored product. Guyot and others (2007) synthesized a yellow dye from phloridzin, a flavonoid, by oxidizing it with PPO and proposed its use as replacement for synthetic colorant, tartrazin. The pigment obtained was a stable yellow colored pigment with only weak degradation at ambient temperature storage. The structure of the phloridzin and the colored pigment formed as a result of enzymatic and subsequent chemical reactions is drawn in figure 1.3.
Figure 1.3. The structure of the (a) phloridzin and (b) the colored pigment produced as a result of PPO activity

Some of the other flavonoids that are acted upon by PPO and produce colored products are catechin (Oszmianski and Lee 1990) and eriodictyol (Jiménez-Atiéñzar and others 2005). Spectral changes produced in the oxidation of eriodictyol by peach PPO were followed over time. A product with $\lambda_{\text{max}} = 390$ nm, corresponding to $\alpha$-quinone was seen to appear before another with $\lambda_{\text{max}} = 475$ nm. Progress curves at this wavelength revealed a lag, length of which varied with enzyme and substrate concentrations. The product absorbing at 475 nm was derived from eriodictyol-$\alpha$-quinone and the production was inhibited by addition of 3-methyl-2-benzothiazolinone hydrazine hydrochloride (MBTH), a potent nucleophilic reagent that reacts with eriodictyol-$\alpha$-quinone to form a dark pink product.

Enzymatic oxidation of catechin was studied at pH 3.0 and 6.0 by monitoring reaction products by HPLC (Guyot and others 1996). The reaction products were
colorless at pH 3.0 but yellow at pH 6.0 with most of the products eluting after catechin, indicating being either more non-polar or bigger in size than catechin. The authors proposed that quinones formed at the two pH conditions may react differently - nucleophilic Micheal 1-4 addition of a phenolic ring onto o-quinone or through coupling of semi quinone radicals, formed by reverse proportionation. Micheal 1-4 addition could be favored by high pH, which increases nucleophilic character of catechin whereas low pH could favor radical mechanisms by increasing the reactivity of semi-quinone radicals.

1.4. Purpose and objectives

Currently avocado seed is an under-utilized resource thus creating a potential problem for disposal (Ramos-Jerz 2007). However there is rich ethnobotanical information on its medicinal usage and also some recent studies pointing to potential bioactive properties. If the seed is used as a colorant, the avocado industry can benefit by getting value from a hitherto unused source. Further if additional functionality (i.e. anticancer or antioxidant activity) can be demonstrated, seed extract may serve as an ingredient in functional foods. The present research is significant because:

- Consumer focus is shifting to functional foods having ingredients derived from natural sources therefore colored avocado seed extract represents potential addition to currently available natural colorants. Food manufacturers need more options for natural colorants because the available natural colorants do not function as well as synthetics under some processing/storage conditions (Pintea 2008), natural color from avocado seed will provide a colorant having different functional properties than common colorants like anthocyanins and carotenoids.
Thus it could be used independently or in conjunction with other natural colorants.

- Food ingredients which serve additional functions are more favored by processors and consumers. Avocado seed extract has potential antioxidant activity, anti-carcinogenic potential and anti-inflammatory properties and thus can give added functionalities to the food, and health benefits when consumed.

**Objective**

The overall goal of the project is to characterize the colored avocado seed extract with respect to structure and function.

**Hypothesis**

I hypothesize that the orange color formed in avocado seed results from a polyphenol oxidase catalyzed reaction and that the resultant colored extract possesses antioxidant, anticancer and anti-inflammatory activities.

Specific objectives and the hypothesis under them are provided below:

Objective 1: To study the kinetics of color production and involvement of enzymes involved in color formation

Hypothesis: Polyphenol oxidase (PPO) is involved in color formation in the seed

Objective 2: To assess the effects of changes in pH, oxygen, temperature and time of storage in the colored extract
Hypothesis: The color is influenced by changes in pH, time of storage, temperature, and absence of oxygen

Objective 3: To assess the antioxidant and anti-carcinogenic activity of the extract
Hypothesis: The colored extract has antioxidant and anti-carcinogenic effects

Objective 4: To assess the anti-inflammatory activity of the extract
Hypothesis: The colored extract has anti-inflammatory activity

1.5. References


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Chapter 2

A COLORED AVOCADO SEED EXTRACT AS A POTENTIAL NATURAL COLORANT

Abstract

There is an increasing consumer demand for, and scientific interest in, new natural colorants. Avocado (*Persea americana*) seed, when crushed with water, develops an orange color in a time-dependent manner. Heat treatment of the seed prevented color development, whereas addition of exogenous polyphenol oxidase (PPO) restored color development. Color formation resulted in a decrease in the concentration of polyphenols indicating utilization for color formation. The orange color intensified as the pH was adjusted from 2.0 to 11.0, and these changes were only partially reversible when the pH was adjusted from 7.5 to 11.0 in the presence of oxygen but completely reversible when the pH was changed in the absence of oxygen. The color was found to be stable in solution form at -18 °C for two months. CASE was added to four products—two fruit juices, milk and a baked product to test its colorant properties. The products showed a dose dependent change in color; however ΔE values and chroma did not always reflect the differences seen visually for all the products. These results suggest that the avocado seed may be a potential source of natural colorant.
2.1. Introduction

Color is one of the most obvious characteristics of a food and, if not appealing, negatively impacts consumer acceptance (Delgado-Vargas and Paredes-Lopez 2003a). Although artificial colorants have a long history of use, and are easy to produce, stable, less expensive and have better coloring properties than natural colorants, consumers have increasingly begun to consider synthetic colorants undesirable. Consequently, there has been increased effort to discover new natural alternatives (Socaciu 2008).

Whereas synthetic colorants generally have similar properties, natural colorants, even if they are of the same shade, may differ in terms of chemistry and functionalities. For example annatto and turmeric differ in their functionalities. Annatto, as norbixin will precipitate at pH below 5.0 whereas turmeric will not (Socaciu 2008). Thus it is crucial to understand the properties of any prospective natural colorant, to decide about its applications. The factors that adversely affect colorant stability include presence of temperature, light, oxidizing and reducing agents, acids or time of storage (Socaciu 2008). There is still a lack of natural colors to completely replace synthetic colorants, and hence, efforts to search more options are needed.

Avocado (Persea americana Lauraceae) is an important tropical crop which is rich in unsaturated fatty acids, fiber, vitamins B and E, and other nutrients (Gomez Lopez 1998). The Hass variety is the most commonly grown. Total U.S. avocado production during the 2004/05 season was 162,721 tons with 90% originating in California (USDA 2006). Mexico, the world’s largest producer, supplied 1.14 million tons in 2007 (Market News-Avocado update, 2008). The seed accounts for 16% of total avocado weight, and is an under-utilized resource (Ramos-Jerz 2007). Ethno-pharmacological studies of the
Aztec and Maya cultures have reported the use of decoctions of avocado seeds for the treatment of mycotic and parasitic infections (Kunow 2003). Seeds have also been reported for use against diabetes, inflammation and gastrointestinal irregularity (Ramos-Jerz 2007; Kunow 2003). The powdered form has been used for skin eruptions and to cure dandruff (Morton and Dowling 1987).

Avocado seeds have more antioxidant activity and polyphenol content than the pulp (Wang and others. 2010; Soong and Barlow 2004). Phytochemical studies on avocado seeds have identified various classes of natural compounds such as phytosterols, triterpenes, fatty acids, furanoic acids, abscisic acid, proanthocyanidins and polyphenols (Leite and others 2009; Ding and others 2007). Wang and others (2010) have reported the presence of catechin, epicatechin, and A- and B-type procyanidin dimers and trimers, tetramers, pentamers and hexamers in the seed.

Our laboratory has observed that avocado seed when ground with water and incubated in the presence of air produces a bright orange color (previously unreported). The aim of the present work was to characterize the color formation, to study the mechanism of color formation and the color properties of the extract.

2.2. Material and Methods

2.2.1. Reagents

Ripened avocado (Persea americana, Hass variety) were sourced locally and stored at 4 °C until used. No change in the seed color was noticed as a result of this storage. Mushroom polyphenol oxidase (tyrosinase), horseradish peroxidase, hydrogen peroxide and tropolone were purchased from Sigma Chemical Co. (St. Louis, MO).
Dimethyl sulfoxide (DMSO) was purchased from EMD Chemicals (Gibbstown, NJ).

Folin Ciocalteu reagent was purchased from Fluka (Buchs, Switzerland). Gallic acid was purchased from Alfa Aeser (Lancaster, PA). All other reagents were of the best commercial grade available.

2.2.2. Preparation of colored avocado seed extract (CASE)

Avocado seeds were separated from the fruit, washed and peeled. Seeds were weighed, cut with a knife and ground in 0.7 vol of deionized (DI) water in a Waring blender for 60 sec at high speed. The resulting paste (pH 6.4) was spread to 1 cm thickness on a flat surface (to enable uniform exposure to air) and incubated at 24 °C for 35 min. The paste was mixed with a spatula 2-3 times during this time. The colored paste was transferred to a beaker, an equal volume of methanol was added, and the mixture was sonicated for 20 min in a Branson 3510 sonicating water bath (Danbury, CT). An additional 2 vol of methanol was added, and the mixture centrifuged at 1200 × g for 10 min. The supernatant was collected and dried under vacuum to remove the methanol. Residual water was then removed by freeze drying, and the resultant powder was stored in a desiccator at -20 °C.

2.2.3. Kinetics of color formation

The kinetics of color formation was studied using two methods: paste was incubated for different time intervals, the color extracted and absorbance of the extract measured or paste was incubated and CIE (International Commission on Illumination) L a b values were read at different time points.
For absorbance measurements, pastes were prepared as described above with the modification that 10 g samples were incubated for 0.5, 2, 5, 10, 15, 20, 25, 30 and 35 min after which they were combined with methanol (3 vol), then sonicated and centrifuged as above. Visible absorbance spectra were recorded immediately (λ = 380 nm to 700 nm) using an Agilent 8453 spectrophotometer (Santa Clara, CA) by placing samples in disposable 1.5 mL cuvettes (Plastibrand, Wertheim, Germany).

To measure L a b values, seeds were weighed, cut and ground in 1 vol DI water in a Waring blender for 60 sec at high speed. The ground paste was spread in a Petridish (CM-A128, Minolta) and Lab values recorded at specified time intervals using a Konica Minolta chromameter CM-3500 d (Ramsey, NJ) in reflectance mode. The paste was mixed thoroughly just before taking measurements. The conditions were a ‘D’ illuminant with a 8° observer. Color difference (ΔE) was calculated using the following formula:

$$\Delta E = \sqrt{(L-L_o)^2 + (a-a_o)^2 + (b-b_o)^2}$$

where L_o, a_o and b_o are L, a and b values of the previous time point respectively.

### 2.2.4. Role of polyphenol oxidase (PPO) in color formation

Avocado seeds were prepared and ground as above. An additional 1.5 vol of DI water was added and mixed, and the paste was kept at 100 °C for 30 min to destroy endogenous enzyme activity. The paste was cooled to 24 °C in a water bath. To 25 g of the cooled paste, 1000, 2500 or 5000 U of mushroom polyphenol oxidase (prepared in phosphate buffered saline : NaCl, 136 mM; KCl, 2.7 mM, Na_2HPO_4, 10 mM; KH_2PO_4, 1.7 mM at pH 6.8) was added and the mixture spread on flat surface as described above. After 35 min, methanol (3 vol) was added to the samples which were sonicated and
centrifuged as described above. Absorbance spectra were obtained as described above. Positive control samples were prepared similarly but were not subjected to heat treatment. Negative control samples were subjected to heat treatment but exogenous PPO was not added.

The involvement of PPO was further confirmed by adding 58 μM tropolone to the seeds while grinding after which the paste was spread on a flat surface for 35 min and processed further. Also, the PPO in respective amounts and tropolone were added together to the inactivated paste and processed as done for only PPO added samples. A high concentration of tropolone was used to ensure complete inhibition of PPO activity.

