



UNIVERSITY
OF MANITOBA

**ANTIOXIDANT POTENTIAL OF SPECIFIC CEREAL GRAIN
FRACTIONS: *IN VITRO* AND *IN VIVO* STUDIES**

By

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DEDICATION

To the pillars of my life: God, my parents and grandmother.
Without you, my life would fall apart.

I might not know where the life's road will take me, but walking with you, God, through this journey has given me strength.

To the loving memory of my grandmother Natefho Mmolai (1924-2005), for sowing in me the seed of discipline, self respect, passion for better things in life.

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Daddy (Tumediso Masisi), you always told me to “reach for the stars.” I think I got one. Thanks for inspiring my life for transformation.
We made it...

DECLARATION

I hereby declare that I KABO MASISI is the sole author of this thesis and have not been accepted in any previous application for any degree. This is a true copy of the original thesis. It is a true record of work done by myself, unless otherwise stated. All sources of information have been duly acknowledged by means of references and copyright licenses for previously published materials have been obtained. The research was conducted in compliance with the ethical standards and guidelines of the University of Manitoba.

THESIS FORMAT

This thesis has been prepared in the manuscript format. Referencing style used is for the Food Chemistry Journal. The thesis has seven chapters of which Chapters 1 and 2 are overall introduction and literature review. The experimental chapters are divided in three sections, Section A (Biochemical Model: Chapter 3), Section B (*In vitro* Model: Chapter 4) and Section C (*In vivo* Model: Chapter 5 and Chapter 6). Chapter 7 provides the general discussion, conclusions and areas for further research.

Experimental Chapters

Section A: Biochemical Model

Chapter 3: Characterization, identification, quantification and antioxidant capacity of carotenoids of aleurone, germ and endosperm fractions of barley, corn and wheat

Section B: In vitro Model

Chapter 4: Carotenoids of aleurone, germ, and endosperm fractions of barley, corn and wheat differentially inhibit oxidative stress.

Section C: In vivo Model

Chapter 5: Dietary corn fractions reduce atherogenesis in LDL receptor knockout mice

Chapter 6: Dietary corn fractions attenuate atherosclerosis in LDL receptor knockout mice by differentially modifying antioxidant enzymes activities and oxidative indicators

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LIST OF ABBREVIATIONS

- AAPH - 2,2'-azobis (2-amidinopropane) dihydrochloride
- ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
- ACVD - Atherosclerotic Cardiovascular Disease
- ANOVA - Analysis of Variance
- AOA - Antioxidant Activity
- AOP - Antioxidant Potential
- ATCC - American Type Culture Collection
- AUC - Area Under the Curve
- CAT - Catalase
- CVD - Cardiovascular Disease
- CAA - Cellular Antioxidant Activity
- CUPRA - Copper Reduction Assay
- DCF - Dichlorofluorescin
- DCFH-DA - Dichlorofluorescin Diacetate
- DMEM - Dulbecco's Modified Eagle Medium
- DPPH - 2,2'-diphenyl-1-picrylhydrazyl
- FBS - Fetal Bovine Serum
- FL - Fluorescein
- FRAP - Ferric Reducing Ability of Plasma
- FOs - Feruloyl Oligosaccharides
- GAE - Gallic Acid Equivalent
- GC - Gas Chromatography

GLIMMIX - Generalized Linear Mixed Models

GPx - Glutathione Peroxidase

GR - Glutathione Reductase

GSSG - Oxidized Glutathione

HAT - Hydrogen Atom Transfer

HDL - High Density Lipoprotein

HPLC - High Performance Liquid Chromatography

hTF - Human Transferrin

H₂O₂ - Hydrogen Peroxide

OH· - Hydroxyl Radical

LDL - Low Density Lipoprotein

LDLr-KO - Low Density Lipoprotein Receptor Knockout

LOX - Lipoxygenase

L-gln - L-glutamine

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide

Na/Pyr - Sodium Pyruvate

NSP - Nonstarch Polysaccharides

ORAC - Oxygen Radical Absorbance Capacity

ox-LDL - Oxidized Low Density Lipoprotein

P/S - Penicillin/Streptomycin

POX - Peroxidase

PBS - Phosphate Buffered Saline

ROO· - Peroxyl Radical

RCT - Reverse Cholesterol Transport

ROS - Reactive Oxygen Species

SET - Single Electron Transfer

SE - Standard Error

SAS - Statistical Analysis Software

$O_2^{\cdot-}$ - Superoxide Anion

SOD - Superoxide Dismutase

TBARS - Thiobarbituric Acid Reactive Substances

TAC - Total Anthocyanin Contents

TC -Total Cholesterol

TCC - Total Carotenoid Content

TOSC - Total Oxyradical Scavenging Capacity

TP - Total Phenolics

TPC - Total Phenolic Content

TRAP - Total Reactive Antioxidant Potential

TG - Triglyceride

TE - Trolox Equivalents

TEAC - Trolox Equivalent Antioxidant Capacity

VLDL - Very Low Density Lipoprotein

WEAX - Water Extractable Arabinoxylans

WHO - World Health Organization

ABSTRACT

Accumulating evidence has suggested that intake of whole grains is inversely associated with oxidative-stress disease. The mechanisms, however, are not completely clear. This study assessed the antioxidant potential of hand-separated aleurone, germ and endosperm fractions of barley (*Hordeum vulgare L.*), corn (*Zea mays*) and wheat (*Triticum spp.*), and anti-atherogenic benefits of hand-separated corn fractions. HPLC analysis confirmed the presence of lutein and zeaxanthin carotenoids (nd-15139 µg/kg) in extracts of cereal grain fractions. The antioxidant properties using 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assays revealed significantly higher ($P<0.001$) antioxidant activity in the germ than in the aleurone and endosperm fractions. Using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced cell loss was effectively reduced by pre-incubating Caco-2, HT-29 and FHs 74 Int cells with carotenoid extracts. Moreover, carotenoid extracts reduced ($P<0.001$) AAPH-induced intracellular oxidation in the cell lines, suggesting significant antioxidant activities. Of the 84 antioxidant pathway genes included in microarray array analysis (HT-29 cells), the expression of 28 genes was enhanced ($P<0.05$).

In *in vivo* study, four groups of male low density lipoprotein receptor knockout (LDLr-KO) mice were fed with the experimental diets supplemented with (3 treated groups) or without (1 control group) 5% (w/w) of each of hand-separated corn fractions for 10 weeks. All diets were supplemented with 0.06% (w/w) dietary cholesterol. Consumption

of aleurone and germ fractions significantly reduced the size and atherosclerotic lesions in the aortic roots as compared to those in the control group ($P<0.003$). This effect was associated with significant reductions in plasma total ($P<0.02$) and LDL ($P<0.03$) cholesterol concentrations as well as an increase in fecal cholesterol excretion ($P<0.04$). Furthermore, abdominal fat mass was significantly reduced by consumption of aleurone ($P<0.03$). Corn fractions also differentially modified antioxidant status. The study suggests cholesterol-lowering and oxidative stress-lowering effects of corn fractions may be beneficial for the prevention of atherosclerosis in LDLr-KO mice. Incorporation of corn fractions boosted the total carotenoid contents (TCC ($P<0.06$), lutein ($P<0.004$) and zeaxanthin ($P<0.002$)) of the diets, but not in the plasma and liver tissues. Therefore, dietary carotenoids of corn fractions do not seem to affect the development of atherosclerosis in LDLr-KO mice. Taken together, aleurone and germ fractions might have therapeutic potential for managing oxidative stress and its associated co-morbidities.

CHAPTER 1

Introduction, Rationale, Hypotheses and Objectives

1.1 Introduction

Several studies have described relationship between the increased cellular reactive oxygen species and the pathogenesis of more than 50 diseases including aging (Romano et al., 2010), atherosclerosis (Stocker & Keaney Jr, 2004), Alzheimer's disease (Simonian & Coyle, 1996), cancer (Klaunig et al., 2004; 2010), and diabetes mellitus (Hayakawa & Kuzuya, 1990; Maritim et al., 2003). Reactive oxygen and nitrogen species are generated from endogenous (normal physiological process) as well as exogenous sources (xenobiotic interaction) (Ho et al., 2013; Rahman, 2007; Sobha & Andallu, 2013) and are removed by cellular antioxidant defenses system (Wang & Jiao, 2000). If not adequately regulated, these highly reactive and unstable molecules may overwhelm the antioxidant defense mechanisms and cause oxidative damage to cell membranes, cellular proteins, lipids and DNA (Poli et al., 2004; Stadman, 1994). During oxygen metabolism, unstable molecules which steal electrons from other molecules are created, causing damage to the cell structures. Oxidative stress occurs when cells are subjected to excess levels of these unstable molecules, known as reactive oxygen species (ROS), or as a result of antioxidant depletion and/or faulty antioxidants. Therefore, antioxidants which can neutralize free radicals may be of central importance in reducing the prevalence of this diseased state (Wang et al., 1996). Antioxidant protection is normally achieved through a balance between pro-oxidants and endogenous and/or dietary antioxidants (Hancock et al., 2001; Klaunig et al., 2004; 2010; Sobha & Andallu, 2013). Dietary antioxidants from natural

sources such as whole cereal grains in particular are of interest as increased dietary intake of these agents may lead to protection against free radical-induced diseases (Adom et al., 2005; Belobrajdic & Bird, 2013; Lee et al., 2015; Yu et al., 2002).

Whole cereal grains are a principal component of all diets, and they significantly contribute to nutrition by supplying energy, protein, vitamin, and other biologically significant chemicals (Slavin, 2004). Accumulating evidence has suggested that intake of whole cereal grains is a protective factor against lifestyle diseases such as diabetes, cardiovascular diseases, and some cancers (Fardet et al., 2008; de Munter et al., 2007; Slavin, 2004). Whole cereal grains consist of different fractions: starchy endosperm (in this thesis, endosperm refers to starchy endosperm), bran, aleurone and germ, which contain phytochemicals. The mainly consumed whole cereal grains in the world include wheat, rice, maize, oats, rye, barley, triticale, millet, and sorghum (Fardet, 2010). In North America, barley, maize (known elsewhere as corn) and wheat are mainly consumed. The major health benefits are attributed to the cereals' unique phytochemical composition (Adom et al., 2005; Belobrajdic & Bird, 2013). Recently, trace amounts of antioxidant phytochemicals have attracted more interest from both food manufacturers and researchers. Among the phytochemicals, carotenoids are known for their role in health promotion by reducing the risks of developing chronic diseases due to their antioxidant properties and other mechanisms (Arathi et al., 2015; Fiedor & Burda, 2014). Carotenoids are among the abundant families of pigments in nature that are responsible for the yellow, orange and red colors in fruits, vegetables and cereal grains, and they form part of the antioxidant system in seeds (Stahl & Sies 2003; Irakli et al. 2011; Howitt & Pogson 2006). Carotenoids have extensive application as antioxidants and therefore, are important for human health. The

carotenoids are referred to as antioxidants because of their capacity to trap not only lipid peroxy radicals, but also singlet oxygen species. Moreover, the antioxidant capacity of carotenoids may also be related to their structure. A large conjugated system such as astaxanthin is known to have higher antioxidant activity (Miki, 1991). Despite the fact that the antioxidant capacity of whole cereal grains has been demonstrated in various *in vitro* and *in vivo* systems, there is still minimal evidence regarding antioxidant potential of cereal grain fractions (aleurone, endosperm and germ) in biochemical and biological systems.

The purposes of this thesis therefore were to first quantify and identify carotenoid extracts and, evaluate the antioxidant potential of these carotenoid extracts of aleurone, endosperm and germ fractions of barley, corn and wheat using biochemical and *in vitro* models. Moreover, the antioxidants and anti-atherogenic effects of aleurone, endosperm and germ fractions of corn were assessed using an animal model with dyslipidemia and atherosclerosis. The primary objectives of this thesis were to evaluate 1) antioxidant potential of carotenoid extracts of aleurone, endosperm and germ fractions of barley, corn and wheat using ABTS, DPPH and ORAC assays; and 2) antioxidant potential against free radical-initiated intracellular oxidation as well as free radical-induced cytotoxicity using intestinal cell lines. In addition, the mechanism by which aleurone, endosperm and germ fractions of corn confer protective effects in a selected animal model with dyslipidemia and atherosclerosis was investigated.

Therefore, the following rationale, hypotheses and objectives were used to guide this research:

1.2 Rationale

Accumulating evidence has suggested that intake of whole grains has a protective role against oxidative stress. The protective roles are attributed to their high levels in phytochemical components and antioxidant activities. However, little is known about the benefits of aleurone, endosperm and germ fractions of barley, corn and wheat on antioxidative status. Therefore, understanding the antioxidant potential and anti-atherogenic benefits of specific cereal grain fractions is of potential importance in aiding the selection of whole grains and their processed fractions for inclusion in healthy diets, and as functional food ingredients in product development. This study was carried out to generate evidence of the effectiveness of hand separated cereal grain fractions (aleurone, endosperm and germ) as antioxidants in biochemical models and, on reducing oxidative stress in human intestinal cell culture models and experimental animals model with dyslipidemia and atherosclerosis. Hand separated fractions were used due to their purity and homogeneity with respect to tissues and biochemical composition, as opposed to mechanically separated fractions which are not homogenous (Brouns et al., 2012).

1.3 Hypotheses and Objectives

Overall Hypothesis

Carotenoids from hand separated aleurone, germ and endosperm fractions of barley, corn and wheat can be effectively extracted and concentrated. They are mainly composed of lutein and zeaxanthin. The antioxidant capacity of hand separated aleurone, germ, and endosperm fractions of barley, corn and wheat can scavenge free radicals biochemically, *in*

vitro and *in vivo* thus protecting against free radical-associated oxidative stress, cytotoxicity and atherosclerosis.

Overall Objectives

1) To extract carotenoids from aleurone, germ and endosperm fractions of barley, corn and wheat and quantify and characterize chemically carotenoids extract, and demonstrate that the antioxidant capacity of these carotenoids extracts can protect against free radical-induced intracellular oxidation and cytotoxicity in cell cultured cells. 2) Furthermore, to assess antioxidant properties and anti-atherogenic effects of corn fractions (aleurone, endosperm and germ) in low density lipoprotein receptor knockout mice.

Specific Hypotheses and Objectives:

Chapter 3: Characterization, Identification, Quantification and Antioxidant Capacity of Carotenoids of Aleurone, Germ and Endosperm Fractions of Barley, Corn and Wheat

Hypothesis: The antioxidant capacities of carotenoid extracts of aleurone, germ and endosperm fractions of barley, corn and wheat are different.

Aim: To evaluate the antioxidant capacities of carotenoid extracts of aleurone, germ and endosperm fractions of barley, corn and wheat using ABTS, DPPH and ORAC assays.

Chapter 4: Carotenoids of Aleurone, Germ, and Endosperm Fractions of Barley, Corn and Wheat Differentially Inhibit Oxidative Stress

Hypothesis 1: Carotenoid extracts of aleurone, germ and endosperm fractions of barley, corn and wheat are not cytotoxic at physiological concentration in different cell culture models (Caco-2, HT-29 and FHs 74 Int).

Aim: To establish the cytotoxicity levels of carotenoid extracts in Caco-2, HT-29 and FHs 74 Int cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay.

Hypothesis 2: Carotenoid extracts of aleurone, germ and endosperm fractions of barley, corn and wheat neutralize free radical-initiated intracellular oxidation as well as free radical-induced cytotoxicity in cell culture models.

Aim 1: To establish the lethal concentration of 50% (LC₅₀) values of AAPH and H₂O₂ radical inducing oxidants in Caco-2, HT-29 and FHs 74 Int cell lines using MTT assay.

Aim 2: To determine the protective effects of carotenoid extracts against AAPH- and H₂O₂-initiated cytotoxicity using MTT assay.

Aim 3: To monitor the manifestation of the antioxidant capacity of carotenoid extracts in suppressing free radical-initiated intracellular oxidation in three distinct cells over time.

Aim 4: To quantitate the protective effect of carotenoid extracts against free-radical-induced cytotoxicity.

Hypothesis 3: Carotenoid extracts of aleurone, germ and endosperm fractions of barley, corn and wheat enhance antioxidant enzymes for human oxidative stress and antioxidant defense.

Aim: To assess expression of antioxidant enzymes in cell line (HT-29) treated with carotenoid extracts using real time PCR for the human oxidative stress and antioxidant defense PCR array.

Chapter 5: Dietary Corn Fractions Reduce Atherogenesis in Low Density Lipoprotein Receptor Knockout Mice

Hypothesis: Adequate intakes of corn fractions (aleurone, endosperm and germ) reduce atherosclerotic lesion development through alterations in lipids profile and inflammatory pathways in LDL receptor deficient (LDLr-KO) mice.

Aim 1: To investigate the effectiveness of consumption of corn fractions on lipid-lowering effects in LDLr-KO mice.

Aim 2: Determine the effect of consumption of corn fractions on excretion of lipids.

Aim 3: To investigate the effectiveness of consumption of corn fractions on the prevention of atherosclerosis lesion development in LDLr-KO mice.

Chapter 6: Dietary Corn Fractions Attenuate Atherosclerosis in LDL Receptor Knockout Mice by Differentially Modifying Antioxidant Enzymes' Activities and Oxidative Indicators

Hypothesis 1: Adequate intakes of corn fractions (aleurone, endosperm and germ) reduce cardiovascular risks through alterations in antioxidant system in LDLr-KO mice.

Aim 1: Determine activities of antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in plasma and liver samples of LDLr-KO mice.

Aim 2: Determine the 1,1-diphenyl-2-picrylhydrazyl-scavenging (DPPH) scavenging radical activity in plasma and liver samples of LDLr-KO mice.

Hypothesis 2: The protective effects of corn fractions against cardiovascular risk factors in LDLr-KO are through antioxidative properties of carotenoids.

Aim: Determine carotenoid levels in the diets and biological samples of LDLr-KO mice fed diets with corn fractions.

Antioxidant potential of aleurone, germ and endosperm fractions of barley, corn and wheat were examined following the models as shown below (**Fig. 1.0**).

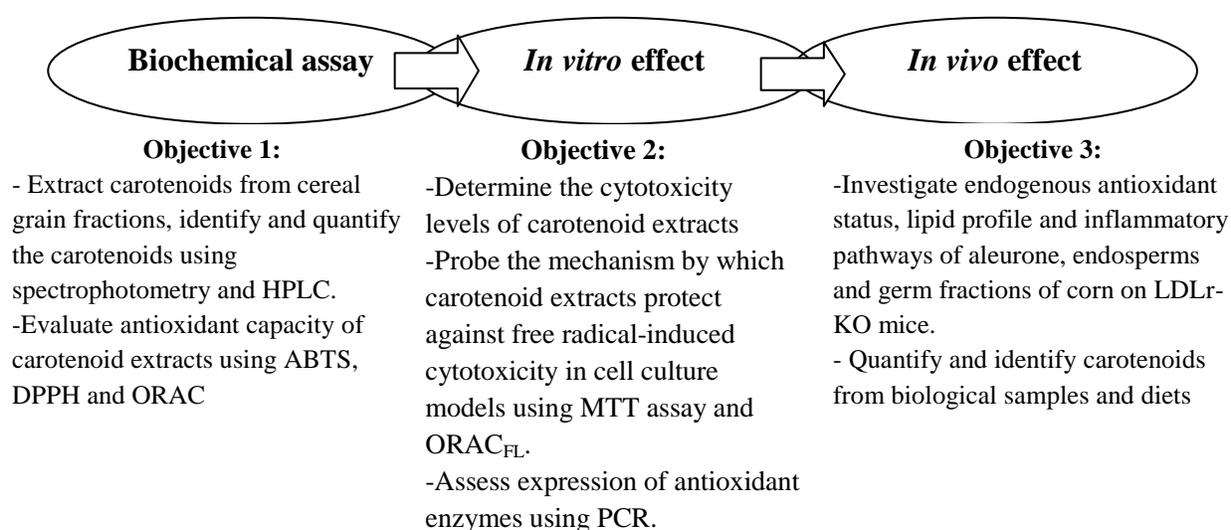


Figure 1.0 Summary of the research models and objectives.

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CHAPTER 2

Literature Review

This chapter briefly describes oxidative stress; oxidative stress on cellular components; oxidative stress on disease: atherosclerotic cardiovascular disease, the major source of morbidity and mortality worldwide associated with oxidative stress; whole grain composition and their phytochemicals; and antioxidant properties of diverse cereal grains.

2.1 Oxidative Stress

Oxidative stress is the imbalance between oxidant and antioxidant in favor of oxidant, leading to the damage of cell components, including DNA, lipids and protein (Sies, 1991). Oxidative stress is caused by excess formation of reactive oxygen species (ROS) in the cell components. Although regulated fluctuations in ROS levels may play important signalling roles, uncontrolled fluctuations in these chemically unstable and reactive molecules may have profound deleterious effects (Droge, 2002). Oxidative stress and its associated pathologies occur due to loss of redox homeostasis, whereby ROS levels remain elevated above normophysiological thresholds (Kaludercic et al., 2014; Winternoun, 2008). Oxidative stress is a complex biological phenomenon that manifests as a result of excessive ROS generation and/or a deficiency in ROS clearance by antioxidant defences. Free radicals such as hydroxyl radicals ($\text{OH}\cdot$) may react with reactive aldehydes, alkanes, lipid epoxide and alcohols initiating a self-propagating reaction known as lipid peroxidation, which can damage cellular membranes and produce cytotoxic lipid end products (Leonarduzzi et al., 2000; Girotti, 2001; Elisia, 2005; Halliwell & Whiteman, 2004). The

cysteine and methionine residues of proteins are also highly susceptible to oxidation, and ROS induced disulfide formation can lead to structural and functional impairment in protein (Droge, 2002; Valko et al., 2007). Modification of DNA by ROS might affect gene expression, and therefore could have deleterious effects when uncontrolled (Winterbourn, 2008). The highly reactive nature of $\text{OH}\cdot$ potentiates its reaction with the purine and pyrimidine bases as well as the deoxyribose backbone of DNA which can ultimately lead to DNA damage (Halliwell & Whiteman, 2004).

2.1.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are considered the damaging agents in living organisms. ROS refers to both radical and non-radical oxygen containing molecules. Radical species such as alkoxyl ($\text{RO}\cdot$), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) peroxy, peroxy ($\text{RO}_2\cdot$), superoxide anion ($\text{O}_2^{\cdot-}$), nitric oxide ($\text{NO}\cdot$) and the hydroxyl ($\text{OH}\cdot$) radicals are collectively defined by unpaired electron (e^-), while non-radical species include hydrogen peroxide (H_2O_2), singlet oxygen ($^1\text{O}_2$) and peroxynitrite (ONOO^-) are similar in that they contain only paired e^- (Bonomini et al., 2008; Dooley et al., 1990; Lushchack, 2014; Ma et al., 1999; Rada et al., 2008; Radi, 2013; Terao & Niki, 1986). However, each ROS is characterized by its own biological and chemical properties (**Table 2.1**), which in turn determines the overall stability of the molecule and its biological and chemical interactions. Superoxide anion ($\text{O}_2^{\cdot-}$) is of particular interest of all the ROS since its formation facilitates generation of a number of other ROS. Therefore, fluctuations of $\text{O}_2^{\cdot-}$ can influence ROS levels in the cells.