2.2.5. Role of peroxidase in color formation

Avocado seeds were prepared, ground and heat inactivated as described above. To 25 g of the cooled paste, 1000, 2500 or 5000 U of horseradish peroxidase (prepared in phosphate buffered saline, pH 6.8) and 1.9 g H$_2$O$_2$ per 1000 U were added. Respective H$_2$O$_2$ and enzyme controls were also prepared. The paste was processed further as described above.

2.2.6. Phenolic content of CASE

To CASE, 50 vol hexane was added and the mixture was shaken for 30 min in an orbital shaker at 600 rpm to remove residual lipids. Hexane was removed by centrifugation at 1200 × g for 10 min. The marc was then extracted with 50 vol of methanol:ethyl acetate (1:1, v/v) for 1 h in the orbital shaker at 600 rpm. The supernatant was separated by centrifugation, and dried under vacuum at 24 °C. The marc was then
extracted with 50 vol of DI water at 600 rpm for 1 h, centrifuged and the aqueous supernatants dried under vacuum at 24 °C for 12 h. Both the methanol : ethyl acetate and water fractions were used for determination of phenolic content by the modified method of Singleton & Rossi (1965). Aliquots of the samples of appropriate dilutions were prepared in methanol and were combined with 790 vol of DI water and 5 vol of Folin–Ciocalteu reagent. The solution was mixed and 15 vol of 15% sodium carbonate solution was added to the sample. Samples were mixed again and the absorbance determined at 765 nm after 2 h. Gallic acid was prepared in methanol and was used as the standard. The total amount of phenolic compounds was expressed as gallic acid equivalent, GAE (mg/g). Uncolored extract, prepared in a method analogous to the colored extract except that methanol was added immediately after grinding the seeds, was also tested for phenolic content for comparison.

2.2.7. Effect of pH on color of CASE

Phosphate buffered saline at pH 2.0, 4.0, 6.0, 7.5, 9.0 and 11.0 was prepared in 20 % aqueous methanol and combined with CASE at a final concentration of 2 mg/ml. The samples were vortexed then centrifuged at 1200 × g for 10 min. Visible absorbance spectra were recorded as described above. Lab values were determined using a Konica Minolta chromameter CR400 (Ramsey, NJ) by placing sample in 1 mm path length rectangular quartz cuvette (Fisherbrand, Pittsburgh, PA) against a white background. A ‘C’ illuminant and a 2° observer were used. For the determination of ΔE at different pH values, \( L_o, a_o \) and \( b_o \) were \( L, a \) and \( b \) of a white surface.
To determine if the effect of pH on the color of the extract was reversible, extract at a concentration of 2 mg/mL in phosphate buffered saline containing 20% aqueous methanol (pH 7.5) was prepared as described above. The visible absorbance spectra were determined. The pH of the solution was adjusted to 11.0 by addition of NaOH and the spectra determined again. The pH was readjusted to 7.5 by addition of HCl and the visible absorbance spectra were recorded a final time. A similar experiment was conducted by adjusting the pH from 7.5 to 2.0 and back to 7.5. Both sets of experiments were also conducted while purging nitrogen through the samples at a rate of 5 ml/sec. This was done to assess the role of oxygen on the effects of pH on color. The amount of dissolved oxygen was measured to be 0.15 ±0.01 mg/l before adding the samples using Thermo-scientific portable DO meter, Orion 087003 (Barrington, IL). The addition of acid and base to change the pH changed the final volume of the solutions by less than 2%.

2.2.8. Color stability

CASE was dissolved in phosphate buffered saline in 20% aqueous methanol at pH 7.5 at a concentration of 2 mg/mL. The samples were centrifuged at 1200 \( \times \) g for 10 min to remove any undissolved solids. The supernatant was transferred to new tubes, sealed and kept at -18 °C, 4 °C, 24 °C and 40 °C. Aliquots of samples were periodically removed and the absorbance at 480 nm and \( \Delta E \) values measured.
2.2.9. Chemical characterization of CASE

Analysis was conducted with a Shimadzu HPLC system (Shimadzu, Columbia, MD) coupled with a Waters triple quadrupole mass spectrometer (Quattro Micro, Waters, Milford, MA) equipped with an electrospray ionization (ESI) probe. The HPLC system consisted of a binary pumping system (LC-10 ADvp), a degasser (DGU-14A), an autosampler (SIL-10vp), a water column heater (TCM model, Waters), a variable-wavelength UV-vis detector 280 nm (Shimadzu, SPD-10a), and a Ultra Aqueous reverse phase C-18 column (2.1 mm X 250 mm, 5 μm particle size). CASE (50 μg) was injected on the RP-18 column and resolved by a gradient according to the following program: 10 to 25% B from 0 to 5 min, 25-45 % B from 5 to 45 min, 45-70% B from 45 to 47 min, 70-90% B from 47-49 min and 90-100% B from 49-54 min at a flow rate of 1 mL/min.

The most abundant colored peak at 440 nm appeared at 17 min and was collected manually. Mass spectrometric ionization conditions were as follows: desolvation temperature, 300 °C; source temperature, 110 °C; capillary voltage, 3.5 kV. The scan range was 80-1000 Da, whereas for sibling ion analysis, the CID was 3.4e-4, collision voltage was 25V, and the sibling ions were scanned over a range of m/z 20-500.

Accurate mass analysis was conducted with a similar Shimadzu HPLC system (Shimadzu, Columbia, MD) coupled to a Waters Q-TOF mass spectrometer (Q-TOF micro) equipped with an electrospray ionization (ESI) probe and lock spray inlet system. Highly accurate mass acquisition of the isolated colored peak was performed by chromatography injection with reserpine as external standard. The isolated peak was injected on the RP-18 column maintained at a temperature of 25 °C using the same eluting conditions as before. Mass spectrometric ionization conditions were as follows:
desolvation temperature, 300 °C; source temperature, 100 °C; capillary voltage, 2.2 kV. For scan mode the scan range was 80-1000 Da.

Combinations of masses with N were removed from the options of structural formula (combinations with only C, O and H considered) because nitrogen was not detected in CASE. Nitrogen analysis on the sample was performed using LECO FP-528 nitrogen composition analyzer (St. Joseph, MI) with detection limit down to 0.1%. 5 mg of CASE was dissolved in DI water, was transferred to vials and analyzed for nitrogen content.

For the determination of $\lambda_{\text{max}}$, a PDA detector (SPD-M10Avp) was employed with other conditions as mentioned above except that 0.1% formic acid was replaced with water.

2.2.10. Using CASE in commercial products

CASE was added to four commercial products - milk, apple juice, grapefruit juice and muffins to determine potential colorant effects. CASE was prepared in water at 200 mg/mL and centrifuged at 1200 $\times$ g to remove any dissolved solids. It was added to milk and juices and mixed to homogeneity. The muffins were prepared by using a commercial white angel cake product as per manufacturer’s instructions with the color added to the batter. To determine $\Delta E$ values of juices and milk, samples were aliquoted into 20 ml scintillation vials, and $L_a b$ values measured using a Konica Minolta chromameter CR 400 by placing the instrument on the filled vial. $L_o$, $a_o$ and $b_o$ values were L, a and b of a white surface.
Chroma ($\Delta C_{ab}$) was also measured using the set up as described above, and calculated using the following formula:

$$\Delta C_{ab} = \sqrt{(a-a_o)^2+(b-b_o)^2}$$

where $a_o$ and $b_o$ are $a$ and $b$ values of the white surface respectively.

2.2.11. Statistical analysis

Results are shown as mean of three independent determinations. Error bars represent the standard error of mean (SEM) or the standard deviation (SD) as indicated in the figure legends. Differences between means were tested for significance by one-way analysis of variance (ANOVA) with Dunnett’s post test significance or two-way ANOVA with Bonferroni post-test as appropriate. Significance was achieved at $p<0.05$. All statistical analyses were performed using Graphpad Prism software (San Diego, CA).

2.3. Results

2.3.1. Kinetics of color formation

The kinetics of color formation in avocado seed were followed by measuring changes in visible absorbance intensity and by determining L a b values and color difference ($\Delta E$, Figure 2.1). Figure 2.1a shows visually how the orange color intensity increased with time. The $\lambda_{max}$ of the color was 480 nm (Figure 2.1b) and absorbance increased over the time course of the experiment in an exponential fashion approaching an asymptote by 20 min (Figure 2.1c). A similar time dependent increase in $\Delta E$ values was observed (Figure 2.1d). Within 0.5 min there was noticeable difference in color compared to water.
Figure 2.1. Kinetics of color formation in avocado seed paste. (a) A visual change in color of the paste was observed with the time of incubation. Representative results of three independent experiments. (b) Spectrophotometric analysis at 35 min showed a λ<sub>max</sub> at 480 nm. (c) Absorption at 480 nm (Mean ±SD of three replicates) and (d) ΔE increased with the time of incubation (mean of two independent experiments).
2.3.2. Role of PPO in color development

We hypothesized that the color development in crushed avocado seed was enzyme dependent and observed that heat treatment prevented color development (negative control, Figure 2.2a) compared to untreated seed (positive control, Figure 2.2a). Since polyphenol oxidase (PPO) is involved in color production in number of fruits and vegetables (Munoz and others 2007), and is present in avocado (Gómez-López 2002), we investigated whether addition of exogenous PPO would restore color formation. Addition of increasing amounts of mushroom PPO to heat-inactivated crushed seed resulted in restoration of color development (Figure 2.2a). The contribution of PPO in color formation was also confirmed by co-addition of 58 μM tropolone and respective amounts of PPO to the inactivated paste which resulted in no formation of color (Figure 2.2a). Also, when tropolone was added to the seeds before grinding, no color formation was observed even after processing the paste similarly (data not shown).
Figure 2.2. Role of polyphenol oxidase and peroxidase in the development of color in avocado seed paste. (a) Role of polyphenol oxidase: The absorbance at 480 nm increased in heat-inactivated avocado paste as increasing amount of exogenous PPO was added. No color formation was observed in samples co-incubated with different doses of PPO and 58 µM tropolone (marked ‘T’ in figure). (b) Role of peroxidase: With the addition of increasing amounts of peroxidase, non-significant increase in absorbance at 480 nm compared to negative control was observed. No effect of co-incubation with tropolone was observed. Mean ±SD of three independent experiments.

Since the enzyme peroxidase can also be involved in oxidative reactions in fruits and vegetables (Chisari and others 2007), its role was explored by adding exogenous enzyme and H₂O₂ to the inactivated paste. On addition of the exogenous peroxidase, an insignificant increase in absorbance at 480 nm, compared to negative control, was observed (Figure 2.2b). Visually, the paste had slight brown color to it. The addition of either H₂O₂ or peroxidase yielded similar results to that of negative control (data not
shown). Thus peroxidase does not contribute to the orange color formation although it produces slight brown color in ground seed.

2.3.3. Phenolic content

In order to assess the impact of color production on seed phytochemistry, the total polyphenol content of the extracts were determined. The phenolic content of the colored extract and the uncolored extract was found to be $219.4 \pm 4.5$ mg/g gallic acid equivalents (GAE) and $283.2 \pm 5.8$ mg/g GAE respectively (Figure 2.3). A higher concentration of phenolics was found in the methanol:ethyl acetate fraction than in the water fraction for both the colored and uncolored extracts (Figure 2.3).