There are a number of sources that contribute to ROS generation in the cells extrinsically and intrinsically. Extrinsic include UV and ionizing radiation that can liberate ROS (Lushchak, 2014; Winterbourn, 2008) leading to unintentional and uncontrolled generation of these species in the cell. While intrinsic source include oxidation of phytochemicals as well as auto-oxidation of compounds, exposure to transition metal can lead to formation of ROS (Siow et al., 2011; Winterbourn & Hampton, 2008). There are also enzymes and enzymatic systems that contribute to the production of these reactive species in the cells. They include nicotinamide adinine dinucleotide phosphate (NDPH), nitric oxide synthase (NOS), xanthine oxidase (X-OX), myeloperoxidase, lipoxygenase, and transition metals (Bonomini et al., 2008; Stocker & Keaney Jr, 2004).

Table 2.1 Chemical and biological properties of ROS

ROS	Membrane permeability	Chemical reactions	Biological reactions	References
$O_2^{\cdot-}$	No	$2O_2^{\cdot-} + 2H^+ \xrightarrow{Fe} H_2O_2 + O_2$ $O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + O_2 + OH^-$ $O_2^{\cdot-} + NO^{\cdot} \rightarrow OONO^-$	Thiols Transition metals Ascorbate	Bonomini et al., 2008; Winterbourn & Hampton, 2008
H_2O_2	Yes	$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe} OH^{\cdot} + O_2 + OH^-$	Thiols Transition metals Methionine	Rada et al., 2008
AAPH	Yes	$A-N=N-A \rightarrow (1-e) A-A + 2eA^{\cdot-} + N_2$ $A^{\cdot-} + O_2 \rightarrow AOO^{\cdot} \rightarrow \text{Attacks tissues}$	DNA Lipid (peroxidation)	Dooley et al., 1990; Ma et al., 1999; Terao & Niki, 1986
OH^{\cdot}	Not applicable		Very reactive and non-discriminatory DNA Carbohydrates Phenylalanine Lipids (peroxidation)	Valko et al., 2007
NO^{\cdot}	Yes	$NO^{\cdot} + O_2^{\cdot-} \rightarrow OONO^-$	protein (nitrosylation)	Radi, 2013; Valko et al., 2007
$ONOO^{\cdot}$	Yes	$OONO^{\cdot} \rightarrow OH^{\cdot} + NO_2^{\cdot}$	Thiols Ascorbate Methionine Tocopherol DNA protein (tyrosine nitration) lipids (peroxidation)	Pacher et al., 2007; Radi, 2013; Valko et al., 2007; Winterbourn & Hampton, 2008

Abbreviations: AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; A is $\text{HClHN}=\text{C}(\text{NH}_2)\text{C}(\text{CH}_3)_2$, e is the efficiency of free radical production; AOO^\cdot , peroxy radical; $\text{O}_2^{\cdot-}$, superoxide anion; H_2O_2 , Hydrogen peroxide; OH^\cdot , Hydroxyl radical; OONO^\cdot , peroxy nitrite; NO_2^\cdot , nitrogen dioxide radical

2.1.2 Antioxidants Defenses

The levels of ROS in the cell can be regulated by many substances. However, antioxidants are substances which are effective against oxidative damage when present in small quantities than the substances they protect (Liao et al., 1994). Antioxidants have been defined as, any substance that, when present at low concentration compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell & Gutteridge, 1995). To avoid accumulation of ROS, the cells are equipped with a broad range of enzymatic and non-enzymatic antioxidant defense mechanisms. All antioxidants share a common property in that they neutralize or minimize the reactivity of ROS by increasing their chemical stability (Hunt & Stocker, 1990). Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione (**Table 2.2**). Superoxide dismutase catalyzes the univalent reduction and oxidation of $O_2^{\cdot-}$ to H_2O_2 (Hamilton et al., 2004; McCord & Fridovich, 1969; Wassmann et al., 2004). Other enzymatic antioxidants including CAT, GPx, GR and glutathione function to decompose H_2O_2 (Hamilton et al., 2004; Nordberg & Arner, 2001; Wassmann et al., 2004). There are also non-enzymatic antioxidant molecules that can directly quench cellular ROS, and they include ascorbic acid (vitamin C) and α -tocopherol (vitamin E) (**Table 2.2**). Although these vitamins increase the stability of ROS by donating their own electrons, their resultant radical species are significantly less reactive (Winterbourn, 2008) and can be neutralized through various dismutation reactions (Parker et al., 1979; Sharma & Baettner, 1993).

Table 2.2 Major endogenous antioxidant defenses

Antioxidant	Type	Substrate	Reaction	Reference
SOD	Enzymatic	$O_2^{\cdot-}$	$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$	Hamilton et al., 2004 Wassmann et al., 2004
CAT	Enzymatic	H_2O_2	$2H_2O_2 \rightarrow O_2 + 2H_2O$	Nordberg & Arner, 2001 Wassmann et al., 2004
GPx	Enzymatic	H_2O_2	$2GSH + H_2O_2 \rightarrow 2H_2O + GSSG$	Nordberg & Arner, 2001 Wassmann et al., 2004
GR	Enzymatic	GSSG	$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$	Nordberg & Arner, 2001
Glutathione	Direct antioxidant (Hydrophilic)	$OH\cdot$ H_2O_2 $LOO\cdot$	H Transfer	Aquilano et al., 2014 Galano & Alvarez-Idaboy, 2011
Ascorbate	Direct antioxidant (Hydrophilic)	$O_2^{\cdot-}$ $OH\cdot$ $ONOO^-$	e^- transfer	Winterbourn & Hampton, 2008 Sharma & Buettner, 1993
Tocopherol	Direct antioxidant (lipophilic)	$OH\cdot$ $ONOO^-$ $ROO\cdot$ $LOO\cdot$	e^- transfer	Winterbourn & Hampton, 2008 Sharma & Buettner, 1993 Packer et al., 1979

Abbreviations: H_2O_2 , hydrogen peroxide; $OH\cdot$, hydroxyl radical; $LOO\cdot$, lipid peroxyl radical; $ONOO^-$, peroxynitrite; $NADP^+$, nicotinamide adenine dinucleotide phosphate; $NADPH$, reduced form of $NADP^+$; $ROO\cdot$, peroxy radicals; GSH , reduced glutathione; $GSSG$, oxidized glutathione; $O_2^{\cdot-}$, superoxide anion.

2.2 Oxidative Stress on Cellular Components

The two major sources of cellular ROS are complex NADH dehydrogenase ubiquinone-ubiquinol reductase and ubiquinol cytochrome c reductase, both part of the mitochondrial electron transport chain (Lenaz, 2001; Stowe & Camara, 2009; Turrens, 2003). The major products of ROS in mitochondria are in the form of superoxide and hydroperoxyl radicals (Stowe & Camara, 2009). Optimum levels of ROS play an important role in cell signaling pathways. However when ROS production increases and overwhelms the cellular antioxidant capacity, it can induce damage of DNA, proteins and lipids, and disrupt thiol redox circuits (Dizdaroglu et al., 2002; Jones, 2008). In the first instance, damage can lead to apoptosis or necrosis. Disruption of thiol redox circuits can lead to aberrant cell signaling and dysfunctional redox control (Jones, 2008).

2.2.1 Cytotoxicity Assay

Theoretically, oxidative stress measurement can be estimated directly by the amount of reactive species present in the cell. Oxidative stress can be measured indirectly by assessing cell viability (McKenna, 2009). The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay is a relatively inexpensive and widely used assay for routine cytotoxicity evaluation. The assay is based on the principle that metabolically active cells reduce yellow tetrazolium salt MTT to an insoluble purple formazan dye that can be quantified by spectrophotometric means (Mosmann, 1983). Mitochondrial succinate dehydrogenase in metabolically active cells transforms the yellow tetrazolium salt to the insoluble formazan dye, whereas non viable cells do not have this property. Trypan blue is another cell viability assay; it is based on the principle that living cells have the ability to

take up the dye while dead cells cannot (Stoddart, 2011). Other cell viability measurement methods include resazurin-based methods, the measurement of up-taking radioactive labeled H-thymidine into cellular DNA, and the reduction resazurin into the resorufin product which is pink and fluorescent (Stoddart, 2011; Yin et al., 2013). In this research, the MTT assay was used.

2.2.2 Cellular Antioxidant Activity Assay

Many antioxidant activity assays exist and most do not measure the *in vivo* activity accurately because they do not account for physiological conditions including pH, temperature, or antioxidant bioavailability. The cellular antioxidant activity (CAA) assay employing the cell-permeable fluorogenic probe dye 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is a cell-based assay for measuring antioxidant activity within a cell in a standard cell culture environment. This environment accounts for the temperature, pH, uptake, metabolism and efficacy of antioxidant in the whole cell (Martins et al., 1991; Rice-Evans et al., 1995). The CAA or DCFH-DA assay is the most commonly used method to detect cellular oxidative stress (Halliwell & Whiteman, 2004; Song et al., 2010; Wolfe & Liu, 2007; 2008; Wolfe et al., 2008). To measure CAA, cells are first cultured in a 96-well black fluorescence cell culture plate. The cells will then be incubated with DCFH-DA. When diffused into the cells, DCFH-DA is deacetylated by cellular esterase to a non-fluorescent substance DCFH. The DCFH will be oxidized to DCF (highly fluorescent) in the presence of ROS. The intensity of fluorescence is proportional to the level of ROS in

the cell. The addition of antioxidant prevents oxidation of DCF, therefore, reducing the fluorescent intensity (Wolfe & Liu, 2007).

2.2.3 Cell lines

Plant derived bioactive compounds have been applied in different cells from human organisms including macrophage cells, monocytic cells and cancer related cells, such as CaCo-2, HT-29, MCF 7, HepG2, H4IIE and prostate cancer cells (Chi et al., 2007; Ferguson et al., 2005; Hu et al., 2003; Kanski et al., 2002; Meyers et al., 2003; Nardini et al., 1995; Wolfe & Liu, 2007; Yang & Liu, 2009; Yuan et al., 2005). The release and absorption of bioactive compounds mostly occurs in the human intestinal system. The intestinal mucosa is the first site for absorption of bioactive compounds followed by the colon where absorption continues. Based on the metabolic pathways of bioactive compounds and their associated antioxidant compounds in the human body, the cell models adopted for investigation of bioactive antioxidants are normally from human gastrointestinal tract. In this thesis, Caco-2, HT-29 and FHs 74 Int were selected as they are widely used as cell models that resemble the human intestinal tract environment. Caco-2 cells are structurally and functionally similar to the intestinal epithelium and are regarded as a simple *in vitro* model for the study of drug transportation and absorption in human intestinal mucosa (Hilger et al., 1990). HT-29, a cell line with epithelial morphology, is regarded as an *in vitro* model for the study of drug transportation, absorption and secretion by intestinal cells (Cohen et al., 1999). Although it is genotypically similar to neonatal enterocytes, FHs 74 Int is a human fetal cell line which has been reported to show mature epithelial-like characteristics (Wagner et al., 1998).

2.3 Oxidative Stress on Diseases

Accumulation of ROS coupled with an increase in oxidative stress has been implicated in the pathogenesis of several diseases. The role of oxidative stress in vascular diseases, diabetes, renal ischemia, atherosclerosis, pulmonary pathological states, inflammatory diseases and cancer has been well established (Klaunig et al., 2004; 2010; Hayakawa & Kuzuya, 1990; Maritim et al., 2003; Stocker & Keaney Jr, 2004). In this thesis I will focus on atherosclerosis, the major source of morbidity and mortality worldwide. Current findings support the hypothesis that oxidative stress plays an important role in the initiation and progression of atherosclerotic cardiovascular disease (Hulthe & Fagerberg, 2002; Stocker & Keaney Jr, 2004).

2.3.1 Cardiovascular Diseases

According to the World Health Organization (WHO) fact sheet of cardiovascular diseases (WHO, 2015), cardiovascular disease (CVD) refers to a group of disorders of the heart and blood vessels, which include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism. CVD is the cause of more than 31% of deaths, not only in developed countries but WHO estimates that low- and middle-income countries are also disproportionately affected. It estimated that 14.1 million people died from CVDs in 2012 and of these deaths 7.4 million were due to coronary heart disease and 6.7 million were due to stroke. The number of mortalities from CVD is estimated to increase to almost 23.6 million people in 2030 (WHO, 2015). In Canada, CVD accounts for one-third of

deaths, more than any other illness and the prevalence is expected to increase in the next decade, predominantly because of increased sedentary lifestyle and an attendant increase of the prevalence of obesity and diabetes mellitus (Genest et al., 2009). CVD is a chronic disease mainly caused by interactions among genetic predisposition, health behavior and the environment. According to the Canadian Heart and Stroke Foundation (CHSF), most significant risk factors include age, sex, family history, tobacco smoking, physical activity, being overweight, diet, blood pressure and diabetes (The growing burden of heart disease and stroke, 2003). These risk factors fall into the modifiable and non-modifiable risk factors. Modifiable risk factors are conditions that can be altered by making certain lifestyle changes and they include tobacco smoking, alcohol abuse, physical inactivity, malnutrition, obesity, high blood pressure, high concentration of dietary fat and blood lipids and high glucose concentration (Buttar et al., 2005; The growing burden of heart disease and stroke, 2003). Non-modifiable risk factors include age, heredity or genetic makeup and type 1 diabetes (Buttar et al., 2005).

2.3.2 Atherosclerosis

Atherosclerosis is the primary underlying pathology of CVD and is characterized by the accumulation of cholesterol deposits in the macrophages in large- and medium-size arteries. This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge the vessel lumen and impede blood flow. The atherosclerosis process may last for decades until an atherosclerosis lesion develops (Hansson, 2005; Stocker & Keaney Jr, 2004).

Elevated levels of blood cholesterol, total and low density lipoprotein (LDL) cholesterol, are considered major risk factors for atherosclerosis (Chen et al., 2008; Glass & Witztum, 2001). Furthermore, epidemiological studies have reflected increasing support for the hypothesis of oxidative modification of atherosclerosis lesion development. LDL oxidations are a key early stage in the initiation of atherosclerosis and oxidative LDL (ox-LDL) and contribute to the progression of atherosclerosis event (Bonomini et al., 2008; Stocker & Keaney Jr, 2004; Young & McEneny, 2001; Vogiatzi et al., 2009). In the early stages of atherosclerosis, endothelial function is impaired and this is strongly correlated with several risk factors. Endothelial dysfunction predisposes to long term atherosclerotic lesion and has been proposed as an important diagnostic and prognostic factor for coronanry syndromes (Stocker & Keaney Jr, 2005). Free oxidative radical production is believed to induce endothelial dysfunction, an initiation step of atherogenesis. Oxidative stress leads to ox-LDL, whose uptake by macrophages is comparable to non-oxidized lipoprotein (Antoniades et al., 2007). Hypercholesterolemia stimulates the production of superoxide anion radicals (O_2^-) from the smooth muscle cells of vessels, an event that leads to increased ox-LDL. Furthermore, the reduction of endothelial-produced nitric oxide (NO) and O_2^- blunts normal endothelial dysfunction as a result of the decreased endothelial NO production. The increased production of ROS reduces the production and consequently the bioavailability of NO, leading to vasoconstriction, platelet aggregation and adhesion of neutrophils to the endothelium (Vepa et al., 1999). Oxidative modification has importance in the oxidative events and redox reactions in the development of vascular diseases. It also includes LDL as the central element that substantially impacts progression of

atherosclerosis. Therefore, lowering oxidative stress and LDL cholesterol may effectively attenuate the development of atherosclerosis.

2.3.3 Cholesterol

Cholesterol is a type of sterol and is a largely hydrophobic molecule. It consists of twenty-seven carbon atoms, forty-six hydrogen and one hydroxyl group (3 β -hydroxyl moiety). It is an unsaturated lipid containing two double bonds. It is an essential component of eukaryotic membranes. Cholesterol plays a crucial role in regulation of membrane fluidity. It increases membrane thickness, modulates the activity of various membrane proteins, and serves as precursor for steroid hormones and bile acids (Burger et al., 2000; Liscum & Underwood, 1995; Mouritsen & Zuckermann, 2004; Pucadyil & Chattopadhyay, 2006; Simons & Ikonen, 2000). It also transports substance through blood from the liver to the tissues (Hanukoglu, 1992). The cholesterol pool in our body comes from two sources, either being absorbed from the diet or synthesized in the body. Approximately 70% of whole body cholesterol is made by the liver and the other 30% comes from the diet. However, a high level of cholesterol in the blood is a significant risk factor for CVD. Some genetic disorders such as familial hypercholesterolemia can raise cholesterol levels (Fahed & Nemer, 2011; Kolovou et al., 2011). Therefore, it is recommended that cholesterol intake should not exceed 300 mg/day level for healthy people and less than 200 mg/day for people with cardiovascular conditions (American Heart Association Nutrition Committee, 2006). Because cholesterol is hydrophobic, it needs lipoprotein to be transported in the blood, of which the most common are chylomicrons, high-density lipoprotein (HDL), low-density lipoprotein (LDL) very-low high density lipoprotein (VLDL).

2.3.4 Chylomicrons

Chylomicrons (CMs) are a type of lipoprotein secreted by the small intestine cells. CMs are very large, spherical particles that consist of 85-92% triglycerides, 6-12% phospholipids, 1-3% cholesterol and 1-2% proteins (Hussain, 2000). Chylomicrons have the lowest protein to lipid ratio and hence have the lowest density of all the lipoproteins. Their main function is to transport fat and triglycerides from the intestinal lumen to the liver and other tissues (Hussain, 2000; Tomkin & Owens, 2011).

2.3.5 High-Density Lipoprotein

High-density lipoproteins (HDLs) are secreted by liver and intestinal cells. The nascent HDLs are disc-shaped, but they become spherical as they acquire free cholesterol from cell membranes and triacylglycerols from other lipoproteins. High-density lipoprotein plays the role of the primary extracellular acceptor. High-density lipoprotein transports cholesterol from the peripheral cells to the liver. The role of HDL is thought to be critical to the reverse cholesterol transport (RCT) (AbuMweis & Jones, 2008). Furthermore, HDLs inhibit oxidation of LDL, inhibit adhesion of monocyte to the endothelium, inhibit apoptosis of vascular endothelial and smooth muscle cells, activate platelets, and stimulate the endothelial secretion of vasoactive substances as well as smooth muscle cell proliferation. These functions related to cholesterol removal from the tissues underlie the inverse relationship between the plasma concentration of HDLs and the incidence of heart diseases (AbuMweis & Jones, 2008; Hersberger & von Eckardstein, 2003). Low HDL cholesterol is an important risk factor for coronary heart diseases (CHD) (Hersberger & von

Eckardstein, 2003). Healthy levels of HDL should be more than 1.0 mmol/L for males and more than 1.3 mmol/L for females (Health Canada, 2013).

2.3.6 Low-Density Lipoprotein and Very-Low Density Lipoprotein

Low-density lipoproteins in blood plasma and their immediate precursors are generally thought to be the major atherogenic lipoproteins (Stewart et al., 1997). The initial indications that LDLs are formed from VLDLs were obtained more than 25 years ago (Havel, 1984; Gitlin et al., 1958; Pierce, 1954), however the mechanism is unknown. Higher levels of LDL-cholesterol and VLDL-cholesterol tend to be associated with greater risks of CVD (Austin et al., 1988). Healthy levels of LDL should be less than 3.5 mmol/L (Health Canada, 2013).

2.3.7 Management of Cardiovascular Diseases

In order to circumvent CVD, patients with elevated cholesterol levels are treated with drugs including statins (Karalis et al., 2012). Another desirable therapeutic alternative is by modification of lifestyle such as dietary regimes, weight reduction and increased physical activity (Jenkins et al., 2005; Mannu et al., 2013). Examples of dietary advices are low contents of saturated fatty acids, a high intake of soluble fiber in the diet (Brown et al., 1999; Kaczmarczyk et al., 2012; Theuwissen & Mensink, 2008; Ripsin et al., 1992; Talati et al., 2009). Other agents reported to lower cholesterol levels are plant sterols (AbuMweis & Jones, 2008; Moghadasian & Frohlich, 1999; Rideout et al., 2012; Xu et al., 2008), soy protein (Anderson et al., 1995; Bakhit et al., 1994; Bakhtiary et al., 2012; Wofford et al., 2012; Teixeira et al., 2000), garlic powder (Isaacsohn et al., 1998; Mader, 1990; Zeng et al., 2012), whole grains (Jensen et al., 2004; Mellen et al., 1994; Steffens et al., 2003), almonds,

and viscous fiber (from oats, barley, psyllium) (Jenkins et al., 2005). Commercial products enriched in physiologically functional ingredients are now available. They are sometimes referred to as “functional foods”, i.e. foods that contribute to beneficial health effects and/or reduce the risk of disease, besides providing energy and essential nutrients (Sirtori et al., 2009). Cereal grains including corn are interesting food candidates in this respect, as they can lower the serum lipids in men with hypercholesterolemia (Shane & Walker, 1995). This is of great interest in prevention and control of atherosclerosis and diabetes.

2.3.8 Animal model for Atherosclerotic CVD

Mice model are the most commonly used animal models in disease investigations. Mice are easy to breed, of low cost maintenance, of well-known genetic background, and they have tissues that can be easily harvested, stored and processed for analysis (deLuna, 2008; Potteaux et al., 2007; Daugherty, 2002; Reardon & Getz, 2001; Breslow, 1996). However, small size and physiological characteristics may be considered as limiting factors. Mice do not develop significant atherosclerosis as a result of high levels of high-density lipoprotein (HDL) and low levels of low-density lipoprotein (LDL) and very-low density lipoprotein (VLDL) (Getz & Reardon, 2006; Zadelaar, 2007). Thus, most of the atherosclerotic mice models used in research are mainly dependent on disruption of normal lipoprotein regulation and metabolism through dietary or genetic manipulations, which allow extensive and mature lesion development. The mice models used previously to study atherosclerosis include low-density lipoprotein receptor knock-out (LDLr-KO) (Sanna et al., 1998), hepatic lipase-knockout (Homanic et al., 1995), human apolipoprotein B₁₀₀ expression (Greeve et al., 1993) and human cholesterol-ester transfer protein expression

(Föger et al., 1999). The LDLr-KO mice are one of the most extensively used dyslipidemia models of atherosclerosis (Kowala et al., 2000; Surendrian et al.; 2013; Sanan et al., 1998). They exhibit elevated levels of LDL and VLDL cholesterol when fed a high fat diet due to the absence of hepatic LDL receptors, which disables hepatic clearance of these circulating lipid particles (Ishibashi et al., 1993). When LDLr-KO mice were fed on regular chow, modest elevation (nearly 5 mmol/ L) of plasma cholesterol was observed when compared to 2 mmol/L in wild type C57BL/6 mice. When high fat cholesterol diet was fed, plasma cholesterol level of LDLr-KO mice was elevated (>25 mmol/ L) and atherosclerosis developed rapidly (Knowles & Maeda, 2000). The plasma lipoproteins profile is similar to that in humans, and in part mimics familial hypercholesterolemia conditions (Getz & Reardon, 2006). LDLr-KO mice generally have lower cholesterol levels and are susceptible to develop atherosclerotic lesions compared to apolipoprotein E deficient mice (deLuna, 2008; Potteaux et al., 2007; Daugherty, 2002; Reardon & Getz, 2001; Breslow, 1996). This mild or modest dyslipidemic profile of LDLr-KO mice is desirable for studies investigating the effects of lipid profile changes on atherosclerotic lesions (Zadelaar et al., 2007).