![Figure 2.3](image.png)

**Figure 2.3.** Comparison of the phenolic content of the colored and the uncolored avocado seed extract. Higher total phenolic concentrations were observed in the uncolored extract. This difference was statistically significant ($p < 0.05$). Mean ±SEM of three independent experiments.
2.3.4. Effect of pH on color

The intensity and the hue of CASE in solution were increased by increasing the pH of the solution. Increases were observed across the visible spectrum, and at pH 11.0, a second visible absorbance maxima ($\lambda = 440$ nm) and a maxima in the near UV portion of the spectra developed (Figure 2.4a). Absorbance at 480 nm increased slightly between pH 2.0 and 9.0, reaching a point of inflection, followed by a rapid increase to pH 11.0 (Figure 2.4).

\[\text{Figure 2.4.} \text{ Effect of pH on the color of the avocado seed extract. (a) Absorption spectra at different pH values show a } \lambda_{\text{max}} \text{ at 480 nm at pH 7.5, 9.0 and 11.0; at pH 11.0 an additional } \lambda_{\text{max}} \text{ at 440 nm was observed. Representative results of three independent experiments (b) Absorption at 480 nm and } \Delta E \text{ values were seen to increase as a function of pH. Mean } \pm SD \text{ of three replicates.}\]

\[\Delta E \text{ also increased as a function of increasing pH (Figure 2.4b). The change in } \Delta E \text{ values was largely due to decreased lightness, } L, \text{ (from 89.1 to 80.0) and increased b (yellowness) (from 2.0 to 24.8) from pH 2.0 to 11.0. The value of a (redness) also}\]
increased but to lesser extent (from -0.1 to 1.8). ΔE less than 1.5 does not represent a significant change in color compared to baseline whereas ΔE greater than 5 represents a readily observable change (Obón and others 2009).

**Figure 2.5.** Effect of pH change and the presence or absence of air on the rehydrated freeze dried extract color (a) When the pH was increased from 7.5 to 11.0, in the presence of oxygen, the absorption intensity increased and two more absorbance maxima emerged at pH 11.0. When pH was decreased back to 7.5, only partial restoration of color character was observed. When pH was increased to 11.0 under a nitrogen atmosphere, an increase was in near UV range was observed. On reducing the pH back to 7.5, complete reversal of absorbance intensities was observed. Representative results of three independent experiments. (b) When pH was reduced from 7.5 to 2.0 and adjusted back to 7.5 –similar effects were observed under both air and nitrogen atmospheres. Representative results of three independent experiments.
To determine if the effect of pH on color was reversible, the pH of the colored avocado seed extract in solution was increased from 7.5 to 11.0 and then reduced back to 7.5. As the pH was increased in the presence of air, an increase in absorbance intensity in the visible region was observed as was the appearance of a new maxima ($\lambda = 393$ nm, Figure 2.5a). When the pH was reduced back to 7.5, absorbance intensity in the near UV range was reversed, but larger differences remained in the visible portion of the spectrum (Figure 2.5a). When similar experiments were conducted under a nitrogen atmosphere, the shape of absorbance spectra at pH 11.0 was different from the spectrum at the same pH under air, and the only changes occurred in the near UV. Changes in the spectrum induced by increasing the pH to 11.0 under nitrogen were also completely reversed when the pH was reduced back to 7.5. Much smaller changes in absorbance spectra were observed when pH was reduced from 7.5 to 2.0 (Figure 2.5b). A complete reversal of the changes was observed both in presence and absence of oxygen when pH was increased back to 7.5.
Figure 2.6. Effect of storage on color of the rehydrated freeze dried extract. (a) Absorbance Intensity at 480 nm and (b) ΔE were determined during storage for 60 days at pH 7.5 at -18 °C (■), 4 °C (●), 24 °C (▲) and 40 °C (▼). Mean ±SD of three replicates.

2.3.5. Stability of the colored extract

At pH 7.5, the color of the extract in 20% aqueous methanol was unstable at 40 °C and a sharp increase in absorbance at 480 nm was observed during the first 12 h of storage after which the intensity decreased (Figure 2.6a). Samples stored at 24 °C showed similar changes although the kinetics were slower. An increase in absorbance occurred until day 10 followed by a subsequent decrease. At both 4 °C and -18 °C, the absorbance
of the samples increased at much slower rates than at the higher temperatures. No significant change was observed until day 60 in the samples stored at -18 °C. The ΔE values of the extract stored at pH 7.5 showed similar trends to those observed spectrophotometrically (Figure 2.6b). For samples stored at -18 °C at pH 7.5, the ΔE value was 1.65 and no visible change in color was discerned. However, samples stored at higher temperatures (4 °C, 24 °C and 40 °C) had ΔE values greater than 5 over the time period of storage study and had noticeable changes in color.

2.3.6. Chemical characterization of CASE

The most abundant colored compound in CASE eluted under our conditions at 17 min. Nitrogen analysis showed no nitrogen present. The monoisotope mass was 602.17 with a predicted structural formula C_{29}H_{30}O_{14} (5 ppm). MS/MS on the compound suggested the presence of a C_6 sugar as indicated by neutral loss of mass 162. The other major neutral loss was ion of m/z = 152. The λ_{max} of the compound were 435 nm (λ_{max1}) and 280 nm (λ_{max2}).
2.3.7. Effect of addition of color to the products

CASE was added to different products to assess the effect of addition on color of the products.

Figure 2.7. Effect on color after addition of CASE to (a) white muffin (b) apple juice (c) grape fruit juice and (d) milk

For the muffins, ΔE was determined for the top surface, the side and the inside. Though ΔE were not different on the top surface (a change of 5 or more is considered a
readily observed or significant change in the ΔE) (Figure 2.8a) a color difference was observed visually (Figure 2.7a). The sample with the highest concentration of CASE was less brown than the control. Because ΔE did not correlate well with the visual differences, the chroma was also calculated and was more representative of visual color changes (Figure 2.8e and f). The differences were higher on the side and inside than those of surface (Figure 2.8).

The juices being acidic (pH of grapefruit juice = 3.3 and apple juice = 3.7), a yellowish color was formed after addition of CASE. CASE when added to the grapefruit juice showed a visual difference in color (Figure 2.7c) but no change in ΔE values (Figure 2.9a). Again changes in chroma corresponded better to visual changes (Figure 2.9b). When added to apple juice, a difference in color was observed visually (Figure 2.7b) but no significant changes in ΔE values or chroma values were observed (Figure 2.10).

For milk samples, the color differences were observed visually as a pink color upon addition of CASE (Figure 2.7d). This color was different than the yellow color obtained in the fruit juices in part because of a higher pH (6.8). Both ΔE and chroma values also increased dose dependently (Figure 2.11).
**Figure 2.8.** Effect of addition of CASE on color of white angel food cake muffins. (a) ΔE value on the surface (b) ΔE values on the side (c) ΔE values inside were measured by cutting the muffins and measuring L, a and b values. (d) Chroma value on the surface (e) Chroma value on the side (f) Chroma values inside. Mean ± SD of two independent experiments.

**Figure 2.9.** Effect of addition of CASE on the color of grapefruit juice (a) ΔE value (b) Chroma value. Mean ± SD of two independent experiments.
2.4. Discussion

In the present study we characterized the color production in avocado seed upon maceration and exposure to air, the mechanism of color formation and the color properties of resultant extract. A stable orange color (CASE) was produced when avocado seeds were ground and incubated at 24 °C. The color production occurred rapidly with a noticeable change produced as early as 0.5 min of incubation. Kinetic analysis showed that intensity of the color formation increased in exponential fashion and
began to approach an asymptote at 20 min. Such kinetics of formation is typical of an enzyme catalyzed reaction. For example, Arias and others (2007) found that pear (*Pyrus communis*) PPO-mediated oxidation of dihydroxyphenylalanine (DOPA) resulted in a similar trend in absorbance at 420 nm. We observed that heat treatment prevented color formation, whereas addition of exogenous mushroom PPO restored color formation. PPO from avocado pulp has been found to be very active and resistant to treatments including heat, making avocado products very prone to enzymatic browning (Gómez-López 2002), but there is scant information on PPO from avocado seed. The orange color formed in the seed may have resulted from particular substrate present in the avocado seed or inhibition of PPO by the products formed during an intermediate stage of the reaction.

PPO catalyzes both the oxygen-dependent hydroxylation of monophenols to their corresponding \( O \)-diphenols and the oxidation of \( O \)-diphenols to their corresponding \( O \)-quinones (Lin and others 2010). The quinones then may polymerize into red, brown or black pigments depending on conditions like the nature and amount of endogenous phenolic compounds, the presence of oxygen, reducing substances, or metallic ions, the pH and temperature, and the activity of the PPO (Dogan and others 2006). Browning reactions in fruits and vegetables occur when tissues are damaged and PPO is released. Here we observed that the color of CASE was stable during the observation window explored and did not produce highly polymerized melanoidins.

The total phenolic content of methanol:ethyl acetate and water extracts of CASE was calculated to be 219.4 mg/g. Soong and Barlow (2004) used ethanol: water at high temperature for extraction of phenolics of avocado seed and calculated the phenolics as 88.2 mg GAE/g. Wang and others (2010) used acetone:water:acetic acid (70:29.7:0.3,
and found the values to be 51.6 mg/g GAE for the seed of Hass variety of avocado, whereas the corresponding values for pulp was only 4.9 mg/g GAE. In the present study, the phenolic content of CASE was 22.5% lower than the content in uncolored extract. The phenolic compounds present in CASE may contribute to functional attributes. The reduction in phenolic content during color development may be due to oxidation of phenolic compounds by the PPO. During processing of fresh grapes to raisins, most of the two major hydroxycinnamic acids and all of the procyanidins and flavan-3-ols were lost. The reasons for this loss were likely to be both enzymatic and non-enzymatic (Karadeniz and others 2000).

Although often associated with undesirable browning reactions, PPO has also been shown to produce attractive colors in some systems. PPO-catalyzed conversion of catechins to theaflavins in the production of black tea is responsible for the characteristic orange color of that beverage (Balentine and others 1997). A yellow-colored product was synthesized through oxidation of ferulic acid with laccase, an enzyme functionally similar to PPO. It was found that in a biphasic system containing ethyl acetate a stable yellow-colored product is formed whereas in an aqueous system browning occurred. This difference was attributed to the incomplete activity of PPO in the medium containing ethyl acetate (Mustafa and others 2005). Catechin was oxidized at pH 7.5 using PPO and it was observed that the absorbance maxima of the product was 430 nm which corresponded to a yellow-colored product (Jiménez-Atiénzar and others 2004). A yellow dye from phlorodzin, a flavonoid specific to apples, was synthesized in the presence of PPO and oxygen (Guyot and others 2007). The product which has a brilliant yellow color
with nuances depending on the pH, can be incorporated into water based foods such as beverages (juices, syrups) and confectionary creams (Socaciu 2008).

The absorbance at 480 nm and the ΔE values of CASE increased as the pH increased, with a rapid increase after pH 8.0. These rapid changes could be occurring due to dissociation or other reactions like ring opening or reactions occurring due to alkaline conditions. This may further suggest presence of phenolic groups in CASE since phenolics demonstrate similar behavior on addition of alkali. Also, this data shows that the colorant from avocado seed may be usable at both low and high pH. Comparatively, anthocyanins, common natural colorant, give a stable hue only at pH values lower than 5 (Delgado-Vargas F & Paredes-Lopez O. 2003b). The color change with increased pH under oxygen was irreversible. This data suggests that the colored compounds produced as a result of oxidation by the PPO may be further oxidized under high pH and oxygen. These observations imply that if the extract is to be used as a food colorant, the pH of the food and the conditions of storage must be considered.

The color of CASE was stable at pH 7.5 for 2 months when stored at -18 °C. At higher temperatures significant changes in the color were observed over shorter time periods. These results suggest that CASE could be used in products stored at low temperatures, but a system for stabilizing the color at higher temperatures may be needed. Since the current studies were conducted in the presence of air, it is possible that CASE would be more stable at higher temperatures if storage conditions included an inert atmosphere. However absence of oxygen will be dependent on the type of food product. Walkowiak-Tomczak and Czapski (2007) observed that in a red cabbage anthocyanin model system, pigment loss was reduced by greater than 50% when samples were stored
in the absence of oxygen. Also, a high water activity solutions were used in this study and may have influenced the stability. The color is expected to have better stability at a lower water activity.

CASE was added to different products and positively affected color suggesting its potential use in food products. The concentrations used for the products were up to 7.68 mg/mL. These concentrations were higher than those typically used for synthetic colorants.

CASE when added to milk turned the color to pink (pH 6.8) and when added to fruit juices (pH 3.3 and 3.7), it made the juices more yellow, an effect most pronounced in the grapefruit juice. The difference in color after addition of CASE to these products is likely due to the different pH of products. Similar results were seen after adding CASE in buffers at different pH (2.0 to 11.0). CASE can be used for products with higher pH, because it is more intense at higher pH however it can also provide yellow hues for products at low pH, albeit a higher concentration will be needed. Other than addition to the products used, it may also be useful in beverage products like vitamin/flavored water where no basal color is present. CASE performed successfully in muffins resulting in noticeable color change at relatively low levels (1.92 mg/mL). These results demonstrate its possible application in products undergoing heat treatment. For example, it can be used in dry baking mixes.

For studying colorant properties in food products, the chroma values were additionally calculated because ΔE did not always represent the visual effects seen. Interestingly, in apple juice both chroma and ΔE values were not changed although a visual change was observed clearly. In the case of muffins, as concentrations of CASE
were increased, though browning on the surface was decreased but orange color due to CASE increased. Because of this neither chroma nor ΔE values changed. It is hypothesized that CASE inhibits browning because of its high phenolic content which blocks the maillard reactions. Milk demonstrated the clearest relationship between visual changes after addition of CASE and changes in chroma and ΔE. Hence our method of measuring color difference may not suitable for all products and further research in this area is needed.

The tentative structural formula of the major colored peak in CASE (at λ= 440 nm) was calculated as C_{29}H_{30}O_{14} (m/z=602.17). MS/MS studies indicated presence of a C_6 sugar. However, further work using NMR is needed to determine the structure of this compound after purification.

Crucial to its proposed color applications in foods is CASE’s safety. A preliminary safety profile of the seed has been conducted in rats (Ozolua and others 2009). An aqueous extract of fresh avocado seed (2, 4, 6, 8, 10 g/ kg) was administered to 6 groups with 4 rats per group by gavage. The animals were fed rat pellet ad libitum and exposed to natural light at room temperature. Animals were handled according to standard protocols for the use of lab animals (NIH, USA: public health service policy on humane care and use of lab animals, 2002). No signs of depression, writhing, diarrhea, hypermobility and aggression were noticed in the treatment groups in the two subsequent weeks. The same group examined sub-chronic toxicity by administering 2.5 g/kg for 28 days. White blood cells, lymphocytes and neutrophils, hemoglobin concentration and platelet count did not differ significantly in the control and treatment groups after 28 days of treatment. Also there were no significant differences between aspartate
aminotransferase and alanine aminotransferase levels in the two groups. Albumin and creatinine concentrations were also comparable in the two groups. Also, the animals did not display any obvious external symptoms of toxicity before they were sacrificed. Further studies with CASE are needed.

2.5. Conclusion

In conclusion, this study examined a colored extract produced enzymatically in avocado seed. Its properties, suitable to addition to products were explored. The color was added to some products to assess effects of its addition. Avocado seeds are not currently commercially useful and represent a large waste stream (Ramos-Jerz 2007). Their application as a source of natural colorants could be of significant commercial value. Because of its high phenolic content; the colored extract may have additional functional attributes which should be explored.

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Chapter 3

ANTIOXIDANT AND ANTI-CANCER PROPERTIES OF COLORED AVOCADO SEED EXTRACT

Abstract

Avocado seeds have been used traditionally for a number of health related indications. Because of its high polyphenol content, we investigated the antioxidant and anti-carcinogenic effects of CASE. Using electron paramagnetic spectroscopy, CASE was found to have radical scavenging capacity (EC_{50} = 42.1 \mu g/mL) and in a model emulsion system, CASE displayed dose-dependent antioxidant activity. The ORAC value of CASE was 2012.1±300 TE/mg. CASE was cytotoxic in four human cancer cell lines (IC_{50} = 19.1-132.2 \mu g/ml) after treatment for 48 h. In LNCaP human prostate cancer cells, CASE induced cell cycle arrest at the G_{0}/G_{1} phase. Down-regulation of cyclin D1 and cyclin E2 was associated with cell cycle arrest. CASE also induced apoptosis dose dependently as indicated by externalization of phosphatidylserine, and cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP). Translocation of NF-\kappaB, a pro-survival signal, was also reduced by treatment with CASE. These results suggest that CASE has potential as an antioxidant and anti-carcinogenic agent. Such potential must be further investigated *in vivo.*
3.1. Introduction

Avocado (*Persea americana*, Lauraceae) is an important tropical crop which is rich in unsaturated fatty acids, fiber, vitamins B and E, and other nutrients (Gomez Lopez 1998). The Hass variety is the most commonly grown and total U.S. avocado production during the 2004/05 season was 162,721 tons with 90% originating in California (USDA 2006). Mexico, the world’s largest producer, supplied 1.14 million tons in 2007 (McLeod 2008). The seed of avocado accounts for 16% of total avocado weight, and is an under-utilized resource (Ramos-Jerz 2007).

Ethno-pharmacological studies of the Aztec and Maya cultures have reported the use of decoctions of avocado seeds for the treatment of mycotic and parasitic infections. The seeds have also been reported for use against diabetes, inflammation and gastrointestinal irregularity (Ramos-Jerz 2007). Topically, powdered seeds have been used for skin eruptions and to cure dandruff (Morton and Dowling 1987). The seed is considered as anti-anemic and diuretic (Duke and Vasquez 1994). A piece of the seed or the decoction put into tooth cavity is suggested to relieve toothache. The seed has also been used historically as a red indelible ink (Morton and Dowling 1987). We have previously reported that when the avocado seed is crushed, a stable orange color develops (Dabas 2011).

Current studies on avocado seed are focused on their chemical composition and the role of these phytochemicals in insecticidal, anti-diabetic and blood pressure reducing effects of avocado seeds (Leite and others 2009; Anaka and others 2009; Ramos-Jerz and others 2007; Ramos-Jerz 2007; Kate and Lucky 2009). Avocado seeds have higher antioxidant activity and polyphenol content than the pulp (Wang and others 2010; Soong
and Barlow 2004). Wang and others (2010) have reported the presence of catechin, epicatechin, and A- and B-type procyanidin dimers and trimers, tetramers, pentamers and hexamers in the seed. Phytochemical studies on avocado seeds have identified various classes of natural compounds including phytosterols, triterpenes, fatty acids, furanoic acids, abscisic acid, proanthocyanidins and polyphenols (Leite and others 2009; Ding and others 2007).

Only one paper has reported the anti-carcinogenic effects of avocado seed and peel. Treatment of MDA-MB-231 human breast cancer cells with the methanolic extract of avocado seed led to induction of apoptosis as measured by increased caspase-3, caspase-7 and PARP cleavage at concentrations greater than 100 µg/mL. DNA laddering was also observed. Similar effects were induced by an extract of peel but at lower concentrations (10 µg/mL) than for seed. The peel showed higher total polyphenols, total flavonoid and higher radical scavenging activity than the seed (Lee and others 2008).

Given the limited information on anti-cancer effects of avocado seed, and the potential usefulness of the colored avocado seed extract (CASE) as a food additive, we sought to investigate the anticancer effects of this extract in vitro. We hypothesized that CASE has antioxidant and anti-cancer activity by virtue of its polyphenol content.

3.2. Material and methods

3.2.1. Reagents

Ripened avocado (Persea americana, Hass variety) were sourced locally and stored at 4 °C until use. The antibodies against cleaved caspase 3, poly (ADP- ribose) polymerase (PARP), cyclin D1, cyclin E2, γH2AX, β actin and NF-κB were purchased...
from Cell Signaling (Danvers, MA). Antibody against cyclin A was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were of the best commercial grade available.

3.2.2. Cell culture

MCF7 human breast cancer, H1299 human lung cancer, LNCaP human prostate cancer, and HT29 human colon cancer cells were purchased from American Type Culture Collection (Manassas, VA). H1299 and LNCaP cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. MCF7 cells were maintained under the same conditions with the addition of 1% sodium pyruvate. HT29 cells were maintained in McCoy’s 5 A medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. All cell lines were maintained in log-phase growth at 37 °C under a humidified CO₂: air (5:95) atmosphere.

3.2.3. Preparation of CASE

CASE was prepared as described previously in chapter 2 (2.2.2). Following homogenization of the seeds and color development, paste was extracted with methanol and dried under vacuum. Stock solutions (200 mg/mL) were prepared in dimethyl sulfoxide and stored at -80 °C.
3.2.4. Radical scavenging and antioxidant activity

The radical scavenging activity of CASE was measured using electron paramagnetic resonance (EPR) by the method of Voest and others (1993) with some modifications. The EPR spectra of TEMPOL were recorded on a Bruker eScan R X-band spectrometer at 37 °C. The EPR microwave power was set to 37.86 mW, the modulation frequency was 86 kHz, and a sweep time of 2.62 s was used. Each time point for a sample was scanned a total of 3 times. CASE was diluted in final concentration from 1µg/mL – 100 µg/mL in phosphate buffered saline (100 mM, pH 7.4) and combined with 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH, 40 mM) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-xyloxy (Tempol, 24 µM). The kinetics of Tempol reduction was studied over 120 min. Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 120 µM) was used as the positive control.

The ability of CASE to prevent lipid oxidation was measured in a 5% corn oil-in-water emulsion in sodium phosphate buffer (pH 7.0) using 1% tween 20 as an emulsifier. CASE was added to the emulsion and incubated at 37 °C for 62 days. Sodium azide (0.02 wt %) was added to emulsions in order to prevent microbiological spoilage. Coarse emulsions were made using a high-speed blender (Brinkmann Polytron, Brinkmann Instruments Inc., Westbury, NY) on high speed for 1 min. Fine emulsions were then quickly prepared by passing coarse emulsions through a twin-stage valve homogenizer (Niro Soavi Panda, GEA Niro Soavi, Hudson, WI) for at least 2 min of continuous run at a pressure of 660 kPa. All emulsions had a mean particle size (d₃₂) of 0.19 ± 0.05 μm, as determined by laser light scattering (Horiba LA 920, Irvine, CA). Emulsions containing 500 µg/mL of CASE were unstable 54th day onwards while emulsions at all other
concentrations were stable throughout the study. Lipid hydroperoxides were measured using a modified method of Chee and others (2005). In brief, samples were mixed with 5 vol (w/v) isooctane/2-propanol (3:1 v/v), vortexed and the organic phase isolated by centrifugation. The organic solvent phase was combined with 14 vol methanol/1-butanol (2:1, v/v) and 0.075 vol, ammonium thiocyanate (3.94 M) and 0.075 vol ferrous iron solution (prepared by mixing 0.132 M BaCl₂ and 0.144 M FeSO₄·7H₂O). After 20 min, the absorbance of the solution was measured at 510 nm. Hydroperoxide concentrations were determined using a standard curve made from cumene hydroperoxide.

### 3.2.5. Oxygen Radical Absorbance Capacity (ORAC) Assay

A Fluoroskan Ascent FL fluorescent plate reader (Thermo Scientific) was used for ORAC measurements. CASE was dissolved in phosphate buffer (10 mM, pH 7.4) at various concentrations (0-100 µg/mL). In each well of a 96-well black opaque plate, 25 µL of sample or phosphate buffer (“blank”) was added to 150 µL of fluorescein (10 nM) in triplicate. The plate was incubated for 30 min at 37°C in the plate reader without shaking. Following incubation, fluorescence was measured (Ex. 485 nm, Em. 520 nm) every 90 seconds for 3 cycles to determine the background signal. AAPH (25 µL, 240 mM) or phosphate buffer (for “Fluorescein only” control) were then added to the wells using a multi-channel pipette, and fluorescence was measured every 90 seconds for 90 minutes. The slope of CASE-treated samples was compared to Trolox and results are expressed as Trolox equivalents.
3.2.6. Growth inhibitory effects

The effect of CASE on cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded (10^4 cells/well) in 96 well plates and allowed to attach overnight. Cells were treated for 12-48 h with medium containing CASE at 37 °C. After treatment, 1 mg/mL MTT was added and conversion to the formazan precipitate was determined spectrophotometrically at 540 nm.