2.4 Whole Grain Composition

Whole grain is a term that abbreviates whole cereal grain. Cereals are species of the grass family that have been domesticated for food and feed production including wheat, maize (also known elsewhere as corn), rye, oats, rice, sorghum, millet and a range of other miscellaneous grasses (Jacobs Jr, & Gallaher, 2004). Wheat and corn are the most

commonly consumed whole grains in North America. The cereal grain have different fractions including starchy endosperm, bran (includes aleurone, hyaline layer, testa, inner and outer pericarp) and germ (Hoseney, 1994). **Figure 2.1** shows the wheat fractions (bran, germ and endosperm) and their bioactive compounds are also summarized in **Table 2.3, 2.4** and **2.5**. The starchy endosperm is the largest component of the grain making 80-85% of the seed, then bran 12-18%, and germ 2-12% (Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Bradolini & Hidalgo, 2012). The starchy endosperm fraction occupies the bulk of cereal grain consisting of nutrients needed during germination. It is mostly composed of starch and protein (Pomeranz, 1988). The second fraction, bran, is a composite multilayer consisting of outer pericarp, inner pericarp, testa, hyaline layer and aleurone layer. The pericarps are composed of empty cells with numerous cross links between the polymer chains (Fincher & Stone, 1986; Pomeranz, 1988). Pericarp makes 3-5% of the kernel. The testa makes ~1% of the kernel and it is rich in lignin and characterized by lipidic compounds which include alkylresorcinol present on the surface of the tissue (Evers & Reed, 1988; Landberg et al., 2008). The hyaline layer makes more than 90% of arabinoxylans which are poorly crossed linked (Barron et al., 2007). The aleurone layer is the outer part of the starchy endosperm. It is attached to the hyaline layer. The aleurone layer makes 5-8% of the kernel and 45-50% of the bran fraction. The aleurone layer is nutritionally rich (Pomeranz, 1988). The third fraction, germ, is highly concentrated with nutrients such as protein, lipids, sugar, minerals and vitamin E, B-group vitamins, carotenoids, flavonoids, phytosterols and policosanols (Pomeranz, 1988; Pietrzak & Collins, 1996; Nyströmet et al., 2007; Eisenmenger & Dunford, 2008; Hidalgo & Brandolini, 2008; Ndolo & Beta, 2013).

Whole grain cereals can protect against obesity, diabetes (Lui et al., 2000; Meyer et al., 2000), cardiovascular diseases (Lui et al., 2000; Kelly et al., 2007) and cancers (Schatzkin et al., 2007) due to the antioxidant properties of numerous compounds, especially those in the bran and germ. Moreover, whole grain is a rich source of methyl donors and lipotropes that may be involved in lipid metabolism and DNA methylation (Fardet, 2010). Other components of whole grains may also protect against oxidative stress related diseases, including vitamins, trace minerals, phytates, phenolic acids, lignans, and phytoestrogens (Slavin, 1999; Webb & McCullough, 2005; Cotterchio et al., 2006).

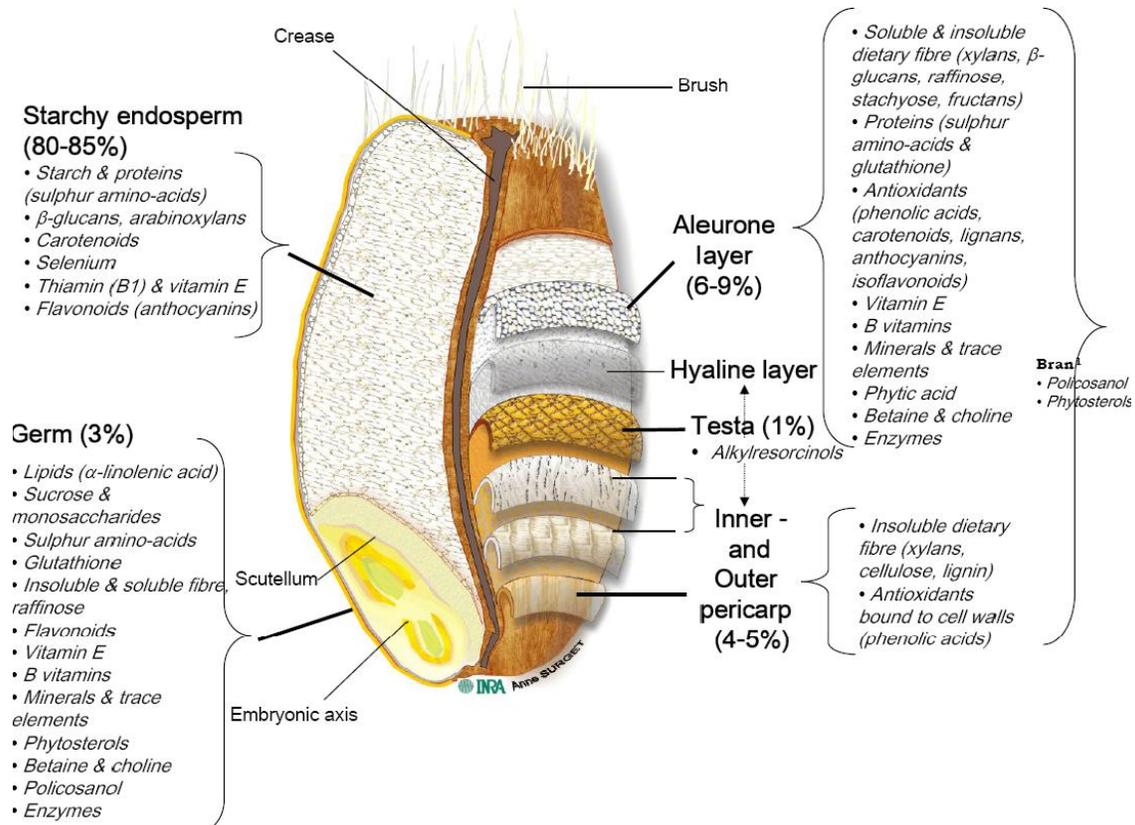


Figure 2.1 The structure of whole wheat with three wheat fractions (bran, germ and endosperm) and their main bioactive compounds (Fardet, 2010; copyright license for use obtained from Cambridge University Press, Nutrition Research Reviews).

2.4.1 The Aleurone Fraction within Whole Grain

The aleurone layer is the innermost layer of the bran and the outer part of the starchy endosperm. It is considered as part of the bran as it is removed from the endosperm with the grain outer layer during milling (Fincher & Stone, 1986; Pomeranz, 1988). The aleurone consists of 1 to 3 layers of thick-walled cells with dense contents and prominent nuclei. The number of layers is a characteristic of cereal species; wheat, rye, oats, corn and sorghum have 1 layer and barley and rice have 3 layers (Stone, 1985; Evers et al., 1999). The aleurone cell wall contains 29% β -glucans, few proteins, and 65% arabinoxylan with a low

arabinose to xylose ratio, and high amounts of esterified ferulic acids monomer (Bacic & Stones, 1981; Rhodes et al., 2002a; 2002b; Saulnier et al., 2007).

The aleurone layer is particularly rich in nutrients (**Table 2.3**). It is mostly composed of dietary fibers (44-50 g/100 g), proteins (~ 15-30%), phenolic compounds (280-7980 µg/g grain), vitamin E (1.2-2 mg/100 g), vitamin B (0.2-90.2 mg/100 g), minerals (8.3-2540 mg/100 g), phytic acids (2-6 g/100 g), lipids (2.8-35.3 mg/100 g), plant sterols (0.13-9.5 mg/100 g), lignins (7-90 mg/100 g), anthocyanins (0.9-48.0 mg/100 g), isoflavonoids (3-10.5 µg/100 g), betaine (230-1553 mg/100 g) and choline (74-210 mg/100 g) (Pomeranz, 1988; Antoine et al., 2002; Amrein et al., 2003; Buri et al., 2004; Surget & Barron, 2005; Fardet, 2010; Brouns et al., 2012; Ndolo & Beta, 2014). Of the total mineral content, 40-60% are found in the aleurone layer reflecting it as a rich source of minerals (Pomeranz, 1988). Furthermore, Ndolo & Beta have reported significant enhanced carotenoid levels in the aleurone of wheat, oats and corn (Ndolo & Beta, 2013). The carotenoid level ranges are 0.15-13.73 mg/100 g (Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Ndolo & Beta, 2013).

Several studies have suggested that intake of fermented wheat aleurone reduces the risk of oxidative stress related diseases (McIntosh et al., 2001; Borowicki, 2010; 2010a; 2010b; Stein et al., 2010; 2011; Price et al., 2012). However, it is unknown whether this effect is only due to fermented wheat aleurone's probiotic/prebiotic nature or whether the aleurone's physicochemical properties also play an important role. Furthermore, Price et al. have indicated wheat aleurone as a marker of antioxidant status (Price et al., 2012). Therefore, it is necessary to clearly understand the mechanism underlying reduced oxidative stress risk through exploring the antioxidant potential of the aleurone fraction.

Table 2.3 Aleurone components

Component	Amount	Potential health protection	References
Carotenoids	0.15-13.7 mg/kg	Visual function protection, stroke, atherosclerosis, lung cancer, skin health, cardiovascular diseases, and skin health	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010; Ndolo & Beta, 2013;
- <i>Wheat</i>	0.37-10.0 mg/kg		
- <i>Barley</i>	0.15-3.28 mg/kg		
- <i>Corn</i>	2.80-13.7 mg/kg	Cancer, cardiovascular disease, neurodegenerative disorder, type 2 diabetes, skin health; anti-aging, hepatoprotective, pulmonary protective, hypertension and obesity	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Antoine et al., 2002; Buri et al., 2004; Surget & Barron, 2005; Fardet, 2010;
Total phenolic acids (<i>Wheat</i>)	280-7980 µg/g grain		
Lignin (<i>Wheat</i>)	2.2-9.0 g/ 100g	Colon cancer, large bowel health, type 2 diabetes, cardiovascular disease	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Glitsø et al., 2000; Fardet, 2010
Vitamin E (<i>Wheat</i>)	1.2-9.5 mg/100 g	Cancer, type 2 diabetes, neurodegeneration, osteoporosis, obesity.	Pomeranz, 1988, Duffus & Cochrane, 1993; Watson, 2003; McCue & Shetty, 2004; Miadokova, 2009; Fardet, 2010

B vitamins (<i>Wheat</i>)		Heart health, mental and brain health, colorectal cancer, asthma attacks, microcytic anaemia, occlusive arterial disease and seborrhoeic dermatitis	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010
B1	0.51-0.80 mg/100 g		
B2	0.21-0.80 mg/100 g		
B3	13.6-36.0 mg/100 g		
B5			
B6	2.2-4.1 mg/100 g		
B8	0.7-1.31 mg/100 g		
B9	0.04-0.2 mg/100 g		
	0.09-0.37 mg/100 g		
Betaine (<i>Wheat</i>)	230-1553 mg/100 g	Cardiovascular diseases, liver and kidney health, colorectal cancer, type 2 diabetes, metabolic syndrome, liver dysfunction	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Graham et al., 2009; Fardet, 2010
Choline (<i>Wheat</i>)	74-210 mg/100 g	Brain development and normal learning and memory functions, weight regulation, fetal development (for example, neural tube), liver dysfunctions (for example, fatty liver), cancer, cardiovascular diseases	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Graham et al., 2009; Fardet, 2010

2.4.2 The Germ Fraction within Whole Grain

The germ is the reproductive part that germinates to grow into a plant. It is composed of the embryo and scutellum. The scutellum is the storage organ of the germ. It consists of four tissues, epithelium, parenchyma, epidermis and provascular tissue (Watson, 2003). The germ stores nutrients and hormones, which are mobilized by enzymes elaborated during the initial stage of germination (Logan et al., 2001).

The barley and wheat germ (embryonic axis and scutellum) represents about 2-4% of total seed weight (Pomeranz, 1988; Duffus & Cochrane, 1993; Bradolini & Hidalgo, 2012), while corn germ represents 10-12% of total seed weight (Watson, 2003). The germ contains about 10-15% lipids (Dubois et al., 1960; Barnes, 1983; Pomeranz, 1988; Posner & Li, 1991), 17-35% proteins (Posner & Li, 1991; Eisenmenger & Dunford, 2008), 0.6-16.0 g/100 g sugars, monosaccharide and sucrose, (Dubois et al., 1960; Pomeranz, 1988), 10-24.7 g/100 g (1.5-4.5%) fiber (Cara et al., 1992; Panfili et al., 2003) and ~4% minerals (Posner & Li, 1991), as well as significant quantities of bioactive compounds such as tocopherols, 23.7-740 mg/kg dry matter, (Barnes 1982; Panfili et al., 2003; Hidalgo & Brandolini 2008), phytosterols, 24-50 mg/kg, (Nyströmet et al., 2007), policosanols, ~10 mg/kg, (Irmak et al., 2006), betaine, 69.2-1395 mg/100 g; choline 152-330 mg/100 g (Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Zeisel et al., 2003; Brandolini & Hidalgo 2012; Fardet, 2010) carotenoid, 0.26-13.8 mg/kg, (Hidalgo & Brandolini 2008; Ndolo & Beta, 2013; **Table 2.4**), thiamin, 0.8-23 mg/kg, (Barnes 1982) and riboflavin, 0.5-10 mg/kg, (Barnes 1982).

Zalatnai et al. (2010) demonstrated that the wheat germ extract prevents colonic cancer in animal studies. However, there is limited literature to support the antioxidative

potential of the germ fraction. Therefore, it is necessary to clearly understand the mechanism underlying reduced oxidative stress risk through exploring antioxidant potential of the germ fraction.

Table 2.4 Germ Components

Component	Amount	Potential health protection	References
Carotenoids	0.26-5.90 mg/kg	Visual function protection, stroke, atherosclerosis, lung cancer, skin health, cardiovascular diseases, and skin health	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Hidalgo & Brandolini 2008; Ndolo & Beta, 2013; Fardet, 2010
- <i>Wheat</i>	0.58-2.82 mg/kg		
- <i>Barley</i>	2.52-5.90 mg/kg		
- <i>Corn</i>	0.26-2.00 mg/kg		
Tocopherols (<i>Wheat</i>)	23.7-740 mg/100 g	Cancers, cardiovascular diseases, type 2 diabetes	Barnes 1982; Pomeranz, 1988; Duffus & Cochrane, 1993; Panfili et al., 2003; Watson, 2003; Hidalgo & Brandolini 2008; Fardet, 2010
Lignin (<i>Wheat</i>)	0.49-1.6 mg/100 g	Colon cancer, large bowel health, type 2 diabetes, cardiovascular disease	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010
Thiamin (<i>Wheat</i>)	0.8-23 mg/100 g	Mental (for example, Korsakoff syndrome and dry Beri Beri), neuronal (for example, neuropathy) and heart (for example, wet Beri Beri) health	Barnes 1982; Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010
Phytosterols (<i>Wheat</i>)	24-50 mg/kg	Cardiovascular diseases, colon, breast and benign prostate cancer, type 2 diabetes.	Barnes 1982; Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010
Riboflavin (<i>Wheat</i>)	0.5-10 mg/100g	Cardiovascular diseases, cancers, vision (for example, corneal opacity and cataract), mental health (for example, neurodegeneration and peripheral neuropathy), skin health	Barnes 1982; Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010

Betaine (<i>Wheat</i>)	69.2-1395 mg/100 g	Cardiovascular diseases, liver and kidney health, colorectal cancer, type 2 diabetes, metabolic syndrome, liver dysfunction	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Zeisel et al., 2003; Brandolini & Hidalgo 2012; Fardet, 2010
Choline (<i>Wheat</i>)	152-330 mg/ 100 g	Brain development and normal learning and memory functions, weight regulation, fetal development (for example, neural tube), liver dysfunctions (for example, fatty liver), cancer, cardiovascular diseases	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Zeisel et al., 2003; Brandolini & Hidalgo 2012; Fardet, 2010

2.4.3 The Endosperm Fraction within Whole Grain

The endosperm is the fraction of importance to human nutrition. The starchy endosperm fraction occupies the bulk of the cereal grain consisting of nutrients needed during germination. The principal contents of the endosperm are starch (75-89%) and protein (7-15%) with very little fiber and carotenoids (except for yellow corn) (Pomeranz, 1988; Surget & Barron, 2005; Fardet, 2010; Brouns et al., 2012; Ndolo & Beta, 2013). The cell wall of wheat starch endosperm comprises of 70% arabinoxylan, 20% 1,3;1,4- β -D-glucan, 7% glucomannan and 4% cellulose (Mares & Stone, 1973a; Bacic & Stone, 1980; Pellny et al., 2012). Wheat starchy endosperm cell wall composition is similar to that of barley. The rice starchy endosperm cell wall composition differs in having 40% glucuronoarabinoxylan and arabinoxylan, 10% pectin, 30% cellulose and 15% glucomannan (Fincher & Stone, 1986). The endosperm of barley, corn, oats and wheat consists of 0.88-32.0 mg/kg of carotenoid. Corn is known to be higher in carotenoid levels

and barley has the lower levels. The carotenoid levels in corn endosperm range from 2-32 mg/kg; wheat 1.5-10 mg/kg; oats 0.5-1.2 mg/kg and barley 0.8-1.5 mg/kg (Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Ndolo & Beta, 2013). The betaine and choline contents are uncertain, with available data indicating 47.0-163.0 mg/100g of choline (Pomeranz, 1988).

Table 2.5 Endosperm components

Component	Amount	Potential health protection	References
Carotenoids	0.04-13.5 mg/kg	Visual function protection, stroke, atherosclerosis, lung cancer, skin health, cardiovascular diseases, and skin health	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Ndolo & Beta, 2013; Fardet, 2010
<i>Wheat</i>	0.04-2.27 mg/kg		
- <i>Barley</i>	0.45-1.12 mg/kg		
- <i>Corn</i>	3.40-13.5 mg/kg		
Phytic acid (<i>Wheat</i>)	24.2-343.0 mg/100 g	Various cancers (for example, colon and breast cancers), type 2 diabetes, cardiovascular diseases, kidney health (renal stone development), Hypercalciuria, acute Pb poisoning, dental caries	Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003;Fardet, 2010
Minerals (mg/100 g)- <i>Wheat</i>			
Copper (Cu)	1.6-3.7	Brain and mental health, bone, tendon and cartilage health, cardiovascular health, cancers	Pomeranz, 1988, Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010
Zinc (Zn)	3.4-23.0	Immunoprotection, brain and mental health, atherosclerosis, cancers (for example, oesophagus), skeletal growth and maturation, olfaction (anosmia), type 2 diabetes, weight regulation (for example, anorexia)	Pomeranz, 1988, Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010
Iron (Fe)	3.5-9.1	Mental health, physical health, bone health	Pomeranz, 1988, Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010
Betaine	-	Cardiovascular diseases, liver and kidney health, colorectal cancer, type 2 diabetes, metabolic syndrome, liver dysfunction	Pomeranz, 1988, Duffus & Cochrane, 1993; Watson, 2003;Fardet, 2010
Choline (<i>Wheat</i>)	47.0-163.0 mg/ 100 g	Brain development and normal learning and memory functions, weight regulation, fetal development, liver dysfunctions, cancer, cardiovascular diseases	Pomeranz, 1988, Duffus & Cochrane, 1993; Watson, 2003; Fardet, 2010

2.4.4 Health Benefits of Whole Grains

Several epidemiological studies have consistently found that intake of whole grain is inversely associated with cancer, cardiovascular diseases (CVD) diabetes and other chronic disorders (de Munter et al., 2007; Fardet, 2010; Jacobs & Gallaher, 2004; ; Kelly at al., 2007; Liese et al., 2003; Lui et al., 2000; Meyer et al., 2000; Sahyoun et al., 2006; Steffen et al., 2003). A few studies have suggested that the bran not the endosperm or germ of whole grains is the major component for disease prevention (de Munter et al., 2007; Holzmeister, 2002; Jensen et al., 2004). Several components concentrated in the bran may work synergistically to bring about these disease prevention benefits (Jensen et al., 2004; Liu, 2004).

Among the phytochemicals in cereal grains, carotenoids are available in the aleurone, germ and endosperm fractions (**Table 2.3-2.5**) and little is known about their antioxidant potential. Cereal grain fractions are rich in carotenoids (0.88-32.0 mg/kg; Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Ndolo & Beta, 2013). Carotenoid quenches singlet oxygen, neutralizing photosensitisers and inhibiting lipid peroxidation. Therefore, carotenoids possess antioxidant potential.

2.4.5 Carotenoids

Carotenoids are natural pigments responsible for the yellow, orange and red colors of fruits, vegetables and grains. They form minor components of cereals (Irakli et al., 2011), with some grains containing higher and other lower carotenoid content as compared to fruits and vegetables (Abdel-Aal et al., 2002; Humphries & Khachik, 2003). Lutein and zeaxanthin are the main carotenoids in cereal grains (Hentschel et al., 2002; Panfili et al.,

2004). Lutein is dominant in oats, barley, spelt and durum wheat, while zeaxanthin is dominant in corn (Panfili et al., 2004).

Carotenoids form part of the natural antioxidant system in seeds (Howitt & Pogson, 2006). They also act as radical scavengers and singlet oxygen quenchers ($^1\text{O}_2$) (Leenhardt et al., 2006; Fiedor et al., 2005; Stratton et al., 1993). Moreover, they effectively scavenge ROS and other free radicals of different origins (Edge & Truscott 2010; Feidor et al., 2012; Galano et al., 2010; Martin et al., 1999; Mortensen et al 1997; Yamauchi et al., 1993), delivering protection against oxidative damage to photosynthetic and non-photosynthetic organisms at all levels of complexity. Generally, there are three major accepted types of reactions of free radical scavenging by carotenoids: (i) electron transfer between the free radical (R^\cdot) and carotenoids, resulting in the formation of a carotenoid cation ($\text{Crt}^{+\cdot}$) or carotenoid anion ($\text{Crt}^{\cdot-}$); (ii) radical adduct formation (R-Crt^\cdot), and (iii) hydrogen atom transfer leading to a neutral carotenoid radical (Crt^\cdot) (Edge & Truscott 2010; El-Agamey et al., 2004). The formed carotenoid radical products can undergo further transformation, producing secondary carotenoid derivatives of different reactivity. Previously, epidemiological studies have indicated that carotenoid-rich foods reduce the risks of degenerative diseases, such as cancer, cardiovascular diseases and age related macular degeneration (AMD) and also maintain skin health (Rice-Evans et al., 1997; Burkhardt & Boehm, 2007; Robertset al., 2009). These studies are on the health benefits of carotenoids in fruits and vegetables, but very limited with respect to cereals and cereal grain fractions, and therefore studies which determine the carotenoids antioxidant potential of cereals or cereal grains fractions are necessary. Undoubtedly, investigations aimed at antioxidant properties of carotenoids of cereal grain fractions will be beneficial and they will aid in the

selection of cereal grain fractions for processing and development of functional food and food ingredients.

2.5 Antioxidant Properties of Diverse Cereal Grains

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Epidemiological and experimental studies have suggested that diet plays a crucial role in prevention of chronic diseases such as heart disease, cancer, diabetes, and Alzheimer's disease (Temple, 2000; Willet, 1994). Consumption of cereal grains has gained popularity with whole grain products being regarded as "healthy foods" because of their potential protection against life-style and diet related disorders such as obesity, diabetes (Fardet et al., 2008; Lui et al., 2000; Meyer et al., 2000), cardiovascular diseases (Fardet et al., 2008; Kelly et al., 2007) and cancers (Fardet et al., 2008; Schatzkin et al., 2007). This effect is thought to be partly due to their phytochemicals that combat oxidative stress. Overproduction of oxidants can cause oxidative damage to large biomolecules such as lipids, DNA and proteins resulting in increased risk for cancer, cardiovascular and other diseases (Temple, 2000; Ames et al., 1993; Wagner et al., 1992). Dietary antioxidants may reduce such oxidative damages to biomolecules through several mechanisms (Duthie et al., 1996; Frag et al., 1991).