3.2.7. H₂O₂ production

H₂O₂ production by CASE under cell culture conditions was measured using the ferrous ion oxidation-xylene orange (FOX) assay. FOX reagent contained 1 mM xylenol orange, 2.5 mM ferrous sulphate and 1.02 M sorbitol in 250 mM sulfuric acid. LNCaP cells (3X10⁵) were grown in 6 well plate. After incubation for 36 h, the cells were treated with CASE at 5, 10, 25 or 50 µg/mL. After incubation for 1, 3, 12, 28 and 48 h, 20 µL of prepared FOX reagent was combined with 140 µL of media. It was mixed, incubated for 30 min and absorbance measured at 560 nm. A standard curve created using known concentrations of H₂O₂ was used to determine H₂O₂ concentration. H₂O₂ concentrations in media treated similarly but without any cells, were also determined.

3.2.8. Cell cycle analysis

To determine the effect of CASE on cell cycle progression of LNCaP cells, cells (1X10⁵) were plated in 75 cm² flasks and allowed to attach for 36 h. The media was replaced with fresh media containing CASE and incubated for 12h. Cells were harvested
by trypsanization, centrifuged at 400 × g for 10 min, washed with phosphate buffered saline (PBS) and fixed with 70% methanol. Cells were then washed with PBS and treated with 500 μg/mL RNAse and 40 μg/mL propidium iodide and incubated at 37 °C for 30 min. The cells were analyzed by flow cytometry on a Coulter Epics XL-MCL (Beckman Coulter, USA).

3.2.9. Induction of apoptosis - externalization of phosphatidylserine

LNCaP cells (1X10^6) were plated in 75 cm² flasks and allowed to attach for 36 h. The media was replaced with the new media containing CASE and incubated for 12h. Surface exposure of phosphatidylserine by apoptotic cells was measured by adding Annexin V-FITC (Fluorescein isothiocyanate) and propidium iodide to harvested cells according to the manufacturer’s instructions using ApoDETECT Annexin V-FITC Kit (Invitrogen, Fredrick, MD). Cells were analyzed using flow cytometry.

3.2.10. Quantification of intracellular reactive oxygen species (ROS)

LNCaP cells (1X10^6) were seeded in 75 cm² flasks and allowed to attach for 36 h. The media was replaced with media containing CASE and after 12 h, the cells were washed with serum free media. Cells were then incubated with 10 μM of dichloro dihydro fluorescein diacetate (DCHF-DA) prepared in medium at 37 °C for 30 min. Cells were then trypsinized, centrifuged and the cell pellet was suspended in media. ROS levels were quantified by flow cytometry.
3.2.11. Western blot analysis

3.2.11.1. Preparation of whole cell lysate

LNCaP cells (1X10^6) were seeded in 75 cm^2 flasks and allowed to attach for 36 h. The media was replaced with media containing CASE at IC_{50} or IC_{60} values. The cells were washed with PBS, scraped off and centrifuged at 1200 × g. Cell pellet was combined with lysis buffer (25 mM 3-(N-morpholino) propanesulfonic acid, 2 mM Ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.5% Nonidet P-40 and 0.02% sodium azide) containing 1:100 phosphatase inhibitor I, phosphatase inhibitor II and protease inhibitor. The samples were mixed and disrupted by freeze thawing.

3.2.11.2. Preparation of nuclear lysate

The cells were treated as above, were scraped and centrifuged at 800 × g for 10 min at 4 °C. The cells were suspended in buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.1% Nonidet P40). The samples were incubated in ice for 10 min and centrifuged at 12000 × g for 2 min at 4 °C. The pellet was resuspended in buffer B (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.4 M NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 25% glycerol). The tubes were then vortexed and incubated on ice for 15 min with mixing every 5 min. They were then centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was removed and used as nuclear fraction.
3.2.11.3. Immuno blots

Loading buffer and 60 µg of protein (20 µg for nuclear extract) was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. After blocking for 1 h, the membrane was incubated with respective primary antibody overnight. The bands were visualized using a fluorescent-conjugated secondary antibody using a Licor Odyssey Infra red system (Lincoln, Nebraska).

3.2.12. Data Analysis

Statistical differences were estimated by one way analysis of variance (ANOVA) followed by Dunnett’s post test. A p value <0.05 was considered statistically significant. Graphpad (La Jolla, CA) was used for statistical analysis. Mean ± SD is presented unless specified otherwise.

3.3. Results

3.3.1. Antioxidant activity

CASE when reconstituted in phosphate buffered saline at pH 7.4, displayed dose dependent radical scavenging activity in the EPR assay (Figure 3.1a). At CASE concentrations greater than 10 µg/mL the Tempol absorption intensity was significantly greater than the vehicle control. The median effective concentration (EC_{50}) for protection of Tempol was 42.1 µg/mL.
Antioxidant activity of CASE. (a) The antioxidant activity of CASE was measured using EPR. Tempol absorbance intensity was determined after incubation for 120 min at 37°C. The Tempol absorbance intensities were normalized to the intensity of control incubated with 120 µM Trolox. Results are mean ±SEM of three independent experiments. (b) Lipid hydroperoxides in oil-in-water emulsion containing CASE at various concentrations were used to assess antioxidant activity in a model food system during storage at 37 °C. Symbols represent negative control (■), 5 µg/mL (●), 15 µg/mL (▲), 50 µg/mL (▼), 100 µg/mL (◄), 250 µg/mL (◇) and 500 µg/mL (◇). Results are represented as mean ±SD.

A dose dependent antioxidant activity was also observed in CASE-supplemented oil-in-water emulsions (Figure 3.1b). Lipid hydroperoxide values were dose-dependently reduced by CASE compared to vehicle control. On day 62, CASE at a concentration of
50 µg/mL and above significantly reduced the hydroperoxides. Hydroperoxide value is indicative of the primary oxidation products formed in the emulsion and thus CASE may inhibit primary oxidation in food systems.

The antioxidant activity was also evaluated using ORAC assay and a value of 2012.1 ± 300 TE/mg of CASE was obtained.

### 3.3.2. Growth inhibitory effects

CASE was dose dependently cytotoxic against four human cancer cell lines (Figure 3.2). The IC\textsubscript{50} values were 19.1, 19.1, 67.6 and 132.2 µg/mL for LNCaP, MCF7, HT29 and H1299 cells, respectively, following treatment for 48 h. LNCaP cell line was selected for further studies because of high sensitivity to CASE. Cytotoxicity was also examined in LNCaP cells for 12 and 24 h treatment and IC\textsubscript{50} values were higher for shorter incubation times (Figure 3.3).
Figure 3.2. Cell survival in selected cancer cell lines after treatment with CASE. The percentage survival was calculated with respect to control after incubation for 48 h. Results are mean ±SEM of three independent experiments.

Figure 3.3. Cell survival in LNCaP cancer cell line after treatment with CASE. The percentage survival was calculated with respect to control after incubation for 12 h (■) and 24 h (●). Results are mean ±SEM of three independent experiments.
3.3.3. Cell cycle arrest

CASE induced cell cycle arrest in LNCaP cells occurred in the G₀/G₁ phase (Figure 3.4). The cells were treated for 12 h with IC₅₀ or IC₆₀ dose of CASE, stained with propidium iodide and analyzed using flow cytometry. The percentage of cells in the G₀/G₁ phase increased by 1.7 fold for the IC₅₀ and 1.84 fold for IC₆₀. A concomitant reduction in cells going to S and G₂ phase was also observed.

![Graph showing cell cycle progression](image)

**Figure 3.4.** Effect of CASE on the progression of cell cycle in LNCaP cells. Cells were treated with only media, IC₅₀ or IC₆₀ for 12 h. Each value represents mean ±SD of three independent experiments.

Western blot analysis showed that cyclin D1 and cyclin E2, two regulators of cell cycle at G₀/G₁, were reduced by treatment with CASE. By contrast, no change in the expression of cyclin A, a regulator of S and G₂/M transition, was observed (Figure 3.5).
3.3.4. Apoptosis

Increased cleavage of caspase 3 and PARP was observed in cells treated for 12 h with CASE (Figure 3.6). These results suggest that CASE induces apoptosis as a way of causing cell death.

As observed in the Figure 3.6, the expression of γH2AX was also increased in the treatment indicating appearance of DNA double strand breaks which serve as signal and activate proteins upstream of γH2AX, thus resulting in its activation.
Figure 3.6. Expression of cleaved caspase 3, cleaved PARP and γH2AX as affected by treatment for 12 h with CASE. Results are representative of three independent experiments.

The treated cells were stained with annexin-FITC and PI to determine the percentage of early apoptotic cells (Figure 3.7). A significant increase from control was observed in both the IC$_{50}$ and IC$_{60}$ treatment.
Figure 3.7. Apoptosis as determined using staining with annexin-FITC/PI. Cells were treated with CASE for 12 h, stained with annexin and PI and analyzed using flow cytometry. Mean ± SD of three independent experiments.

No H₂O₂ production was observed in the media treated with CASE (up to a concentration of 50 μg/mL) with or without LNCaP cells, up to 48 h of incubation (data not shown). This indicates that H₂O₂ is not involved in cell death. No increase in intracellular ROS was observed as a result of the treatment hence negating the possibility of free radicals being involved in cell death (data not shown).
3.3.5. Effect on nuclear factor kappa B (NF-κB) translocation

The translocation of NF-κB to nucleus was high in the control cells and was reduced as a result of the treatment (Figure 3.8). In comparison relatively smaller difference in the expression of total NF-κB was noticed. The relative expression of nuclear NF-κB to total NF-κB was reduced significantly as a result of the treatment.

![Figure 3.8. Expression of NF-κB in the nuclear extracts as affected by treatment for 12 h with CASE. Total NF-κB expression is also shown for comparison. β-actin and Histone H3 were used as loading controls for total and nuclear fractions respectively. Results are representative of three independent experiments.](image)

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<th>Control</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;60&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NF-κB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nuclear NF-κB</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Histone H3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio Nuclear NF-κB /Total NF-κB</td>
<td>0.016</td>
<td>0.003</td>
<td>0.006</td>
</tr>
</tbody>
</table>

3.4. Discussion

CASE was examined for antioxidant and anti-carcinogenic effects. CASE showed significant radical scavenging activity in aqueous buffer and antioxidant activity in an oil-in-water emulsion. The ORAC value was found to be 2012.1 ± 300 TE/mg. These data suggest that CASE may prevent oxidation when added into food systems. Indeed an
acetone: water (70:30, v/v) extract from 10 g of avocado seed was added to 1 kg porcine patties, the product stored at 5 °C for 15 days and color (Lightness, L*, redness, a*, and yellowness, b*), thiobarbituric acid reactive substance (TBARS) and protein hydrazones were measured. The samples with avocado seed had better color preservation and also lower TBARS and hydrazones values on day 15 than the controls (Rodríguez-Carpena and others 2011).

The antioxidant activity of seeds has been correlated to phenolic content of the seeds (Soong and Barlow 2004; Wang and others 2010). Antioxidant activity of an acetone extract of the seed gave an ORAC value as 428.8 TE/g fresh weight whereas the ORAC value for the pulp was 11.6 TE/g. The same study found that the seed has 10.5 times phenolic content that that of the pulp (Wang and others 2010).

CASE showed cytotoxic effects against four human cancer cell lines with IC\textsubscript{50} values of 19.1 to 132.2 μg/mL after 48 h of treatment. H1299 lung and HT29 colon cancer cells were relatively resistant whereas LNCaP prostate and MCF7 breast cancer were more sensitive. Both sensitive cell lines are hormone sensitive cancers. This may suggest a molecular mechanism for the inhibitory effects. The phenolics in CASE may be responsible for the anti-carcinogenic effects, in addition to contributing to antioxidant effect. This hypothesis has been proposed for the avocado seed extracts by Lee and others (2008).

CASE inhibited the cell cycle progression in the G\textsubscript{0}/G\textsubscript{1} phase of LNCaP cancer cells. Deregulation of the G\textsubscript{1} to S-phase transition is implicated in the pathogenesis of most human cancers, and D- and E-type cyclins act as positive regulators of this critical
transition (Masamha and Benbroo 2009). In this study, the expression of cyclin D1 and cyclin E2 were reduced as a result of treatment with CASE.

CASE treatment induced apoptosis as demonstrated by increased cleaved caspase-3 and cleaved PARP after 12 h incubation. Caspases belong to cysteine proteases family and are involved in proteolytic cleavage of a variety of cytoplasmic and nuclear proteins during apoptosis. Once activated caspases-8 and 9 cleave and activate effector caspase like caspase -3,6 and 7 which in turn cleave a wide array of proteins required for cell integrity resulting in cell death (Dorsey and others 2008). PARP is involved in DNA repair and is activated by binding to DNA strand breaks, and is cleaved and inactivated by caspase 3 (Masutani and others 2005). Lee and others (2008) using doses up to 200 μg/mL of methanolic extract of avocado seed, observed increased cleavage of caspase-3 and PARP in MDA-MB-231 cells indicating apoptosis. CASE increased externalization of phosphatidylserine. Appearance of phosphatidylserine on the outer surface of cell is an early marker of apoptosis.

Because polyphenols have been previously shown to exert pro-oxidant effects, we investigated whether CASE could work by inducing oxidative stress in cancer cells. The phosphorylation of H2AX is a marker of oxidative stress and double strand DNA breaks. CASE treatment enhanced phosphorylation of H2AX at the sites of DNA damage in response to formation of double strand breaks. The resultant effect is promoting the rejoining of double strand breaks by non-homologous end-joining (NHEJ). This indicates that CASE caused damage to the DNA which resulted in activation of γH2AX (Burma and others 2001).
The expression of NF-κB was reduced in the nucleus as a result of treatment with CASE. Constitutive activation of NF-κB has been reported in several types of cancer including in prostate cancer (Wong and others 2009). Targets of NF-κB include genes relating to cell proliferation, apoptosis and immune response. Thus reduced expression of this transcription factor may be contributing to apoptosis and cell cycle inhibition and thus provides mechanistic information on these effects. Interestingly, CASE did not produce H$_2$O$_2$ under cell culture conditions or induce intracellular oxidative stress. These results suggest that CASE may induce γH2AX by another mechanism perhaps by inhibition of topoisomerase II.

CASE has direct applications in food systems as a natural colorant. Today’s consumer wants foods that not only satisfy hunger but have additional health benefits. Some natural colors have proved to provide these additional benefits. For example anthocyanins are colorants that have antioxidant and anti-cancer activity (Duangmal and others 2008). CASE may represent a novel functional ingredient. However further studies on safety and in vivo efficacies are needed.

3.5. Conclusion

This study evaluated the antioxidant and anti-cancer effects of CASE. CASE was found to have radical scavenging activity and antioxidant activity in a model food system. In addition, CASE exhibited anti-cancer effects by causing apoptosis and inhibiting the cell cycle progression in prostate cancer cells. Such attributes suggest CASE as a new functional ingredient.
3.6 References


USDA. 2006. Avocado situation and outlook for selected countries. World Horticultural Trade & U.S. Export Opportunities.


Chapter 4

ANTI-INFLAMMATORY PROPERTIES OF A COLORED AVOCADO SEED EXTRACT

Abstract

Chronic inflammation contributes to many diseases including cancer, cardiovascular diseases and arthritis. The anti-inflammatory potential of CASE was explored based on ethnobotanical use of avocado seed for inflammatory diseases. Treatment of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage cells with CASE for 24 h reduced the production of pro-inflammatory cytokines, IL-6, TNFα and IL-1β. NO production was reduced in a dose dependent manner, and the reduction was associated with a decrease in the protein expression of inducible nitric oxide synthase (iNOS). Whereas PGE₂ production was significantly reduced by CASE, no change in the protein expression of Cyclooxygenase -2 (COX-2) was observed. CASE did inhibit the activity of purified secreted PLA₂ (IC₅₀= 36 μg/mL). Kinetic analysis indicated that the inhibition was non-competitive with respect to substrate concentration. Nuclear translocation of NF-κB to the nucleus was reduced by treatment with CASE, and this inhibition may underlie the effects of CASE on iNOS and cytokine expression. These results suggest that CASE may represent a source of anti-inflammatory compounds which can be exploited as functional food ingredients or as a lead compounds for pharmaceutical development.
4.1. Introduction

Although inflammation plays role in host defense mechanism against injury and infection, inadequate resolution of inflammatory responses can contribute to chronic disorders including atherosclerosis, arthritis and cancers (Porta and others 2009). Non-steroidal anti-inflammatory agents have therefore been investigated as a means of reducing the incidence and mortality of various cancers and cardiovascular diseases as well as to improve symptoms of arthritis (Mantovani and others 2008).

Cytokines are small non-structural proteins primarily involved in host responses to disease or infection. They serve as messengers between cells and on release bind to receptors on the surface of immune cells resulting in the activation of a series of intracellular kinases and multiple transcription factors (Mantovani and others 2008). These transcription factors increase the expression of various proteins leading to recruitment of immune cells to the site of injury. The production of cytokines is reduced to normal levels upon resolution of infection. Thus, cytokines are critical for functioning of body’s defense mechanism (Gabey 2006). However, under chronic inflammatory conditions, they are continuously formed leading to accumulation of pro-inflammatory mediators which then signal the body to send inflammatory cells at the site of production even in the absence of any external insult (Grivennikov and Karin 2011). By blocking these cytokines, it may be possible to control the symptoms and slow disease progression in patients with inflammatory conditions including cancer, rheumatic arthritis and diabetes (Cronstein 2007).

IL-1β modulates the microenvironment to the benefit of tumor growth, invasion and activation of proteolytic enzymes. IL-1β has a broad range of regulatory functions
including regulating the expression of COX-2, iNOS, and of PLA2. Prolonged production of IL-1β is implicated in a variety of pathological conditions including rheumatoid arthritis and inflammatory bowel disease. Enhanced transcription of IL-1β is induced by signaling through multiple transcription factors including NF-κB. TNFα and IL-6 activate NF-κB and STAT3 signaling which render malignant cells resistant to apoptosis and enhance tumor cell proliferation. IL-1β and TNFα also induce their own expression causing an amplification loop of the inflammatory response (Apte and Voronov 2002). Both also stimulate the expression of pro-inflammatory genes like IL-6, IL-8 and COX-2 (Nonn and others 2007).

The transcription factor, NF-κB has an important purpose in relaying inflammatory signals from the cell surface. Inactive NF-κB is localized in the cytoplasm and on stimulation by LPS, TNFα, IL-1β or cellular stress, the inhibitors of NF-κB are degraded. As a result it is translocated from cytosol to nucleus and binds to its cognate DNA binding sites and results in formation of pro-inflammatory products like PGE₂, NO and other cytokines. The inappropriate regulation of NF-κB and its dependent genes have been associated with various pathological conditions including toxic septic shock, graft vs host reaction, acute inflammatory conditions, acute phase viral replication, radiation damage, atherosclerosis and cancer (Aggarwal and Bharti 2003).

Inducible Nitric Oxide Synthase (iNOS) is induced in response to infection, inflammatory stimuli and oxidative environment and catalyzes the oxidative deamination of l-arginine to produce NO, a potent pro-inflammatory mediator. NO can act as both initiator of carcinogenesis as well as a tumor promoter (Surh and others 2001). NO can react with superoxide radicals to form peroxynitrite, which is highly toxic to cells. These
products contribute to inflammation and play a role in the development of various diseases (Mantovani and others 2008).

The enzyme phospholipase A₂ (PLA₂) releases arachidonic acid from the sn-2 position of membrane phospholipids and plays an important role in inflammation (Sudhir 2005). PLA₂ is present as secretory (sPLA₂), cytosolic (cPLA₂) and calcium-independent PLA₂ (iPLA₂) forms. cPLA₂ is highly expressed in response to pro-inflammatory stimuli including bacterial lipopolysaccharide (LPS), cytokine and others. cPLA₂ is associated with allergic rhinitis, rheumatoid arthritis and septic shock. The arachidonic acid released from membrane phospholipids can be converted into prostaglandins and leukotrienes by COX-2 and lipoxygenase (LOX), respectively. These mediators control intensity and duration of pain and also occurrence of fever and swelling of affected area (Yoon and Baek 2005).

Avocado (Persea americana, Lauraceae) is an important tropical crop with Hass variety commonly grown in the US. The seed, which accounts for 16% of total avocado weight is an under-utilized resource. Traditionally the seed was used for both medicinal and non-medicinal purposes for example decoctions have been used for treatment of mycotic and parasitic infections, against diabetes and for curing inflammation (Ramos-Jerz 2007). Additionally the seed was used as a red indelible ink (Morton and Dowling 1987). We have previously reported that when the avocado seed is crushed, a stable orange color develops (Dabas 2011).

Current studies on avocado seed focus on their chemical composition and the role of these compounds in the putative anti-cancer, insecticidal, anti-diabetic and blood pressure reducing and skin improving effects of seed extracts (Kate and Lucky 2009;
Leite and others 2009; Anaka and others 2009; Ramos-Jerz and others 2007; Lee and others 2008). Phytochemical studies on avocado seeds have identified various classes of natural compounds such as phytosterols, triterpenes, fatty acids, furanoic acids, abscisic acid, proanthocyanidins and polyphenols – being the major class (Leite and others 2009; Ding and others 2007). Avocado seeds have higher antioxidant activity and polyphenol content than the pulp (Wang and others 2010; Soong and Barlow 2004).

Lipidic polyols purified from avocado seed were found to inhibit sPLA$_2$. The active compounds were identified as 1-acetoxy-2,4-dihydroxy-n-heptadeca-16-en (olefin A), 1,2,4-trihydroxy-n-heptadeca-16-en (olefin B), 1-acetoxy-2,4-dihydroxy-n-heptadeca-16-yn (acetylene A) and 1,2,4-trihydroxy-n-heptadeca-16-yn (acetylene B). The concentrations of these compounds to inhibit approximately 98% of PLA$_2$ activity, when used independently, were: 12.2 mM Olefin A, 7.5 mM acetylene A, 0.17 mM olefin B or 0.08 mM acetylene B (Etozioni 2003).

A hexane extract containing polyhydroxylated fatty alcohols derived from avocado seed and pulp, when applied to human keratinocytes before and after UV B irradiation, reduced secretion of IL-$\alpha$-6 and PGE$_2$. Cell viability was also higher in the treated UV B-irradiated cells than controls. Treatment of human skin explants with polyhydroxylated fatty alcohols reduced the numbers of sunburnt cells and enhanced DNA repair. 1-acetoxy-2,4-dihydroxy-hepta-dec-16-ene and 1-acetoxy-2,4-dihydroxy-hepta-dec-16-yne were identified as the major active constituents in this extract (Rosenblat and others 2010).

Given the limited information on anti-inflammatory effects on avocado seed, there is a clear need of more studies to evaluate these effects. In the present study, we
examined the effect of the colored avocado seed extract (CASE) on LPS-induced inflammatory responses of RAW 264.7 murine macrophages.

4.2. Material and methods

4.2.1. Reagents

Ripened avocado (*Persea americana*, Hass variety) were sourced locally and stored at 4 °C until use. The ELISA for PGE$_2$ was obtained from Cayman (Ann Arbor, MI). ELISAs for IL-6, IL-1β and TNFα were obtained from R&D systems (Minneapolis, MN). Lipopolysaccharide and Greiss reagent were obtained from Sigma (St. Louis, MO). The phospholipase A$_2$ enzyme assay was purchased from Invitrogen (Carlsbad, CA). Antibodies against NF-κB, iNOS, COX2, histone H3 and β actin were purchased from Cell Signaling (Danvers, MA). All other reagents were of the highest grade commercially available.