The importance and health benefits of regular cereal grain consumption in the prevention of chronic diseases are a focus for many research laboratories. Current research

data suggest that whole-grains contain more antioxidant phytochemicals than was previously reported (Liu, 2007). This section discusses research data pertaining to antioxidant activities of cereal grains produced by *in vitro* and *in vivo* studies. Such information has been summarized here for a better understanding of antioxidant properties of commonly consumed cereal grains (wheat, corn, rice, barley, sorghum, rye, oats and millet).

2.5.1 Antioxidants in Cereal Grains

Cereal grains have a long history as major sources of staple foods worldwide. Whole grain cereals are good sources of phenolic compounds which include derivatives of benzoic and cinnamic acids, anthocyanidins, quinines, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds (Bellido & Beta, 2009; Jang & Xu, 2009; Hosseinian et al., 2008; Lloyd et al., 2000; Shahidi & Naczk, 1995; Thompson, 1994). Grains contain tocotrienols and tocopherols (Thompson, 1994) and oryzanols (Lloyd et al., 2000). This wide range of phytochemicals has been recognized to support overall health through their antioxidant potential. The protective function of antioxidants in the body is through balanced oxidative stress free radicals. Cereal grains antioxidants are thought to act as direct free radical scavengers, cofactors of antioxidant enzymes or as indirect antioxidants (Fardet et al., 2008). Free radical compounds result from normal metabolic activities as well as from the diet and environment. Excess amounts of these reactive substances can cause oxidative damage to biomolecules such as lipids, protein and DNA (Temple, 2000) resulting in an increased risk of chronic disease and contributing to general inflammatory response and tissue damage (Slavin, 2003; Young & Woodside, 2001).

Reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide, peroxy radical, hydroxyl radical, singlet oxygen and peroxynitrite are produced during cellular metabolic processes (Sánchez-Moreno, 2002). Several studies suggested a strong association between elevated levels of these molecules and the pathogenesis of several chronic diseases through the process of oxidative stress (Leavy, 2014; Nogueira & Hay, 2013; Sugamura & Keaney Jr, 2011). ROS are involved in a variety of physiological and pathological processes, including cell signaling transduction, cell proliferation, differentiation, apoptosis, as well as ischemia-reperfusion, inflammation and many neurodegenerative disorders (Ames et al., 1993; Bland, 1995). The process of ROS generation/production occurs when there is an imbalance between pro-oxidants and antioxidants leading to oxidative stress (Qingming et al., 2010). The process of oxidation in cells is regulated by antioxidants, which delay or prevent cellular damage. Antioxidant protection is normally achieved through a balance between pro-oxidants and endogenous and/or dietary antioxidants (Sobha & Andallu, 2013; Hancock et al., 2001).

The dietary antioxidants (minerals, trace elements, vitamins, carotenoids, polyphenols, alkylresorcinols, betaine, choline, sulphur amino acids, phytic acids, lignans and avenanthramides) of whole grain cereals are primarily located in the bran and germ fractions (Fardet, 2010). Different antioxidant mechanisms have been attributed to cereal grain phytochemicals. These include prevention of oxidation of polyunsaturated lipids (vitamin E), reduction of the concentration of plasma homocysteine (vitamin B9, betaine, choline), acting as cofactor of antioxidant enzymes superoxide dismutase, glutathione peroxidase and thioredoxine (Zn, Fe, Se, Cu, Mn) or stabilization and delocalization of unpaired electron (vitamin E, polyphenols, alkylresorcinols). Phenolic acids (*p*-coumaric

acid, ferulic, syringic, sinapic and vanillic) are also believed to chelate transition metals as well as activate or repress particular genes, while sulphur-containing amino acids (cysteine and methionine) contribute to the synthesis of a major endogenous antioxidant glutathione (Cukelj et al., 2010; Fardet et al., 2008). Whole cereal grain antioxidants are water and fat soluble, providing protection through the entire digestive tract (Slavin, 2003).

2.5.2 *In Vitro* Cereal Grain Antioxidant Activity

The antioxidant capacity of different cereals is evaluated based on two chemical antioxidant assays, namely electron transfer (ET) and hydrogen atom transfer (HAT) methods. The ET method is characterized by change in color during the reduction of oxidants. HAT involves competition between the antioxidant and substrate (probe) for free radicals (Huang et al., 2005). Examples of ET assays include trolox equivalent antioxidant capacity (TEAC), the ferric reducing ability of plasma (FRAP) assay, copper reduction assay (CUPRAC), and 2,2,-diphenyl-1-picrylhydrazyl (DPPH) assay. The HAT assay includes the crocin bleaching assay, oxygen radical absorbance capacity (ORAC) and total peroxy radical trapping antioxidant parameter (TRAP) (Huang et al., 2005).

2.5.2.1 Wheat

Yu et al. (2013) investigated the antioxidant properties of refined and whole wheat flour and their resultant bread. Their data showed that whole wheat flour and bread were superior to refined flour and bread in regard to their *in vitro* antioxidant properties. Žilić et al. (2012) studied the distribution of phenolic compounds and yellow pigments in wheat grains and their relation to the total antioxidant capacity of bran and debranned flour. The

color intensity of yellow pigments, the activity of lipoxygenase (LOX) and peroxidase (POX) enzymes were also measured. Their results showed that bran fraction contains significantly high concentrations of phenolic acids, flavonoids and yellow pigments. The LOX activity was concentrated in endosperm and embryo, while the POX activity was mostly concentrated in the bran fraction. Therefore, their results suggest that the bran fraction of wheat would potentially provide natural antioxidants. Liu et al. (2010) used the DPPH assay to investigate antioxidant activities of various colored wheat grains and their phenolic compounds. The results showed that Charcoal purple wheat had the highest antioxidant activity (up to 6899 $\mu\text{mol}/100\text{ g}$) followed by red Fife wheat, yellow Luteus wheat while white AC vista wheat had the lowest antioxidant activity. The antioxidant activity was positively correlated to phenolic contents in these grains. The major phenolic composition identified in wheat grains consisted of phenolic acids, flavonoids, and anthocyanins. Ferulic acid was reported as the most dominant phenolic acid (74-87 mg/100g). Vanillic, *p*-coumaric, and sinapic acids were found in moderate levels (about 2 mg/100g), whereas caffeic acid was present in the least amount (< 1 mg/100g). Purple wheat, compared with colored wheat grains, was distinguished by higher content of vanillic acid (> 2.6 mg/100g) and ferulic acid (> 81 mg/100g). The flavonoids ranged from 22-103 mg/100g in purple wheat, 13 mg/100g and 11 mg/100g for yellow and red wheats respectively, and 9.6 mg/100g for white wheat. Anthocyanin contents were only present in purple wheats ranging from 2.5 to 23.5 mg/100g. Verma et al. (2008) measured the free, bound, and total phenolic contents and antioxidant activities in the bran of 51 wheat cultivars belonging to eight Western Canadian spring wheat market classes grown in replicated trials at Saskatoon, Saskatchewan, Canada. The free phenolic content ranged

from 854 ± 265 to 1754 ± 240 $\mu\text{g/g}$ of bran gallic acid equivalent (GAE). Saponification followed by a liquid-liquid solvent extraction released bound phenols ranged from 2305 ± 438 to 5386 ± 928 $\mu\text{g/g}$ of bran GAE, contributing 66-82% of the total wheat bran phenolic content. Total phenolic acids ranged from 3406 ± 32 to 6702 ± 20 $\mu\text{g/g}$ of bran GAE, with an average of 5197 ± 804 $\mu\text{g/g}$ of bran GAE. Antioxidant activity was determined as % discoloration = $(1 - [(\text{absorbance of sample at 30 min}) / (\text{absorbance of control at time 0})])$ and ranged from 11.9 ± 2.6 to $20.1\pm 0.5\%$, while the overall average was $15.6\pm 2.2\%$. Based on varietal means, antioxidant activity correlated with free, bound and total phenolic contents (TPC; $r = 0.8$, $P < 0.05$). Esposito et al. (2005) investigated the antioxidant activity of durum wheat products with the ABTS 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) method. The data suggest that the antioxidants of durum wheat differ in three bran layers and increases in fractions with reduced granulometry. Their antioxidant activities were well comparable to known antioxidants of common food stuffs such as red wine, tomato or peach, likely due to the presence of fiber-bound phenol compounds. Martinez-Tome et al. (2004) evaluated the antioxidant capacity of cereal brans using the TEAC method. The data showed weaker oat bran antioxidant activity as compared to different wheat bran products. Phenolic acid composition, tocopherol contents, carotenoids profile, and total phenolic contents were examined for determination of the phytochemical composition of wheat bran and related antioxidant activity (Zhou et al., 2004). The data suggest that wheat and wheat bran from different countries may differ in their antioxidant potentials.

Anson et al. (2010) investigated antioxidant and anti-inflammatory capacity of bioaccessible compounds from wheat fractions after gastrointestinal digestion. In their

study, the bioaccessible compounds from aleurone, bran and flour were obtained from a dynamic *in vitro* model of the upper gastrointestinal tract. They found that bioaccessible compounds from aleurone had the highest antioxidant capacity and provided prolonged anti-inflammatory effect as compared to bran and flour. Bhanja et al. (2009) investigated the TPC and antioxidant potential of ethanolic extract of non-fermented and fermented wheat grains with two filamentous fungi (*Aspergillus oryzae* and *Aspergillus awamori* nakazawa). They found that total phenolic content and antioxidant property of wheat (54% ethanol extraction) was drastically increased when fermented with *Aspergillus oryzae* and *Aspergillus awamori* nakazawa. Moore et al. (2007) evaluated the potential of solid-state yeast fermentation ability to improve the extractable antioxidant properties of wheat. The results demonstrated that solid state yeast treatment significantly increased releasable antioxidant properties ranging from 28 to 65, 0 to 20, 23 to 19, 0 to 25, 50 to 100 and 3 to 333% for scavenging capacity against ORAC, ABTS, DPPH TPC, and phenolic acids, respectively.

2.5.2.2 Rice

Shao et al. (2014) investigated total phenolic contents, antioxidant capacity by using DPPH and ORAC assays, and phenolic acids in fractions of white (unpolished), red, and black rice after flowering and maturity, as well as the distribution of phenolic acids and anthocyanins in endosperm, embryo, and bran of white, red, and black rice. Their study showed total phenolic contents (TPC) of white rice (15-33 mg/100 g) and red rice (67-422 mg/100 g) were higher at 1-week development than at late stage, and black rice (57-82

mg/100 g) had higher antioxidant activity at maturity (Shao et al., 2014). Shao et al (2014) also demonstrated that rice bran had higher TPC (7.4 mg GAE/g average) and contributed to 60%, 86% and 84% of phenolics in white, red and black rice whole grain, respectively. The average TPC of the embryo and endosperm were 2.8 and 0.1 mg GAE/g accounting for 17% and 23%, 4% and 10% and 7% and 9% in white, red and black rice, respectively. The antioxidant capacity followed similar trend to TPC. Deng et al. (2012) used spectrophotometric methods to investigate antioxidant properties and lipophilic and hydrophilic phenolic contents in 24 cereal grains from China. Their results showed that cereals have diverse antioxidant capacities. Phenolic compounds gallic acid, kaempferol, quercetin, galangin and cyanidin 3-glucoside were widely found in the cereals tested. The pigmented cereals, such as black rice, red rice, and purple rice had the highest antioxidant capacities and total phenolic contents among tested cereals.

Qiu et al. (2009 and 2010) used DPPH and ORAC assays to evaluate antioxidant properties of wild rice. In both studies, their results showed that wild rice had higher antioxidant activity than white rice (control), most likely due to their high phenolic acid contents. Zhang et al. (2010) determined the phytochemical profiles and antioxidant activity of rice bran samples from 12 diverse varieties of black rice. Their data showed that there are significant differences in phytochemical content and antioxidant activity among the different black rice varieties. Black rice bran has higher content of phenolics, flavonoids, and anthocyanins as well as higher antioxidant activity compared to white rice. The phenolic, flavonoids and anthocyanins of black rice bran are mainly present in free form.

Bhanja et al. (2008) investigated a self-designed new bioreactor (NB) for improvement of phenolics and antioxidant activity in rice koji and compared the results with solid-state

fermentation (SSF). Rice fermented in the NB resulted in higher yield of phenolics and DPPH compared to SSF and control. This might be due to higher titre values of β -glucosidase (62.7%) and α -amylase (40.7%) in the extraction media of NB compared to SSF.

2.5.2.3 Other Cereal Grains

Kljak and Grbeša (2015) evaluated the relationship between carotenoid content and antioxidant activity in six Croatian commercial high-yield corn hybrids using TEAC, ABTS and thiobarbituric acid reactive substances (TBARS) systems. Their data showed that the corn hybrids varied in carotenoids content and antioxidant activity. Antioxidant activity in both assays increased linearly with total carotenoid content. Ndolo and Beta (2013) investigated carotenoid antioxidant activity using DPPH scavenging activity on aleurone, endosperm and germ fractions of barley (purple and regular), yellow corn, oats and wheat (purple and regular). The data suggested that antioxidant activity of carotenoids from the germ fraction showed higher scavenging activity compared to aleurone and endosperm. Malunga and Beta (2015) analyzed the antioxidant capacity of water-extractable nonstarch polysaccharides (NSP) and feruloylated arabinoxylans (WEAX) from commercial barley, wheat and wheat fractions (germ, bran, and aleurone) using DPPH, ABTS and ORAC assays. Their data showed that NSP and WEAX exhibited antioxidant activity. The results demonstrated that arabinoxylan content, TPC, xylose and degree of substitution influenced NSP and WEAX antioxidant capacity. Gamel and Abdel-Aal (2012) investigated phenolic acid composition and antioxidant capacity against DPPH and ABTS

radicals and inhibition of oxidation of human low density lipoprotein (LDL) cholesterol of selected Canadian and Egyptian barley whole grain flours and four pearling fractions. The data showed significant variations among barley wholegrain flour and pearling/milling fractions in terms of phenolic acid composition and antioxidant capacity. Awika et al. (2003) analyzed the antioxidant activity and phenolic content of less common grains such as sorghum. Phenolic contents of different cultivars of sorghum, sorghum bran, baked and extruded products had higher correlation with their antioxidant activity. Adom and Liu (2002) used total oxyradical scavenging capacity (TOSC) assay to investigate soluble, conjugated and insoluble bound forms of phytochemicals in uncooked wheat, oats, corn and rice as well as their antioxidant activity. The major portion of phenolics was found to exist in the bound form. Corn had the highest antioxidant activity followed by wheat, oats and rice, the activity of which was positively correlated to higher phenolic contents in these cereals. Bound phytochemicals were the major contributors to the total antioxidant activity. Miller et al. (2000) used DPPH assay to show that average antioxidant activities of cereal and cereal products (measured as Trolox equivalents) is higher (between 1200 and 3500 mmol TE/100 g for fresh products) than that of common fruits (1200 mmol TE/100 g) and vegetables (400 mmol TE/100 g), but lower than that of common berries (3880 mmol TE/100 g). The antioxidant capacity of the wheat bran fraction was 8500 mmol TE/100 g and that of the germ was 5000 mmol TE/100 g. Therefore, whole grains have higher total phenolic content and antioxidant capacity than some of the common fruits and vegetables (Lui, 2007).

Bhanja Dey and Kuhad (2014) evaluated the antioxidant potential of various filamentous fungi-fermented products derived from different whole grain cereals (wheat,

brown rice, oats and maize). The fermented products showed high efficiency for the improvement of water soluble TPC and antioxidant properties including ABTS and DPPH. Fermented wheat showed 14-fold improvement in TPC, 6.6 and 5-fold enhancement of DPPH and ABTS respectively. This study demonstrates that fermented wheat can be a powerful source of natural antioxidants. Gong et al. (2013) compared the *in vitro* procedure of antioxidant extraction with the Quencher method and water as an extracting solvent. Total antioxidant capacity values of cereal grains obtained using the *in vitro* procedure were 1.8-10.3 times higher than the Quencher procedure and 3.5-10.5 times higher than water extract. This indicates that the *in vitro* gastrointestinal digestion procedure is more useful in screening of grains, and assessing for their health benefits compared to the Quencher procedure and water extraction. Prajapati et al. (2013) evaluated the total phenol content, flavonoids, flavonol and antioxidant activity of five cereals (wheat, pearl millet, rice, maize and sorghum) by *in vitro* digestion and chemical extraction along with the effect of cooking. Their results showed higher values of total phenolics, flavonoids, flavonols as well as increase in antioxidant activity measured by FRAP method in *in vitro* digesta (enzymatic extract) of cereals compared to their chemical extracts. Cooking of cereal also resulted in increased total antioxidant capacity and enhanced phenolics, flavonoid and flavonol content. Based on this study, antioxidant components like phenolic compounds, flavonoids and flavonols of selected cereals were affected by *in vitro* digestion. Pazinato et al. (2013) evaluated the effect of different processes-defatting, protein concentration, thermal treatment, hydrolysis with Alcalase and *in vitro* digestion on the antioxidant capacity of amaranth seeds. The combination of protein concentration and hydrolysis with Alcalase led to the product with high antioxidant activity as evaluated by DPPH and ORAC.

After *in vitro* digestion, protein concentrate and its hydrolysate showed similar antioxidant capacity. Cai et al. (2012; 2014) evaluated the potential of solid-state fermentation for the improvement of TPC and antioxidant potential of oat. They found that fermentation increased TPC and antioxidant activity. Đorđević et al. (2010) evaluated the influence of fermentation by *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae* on antioxidant activities and total phenolic content of 4 cereals (buckwheat, wheat germ, barley and rye) and compared them to their unfermented counterparts. Total phenolic content increased significantly and antioxidant activities were enhanced through fermentation by lactic acid bacteria and yeast.

2.5.3 Intracellular Antioxidant Activity of Cereal Grains

Masisi et al. (2015) investigated antioxidant potential of carotenoids from aleurone, germ, and endosperm of barley, corn, and wheat. The antioxidant properties using DPPH, ABTS and ORAC assays revealed significantly higher antioxidant activity in the germ than in the aleurone and endosperm fractions. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, 2,2'-azobis (2-amidinopropane)dihydrochloride (AAPH)-induced cell loss was effectively reduced by pre-incubating Caco-2, HT-29, and FHs 74 Int cells with carotenoid extracts. The data showed that carotenoids of germ, aleurone, and endosperm fractions improve biochemical and intracellular antioxidant activity. Hirawan et al. (2011) investigated the properties of whole purple wheat, unpolished red rice and partially polished red rice infant cereals. ORAC, TPC, total anthocyanin contents (TAC) and cellular antioxidant activity (CAA) were measured. Home- and laboratory-made unpolished red rice had higher TPC and peroxy radical

scavenging activity than purple wheat infant cereals did; the latter had higher TAC. Pigmented infant cereals were found to have higher TPC, TAC and ORAC than commercial ones. Anthocyanins were identified in whole purple wheat but not in unpolished red rice. Purple wheat infant cereal had higher CAA than unpolished rice. Whole purple wheat infant cereals showed higher antioxidant activity than the commercial infant cereals did. Stein et al. (2010) investigated the effects of fermented wheat aleurone on the expression of genes involved in stress response, toxicity, activity of drug-metabolising enzymes and anti-genotoxic potential. They also investigated the protection against H₂O₂-induced DNA damage in HT29 cells. The data showed that fermented aleurone significantly induced mRNA expression of *CAT*, *GSTP1* and *SLUT2B1* (HT29) and *GSTP1* (epithelial stripe). The enzyme activity of GST (HT29) and CAT (HT29, epithelial stripes) were also increased. DNA damage induced by H₂O₂ was significantly reduced by the fermented aleurone.

2.5.4 *In Vivo* Cereal Grain Antioxidant Activity

Although *in vitro* experiments indicate great antioxidant abilities of the whole grain cereals, it is questionable if the methods underestimate physiological antioxidant capacity. Several studies have been done in order to evaluate bioactivity of cereal grain phytochemicals, mostly on animals and human.

2.5.4.1 Animal Studies

Qingming et al. (2010) evaluated the antioxidant activity of malt extract from barley *in vivo* (male Kunming mice). Scavenging effects on the hydroxyl and superoxide radicals, and protection against reactive oxygen species induced lipid, protein and DNA damage

were evaluated. The D-galactose induced Kunming mice were used to evaluate the ability of malt extract to behave as an antioxidant. The extract exhibited high antioxidant activities in both *in vitro* and *in vivo*, evidenced by its ability to scavenge hydroxyl- and superoxide-radicals, high reducing power, and protection against macromolecular (lipid, protein and DNA) oxidation damage. Malt extract prevented the decrease of antioxidant enzyme activities, decreased liver and brain malondialdehyde levels and carbonyl contents and improved total antioxidant capacity in D-galactose-treated mice. Wang et al. (2010) investigated the protective effect of wheat feruloyl oligosaccharides (FOs) against oxidative stress in rat plasma. The oxidative markers (oxidized glutathione and malondialdehyde) and the activity of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) in plasma of rats fed standard diet supplemented with 1% FOs were evaluated. The anti-radical capacity of rat plasma after ingestion of 0.5 mg FOs was measured using AAPH as the free radical inducer. The results suggested that FOs enhanced plasma antioxidant enzyme activity and lowered oxidized glutathione and malondialdehyde levels for the group supplemented with FOs compared to the control. Moreover, FOs-supplemented diet resulted in resistance to AAPH-induced hemolysis than was the control group.

Zhang et al. (2009) studied the effect of replacing white rice and processed wheat starch with wild rice as the chief source of dietary carbohydrates in rats fed a high fat diet/cholesterol diet. The rats fed wild rice were found to suppress the build-up of oxidative stress by improving antioxidant capacity, increasing superoxide dismutase (SOD) activities and reducing malondialdehyde concentrations, both in serum and liver tissue of the rats. Another study on the potential cardiovascular benefits of wild rice in male and female

LDL-receptor-deficient (LDLr-KO) mice showed that wild rice diet has protective roles in improving plasma lipids profile in LDLr-KO mice (Surendrian et al., 2013). Guo et al. (2007) evaluated the effect of an anthocyanin-rich extract from black rice on hyperlipidemia and insulin resistance in fructose-fed rats. Dietary supplementation with the anthocyanin-rich extract (5 g/kg of high-fructose) prevented the development of fructose-induced insulin resistance. In addition, rats supplemented by the extract exhibited lower oxidative stress than the fructose-fed control animals, as indicated by the lower concentrations of plasma TBARS and blood oxidized GSH. Fardet et al. (2007) investigated the metabolic responses of two groups of rats fed two diets (whole-grain wheat flour, WGF and refined wheat flour, RF) for two weeks each in a crossover design. Their metabolic approach showed that consumption of WGF produced metabolic changes, some of which may protect the organism against oxidative stress. Álvarez et al. (2006) fed healthy mice for 5 weeks with cereal fraction (wheat germ, buckwheat flour, rice bran and wheat middlings) to investigate supplementation effect. The results showed that all cereal fractions caused improvement of the leukocyte parameters including chemotaxis capacity, microbicidal activity, lymphoproliferative response to mitogens, interleukin-2 (IL-2) and tumor necrosis factor (TNF) release, as well as oxidized glutathione (GSSG), GSSG/reduced glutathione (GSH) ratio, CAT activity and lipid oxidative damage. The observation was similar among the cereal fractions. These effects are due to the antioxidant activity of polyphenols naturally present in cereals.