4.2.2. Preparation of CASE

CASE was prepared as described previously (chapter 2, 2.2.2). Following homogenization of the seeds and color development, the paste was extracted with methanol and dried under vacuum. Stock solutions (200 mg/mL) were prepared in dimethyl sulfoxide and stored at -80 °C.
4.2.3. Cell culture and viability

RAW 264.7 cells were maintained in log-phase growth with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under a humidified CO₂: air (5:95) atmosphere.

The effect of CASE on cell viability was determined using the MTT assay. In brief, cells were seeded (10⁴ cells/well) in 96 well plates and allowed to attach overnight. The cells were co-treated with 1 μg/ml LPS and CASE for 24 h. After CASE treatment, cells were combined with MTT and absorbance read at 540 nm. The viability of treated cells was normalized to LPS-only treated controls.

4.2.4. Modulation of inflammatory cytokines

RAW 264.7 cells were seeded (10⁴ cells/well) in 96 well plates and allowed to attach overnight. Cells were then co-treated with CASE and 1 μg/mL LPS for 24 h in serum complete medium. IL-6, IL-1β and TNFα levels in the medium were determined by ELISA as per manufacturer’s instructions. The levels of these cytokines were compared to unstimulated cells and LPS stimulated control cells.

4.2.5. NO production

RAW 264.7 cells were plated and stimulated as above. The quantity of nitrite in the culture medium of CASE-treated cells was measured after 24 h as an indicator of NO production using Griess reagent. Briefly 50 μl of cell culture medium was mixed with 50 μL of Griess reagent, the mixture was incubated at room temperature for 20 min and
absorbance at 540 nm was measured. The values were expressed as percentage of LPS-only treated cells.

4.2.6. Prostaglandin E₂ production

PGE₂ levels in media of CASE/LPS co-treated and LPS-stimulated RAW 264.7 cells were measured using a PGE₂ EIA monoclonal ELISA kit (Cayman Co., Ann Arbor, MI).

4.2.7. Phospholipase A₂ inhibition Assay

Inhibition of sPLA₂ was examined using a commercially available fluorometric enzyme method. Buffered PLA₂ solution (4 U/ well, pH 8.9) and CASE were combined in a 96-well plate. A fluorogenic PLA₂ substrate (Red/Green boron-dipyrromethene {BODIPY} PC-A₂, 1.5 μM) was dispensed to each well to start the reaction. After incubation at room temperature in the dark for 10 min, fluorescence was determined at \( \lambda_{ex} = 485 \text{ nm} \) and \( \lambda_{em} = 538 \text{ nm} \) (Fluoroskan Ascent FL, ThermoFisher Scientific Inc.). Kinetic analysis of inhibition by PLA₂ was carried out similarly but with the following modification. The concentration of CASE was held constant while the substrate concentration (0.5-4 μM) was varied.

4.2.8. Western blot

4.2.8.1. Preparation of whole cell lysate

RAW 264.7 cells (10⁶) were seeded in 75 cm² flasks for 36 h. The media was replaced with media containing 1 μg/mL LPS and CASE at 5 or 6 μg/mL and incubated
for 24 h. The cells were washed with PBS, scraped off and centrifuged at 1200 \( \times \) g. The cell pellet was combined with lysis buffer (25 mM 3-(N-morpholino) propanesulfonic acid, 2 mM Ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.5% Nonidet P-40 and 0.02% sodium azide) containing 1:100 phosphatase inhibitor I, phosphatase inhibitor II and protease inhibitor. The samples were mixed and disrupted by freeze thawing.

### 4.2.8.2. Preparation of nuclear lysate

The cells were treated as above, and scraped and centrifuged at 800 \( \times \) g for 10 min at 4 °C. The cell pellet was suspended in buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mM MgCl\( _2 \), 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.1% Nonidet P40, incubated on ice for 10 min and centrifuged at 12000 \( \times \) g for 2 min at 4 °C. The pellet was resuspended in buffer B (10 mM HEPES, pH 7.9, 1.5 mM MgCl\( _2 \), 0.4 M NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 25% glycerol). The tubes were then vortexed and incubated on ice for 15 min with mixing every 5 min. They were then centrifuged at 10,000 \( \times \) g for 10 min at 4 °C. The supernatant was removed and used as nuclear fraction.

### 4.2.8.3. Immuno blots

Protein (60 μg total or 20 μg for nuclear extract) was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. After blocking for 1 h with blocking buffer (Li-Cor, Lincoln, NE), the membrane was incubated with respective primary antibody overnight. The bands were visualized using a
fluorescent-conjugated secondary antibody using a Licor Odyssey infra red system (Lincoln, Nebraska).

4.2.9. Data Analysis

For kinetic analysis of PLA₂, Michaelis Menten plots were generated using GraphPad Prism (San Diego, CA), and the maximum velocity (V_max), Michaelis Menten constant (K_m), and mode of inhibition were determined from those plots. All other data were analyzed by one way analysis of variance (ANOVA) with Dunnett’s post test. P values <0.05 were considered as statistically significant. Data are presented as the mean ±SD unless otherwise specified.

4.3. Results

4.3.1. Cytotoxicity

The viability of RAW 264.7 cells was determined after co-incubation with LPS and CASE for 24 h. At concentrations of CASE less than or equal to 6 µg/mL, cell viability was greater than 80% (data not shown). Hence only concentrations up to 6 µg/mL were used for further experiments.

4.3.2. Inhibition of pro-inflammatory cytokines and nitric oxide production by CASE

The levels of IL-6, IL-1β and TNFα were measured in the media of LPS-stimulated RAW 264.7 macrophages after co-treatment with CASE for 24 h. A dose dependent reduction in the concentration of IL-6 was observed with a significant
reduction at concentrations greater than 5 μg/mL (Figure 4.1). The levels of IL-1β and TNFα were significantly reduced by treatment with all the concentrations of CASE (Figure 4.1).

![Graphs showing cytokine levels](image)

**Figure 4.1.** Effect of CASE on the production of inflammatory mediators by RAW 264.7 cells stimulated with LPS. Cytokine and NO levels were measured in the supernatant of cells treated with CASE. Mean ± SD of three independent experiments. ‘NC’ indicates non-LPS stimulated control.

NO production by LPS-stimulated RAW 264.7 cells co-treated with CASE was assessed using the Greiss reagent. CASE inhibited the formation of nitrite in a concentration dependent manner with significant reduction at concentrations greater than or equal to 5 μg/mL (Figure 4.1). Western blot analysis showed that CASE dose-dependently reduced the protein expression of iNOS in LPS-stimulated RAW 264.7 cells after 24 h treatment (Figure 4.2).
Figure 4.2. Effect of CASE on the protein expression of iNOS in LPS-stimulated RAW 264.7 cells. β-actin was used as loading control. Cells were treated with CASE for 24 h. Results are representative of three independent experiments.

4.3.3. Inhibition of PGE$_2$ production by CASE

PGE$_2$ production by RAW 264.7 cells was assessed after co-incubation with 1µg/mL LPS and CASE for 24 h. It was observed that the PGE$_2$ production was significantly reduced by treatment with 6 µg/mL CASE compared to LPS-stimulated controls (Figure 4.3a). Western blot analysis of COX-2 showed that CASE did not affect its expression (results not shown).

4.3.4. Inhibition of PLA$_2$ activity by CASE

CASE dose dependently inhibited the activity of secreted PLA$_2$ in a cell-free system. The IC$_{50}$ of CASE was 36 µg/mL (Figure 4.3b). Kinetic analysis showed that CASE significantly reduced the $V_{\text{max}}$ but had no significant effect on $K_m$ of PLA$_2$ (Figure 4.3c; Table 1). These results suggest that CASE inhibits PLA$_2$ in a non-competitive manner with respect to substrate concentration.
**Figure 4.3.** (a) The effect of CASE on the production of PGE$_2$ in RAW 264.7 cells stimulated with LPS for 24 h. Mean ± SD of three independent experiments. (b) The effect of CASE on the activity of pure secreted PLA$_2$. Symbols represent mean ± SD (n=5) (c) Kinetic analysis of CASE inhibition of secreted PLA$_2$. Concentrations of CASE used were (●) nil, (■) 36 µg/mL and (▲) 72 µg/mL. Inhibitory kinetics were determined using Michelis-Menton analysis.

**Table 1.** Kinetic analysis of inhibition of secreted PLA$_2$ by CASE*

<table>
<thead>
<tr>
<th>CASE (µg/mL)</th>
<th>0</th>
<th>36</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_m$ (µM substrate)</td>
<td>1.2 ±0.1$^a$</td>
<td>1.2 ±0.1$^a$</td>
<td>1.2 ±0.3$^a$</td>
</tr>
<tr>
<td>V$_{max}$ (pmol/min/mg protein)</td>
<td>145.8 ±15.6$^a$</td>
<td>92.6 ±3.9$^b$</td>
<td>49.5 ±3.9$^c$</td>
</tr>
</tbody>
</table>

*Values in the same row not sharing a common superscript letter are significantly different (p < 0.05).

**4.3.5. Effects on NF-κB translocation**

The nuclear localization of NF-κB is a critical signal in LPS-induced inflammation. Co-incubation with CASE reduced nuclear levels of NF-κB, but had no effect on total NF-κB expression (Figure 4.4).
Figure 4.4. Effect of CASE on nuclear translocation of NF-κB in LPS-stimulated RAW 264.7 cells. Nuclear and total levels NF-κB were determined by western blot. β-actin and Histone H3 were used as protein loading controls for total and nuclear protein respectively. Representative results of three independent experiments.

4.4. Discussion

This study was carried out to assess the anti-inflammatory effects of CASE in LPS-stimulated RAW 264.7 murine macrophage cell line. On stimulation by LPS, macrophages produce pro-inflammatory cytokines, eicosanoids and NO. These inflammatory mediators play a key role in the progression of inflammatory diseases (Gabey 2006). Treatment with CASE reduced production of TNFα, IL-1β and IL-6. IL-1β induces the expression of COX-2 and iNOS. TNFα although binds to different receptors than IL-1β, the post receptor events are similar, and both activate a similar portfolio of genes. IL-6 also triggers the formation of other inflammatory markers.
including eicosanoids and NO and as a result there is growing interest in developing anti-IL-6 agents (Gabey 2006). Inhibition of these cytokines by CASE indicates that the formation of downstream inflammatory products may also be inhibited.

CASE inhibited LPS-induced NO production. NO is a key inflammatory mediator and inhibition of NO production has an anti-inflammatory effect. CASE reduced the expression of iNOS providing a mechanism of the observed decrease of NO. The promoter region of iNOS gene contains several binding sites for transcriptional factors such as NF-κB and activator protein-1 (AP-1) as well as other proteins. LPS-stimulated induction of iNOS is mediated through NF-κB (Chen and Wang 1999).

CASE inhibited the formation of PGE₂, a pro-inflammatory eicosanoid, but did not affect COX-2, its biosynthetic enzyme. CASE also inhibited the activity of sPLA₂. CASE-mediated inhibition of PGE₂ production may result from inhibition of PLA₂. Alternatively it could be the result of inhibition of COX-2 activity. PLA₂ catalyzes the release of arachidonic acid from phospholipids. Arachidonic acid is then metabolized through the COX pathway to prostaglandins and through the LOX pathway to leukotrienes. COX-2 is a known drug target with clinically useful inhibitors, whereas inhibitors of PLA₂ are currently sought as potentially useful anti-inflammatory agents (Fiancette 2011). sPLA₂ was used in our study whereas cPLA₂ is up regulated as a result of LPS-stimulation. It is thus not confirmed if activity of cPLA₂ will be affected by CASE.