A study on black rice by Chiang et al. (2006) showed that black rice extract significantly increased superoxide dismutase and catalase activities by 161.6% and 73.4%, respectively. Hsieh et al. (2005) evaluated the effect of oxidative damage to DNA by

feeding rice bran oil to streptozotocin-induced diabetes rats. They measured the levels of 8-hydroxy-2'-deoxyguanosine (8-OhdG) oxidative DNA damage in various tissues of rats with streptozotocin-induced diabetes. There were significant reductions in mtDNA 8-OhdG levels in the liver, kidney, and pancreas of these rats treated with rice bran oil as compared to those in diabetic rats without intervention. Another study using rabbits showed that black rice diet significantly decreased aortic 8-OhdG and serum and aortic malondialdehyde, hence indicating decreased oxidative stress (Ling et al., 2002). In a study conducted by Hegde et al. (2005) alloxan-induced diabetic rats were fed a diet supplemented with 55% (w/w) finger millet and kodo millet. Blood glucose, cholesterol, enzymatic and nonenzymatic antioxidants, lipid peroxides in plasma, and glycation of tail tendon collagen were measured. Feeding diabetic rats diet supplemented with millet restored the levels of enzymatic (GSH, vitamin E and C) and nonenzymatic antioxidants (SOD, CAT, GPx and glutathione reductase) and lipid peroxide which had been reduced by their diabetic condition. Blood sugar and total cholesterol were lowered in rats fed diets formulated with millet. Another confirmation for postulating that the beneficial effects could involve mechanisms related to free-radical scavenging activity is the inhibition of glycation of rat-tail tendon collagen in the millet-fed diabetic rats. Chen et al. (2004) examined the bioavailability of avenanthramides and phenolics from oats using BioF1B hamsters. Absorbed oats phenolics did not change the resistance of hamster LDL particles against Cu^{2+} -induced oxidation until ascorbic acid was introduced to the assay mixture.

2.5.4.2 Human studies

The effects of a diet high in wheat aleurone on plasma antioxidants status, markers of inflammation and endothelial function were studied by Price et al. (2012). Seventy-nine healthy, older, overweight participants incorporated either aleurone-rich cereal products (27 g aleurone/d), or control products balanced for fiber and macronutrients, into their habitual diets for 4 weeks. Fasting blood samples were collected at baseline and on day 29. The results showed that, compared to the control, consumption of aleurone-rich products provided substantial amounts of micronutrients and phytochemicals which may function as antioxidants. However, no changes were found in other markers of inflammation, antioxidant status or endothelial function. Söderholm et al. (2012) evaluated the effect of none versus a high intake of rye bread on the oxidation resistance of LDL in healthy humans while otherwise on a habitual diet. Their data showed that intake of rye in four weeks improved significantly resistance of LDL. However, they were unable to show rye-originating substances in LDL responsible for the enhancement. Antioxidant ability of capsules containing oats avenanthramides was evaluated in 120 healthy individuals (60 women and 60 men). The materials were randomly assigned to four groups, which consumed 4 basal corn oil capsules (placebo), 4 capsules containing 1.56 mg of oat avenanthramides-enriched extract (OAEs) and 8 capsules containing 3.12 mg of OAEs or without treatment (control) for 1 month. Plasma lipid peroxides and antioxidant status were measured. For the 8 capsules group, the level of serum SOD and reduced glutathione hormones were significantly increased by 8.4 and 17.9%, respectively ($P < 0.05$) and malondialdehyde level significantly decreased by 28.1%. The total cholesterol, triglyceride, and low density lipoprotein cholesterol levels were lowered by 11.1, 28.1 and 15.1%,

respectively ($P < 0.05$), while the high density lipoprotein cholesterol level was increased by 13.2%. Oat extracts containing avenanthramides possess a high antioxidative activity in humans (Liu et al., 2011). A study on bioavailability and bioactivity of avenanthramides in six healthy older adults showed an increased antioxidant capacity after consuming skim milk containing avenanthramides (Chen et al., 2007). A study by Wang et al. (2007) investigated the influence of black rice pigment fraction (BRF) supplementation in sixty patients with coronary heart diseases (CHD) for six months. After six months of intervention, compared to white rice pigment fraction supplementation, BRF supplementation greatly enhanced plasma total antioxidant capacity. Physiological consequences of using white rice or mixed rice were evaluated in overweight Korean women. The data showed that plasma GPx activity increased by 15% when subjects consumed brown and / or black rice for 6 weeks (Kim et al., 2008).

Price et al. (2008) evaluated total phenolics (TP) and antioxidant potential (AOP) in plasma and urine of humans following consumption of a single meal of unprocessed wheat bran or a refined cereal (ground white rice). Their data showed that wheat bran phenolics are relatively well absorbed and may enhance antioxidant status. The potential antioxidative effect of rye bran intervention was investigated by measuring LDL susceptibility to copper oxidation *ex vivo*. Rye bran intervention had no influence on lag time or propagation rate of the LDL oxidation *ex vivo* (Harder et al., 2004). Research conducted in 76 male patients with coronary artery disease (CAD) during 16 weeks showed that replacement of refined rice with whole grain powder resulted in significant beneficial effects on glucose, insulin, and homocysteine concentrations and lipid peroxidation in CAD patients (Jang et al., 2001). **Table 2.6** summarizes findings on *in vivo* antioxidant activity of cereal grains.

2.5.5 Summary

Cereal grains remain a staple component of diets worldwide. They provide significant levels of bioactive phytochemicals including folates, phenolic acids, carotenoids, betaine, choline, sulphur amino acids, phytic acid, lignins, avenanthramides and alkylresorcinols. These phytochemicals are unevenly distributed between the various tissues and cell types of the grains. *In vitro* and *in vivo* studies have shown that cereal grain phytochemicals may improve antioxidant capacity, and thus have the potential to mitigate oxidative stress hence delaying the onset of some chronic diseases.

Antioxidant properties of cereal grains polyphenols (mainly ferulic and phenolic acid) have been previously shown to contribute to contributors to antioxidant status of cereal grains. However, antioxidant status of carotenoids of cereal grains or cereal grain fractions has not been well explored. Therefore, this study will explore the antioxidant potential of carotenoids of cereal grain fractions using biochemical, *in vitro* and *in vivo* models. Such findings will provide novel information on the health benefits of specific fractions of the cereal grains and will assist in development of healthy functional products from these cereal grain fractions.

Table 2.6 *In vivo* antioxidant activity of cereal grains

Cereal grains	Potential bioactive compounds	Observed biological activity	Animal model	References
Millet	Phenolics	Scavenged hydroxyl-and superoxide-radicals, high reducing power, and protect against macromolecular (lipid, protein and DNA oxidation damage).	D-galactose-induced mouse aging model	Qingming et al., 2010
	Phenolics, tannins and phytates	Improvement in the enzymatic (glutathione, Vitamin E and C) and non enzymatic antioxidants (SOD, CAT, GPx, glutathione reductase).	Alloxan-induced diabetic rats	Hedge et al., 2005
Oats	Avenanthramides and Phenolics	Absorbed oats phenolic did not change the resistance of hamster LDL particles against CA ²⁺ -induced oxidation until ascorbic acid was introduced.	BioF1B hamster	Chen et al., 2004
		Serum SOD and reduced glutathione hormones increased by 8.4 and 17.9% respectively; malondialdehyde decreased by 28%; TC, TAG, and LDL cholesterol were lowered by 11.1, 28.1, 15.1% respectively	Healthy human	Liu et al., 2011
		Increased antioxidant capacity.	Healthy old adults	Chen et al., 2007
Black rice	Anthocyanin	Prevented development of fructose-induced insulin resistant; lowered plasma TBARS concentration and blood oxidized glutathione	Fructose-fed rats	Guo et al., 2007
		Increased SOD and CAT activities by 161.1% and 73.4% respectively	C57BL/6 mice	Chiang et al., 2006
		Decreased aortic 8-OhdG and serum and aortic malondialdehyde	Rabbits	Ling et al., 2002
		Enhanced plasma TAC, reduced plasma levels of vascular cell adhesion molecule-1, soluble CD40 ligand and high sensitive C-reactive protein	CHD human	Wang et al., 2007
Brown rice		Plasma GPx activity increased by 15%	Over weight Korean women	Kim et al., 2008
Wild rice		Increased SOD and reduced malondialdehyde concentration, in serum and liver	Male sprague-dawley rats	Zhang et al., 2009

Wild rice	Phenolics	Increased SOD and CAT activities	LDL-receptor-deficient mice	Surendrian et al 2013
Rice bran	Polyphenol	Improved GSSG, GSSG/GSH ratio, catalase activity and lipid oxidative damage	Healthy mice	Álvarez et al., 2006
Rye	Ferulic acid	Improved oxidation resistance of LDL	Healthy human	Söderholm et al., 2012
		Significant reduction in mtDNA 8-OhdG levels in liver, kidney and pancreas	Streptozotocin-induced diabetes rats	Hseih et al., 2005
		Increased urine ferulic acid but did not change measurable antioxidative effect on the subject's LDL	Healthy human	Harder et al., 2004
Wheat (Bran germ and flour)	Phenolics	Decreased transit time and Increased TBARS	Obese rat	Kim et al., 2012
		Consumption of aleurone-rich products provided substantial amounts of micronutrients and phytochemicals which may function as antioxidant	Overweight human	Prince et al., 2012
		Improved GSSG, GSSG/GSH ratio, catalase activity and lipid oxidative damage	Healthy mice	Álvarez et al., 2006
		Increased SOD and reduced malondialdehyde concentration, in serum and liver	Male sprague-dawley rats	Zhang et al., 2009
		WGF produced metabolic change	Rats	Fardet et al., 2007
		Enhanced antioxidant status	Healthy human	Price et al., 2008

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SECTION A: BIOCHEMICAL MODEL

CHAPTER 3

Characterization, Identification, Quantification and Antioxidant Capacity of Carotenoids of Aleurone, Germ and Endosperm Fractions of Barley, Corn and Wheat

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3.1 Abstract

Total carotenoid content (TCC) and carotenoid composition were analyzed using spectrophotometry and HPLC. Furthermore, antioxidant properties of carotenoid extracts of aleurone, germ and endosperm fractions of barley, corn and wheat were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. Total carotenoid content differed significantly among fractions from different cereal grains ($P < 0.001$). TCC were highest in germ fractions of the cereal grains (57.3-60.3 mg/kg) and lower in aleurone and endosperm of barley (28.6 and 28.9 mg/kg respectively). Corn germ had the highest concentration of carotenoid compared to the other samples ($P < 0.001$). HPLC analysis confirmed the presence of lutein and zeaxanthin carotenoids (nd-15139 $\mu\text{g}/\text{kg}$) in extracts of cereal grain fractions. The antioxidant properties using DPPH, ORAC and ABTS assays revealed significantly higher ($P < 0.001$) antioxidant activity in the germ than in the aleurone and endosperm fractions. Our findings suggest that carotenoids of germ, aleurone and endosperm fractions had significantly higher carotenoid levels and improved antioxidant capacity, and these features would lead to a greater potential to mitigate oxidative stress.

3.2 Introduction

Reactive oxygen species (ROS) are generated from endogenous (cellular oxygen metabolism) as well as exogenous sources such as tobacco smoke, pollutants, ultraviolet radiation and ionizing radiation (Ho et al., 2013; Sobha & Andallu, 2013). These molecules attract electrons from other molecules, and, if not adequately regulated, can cause damage to cell membranes, cellular proteins, lipids and DNA (Young & Woodside, 2001). Oxidative stress occurs when cells are subjected to excess levels of these unstable ROS. Several studies have described the relationship between increased cellular ROS and the pathogenesis of chronic diseases, including cancer (Nogueira & Hay, 2013), inflammation (Leavy, 2014), cardiovascular diseases (Sugamura & Keaney Jr, 2011) and age related macular degeneration (Khandhadia & Lotery, 2010).

The process of oxidation in cells is regulated by antioxidants, which delay or prevent cellular oxidation. Antioxidant protection is normally achieved through a balance between pro-oxidants and endogenous and/or dietary antioxidants (Sobha & Andallu, 2013; Hanock et al., 2001). Pathogenesis is most prominent when there is antioxidant depletion, inadequate supply of dietary antioxidants and/or faulty antioxidants in cells, which compromise the body's ability to regulate cellular oxidation. Some of the dietary nutrients with significant antioxidant potential are phytochemicals from fruits, vegetables and cereal grains (Krinsky & Johnson, 2005; Tapeiro et al., 2005). Research has shown that phytochemicals, including carotenoids, are associated with reducing the risk of cancer, cardiovascular disease, inflammation, cataracts, and macular degeneration through the inhibition of cell proliferation (Sobha & Andallu, 2013; Adom et al., 2005).

Carotenoids are a class of natural pigments that form part of the antioxidant system in seeds (Stahl & Sies, 2003; Gentili & Caretti, 2011). They are responsible for the yellow-orange color of many foods. They are widely distributed in fruits (e.g., cantaloupe melon and apricots), vegetables (e.g., carrots, tomato and spinach), cereal grains (e.g., corn, wheat and barley) and are also added as additives in processed foods (flour and breakfast cereal). Carotenoids are classified into two groups, carotenes which contain carbon and hydrogen only and oxocarotenoids (xanthophylls) which carry at least one oxygen atom (Tapeiro et al., 2005). Xanthophyll carotenoids, which consist of lutein and zeaxanthin, are recognized for their antioxidant properties (Gentili & Caretti, 2011; Leenhardt et al., 2006; Miller et al., 1996). The carotenoids are so called antioxidants because of their capacity to trap lipid peroxy radicals, singlet oxygen species and triplet energy levels (Palozza & Krinsky, 1992). Moreover, the antioxidant capacity of carotenoids has been shown to be strongly dependent on their redox properties (Polyakov et al., 2001).

In cereals, carotenoids are minor components (Irakli et al., 2011), with some grains containing higher and other lower content of carotenoids compared to fruits and vegetables (Abdel-Aal et al., 2002; Humphries & Khachik et al., 2003). The main carotenoids in cereal grains are lutein and zeaxanthin (Hentschel et al., 2002; Panfili et al., 2004). Their extensive conjugated double bond system makes them effective radical scavengers (Cooke et al., 2002) and inhibits free radical propagation reactions such as lipid peroxidation. Lutein is dominant in oats, barley, spelt and durum wheat whereas zeaxanthin is the main component in corn (Panfili et al., 2004). Carotenoids content, composition and distribution in cereal grains have been well studied (Abdel-Aal et al., 2002; Hentschel et al., 2002; Abdel-Aal et al., 2007; Hidalgo et al 2006; Kurilich & Juvik, 1999; Ndolo & Beta, 2013).

However, there is limited information on antioxidant potential of carotenoids from cereal grain fractions. Therefore, we hypothesize that carotenoids of aleurone, germ and endosperm (also referring to starchy endosperm) fractions of barley, corn and wheat have antioxidant potentials. The main objective of this study was to extract carotenoids from aleurone, germ and endosperm fractions of barley, corn and wheat, identify and quantify carotenoid extracts and evaluate their antioxidant capacities using ABTS, DPPH and ORAC assays.

3.3 Materials and Methods

3.3.1 Chemicals and Materials

HPLC grade acetonitrile, hexane, methyl-butyl ether (MtBE) 1-butanol and methanol were acquired from Fisher Scientific (Whitby, ON, Canada). Carotenoid standards, lutein (98.2%) and zeaxanthin (95.8%) were acquired from Chromadex Inc (Santa Ana, CA, USA), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; $\geq 98\%$), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) (95%) and butylated hydroxytoluene (BHT) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were acquired from Fisher Acros Organics (Morris Plains, NJ, USA).

3.3.2 Samples

Three cereal grains, barley (regular), corn (flint) and wheat (MSUD8006) as described by Ndolo & Beta (2013) were used. These samples were used throughout the study.

3.3.3 Sample Preparation

The grains were hand dissected to separate the aleurone, germ and endosperm according to the procedure described by Stewart, Nield & Lott (1988) with further modifications (Ndolo & Beta, 2013). The germ and grains ends were removed with a sharp scalpel and the remaining grain was cut lengthwise into half. The degermed grains were soaked in 0.1% (v/v) sodium hypochlorite (NaClO) for 15-20 min to sterilize the surfaces and rinsed using sterile deionized water. The seeds were then placed in 10 cm petri dishes lined with 2 ashless filters, moistened with 10 mL of sterile deionized water. The petri dishes were wrapped in aluminum foil and kept at 20 °C for 48 h. The aleurone and endosperm were separated using a scalpel and stored at -20 °C. Samples were freeze dried thereafter and ground using a multi-use blade grinder, model PCC 770 (Loblaw's Inc. MO, Canada) to pass through a 0.5 mm sieve. The ground samples were stored at -20 °C before extraction and analysis. **Appendix I** shows aleurone, endosperm and germ fractions of barley and wheat.

3.3.4 Extraction of Carotenoids

The extraction method of Cha et al. (2008) was further modified for this assay. One-gram samples (aleurone, germ and endosperm) were extracted in a 100 mL ethanol solution (95%) containing 0.1% (w/v) butylated hydroxytoluene (BHT) at room temperature in the dark. After 3 h of extraction under continuous shaking using water bath (VWR, Radnor, PA, USA), the mixtures were filtered through a Whatman no. 1 filter paper. Still at room temperature and in the dark, 120 µL of 100% potassium hydroxide (KOH) was added to 5 mL of the contents for saponification. After saponification for 1 h, 3 mL of hexane was

added to partition carotenoids. The mixture was shaken for 1 min and then diluted with 3 mL of water. The mixture was allowed to stand in an amber separatory funnel to clearly separate the two phases. The upper layer containing carotenoids was collected, and the residue was repeatedly extracted until it contained no trace of carotenoids. The carotenoid extracts were concentrated to dryness at 35 °C using rotary evaporation (IKA RV10, IKA® Works Inc., Wilmington, NC, USA).

3.3.5 Preparation of Test Solutions

For the biochemical model, the concentrated carotenoid extracts were redissolved in 5 mL methanol/saturated butanol to prepare test solutions.

3.3.6 Determination of Total Carotenoid Content (TCC)

The extracted carotenoids were reconstituted with water saturated butanol and transferred to semi-micro quartz cuvette and absorbance measured at 450 nm using Ultrospec 1100 pro, UV/Visible spectrophotometer (Biomicon Ltd. Cambridge CB4 QFJ, England). All analyses were conducted in triplicates and expressed as μg xanthophylls equivalent/g sample

$$C = \frac{(5 \times A)}{S \times W} \left[\frac{\mu\text{g}}{\text{g}} \right] \quad [1]$$

where C = xanthophyll content, $\mu\text{g/g}$; A = absorbance reading; S = regression coefficient (the number that expresses the relationship which is created based on concentration of xanthophyll working standard solutions in $\mu\text{g/mL}$ and the absorbance); 5 = dilution factor (the factor based on the total extracted volume of 5 mL) and W = sample weight, g (Abdel-Aal et al., 2007).

3.3.7 HPLC Analysis of Carotenoids

Carotenoids were quantified by HPLC, using external calibration of a standard mix (lutein and zeaxanthin). Freshly extracted samples were filtered through 0.45 µm nylon disc filter into a brown HPLC vial. The quantification of carotenoids was done according to methods from Ndolo & Beta (2013) and Abdel-Aal et al (2007) with some modifications. The quantification of carotenoids was performed by HPLC (Waters 2695) with a photoiodide array detector (PDA) (Waters 996) and autosampler (Waters 717 plus) (Waters, Milford, MA, USA). YMC™ carotenoid S-3, 3 µm packing, 4.6 × 100 mm column (Waters, Milford, MA, USA) was used. The column was operated at 35 °C, 20 µL of sample was injected by autosampler and eluted with a gradient system consisting of (A) methanol/methyl tert-butyl ether/milli-Q water (81:15:4 v/v/v) and (B) methyl tert-butyl ether/methanol (90:10 v/v). The flow rate was set at 1 mL/min. The gradient was programmed as follows: 0-9 min, 100-75% A; 10-12 min 0% A; 12-13 min 0-100% A; and 13-20 min 100% A. The separation of carotenoids were detected and measured at 450 nm. Carotenoids in samples were identified based on retention times of standards. Six concentrations in the range of 0.05-5.0 µg/mL per 20 µL were prepared for lutein and zeaxanthin to generate regression equations for quantification.

3.3.8 Determination of DPPH Radical Scavenging Activity

The DPPH method of Brand-William et al. (1995) as modified by Li et al. (2005) was further modified for this assay. Sixty µM DPPH radical solution was freshly prepared from a stock solution of 300 µM. Extracts (100 µL) were reacted with 3.9 mL of the DPPH radical solution for 30 min. The absorbance at 515 nm was measured against a blank of

pure methanol using Ultraspec 1100 pro, UV/Visible spectrophotometer (Biomicon Ltd. Cambridge CB4 QFJ, England). The analysis was conducted in quadruplicates. Concentrations ranging from 100-800 μM were used for the trolox standard curve. Antioxidant activity was calculated as a % DPPH radical scavenging activity as:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \left[\frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}} - A_{\text{blank}}}\right]\right) \times 100 \quad [2]$$

where A_{control} is the absorbance of DPPH radical in methanol at 0 min, A_{sample} is the absorbance of DPPH radical for the sample extract or standard at 30 min. A calibration curve of % DPPH decolorization obtained from different concentration of trolox was used to quantify the antioxidant capacity of the extracts. Antioxidant capacity was expressed using Trolox equivalents (TE) per gram of dry samples.

3.3.9 Determination of ABTS Radical Scavenging Activity

The ABTS method of Pellegrini et al. (1999) was further modified for this assay. Seven mM of ABTS reagent and 2.45 mM of potassium persulfate solution were mixed and allowed to stand in the dark at room temperature for 16 h before use. Prior to assay, the solution was diluted in methanol to give an absorbance at 734 nm of 0.7 ± 0.02 . Extracts (100 μL) were reacted with 3.9 mL of the ABTS radical solution for 30 min. The absorbance at 734 nm was measured against a blank of pure methanol using Ultraspec 1100 pro, UV/Visible spectrophotometer (Biomicon Ltd. Cambridge CB4 QFJ, England). The analysis was conducted in quadruplicates. Concentrations ranging from 100-800 μM were used for the trolox standard curve. Antioxidant activity was calculated as a % ABTS radical scavenging:

$$\text{ABTS radical scavenging activity (\%)} = \left(1 - \left[\frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}} - A_{\text{blank}}}\right]\right) \times 100 \quad [3]$$

where A_{control} is the absorbance of ABTS radical in methanol at 0 min, A_{sample} is the absorbance of ABTS radical for the sample extract or standard at 30 min. A calibration curve of % ABTS decolorization obtained from different concentration of trolox was used to quantify the antioxidant capacity of the fractions extracts. Antioxidant capacity was expressed using Trolox equivalents (TE) per gram of dry samples.

3.3.10 Determination of Oxygen Radical Absorbance Capacity

The determination of oxygen radical absorbance capacity (ORAC), first developed by Cao et al. (1993) was conducted according to Huang et al. (2005) and Li et al. (2007). A FLx800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) was used with fluorescence filters for an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. The plate reader was controlled by KC4 3.0 software (version 29). The instrument was monitored by KC4 3.0 software, version 29. Dilutions of barley, corn and wheat extracts and trolox standard were done manually. A volume of 300 μL each of buffer solution (blank) and diluted sample solution and trolox standard was transferred to a 96-well flat bottom polystyrene microplate according to their allocated wells. Trolox standards of 12.5, 25, 50 and 100 μM were used. The remainder of the procedure was conducted according to Li et al. (2007). Antioxidant capacity was calculated based on the method by Huang et al. (2005). Regression equation between trolox concentration and the net area under the fluorescence decay curve was constructed. The analysis was conducted in quadruplicates. The formula to obtain the area under curve (AUC) was:

$$\text{AUC} = 0.5 + \frac{f_1}{f_0} + \dots \frac{f_i}{f_0} + \dots \frac{f_{49}}{f_0} + \dots 0.5 \frac{f_{50}}{f_0} \quad [4]$$

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i min.

Relative ORAC value was calculated according to the formula:

$$\text{Relative ORAC Value} = \frac{\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}}{\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}}} \left(\frac{\text{Molarity of trolox}}{\text{Molarity of sample}} \right) \quad [5]$$

3.3.11 Statistical Analysis

Data obtained were analyzed using one-way analysis of variance (ANOVA) with GLIMMIX Procedure of SAS, release 9.3 (SAS 2014; SAS Institute). Sample means were compared using Tukey-Kramer multiple comparison procedure and data were considered significantly different when $P < 0.05$. All results are presented as mean \pm standard error.

3. 4 Results and Discussion

3.4.1 Total Carotenoid Content in Aleurone, Endosperm and Germ of Barley, Corn and Wheat

Figure 3.1 shows total carotenoid content (TCC) expressed as xanthophyll equivalent (mg/kg) in cereal grain fractions. TCC differed significantly among fractions from different cereal grains ($P < 0.001$). TCC were highest in germ fraction of the cereal grains (57.3-60.3 mg/kg) and lower in aleurone and endosperm of barley (28.6 and 28.9 mg/kg respectively). Corn germ had the highest concentration of carotenoid compared to the other samples ($P < 0.001$). The higher content of TCC in germ could be attributed to the presence of yellow

carotenoid pigments. Our results are consistent with studies by Panfili et al. (2004) and Ndolo and Beta (2013) indicating higher carotenoid levels in germs of barley and wheat.

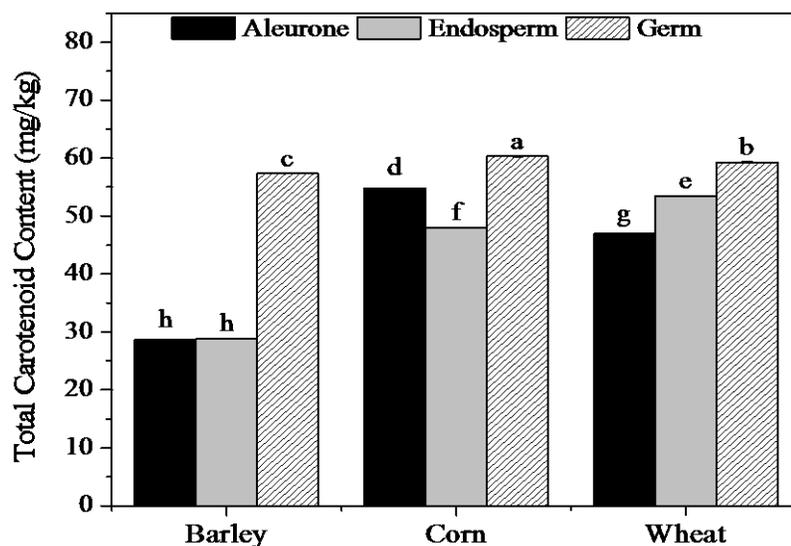


Figure 3.1 Total carotenoid content (mg/kg) in aleurone, endosperm germ fractions of barley, corn and wheat. Data presented are mean \pm standard error ($n = 3$). Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

3.4.2 Carotenoids Analysis in Aleurone, Endosperm and Germ of Barley, Corn and Wheat

The short method was chosen assuming BHT-ethanol extracted mainly polar carotenoids unlike non-polar ones, which are found in minimal amount in cereal grains. Carotenoid identification was accomplished by comparing the retention times (t_R) in the

sample and those of external standards and the UV-visible absorption spectra from literature. Two peaks were identified for lutein and zeaxanthin, the primary carotenoids found in cereal grains and products (Panfili et al., 2004; Fratianni et al., 2005). Quantitative data were calculated from the linear calibration curves. Representative cereal grain fractions quantified by HPLC are shown in **Appendix II**. Lutein levels varied significantly ($P < 0.01$) in all cereal grain fractions. It ranged from 0-1328 $\mu\text{g}/\text{kg}$ in barley; 72-431 $\mu\text{g}/\text{kg}$ in wheat and 72-1369 $\mu\text{g}/\text{kg}$ in corn fractions ($P < 0.01$) (**Table 3.1**). The endosperm fraction of corn had higher lutein content (1369 $\mu\text{g}/\text{kg}$) followed by germ fraction of barley (1328 $\mu\text{g}/\text{kg}$) and with no detection in the endosperm of barley. Zeaxanthin levels also varied significantly ($P < 0.01$) in all cereal grain fractions. It ranged from nd-15139 $\mu\text{g}/\text{kg}$ in barley; 358-13671 $\mu\text{g}/\text{kg}$ in corn and 7-215 $\mu\text{g}/\text{kg}$ in wheat. The germ fraction of barley had higher zeaxanthin content (15139 $\mu\text{g}/\text{kg}$) followed by endosperm fraction of corn (13671 $\mu\text{g}/\text{kg}$) and with no detection in the endosperm of barley. Corn fractions had higher levels of zeaxanthin than barley and wheat; and even higher than whole meal as previously shown by Ndolo and Beta (2013). Zeaxanthin has been reported to be concentrated in the germ fraction of wheat (Panfili et al., 2004) and corn endosperm (Kean et al., 2008). In the present study, zeaxanthin was also reported as the dominant carotenoid in the aleurone layer in wheat consistent with results reported by Ndolo and Beta (2013). In this study, lutein and zeaxanthin were unevenly distributed in grain fractions and were not detected in the endosperm of barley. Differences in lutein and zeaxanthin levels would be attributable to genetic differences and growing locations which affect chemical composition of the cereal grains (Panfili et al., 2004; Kean et al., 2008).

Table 3.1 Carotenoid composition in aleurone, endosperm germ fractions of barley, corn and wheat ($\mu\text{g}/\text{kg}$) by HPLC

Grain Fractions	Lutein	Zeaxanthin
Barley		
Aleurone	112 \pm 5.5 ^c	12 \pm 1.5 ^f
Endosperm	nd	nd
Germ	1328 \pm 3.8 ^a	15139 \pm 4.6 ^a
Corn		
Aleurone	161 \pm 4.5 ^c	358 \pm 2.4 ^d
Endosperm	1369 \pm 8.7 ^a	13671 \pm 4.7 ^b
Germ	72 \pm 7.7 ^c	989 \pm 6.7 ^c
Wheat		
Aleurone	72 \pm 3.2 ^c	212 \pm 1.8 ^e
Endosperm	155 \pm 2.9 ^c	7 \pm 0.7 ^f
Germ	431 \pm 3.3 ^b	215 \pm 3.0 ^e

Values presented are mean \pm standard error ($n = 3$). Data in the same column with the same superscript are not significantly different at $p < 0.05$ (Tukey-Kramer's multiple range test).

3.4.3 DPPH Antioxidant Activity

DPPH scavenging activity is based on discoloration as a result of reduction of DPPH free radical by antioxidant (Brand-Williams et al., 1995). Our results (**Table 3.2**) suggest higher antioxidant activity of aleurone of corn and barley than that of wheat, germ of barley than that of corn and wheat, endosperm of corn than that of barley and wheat ($P < 0.01$). The antioxidant activity of the fractions ranged from 4-77 for barley, 37-71 for corn and 25-50 μM trolox equivalent for wheat (**Table 3.2**). Barley germ had higher DPPH free radical quenching activity with barley endosperm having the lowest activity ($P < 0.01$). The low DPPH scavenging activity in the endosperm of barley may be attributed to the low TCC and absence of lutein and zeaxanthin. According to a study by Mortensen and Skibsted

(1997) on the role of carotenoid structure in radical scavenging reaction, zeaxanthin is more reactive than lutein although their order of reactivity only shows slight differences. DPPH scavenging activity in germ fractions ranged from 37.1 μM for wheat to 76.8 μM for barley in aleurone fractions from 44.4 μM for wheat and 71.0 μM for corn.

3.4.4 ABTS+ Antioxidant Activity

ABTS+ scavenging activity is based on the ABTS+ radical losing its blue-green color when reduced to ABTS at 734 nm (Pellegrini et al., 1999). The ABTS scavenging activity ranged from 32-68 for barley, 45-53 for corn and 42-44 μM trolox equivalent for wheat fractions (**Table 3.2**). Our results suggest that the germ of barley had the higher antioxidant activity compared to that of corn and wheat ($P < 0.01$). Furthermore the results suggest the higher antioxidant activity of aleurone of barley and corn than of wheat, and endosperm of corn than that of wheat and barley. Germ fractions had scavenging activity ranging from 43 for wheat to 68 $\mu\text{M TE/g}$ for barley; aleurone fractions 42 for wheat and 52 $\mu\text{M TE/g}$ for barley; endosperm fractions 32 μM for barley to 53 $\mu\text{M TE/g}$ for corn (**Table 3.2**).

3.4.5 ORAC Values

ORAC assay is based on the principle that antioxidants will prevent peroxy radicals generated by AAPH from decaying the fluorescence intensity of fluorescein (Cao et al., 1993). The ability of a compound to delay the loss of fluorescence intensity over time is a measure of antioxidant capacity. Our results show that ORAC values ranged from 18-37 μM trolox equivalent (TE)/g (**Table 3.2**). ORAC values were generally low in endosperm (18.3-33.8 $\mu\text{M TE/g}$) compared to germ and aleurone fractions. Among aleurone fractions, barley had the lowest ORAC values. In contrast, ORAC values of germ fractions were

highest ranging from 29 $\mu\text{M TE/g}$ for wheat and 37 $\mu\text{M TE/g}$ for corn. ORAC values were also higher in aleurone fractions: 25 $\mu\text{M TE/g}$ for wheat and 31 $\mu\text{M TE/g}$ of for corn, likely due to variation in zeaxanthin levels (**Table 3.1**).

Table 3.2 Antioxidant capacity ($\mu\text{M Trolox equivalent}$) per 1 g of dry samples

Fractions	DPPH	ABTS	ORAC
Barley			
Aleurone	68.0 ± 1.4^b	51.7 ± 0.7^b	27.7 ± 1.8^{bc}
Endosperm	4.3 ± 0.6^g	32.0 ± 2.7^d	18.3 ± 1.6^d
Germ	76.8 ± 0.2^a	67.6 ± 1.7^a	33.0 ± 3.4^{ab}
Corn			
Aleurone	71.0 ± 0.6^b	47.8 ± 2.1^{bc}	30.5 ± 3.5^{abc}
Endosperm	66.2 ± 1.2^b	53.3 ± 3.5^b	33.2 ± 3.5^{ab}
Germ	37.1 ± 1.3^e	43.5 ± 2.0^c	37.3 ± 1.2^a
Wheat			
Aleurone	44.4 ± 0.5^d	41.6 ± 1.0^c	24.7 ± 2.1^{cd}
Endosperm	25.1 ± 1.1^f	43.9 ± 2.5^c	33.8 ± 3.3^{ab}
Germ	50.1 ± 1.1^c	43.3 ± 3.3^c	29.3 ± 1.3^{bc}

Values presented are mean \pm standard error ($n = 4$). Data in the same column with the same superscript are not significantly different at $p < 0.05$ (Tukey-Kramer's multiple range test).

3.5 Conclusion

Generally, lutein and zeaxanthin and TCC were unevenly distributed across the grain fraction. For all cereal grain fractions, the germ had significantly ($P < 0.001$) higher levels of TCC than aleurone and endosperm (corn > wheat > barley). In barley and wheat, lutein and zeaxanthin were concentrated in the germ unlike in corn where they are concentrated in endosperm. Carotenoid extracts of aleurone, starchy endosperm and germ of barley, corn and wheat had differential antioxidant capacities as measured by DPPH, ABTS and ORAC assays.

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SECTION B: *IN VITRO* MODEL

CHAPTER 4

Carotenoids of Aleurone, Germ, and Endosperm Fractions of Barley, Corn and Wheat Differentially Inhibit Oxidative Stress

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4.1 Abstract

Reactive oxygen species (ROS) and free radical damage to biomolecules are implicated in the etiology of many chronic diseases. Dietary antioxidants which inactivate ROS and provide protection from oxidative damage have the potential to mitigate and even prevent such diseases. Accumulating evidence has suggested that intake of whole grains may play a role in prevention of oxidative stress related diseases. We hypothesize carotenoid extracts of aleurone, germ and endosperm fractions of barley, corn and wheat neutralize free radical-initiated intracellular oxidation as well as free radical-induced cytotoxicity in cell culture models. This study evaluated carotenoids extracts antioxidant potential against free radical-initiated intracellular oxidation as well as free radical-induced cytotoxicity using DCFH-DA probe and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, respectively. Using MTT assay, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced and hydrogen peroxide (H₂O₂)-induced cell loss was effectively reduced by pre-incubating Caco-2, HT-29 and FHs 74 Int cells with carotenoid extracts. Moreover, carotenoid extracts reduced ($P < 0.001$) AAPH-induced intracellular oxidation in the cell lines, suggesting an antioxidant activity. Of the 84

antioxidant pathway genes included in microarray array analysis (HT-29 cells), the expression of 28 genes were enhanced ($P<0.05$). Our results suggest that carotenoids of germ, aleurone and starchy endosperm fractions improved cellular antioxidant capacity differently, and thus have the potential to differentially mitigate oxidative stress. These results indicate that corn endosperm, aleurone and germ of corn, barley and wheat might be useful functional ingredients in curbing oxidative stress, hence preventing human pathogenesis.

4.2 Introduction

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen including singlet oxygen, superoxide anion radicals, peroxide anions, and hydroxyl radicals which are involved in the etiology of many diseases as indicated by the signs of oxidative stress seen in those diseases (Bonomini et al., 2008; Lushchack, 2014; Rada et al., 2008; Radi, 2013; Terao & Niki, 1986). They can be generated during normal cellular respiration, by activated leucocytes as part of the immune response, and by exogenous oxidants such as air pollution and cigarette smoke (Finkel & Holbrook, 2000). They have an undoubted capability to be harmful by their action on vital cellular components including lipids, proteins and DNA (Temple, 2000). Antioxidant protection is normally achieved through a balance between pro-oxidants and endogenous and/or dietary antioxidants (Hancock et al., 2001; Klaunig et al., 2004; Klaunig et al., 2010; Sobha & Andallu, 2013). Fortunately, natural antioxidants, including phenolic compounds, vitamins, and carotenoids, are proven to be effective nutrients in the prevention of these oxidative stress related diseases.

Epidemiological evidence indicates that nutrition plays an important role in the modulation of oxidative stress leading to chronic diseases. Cereal grains are a principal component of all diets, and they significantly contribute to nutrition by supplying energy, protein, vitamin, and other biologically significant chemicals (Slavin, 2004). A meta-analysis study by Haas et al. (2009) alluded to the possibility that whole grain can reduce the risk of chronic disorder due to its high fiber content. However, the important question is whether it is just fiber that could be beneficial in the fight against chronic diseases, or are the other components of whole grains also beneficial? Cereal grains consist of different fractions: starchy endosperm, bran, aleurone and germ, which contain phytochemicals. The mainly consumed cereal grains in the world include wheat, rice, maize/corn, oats, rye, barley, triticale, millet, and sorghum (Fardet, 2010). In North America, corn, barley and wheat are mainly consumed. The major health benefits are attributed to their unique phytochemical compositions (Belobrajdic & Bird, 2013; Ndolo & Beta, 2013; 2014). Recently, these trace amounts of antioxidant phytochemicals, including carotenoids have attracted more interest from both food manufacturers and researchers. Carotenoids are among the abundant families of pigments in nature that are responsible for the yellow, orange and red colors in fruits, vegetables and grains, and they form part of the antioxidant system in seeds (Stahl & Sies, 2003; Irakli et al., 2011; Howitt & Pogson, 2006). Carotenoids have extensive application as antioxidants and therefore, are important for human health. The carotenoids are so called antioxidants because of their capacity to trap not only lipid peroxy radical, but also singlet oxygen species. Moreover, the antioxidant capacity of carotenoids may also be related to their structure. A large conjugated system such as astaxanthin is known to have higher antioxidant activity (Miki, 1991). However, the antioxidant potential of carotenoids

of aleurone, germ, and endosperm fractions of barley, corn and wheat has not been studied. We hypothesize that carotenoid extracts of aleurone, germ and endosperm fractions of barley, corn and wheat neutralize free radical-initiated intracellular oxidation as well as free radical-induced cytotoxicity in cell culture models. This study, therefore, quantitates the protective effect of carotenoid extracts against free-radical-induced cytotoxicity using three distinct cell lines.

4.3 Materials and Methods

4.3.1 Chemicals and Materials

2,2'azobis (2-amidinopropane) dihydrochloride (AAPH) was acquired from Sigma-Aldrich (St. Louis, MO, USA). The fluorescent dye, 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H₂DCFDA) was acquired from Invitrogen (Eugene, OR, USA). Fluorescein and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were acquired from Fisher Acros Organics (Morris Plains, NJ, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), hybrid-care medium, Caco-2, HT-29 and FHs 74 were acquired from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cell culture medium, Dulbecco's modified Eagle's medium (DMEM) was acquired from Sigma-Aldrich (St. Louis, MO, USA), as were Dulbecco's phosphate buffer solution (PBS), L-glutamine ($\geq 99\%$), sodium pyruvate ($\geq 99\%$), epidermal growth factor (EGF) and transferrin human (hTF; $\geq 98\%$). Penicillin/streptomycin was acquired from Gibco BRL (Burlington, ON, Canada). Trypsin 0.05% was acquired from Thermo Scientific (Logan, UT, USA). RNeasy mini kit was obtained from Qiagen (Mississauga, ON, Canada). RT²

First Strand kit and RT² Profiler array (PAHS-065Y) were obtained from SABioscience (Mississauga, ON, Canada). The 25 cm² polystyrene flask or Transwell[®] and 96-well flat bottom polystyrene microplate were purchased from Corning Incorporated (Ithaca, NY, USA).

4.3.2 Samples

Barley, corn and wheat were as described in **Chapter 3**.

4.3.3 Sample Preparation

Aleurone, endosperm and germ fractions of barley, corn and wheat were prepared as described in **Chapter 3**.

4.3.4 Extraction of Carotenoids

Carotenoids extracts of aleurone, endosperm and germ fractions of barley, corn and wheat were prepared as described in **Chapter 3**.

4.3.5 Preparation of Test Solutions

For the *in vitro* model, stock solutions of carotenoid extracts were prepared by reconstituting concentrated carotenoid extracts in 10% DMSO with PBS. The final concentration of DMSO was less than 1%. The original carotenoid stock solutions were 200 mg/mL which were further diluted to working solutions of 100, 20, 5, 0.5 mg/mL with PBS.

4.3.6 Cell Culture Assay

Three *in vitro* models were used in this study: Caco-2, HT-29 and FHs 74 Int. Caco-2 is structurally and functionally similar to human intestinal epithelium, which is the first to be

in contact with food. Caco-2 are regarded as a simple *in vitro* model for the study of drug transportation and absorption in human intestinal mucosa (Hilgers et al., 1990). HT-29, a cell line with epithelial morphology, is used to study drug transportation, absorption and secretion by intestinal cells (Lesuffleur et al., 1990). The Caco-2 and HT-29 cell line used in this study were differentiated. These cell lines when left undifferentiated are cancer cells. The parental cell line, originally obtained from human colon adenocarcinomas of different ages and sex (Caco-2: from 72 year old male Caucasian; HT-29: from 44 year old female Caucasian) undergoes in culture a 21-day process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and biochemical characteristics of the mature small intestine enterocytes (Cohen et al., 1999; Pinto et al., 1983; Sambuy et al., 2005). Furthermore, HT-29 undergoes a differentiation in polarized monolayers of mucus-secreting and/or absorptive cells (Fantini et al., 1986). Therefore, due to these characteristics HT-29 was a better cell line for this model of study, with Caco-2 and FHs-74 Int used to verify the data. FHs 74 Int is a primary human fetal intestine cell culture which does not form confluent, polarized monolayers in culture, yet is otherwise genotypically similar to neonatal enterocytes. The FHs 74 Int was originally obtained from normal small intestine of a female, 3 to 4 months gestation. It has been reported to show mature epithelial-like characteristics (Wagner et al., 1998).

All three cell lines were grown in 25 cm² polystyrene flask or Transwell[®] culture chambers. The Caco-2 and HT-29 were maintained in DMEM with D-glucose content supplemented with 10% FBS, penicillin (100 kU/L), streptomycin (100g/L), 200 mM L-glutamine, 100 mM sodium pyruvate, and hTF (**Table 4.1**). FHs 74 Int cells were cultured using Hybri-Care medium with 10% FBS, penicillin (10 kU/L), streptomycin (100g/L) and

EGF (40 µg/L) (**Table 4.1**). Cells were sub-cultured after reaching confluence using trypsin (0.05%). Cells were maintained at 37 °C in a 5% CO₂ humidified incubator for optimal growth.

Table 4.1 Essential supplements for maintaining cell lines

Name	Function
Dulbecco's Modified Eagle Medium (DMEM with high glucose content)	Cell culture medium (Caco-2 and HT-29)
Hybri-Care Medium	Cell culture medium (FHs 74 Int)
Fetal Bovine Serum (FBS), 10%	A serum-supplement provides growth promoting and survival enhancing factors to cells in culture
Penicillin/Streptomycin (P/S), 10 kU/mL pen, 10 mg/mL strep	Antibiotics prevent bacterial contamination
L-glutamine (L-gln), 200 mM	A source of cellular energy, next to glucose
Sodium pyruvate (Na/Pyr), 100 mM	An additional source of energy for cells
Human transferrin (hTF), 1 mg/mL	An agent that binds and delivers ions in cell culture

4.3.7 Cytotoxicity Assay

Aleurone, germ and endosperm fractions of barley, corn and wheat were tested for their cytotoxicity using the MTT colorimetric assay according to the protocol provided by the manufacturer with some modifications. The flow chart of the cytotoxicity assay is displayed in **Figure 4.1**. Caco-2, HT-29 and FHs 74 Int cell suspensions (100 µL) were seeded in a 96 well plate and maintained until confluent (a concentration of 1×10^5 /mL) for 72 h. Following cell confluence, medium from all wells were aspirated. Then 100 µL of 20, 5 and 0.5 mg/mL sample extract and 100 µL of PBS for control wells were added. Blank

wells contained PBS only. Cells were treated and left in the incubator for 4 h. MTT reagent (10 μL) was added to all wells, including the control wells, and left in the incubator for 3 h. After 3 h, dots of purple precipitates were visible under the microscope and all 110 μL of treatment solution was then aspirated, and 100 μL of detergent reagent was added to all plates including the control. The plates were swirled gently. The plates were covered with aluminum foil and left in the dark for 15 min at room temperature. After 15 min, plate covers were removed and absorbance measured at 560 nm using an Opsy MR 96-well reader (Dynex Technologies, Chantilly, VA, USA). Cytotoxicity level (%) was then calculated by comparing absorbance of samples to that of controls. Analysis was done in quadruplicates.