The nuclear translocation of NF-κB was reduced by treatment with CASE. NF-κB is a transcription factor which in the resting state is bound via non covalent interactions to inhibitor of Kappa B (IκB) and sequestered in the cytoplasm. Treatment with LPS causes
phosphorylation of IκB and subsequently they get degraded allowing NF-κB to enter nucleus and induce gene expression. NF-κB is involved in regulating many aspects of cellular function, including immune response. The expression of inflammatory cytokines, adhesion molecules, angiogenic factors COX-2 and iNOS are all regulated by NF-κB (Porta and others 2009). NF-κB therefore represents a possible pathway through which CASE may exert its anti-inflammatory effects. Modulation of NF-κB signaling may account for the effects of CASE on cytokine production and iNOS expression. The effects on PGE₂ and PLA₂, however, are likely due to an alternative mechanism since COX-2 expression was unchanged by CASE.

4.5. Conclusion

This study characterized the in vitro anti-inflammatory effects of a colored extract obtained from avocado seed. The extract was found to reduce the production of pro-inflammatory mediators by stimulated macrophage cells. Anti-inflammatory medications such as NSAIDs and steroids are commonly used to treat various diseases. CASE represents a potential source for novel anti-inflammatory compounds that can be developed as functional food ingredients or as pharmaceuticals.

4.6. References


Chapter 5

CONCLUSION AND FUTURE WORK

This study characterized a colored avocado seed extract (CASE) with respect to its colorant properties and possible applications as well its potential beneficial biological effects. Natural colors are gaining importance with consumer’s increasing preference for natural ingredients, and this makes my work on CASE increasingly relevant.

The color was shown to be produced as a result of activity of polyphenol oxidase on as yet unknown substrate(s). The orange color of CASE intensified as the pH increased and these changes were only partially reversible in the presence of oxygen but completely reversible when the pH was adjusted and readjusted in the absence of oxygen. At both -18°C and 4°C, the color of CASE was relatively stable whereas at elevated temperature it was not.

CASE was successfully incorporated into milk, fruit juices and a baked product resulting in noticeable color differences in all products. The above results suggest that CASE can be added to different products. It can also be used in non-food applications.

CASE was found to have radical scavenging activity and antioxidant activity in a model food system. The ORAC value of CASE was 2012.1 ± 300 TE/mg. This ORAC value was higher than ORAC values for pulp and comparable to values for other fruits as seen from literature. CASE can serve additional function of maintaining food quality.

CASE inhibited the growth of human cancer cell lines (lung, colon, breast, and prostate) under in vitro conditions with prostate cancer cells being the most sensitive to
the effects. The effects were mediated by inhibition of cell cycle progression (at G₀/G₁) and induction of apoptosis.

CASE treatment reduced the formation of cytokines (IL-6, IL-1β and TNFα) in LPS-stimulated murine macrophage cells in culture. Additionally CASE reduced the formation of NO and prostaglandin E₂, both key mediators in inflammatory response. These effects may be the result of CASE-mediated inhibition of nuclear translocation of NF-κB.

Overall, these results suggest that the avocado seed may be a potential source of natural colorant which could impart additional functions including antioxidant, cancer preventive activity and additionally anti-inflammatory effects. At present seeds do not find any uses and their disposal is an extra cost to the processors and a burden on the environment. However, using seeds to make CASE can lead to financial benefits and also can be good for environment. Also, the uncolored seed extract was found to have antioxidant and anti-cancer activity (Appendix II). These results are important because despite (the pulp) being used in large quantities, not many studies on avocado seed have been done.

Suggestions for the future work are as follows:

- The major colored compounds should be isolated and identified – The biggest colored peak on the HPLC chromatogram should be isolated and identified.
- It is crucial to carry out safety study on the colored extract for commercial use in products. Safety study has been done on the uncolored extract but it is important to test safety of CASE using a suitable animal model.
• The anticancer and anti-inflammatory effects were shown under cell culture conditions on select cell lines. However these effects need to be demonstrated in living systems to prove its efficacy for human use.

• The active compounds responsible for the anti-cancer and anti-inflammatory attributes should be identified. A bioactive guided fractionation on CASE should be the next step for studying these compounds. The isolated compounds should be tested as pharmaceuticals.

• Effects of CASE on other chronic diseases, for example metabolic syndrome should be explored. Phenolics are being studied for prevention of many diseases, including diabetes, cardiovascular diseases and obesity. CASE owing to its high phenolic content may be a candidate for studying the ability to prevent these diseases especially since some studies carried out on seed extract have shown beneficial effects in animal models.
CHAPTER 2 - SUPPLEMENTAL DATA

Isolation and identification of colored compound/s

Flash silica gel chromatography was performed to fractionate CASE. CASE (10 g) was mixed with silica gel (1:3 wt/wt) and methanol (1:5 wt/vol) was added. This mixture was allowed to dry in air for 24 h with regular stirring. The glass column (length =24.1’, radius=1.0’) was filled with approximately 500 g of silica. CASE was placed on the top of column and solvents added sequentially as follows: 1 L hexane, 1 L ethyl acetate (EtOAc) and increasing concentrations of methanol (MeOH) in EtOAc (5-100%) in 5% increments. Fractions were collected and dried under vacuum.

Of the total eluted mass of 7557.6 mg, the fraction 20 MeOH:80 EtOAc had the highest colored mass (2365 mg) followed by 15 MeOH:85 EtOAc (1088.5 mg). Fractions after this, although red in color were present in small quantities (less than 61 mg/fraction). The fractions eluting after 50 MeOH:50 EtOAc were predominantly brown in color.

Fractions eluted with 15 MeOH:85 EtOAc were collected as 5, 200 ml fractions and so five sub-fractions were collected. 20 MeOH:80 EtOAc were collected as 10, 100 ml sub-fractions. These were re-combined based on thin layer chromatography (TLC) profiles. Sub-fractions from both these combinations gave similar profiles on C18 column using method described in section 2.2.9. The fifth sub-fraction eluted from 15 MeOH:85
EtOAc -5 (451.4 mg) was further resolved by HPLC with a C18 analytical column (Restek, ultra aqueous, 250 mm X 10 mm) using the method described in section 2.2.9 at a flow rate of 1 mL/min (Figure A.1). The most abundant colored peak ($\lambda_{\text{max}} = 440 \text{ nm}$) was collected manually. Pooled fractions were dried under vacuum to yield 1.1 mg of isolate. The peak was re-analyzed by HPLC to assess the purity of HPLC conditions. Although significant improvement in purity was observed, other peaks were observed at 6 min and between 13-17 min. Based on absorbance at 280 nm of the peak at $t_R = 18 \text{ min}$, the purity of the compound is approximately 70%. The isolate was dissolved in deuterated water and NMR was performed (Figure A.3 and A.4). However the structure could not be determined from this data because of presence of multiple compounds. Infra Red spectroscopy was also performed on the isolated peak (Figure A.5). A pellet of dried sample was prepared with KBr and an IR spectrum was measured. $\text{C=O (1630 cm}^{-1}), \text{C-O (1360, 1280, 1100 cm}^{-1})$ and OH (3400 cm$^{-1}$) groups may be present in the structure.
Figure A.1. Chromatogram of the fraction 15 MeOH:85 EtOAc-5. The conditions have been described in the section 2.2.9. The chromatogram was obtained at two wavelengths - 280 nm (blue line) and at 440 nm (pink line). The intensity at 440 nm was intensified 10 times for better visibility.

Figure A.2. Chromatogram of the isolated colored peak from the fraction 15 MeOH:85 EtOAc-5. The conditions have been described in the section 2.2.9. The chromatogram was obtained at two wavelengths - 280 nm (blue line) and at 440 nm (pink line). The intensity at 440 nm was intensified 10 times for better visibility.
Figure A.3. Proton NMR spectra of isolated colored peak from the fraction 15 MeOH:85 EtOAc-5.
Figure A.4. Carbon NMR spectra of isolated colored peak from the fraction 15 MeOH:85 EtOAc-5.
Figure A.5. Infrared spectra of isolated colored peak from the fraction 15 MeOH:85 EtOAc-5.
Appendix B

CHAPTER 3 - SUPPLEMENTAL DATA

Antioxidant and anti-carcinogenic activity of the uncolored seed extract

An uncolored extract was prepared in a method analogous to CASE except that methanol was added immediately after grinding the seeds so that color did not form. This extract was also tested for antioxidant and anti-carcinogenic effect. The radical scavenging activity was determined using the EPR assay as described in section 3.2.4 and ORAC assay as mentioned in section 3.2.5. The cytotoxic effects were evaluated as per section 3.2.6.

Antioxidant activity

The uncolored extract when reconstituted in PBS at pH 7.4 displayed dose dependent radical scavenging activity in the EPR assay (Figure A.1). The EC$_{50}$ for protection of Tempol was 16.8 μg/mL. This value was significantly lower from the EC$_{50}$ value for CASE (42.1 μg/mL). However, the ORAC value for the uncolored extract (2300.0 ± 200.1 TE/mg) was not significantly different from that of CASE (2012.1 ± 300 TE/mg).
**Figure B.1.** Antioxidant activity of the uncolored extract was determined using EPR. Tempol absorbance intensity was determined after incubation for 120 min at 37 °C. The Tempol absorbance intensities were normalized to the intensity of control incubated with 120 μM Trolox. Mean ± SEM of three independent experiments.

**Cytotoxic effects**

The uncolored avocado seed extract dose-dependently inhibited the growth of all of the cancer cell lines after 48 h treatment (Figure A.2). LNCaP cells were the most sensitive cell line (IC$_{50}$ = 4.6 µg/mL) whereas H1299 cells were the least sensitive cell line (IC$_{50}$ = 53.6 µg/mL). LNCaP, H1299, and HT29 cells were more sensitive to the uncolored extract (IC$_{50}$ = 4.6 – 53.6 µg/mL) than CASE (IC$_{50}$ = 19.1 – 132.2 µg/mL). By contrast, MCF7 (breast cancer) cells were nearly equally sensitive to both extracts (IC$_{50}$ = 19.1 µg/mL vs. 27.6 µg/mL for CASE vs. the uncolored extract).
Figure B.2. Viability of selected cancer cell lines after treatment with the uncolored avocado seed extract. The percentage survival was calculated with respect to control after incubation for 48 h. Cell lines are represented as: (○) LNCaP, (●) MCF7, (▲) HT29 and (■) H1299. Results are mean ±SEM of three independent experiments.

Avocado seed, despite having a number of traditional uses does not find uses currently and hence is a waste product and a potential disposal problem. Our results, as well as those of previous researchers, show that the seed extract is a rich source of phenolics and has functional attributes including high antioxidant and anti-cancer activity.
VITA

DEEPTI DABAS

EDUCATION

- Ph. D. in Food Science, Department of Food Science, The Pennsylvania State University (PSU). (2007-2012)
- M. S. Food Science and Technology, CCS Haryana Agricultural University, India. (1999-01)
- B. A. Sc. (Bachelor of Applied Sciences) Food Technology, College of Applied Sciences, India. (1996-99)

EXPERIENCE

- Scientist-D, National Mission on Bamboo Applications, Department of Science and Technology, India (2003-07)
- Food Technologist in R&D division of TOPS Foods Private Limited, New Delhi, India (2001-02)

SELECT PUBLICATIONS AND PRESENTATIONS

- Presented a talk titled ‘Colored avocado seed extract with radical scavenging and anti-carcinogenic activity’ in 241th American Chemical Society National Meeting (2011)
- Presented a talk titled ‘A colored avocado seed extract with antioxidant and anti-carcinogenic effects’ at Center for Food Manufacturing meeting, Department of Food Science, PSU (2011)
- Presented a paper on ‘Community level shoot processing technology for bamboo shoots’ at ‘Development of Commercial Agriculture and Food Processing Industries’ conference, India (2005)

SELECT AWARDS

- Sponsored registration for attending annual American Chemical Society (ACS) meeting (2011)
- First prize in Annual Graduate and Undergraduate Research Expo, PSU (2011)
- Robert D. and Jeane L. McCarthy Memorial Graduate Scholarship, PSU (2010)
- Junior Research Scholarship (JRF), India (1999-01)