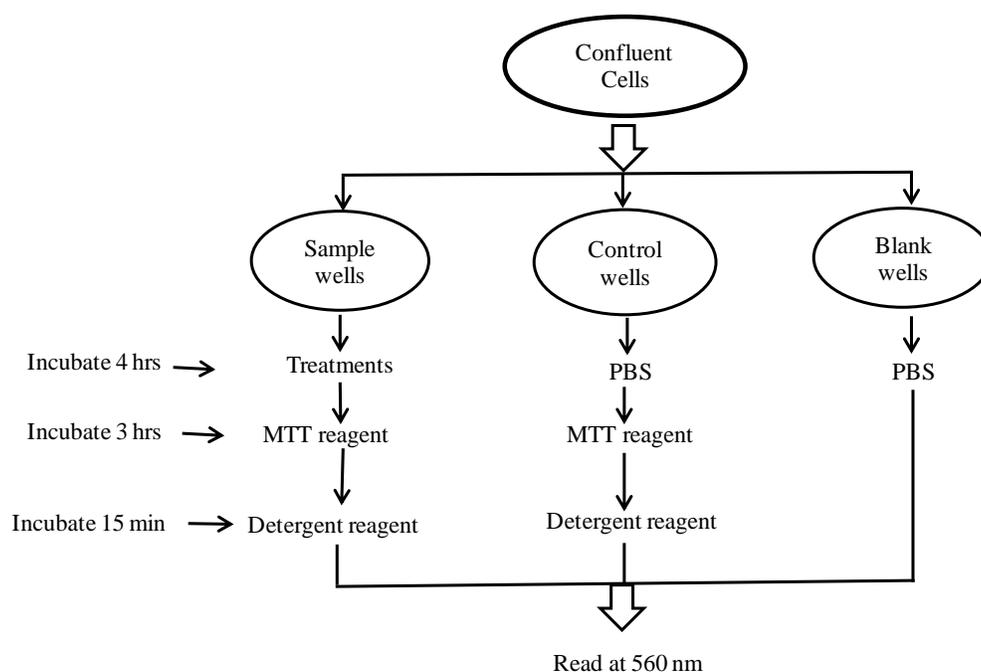


Figure 4.1 Flow chart of cytotoxicity using MTT assay

4.3.8 Cytoprotective Activity Against AAPH-Induced Oxidation

The cytoprotection of extracts of cereal grain fractions against AAPH-induced oxidation was determined by MTT assay according to Gliwa et al (2011) with some modifications. The flow chart of the cytoprotective activity assay is displayed in **Figure 4.2**. Caco-2, HT-29 and FHs 74 Int cells suspensions (100 μ L) were seeded in 96-well plates and maintained until confluent (a concentration of 1×10^5 /mL) for 72 h. Following cell confluence, medium from all wells were aspirated and then 100 μ L of 20, 5 and 0.5 mg/mL sample extract were added to appropriate sample wells and 100 μ L of PBS was added to control wells. Blank wells contained PBS only. After 2 h of incubation, 10 μ L of 3 mM AAPH was added to sample and AAPH positive control wells. Cells were incubated for another 4 h. After that, MTT reagent was added to all except the blank wells and the plates were incubated for 3 h until the intracellular punctate purple color was visible under the microscope. Detergent reagent was then added to all except the blank wells. The plates were swirled gently. The plates were covered with aluminum foil and left in the dark for 15 min at room temperature. After 15 min plate covers were removed and the plate was measured for absorbance at 560 nm using an Opsys MR 96-well reader (Dynex Technologies, Chantilly, VA, USA). Cytotoxicity level (%) was then calculated by comparing absorbance to that of controls. Analysis was done in quadruplicates.

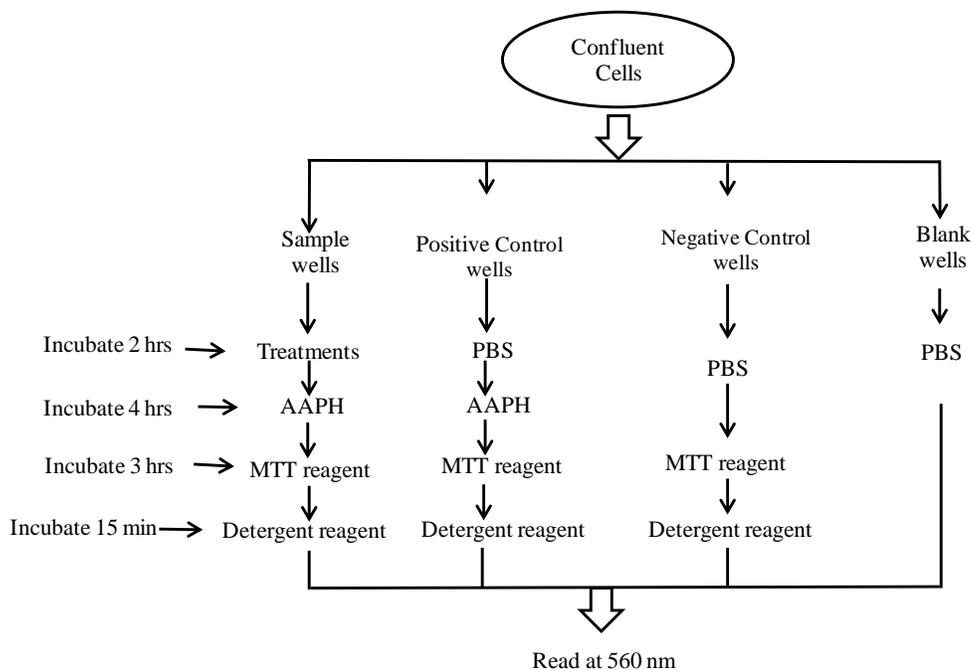


Figure 4.2 Flow chart of cytoprotective activity assay against AAPH induced oxidation

4.3.9 Cytoprotective Activity Against H₂O₂-Induced Oxidation

The cytoprotective activity against H₂O₂-induced oxidation assay was performed as described in 4.3.8, but 1 mM of H₂O₂ was used instead of 3 mM of AAPH. The flow chart of cytoprotective activity assay is displayed in **Figure 4.3**. Analysis was done in quadruplicates.

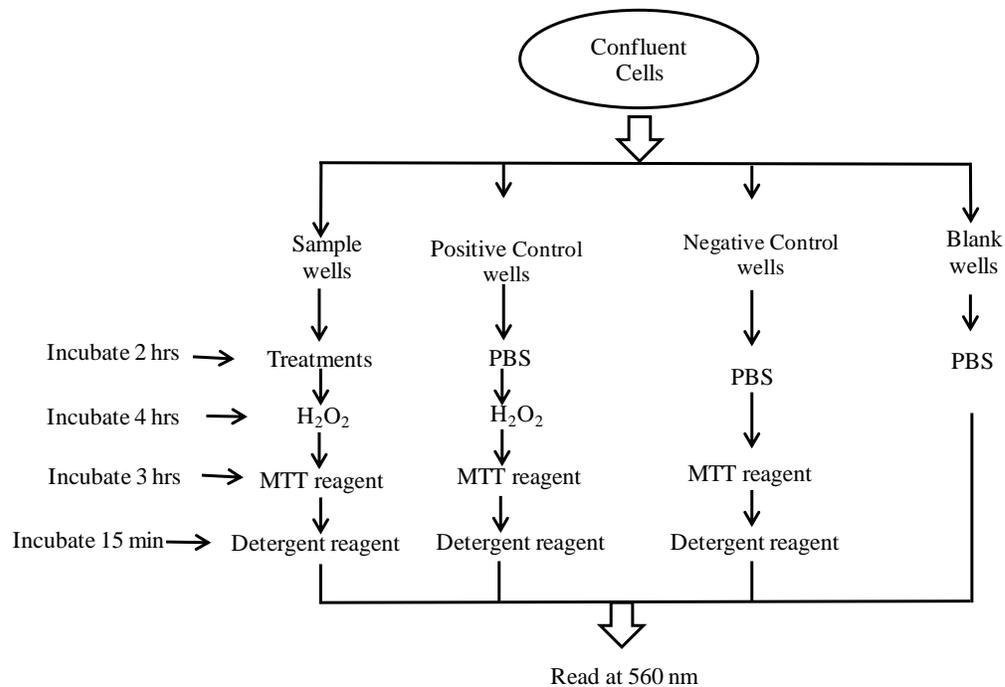


Figure 4.3 Flow chart of cytoprotective activity assay against H₂O₂ induced oxidation

4.3.10 Intracellular Oxidative Stress

Determination of intracellular oxidative stress was based on the oxidation of CM-H₂DCFDA to yield an intracellular-trapped fluorescent compound. Aleurone, germ and endosperm fractions of barley, corn and wheat were then tested for their effect on cellular oxidative status according to Wolfe and Liu (2007) with some modifications. Caco-2, HT-29 and FHs 74 Int cell suspensions (100 μ L) were seeded in a 96 well plate and maintained until confluent (a concentration of 1×10^5 /mL) for 72 h. The media from all wells were aspirated and 100 μ L of 20, 5 and 0.5 mg/mL sample extract and 100 μ L of PBS for positive control wells were added. Blank wells contained PBS only. The cells were treated and left for 1 h. At 30 min of incubation, 100 μ L of 10 μ M CM-H₂DCFDA solution was added to all wells. The final concentration of CM-H₂DCFDA was then 5 μ M. After 1 h of

incubation, all 200 μL of treatment solutions was removed and 100 μL of 500 μM AAPH dissolved in PBS was added to all wells except the negative control (100 μL of PBS was added instead). The plates were then read immediately by Fluoroskan Ascent FL 96-well plate reader (ThermoLabsystems, Franklin, MA, USA). Temperature was set at 37 $^{\circ}\text{C}$, emission wavelength at 527 nm, and excitation wavelength at 485 nm, and measurements were taken every 30 min for 2 h. The analysis was done in quadruplicates. The CAA value was calculated by integrating area under the curve (AUC) for each sample and standard using final assay values and their linear regression formula below:

$$\text{AUC} = 1 + \frac{\text{RFU}_1}{\text{RFU}_0} + \frac{\text{RFU}_2}{\text{RFU}_0} + \frac{\text{RFU}_3}{\text{RFU}_0} + \dots + \frac{\text{RFU}_{120}}{\text{RFU}_0} \quad [1]$$

RFU_0 = relative fluorescence value of time zero

RFU_x = relative fluorescence value of time point

The AUC values were used to determine CAA values according to the formula:

$$\text{CAA Value} = 100 - \left(\frac{\text{AUC}_{\text{Antioxidant}}}{\text{AUC}_{\text{Control}}} \right) \times 100 \quad [2]$$

4.3.11 Microscopic Imaging of Real Time Cells Oxidizing 2',7'-Dichlorodihydrofluorescein Diacetate (H_2DCFDA)

Microscopic imaging of real time cells oxidizing H_2DCFDA in the presence of AAPH was conducted. Specifically, HT-29 cell line were grown, trypsinized, washed and plated onto a 22 mm^2 sterile cover slide. Cells were left to grow to 90% confluence in Inoue chamber at 37 $^{\circ}\text{C}$ in a 5% CO_2 humidified incubator for 48 h. The medium from the cover slide was aspirated and the cover slide and main body were joined using GE Bayer Silicones to make a chamber. Hundred μL of 5 mg/mL sample extracts and 100 μL of PBS

for the control chamber were then added. The cells were incubated for 30 min at 37 °C in a 5% CO₂ humidified incubator. DCFH-DA solution (100 µL of 10 µM) was then added to the chambers. After 1 h of incubation, all 200 µL of treatment solutions were aspirated and 100 µL 500 µM AAPH dissolved in PBS was added to all chambers. The cells were then viewed with fluorescent microscope (Olympus IX-70) connected to a Xenon Arc Lamp (Sutter DG4), computer and, camera (Hamamatsu C4880-80). The combination wavelengths setting was used and was set at Fura2 emission, 510 nm, and BCF emission, 531 nm, and images were captured every 30 min for 2 hrs.

4.3.12 Microarray Analysis

Homogenization: RNA from HT-29 cells treated with 20 mg/mL of carotenoid extract of corn fractions for 3 hrs was isolated using TRIZOL reagent and RNeasy mini kit (Qiagen Inc, Toronto, ON, Canada) according to manufacturers' instructions. The cells were lysed by adding 1.5 mL of TRIZOL reagent followed by 30 sec vortexing using a mini vortexer (VWR, Mississauga, ON, Canada) and 5 min incubation at room temperature. Thereafter, 0.3 mL of chloroform was added followed by vigorous shaking for 30 seconds and 3 min incubation at room temperature. The homogenate was centrifuged using a Centrifuge 5804 R (Eppendorf Canada, Mississauga, ON, Canada) at 12,000 g for 15 min at 4 °C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL reagent used for homogenization.

RNA Precipitation: The RNA was precipitated from the aqueous phase by mixing with 0.75 mL isopropyl alcohol per 1.5 mL of TRIZOL reagent used for the initial homogenization. The samples were incubated at -20 °C for 1 hr and transferred to -80 °C overnight and centrifuged at 12,000 g for 20 min at 4 °C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

RNA Wash: The supernatant was removed. The RNA pellet was washed once with a series of 1 mL, 550 µL, and 450 µL of 75% ethanol. The samples were then mixed by vortexing and centrifuged at 10,000 g for 2 min at 4 °C for each series of cleaning followed by discarding the ethanol. The sample was vacuum dried using a Savant™ DNA SpeedVac™ Concentrator (Thermo Scientific, Suwanee, GA, USA) for 3 min (not completely drying the pellet) followed by reconstituting with 60 µL of molecular biology graded water (DNase, RNase and protease free). Pellet reconstitution was done in a cold room.

RNA Cleanup: RNeasy Mini Protocol for RNA Cleanup was used for cleaning the extracted RNA according to manufacturers' instructions. Purity of the extracted RNA was assessed by measuring the 260/280 nm absorbance ratio using NanoDrop UV-Vis 2000 Spectrophotometer (NanoDrop products, Thermo Scientific, Wilmington, DE, USA). Isolated RNA was stored at -80 °C until analysis.

Quantitative Real-time Polymerase Chain Reaction Analysis: An equal amount of RNA (500 ng) was reverse transcribed to cDNA using MyCycler Thermal Cycle (BioRad, Foster, CA, USA) and RT² First strand kit (Qiagen Inc, Toronto, ON, Canada) according to the manufacturer's instructions. Samples were diluted in quantitative polymerase chain reaction (qPCR) master mix (RT² SYBR Green; Qiagen Inc, Toronto, ON, Canada)

according to the supplier's directions and pipetted into 96-well PCR array plates. Real-time PCR for the human oxidative stress and antioxidant defense PCR array (PAHS-065Y; Qiagen Inc, Toronto, ON, Canada) consisting of 84 antioxidant enzymes with 3 RT controls and 3 positive PCR controls, a genomic contamination control, and 5 constitutively expressed housekeeping genes were performed according to the manufacturer's instructions using ABI 7500 standard Real-time PCR system (Applied Biosystems, Foster, CA, USA) with the following cycle conditions: 95 °C for 3 min followed by 40 cycles of 95 °C for 3 s, 60 °C for 20 s and 72 °C for 32 s and, 95 °C for 15 s followed by 60 °C for 1 min and 95 °C for 15 s. Three independent replicates of each treatment with or without aleurone, germ and endosperm fractions of corn were performed and data analyzed using software from the SABioscience website (2014).

4.3.13 Statistical Analysis

Data obtained were analyzed as complete randomized design (CRD)-factorial plus control using the one-way analysis of variance (ANOVA) with GLIMMIX Procedure of SAS, release 9.3 (SAS 2014; SAS Institute). Data obtained from microarray analyses were analyzed using a 2-tailed *t* test assuming equal variance. Sample means were compared using Tukey-Kramer multiple comparison procedure and data were considered significantly different when $P < 0.05$. All results are presented as mean \pm standard error.

4.4 Results and Discussion

4.4.1 Cytotoxicity Assay

The MTT colorimetric assay evaluated the cytotoxicity of aleurone, germ and endosperm fractions of barley, corn and wheat. The cytotoxicity assay was performed to confirm that the sample treatments had minimal negative effect on the viability of the cell line. Compared to the untreated control, extracts were not cytotoxic to Caco-2 (84-115% live cells; **Figure 4.4a; Table 4.2**). Analysis of variance of cytotoxicity data from factorial plus control showed no significant differences between the interactions, but showed significant effects between fractions ($P < 0.02$) which did not pose any toxicity (**Figure 4.4; Table 4.2**). An extract is considered toxic if it is lethal to 50% of the organisms exposed to it in a toxic test. Therefore, our results indicate that different fractions, concentrations and sources had no negative effect on cell viability. Similar trends were observed with FHs 74 Int and HT-29 cell lines (**Figure 4.4b,c**). There was no nutrient impact described by the cytotoxicity assay; however, the cytoprotective and intracellular oxidative stress assays further explored antioxidant activity of the sample treatment with different concentrations (0.5, 5 and 20 mg/mL). In order to determine the optimal concentration of AAPH to induce cellular oxidative stress in Caco-2, HT-29 and FHs 74 Int, we constructed dose dependent curves. AAPH concentrations of 0, 1, 3, 5, 10, 15, 20, 25 mM prepared in PBS were incubated with confluent cells in 96 well plates for 4 h. Cell death by AAPH-induced oxidation was determined by MTT assay and expressed as cell viability (%). An escalating concentration of AAPH from 0 to 25 mM resulted in an increasing cell loss in all the cell lines. After 4 h of treatment with AAPH, up to 45-55% loss of cell viability was observed (**Figure 4.5a**). Different LD₅₀ (the concentration of a

substance that is lethal to 50% of the organisms exposed to it in a toxic test) obtained from concentration-response curves of the three cell lines indicated Caco-2 cells ($LD_{50} = 42.9$) are more sensitive than FHs 74 Int ($LD_{50} = 37.3$) and HT-29 cells ($LD_{50} = 22.8$) in the presence of AAPH. On the basis of the results, 3 mM of AAPH was selected to stimulate sufficient levels of oxidation in the subsequent study.

On the other hand, H_2O_2 concentrations of 0, 1, 3, 5, 10 mM prepared in PBS were incubated with confluent cells in 96 well plates for 4 h. Cell death by H_2O_2 -induced oxidation was determined by MTT assay and expressed as cell viability (%). An escalating concentration of H_2O_2 from 0 to 10 mM resulted in an increasing cell loss in all the cell lines. After 4 h of treatment with H_2O_2 , up to 45-55% loss of cell viability was observed (**Figure 4.5b**). On the basis of the results, 1 mM of H_2O_2 was selected to stimulate sufficient levels of oxidation in the subsequent study.

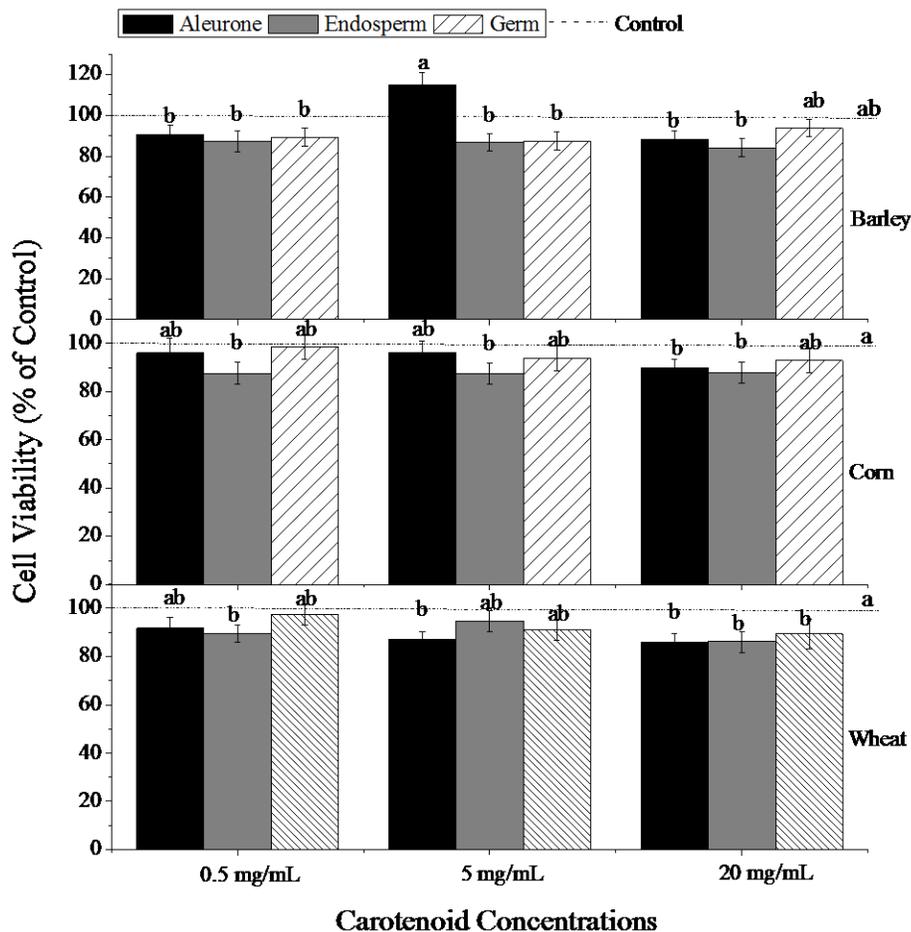


Figure 4.4a Cytotoxicity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL in confluent Caco-2 cell line measured by MTT assay. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

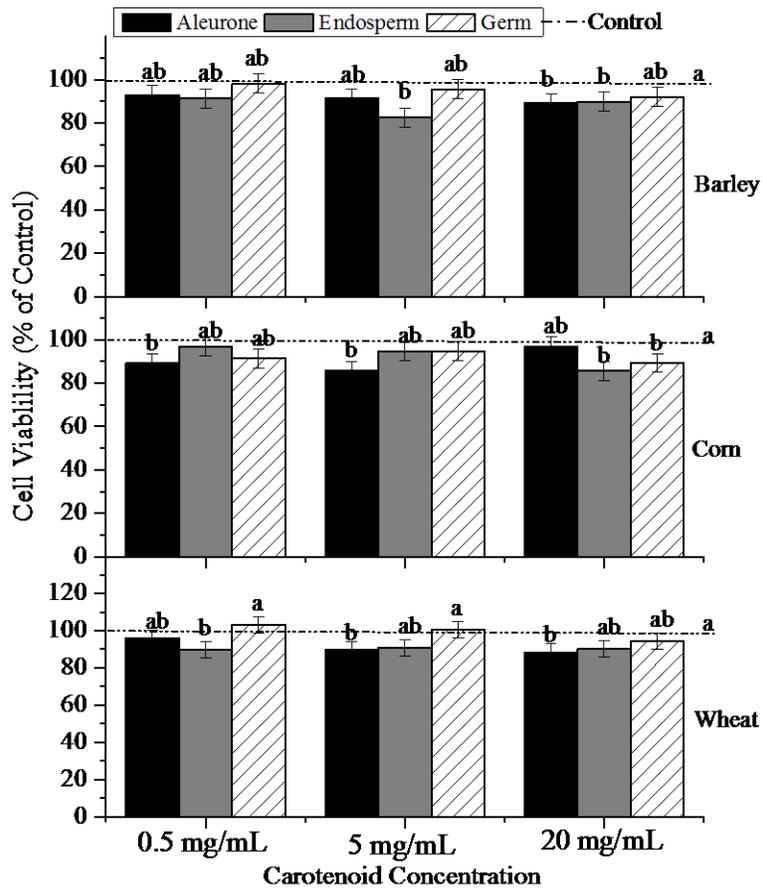


Figure 4.4b Cytotoxicity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL in confluent HT-29 cell line measured by MTT assay. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

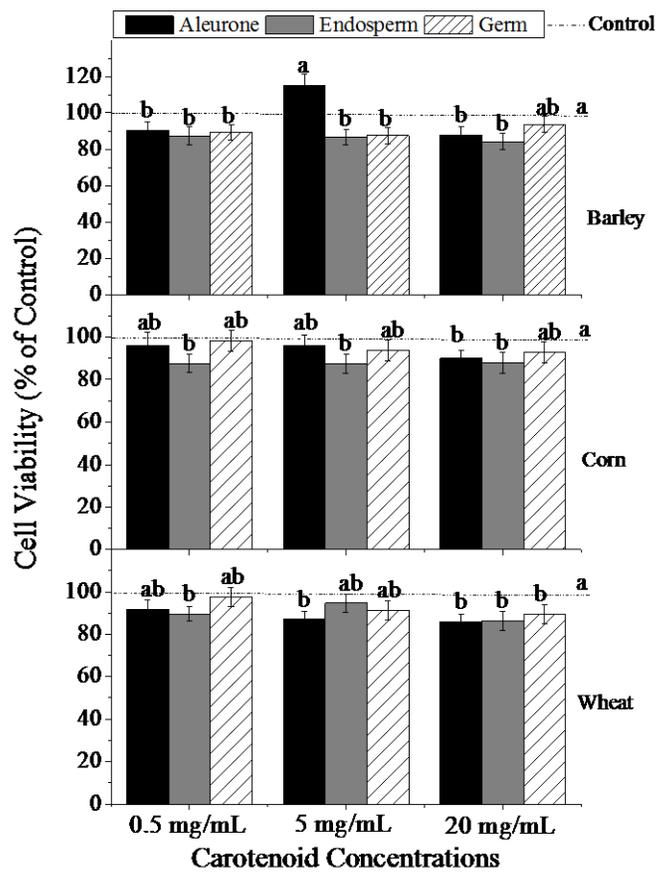


Figure 4.4c Cytotoxicity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL in confluent FHs 74 Int cell line measured by MTT assay. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

Table 4.2 ANOVA table of cytotoxicity and cytoprotective activity of aleurone, germ and aleurone fractions of barley, corn and wheat as measured by MTT assay

	Cytotoxicity activity	Cytoprotective activity against AAPH	Cytoprotective activity against H ₂ O ₂
		—P-value—	
source	0.65	0.85	<0.0001
fraction	0.02	0.11	0.006
Source*fraction	0.05	<0.0001	<0.0001
concentration	0.07	0.17	0.0002
source*concentration	0.35	<0.0001	0.0009
fraction*concentration	0.12	<0.0001	0.0003
Source*fraction*concentration	0.09	<0.0001	0.01

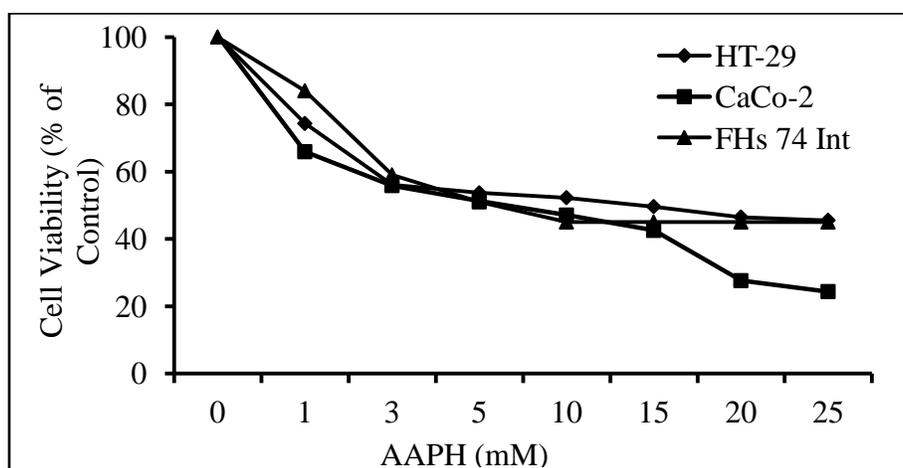


Figure 4.5a Cytotoxicity of AAPH peroxy radical generated at 0 to 25 mM in Caco-2, HT-29 and FHs 74 Int cell measured by MTT assay

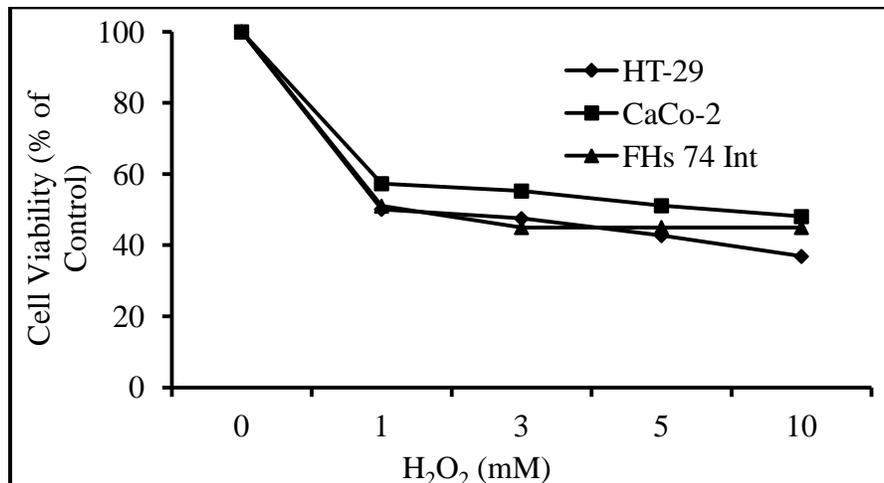


Figure 4.5b Cytotoxicity of H₂O₂ peroxy radical generated at 0 to 10 mM in Caco-2, HT-29 and FHs 74 Int cell measured by MTT assay

4.4.2 Cytoprotective Activity of Aleurone, Endosperm and Germ extracts of Barley, Corn and Wheat against AAPH-induced cytotoxicity

Based on the cytotoxicity results, 0.5, 5 and 20 mg/mL of aleurone, endosperm and germ fractions extracts were applied against 3 mM AAPH-induced oxidative damage in Caco-2, HT-29 and FHs 74 Int cells. Analysis of variance of AAPH-induced cytotoxicity data demonstrated insignificance for the main factors but showed significant effects for all the interactions ($P < 0.0001$; **Table 4.2**). This therefore, indicated different sources, fractions and concentrations attenuate free radical toxicity differently. The cell viability increased from 45.5% (cell treated with 3 mM of AAPH only) to a maximum of 95.1% (cell pre-treated with 0.5, 5 and 20 mg/mL of aleurone, germ and endosperm extracts followed by 3 mM of AAPH) (**Figure 4.6a; Table 4.2**). Similarly, 0.5, 5 and 20 mg/mL of extracts suppressed the AAPH initiated oxidation damage in Caco-2 and FHs 74 Int cells (**Figure 4.6b,c; Table 4.2**). Elisia and Kitts (2008) have previously indicated that AAPH-

initiated loss of cell viability in Caco-2 cells is partially attributed to the inductive effect of AAPH on cell apoptosis. AAPH under thermal decomposition produces peroxy radicals in a cultured cell, and this will increase lipid peroxidation, damage cell membrane integrity, and eventually induce cell apoptosis (Kulkarni et al., 2008). In this study, all concentrations of cereal grain fractions displayed effective protection in Caco-2, HT-29 and FHs 74 Int cells against AAPH-induced cell loss. Thus carotenoid extracts from cereal grain fractions may function as a free radical cleaner and prevent the formation of apoptosis cells caused by AAPH oxidation.

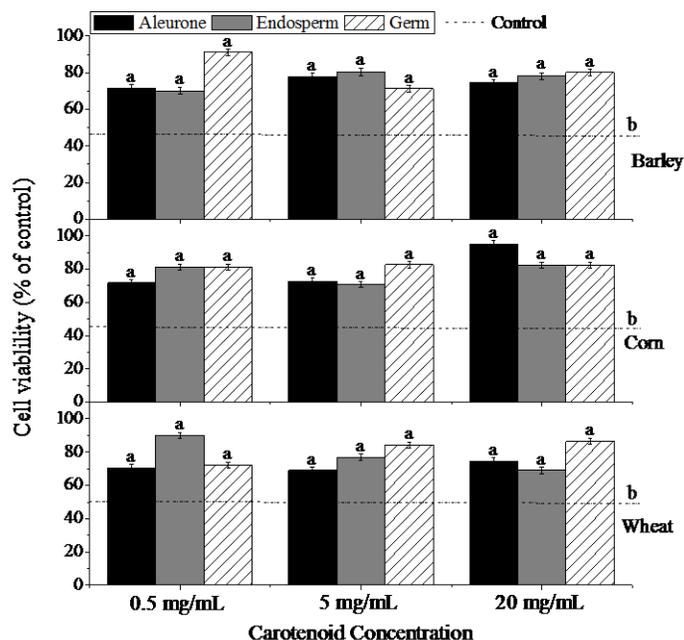


Figure 4.6a Cytoprotective activity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, n = 4) in confluent HT-29 cell line against AAPH-induced oxidation. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

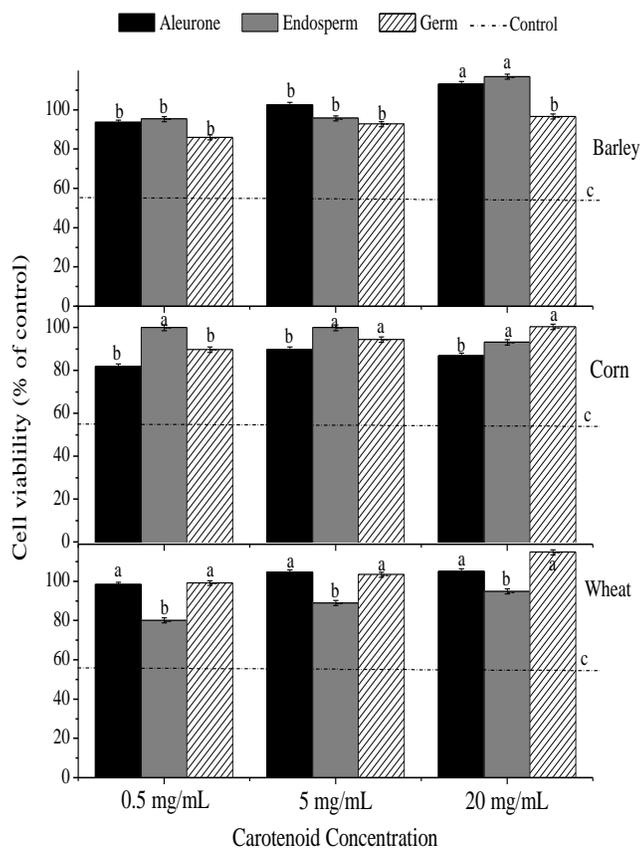


Figure 4.6b Cytoprotective activity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, $n = 4$) in confluent Caco-2 cell line against AAPH-induced oxidation. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

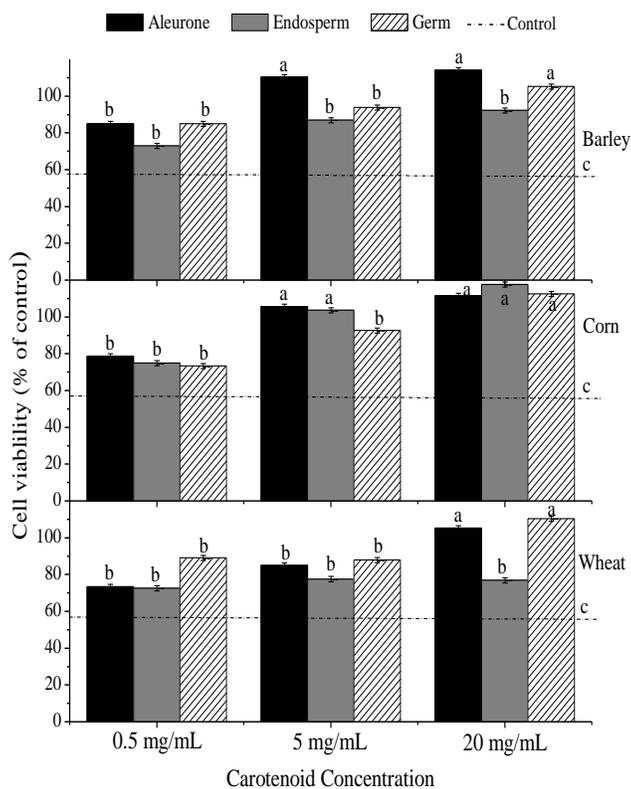


Figure 4.6c Cytoprotective activity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, n = 4) in confluent FHs 74 Int cell line against AAPH-induced oxidation. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

4.4.3 Cytoprotective Activity of Aleurone, Endosperm and Germ extracts of Barley, Corn and Wheat against H₂O₂-induced cytotoxicity

Based on the cytotoxicity results, 0.5, 5 and 20 mg/mL of aleurone, endosperm and germ fraction extracts were applied against 1 mM H₂O₂-induced oxidative damage in Caco-2, HT-29 and FHs 74 Int cells. Analysis of variance of H₂O₂-induced cytotoxicity data was not significant for the main factors but showed significant effects for all the interactions ($P < 0.01$; **Table 4.2**). This therefore, indicated different sources, fractions and concentrations attenuate free radical toxicity differently. The cell viability increased from 50% (cell treated with 1 mM of H₂O₂ only) to a maximum of 95.1% (cell pre-treated with 0.5, 5 and 20 mg/mL of aleurone, germ and endosperm extracts followed by 1 mM of H₂O₂) (**Figure 4.7a; Table 4.2**). Similarly, 0.5, 5 and 20 mg/mL of extracts suppressed the H₂O₂ initiated oxidation damage in Caco-2 and FHs 74 Int cells (**Figure 4.7b,c; Table 4.2**). Most concentrations of cereal grain fractions displayed effective protection in Caco-2, HT-29 and FHs 74 Int cells against H₂O₂-induced cell loss except 20 mg/mL which showed no change in FHs 74 Int cells. Thus carotenoid extracts from cereal grain fractions may function as free radical cleaners and prevent the formation of apoptosis cells caused by H₂O₂ oxidation.

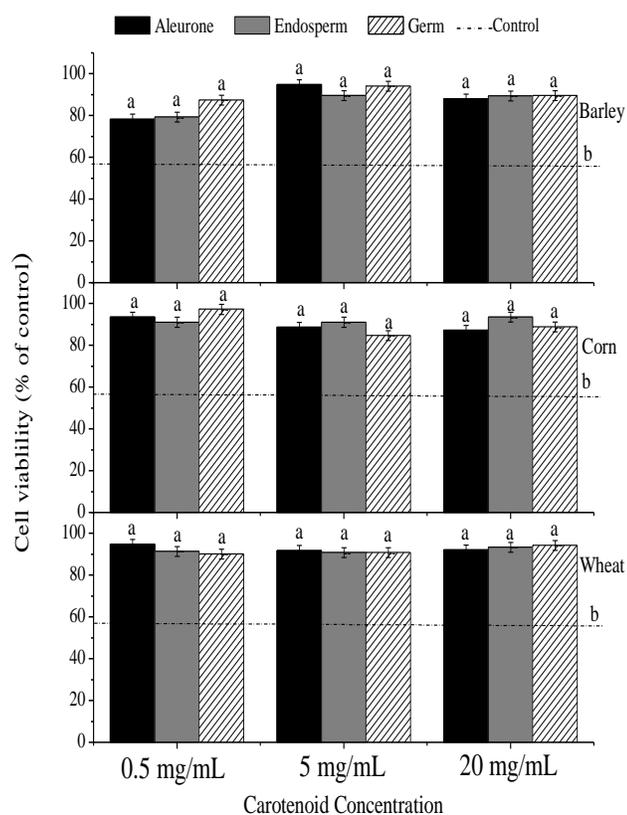


Figure 4.7a Cytoprotective activity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, n = 4) in confluent HT-29 cell line against H₂O₂ induced oxidation. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

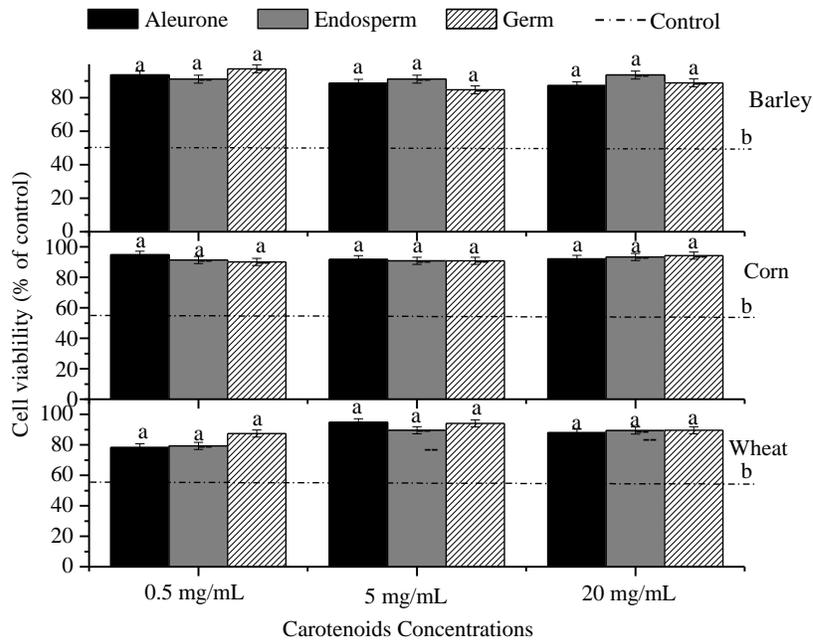


Figure 4.7b Cytoprotective activity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, $n = 4$) in confluent Caco-2 cell line against H_2O_2 induced oxidation. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

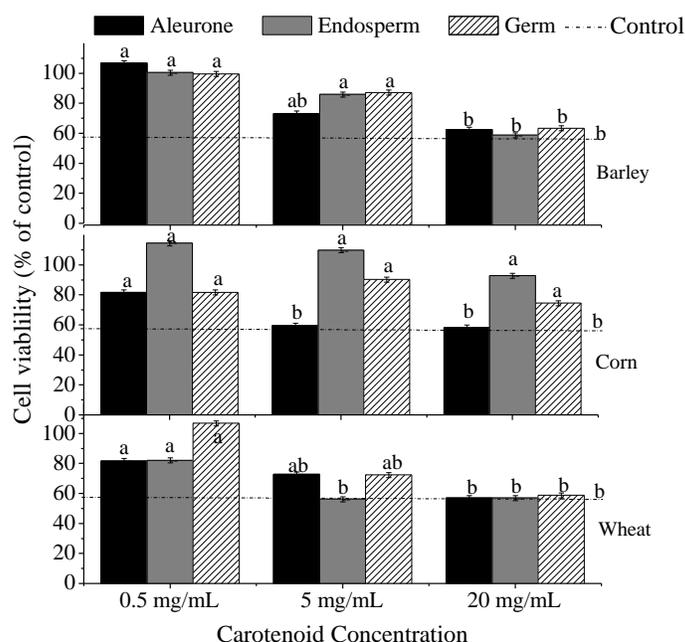


Figure 4.7c Cytoprotective activity of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, $n = 4$) in confluent FHs Int cell line against H_2O_2 induced oxidation. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

4.4.4 Intracellular Oxidative Stress

CM- H_2DCFDA is a fluorogenic dye for measuring hydroxyl, peroxy and other ROS activity within the cell. After diffusion into the cell, CM- H_2DCFDA is deacetylated by cellular esterases to a non-fluorescent compound which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF is a highly fluorescent compound that can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm, respectively. The reaction of sample-treated cell lines against APPH-induced free

radical oxidation is shown in **Figure 4.8-4.10**. AAPH induced the oxidation of the dye CM-H₂DCFDA to DCF. The fluorescence intensity of DCF was measured to represent the rate of oxidation. Positive (+) controls showed the fluorescence intensity of the AAPH oxidation over time, while negative (-) control were cells to which AAPH was not added. They were used to illustrate conditions devoid of any oxidation inducer. Cell lines treated with cereal fractions carotenoid extracts showed the inhibition of oxidation as their fluorescence intensities were higher than the negative control but less than the positive control over time. CAA units were then calculated (equation 1 and 2) based on the fluorescence intensity of the fraction extracts and AAPH-treated cells. In Caco-2 cell line, CAA ranged from 5-55 CAA units (**Figure 4.8**). Wheat fraction always showed the highest antioxidant activity whereas barley fractions showed the lowest CAA units. In FHs 74 Int cell line, CAA ranged from 15-63 CAA units (**Figure 4.9**). Barley germ at 0.5 mg/mL showed higher CAA units than wheat which had the lowest. Corn endosperm had highest antioxidant activity while wheat had the lowest CAA units. Barley and wheat aleurone fractions at 20 mg/mL showed highest and the lowest CAA units, respectively. Using HT-29 cell line, CAA ranged from 5-38 CAA units (**Figure 4.10**). Corn fraction always showed the highest antioxidant activity whereas barley fractions showed the lowest CAA units. On the basis of current study, all tested doses inhibited ROS as shown by increase in CAA units ($P < 0.05$).

Figure 4.11 shows the fluorescent intensity of HT-29 cells exposed to AAPH-induced oxidation. Corn fractions at concentration of 5 mg/mL were selected to display the inhibitory effect of digested extract. **Figure 4.11 (a)-(c)** showed the intensity level of cells incubated with sample treatment. The fluorescence gradually increased and finally was

maintained at a constant level. **Figure 4.11 (d)** exhibited the positive control. The fluorescence intensity increased rapidly and was higher than that of sample treatments. This thereby indicated the ability of corn fractions extracts to reduce AAPH-induced cellular oxidation.

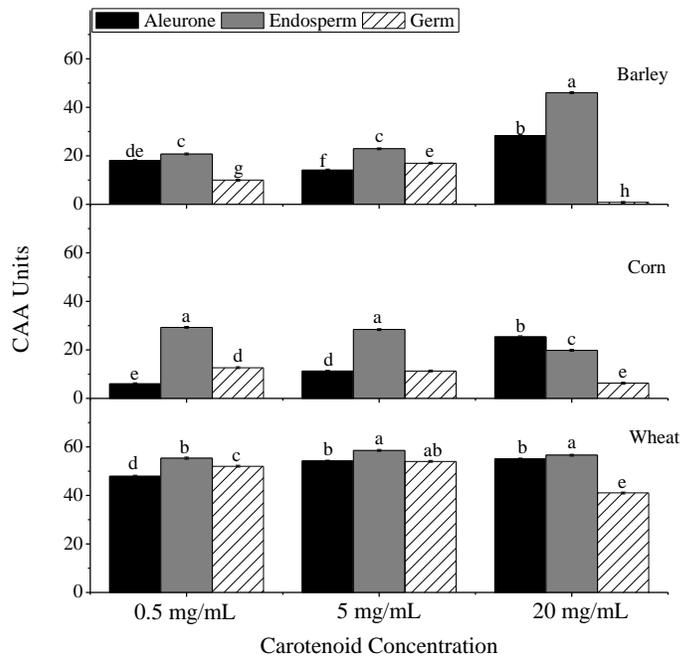


Figure 4.8 CAA values of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, $n = 8$) using Caco-2 cell line. Different letters indicate significant differences ($p < 0.05$), Tukey-Kramer's multiple range test) among fractions for each cereal.

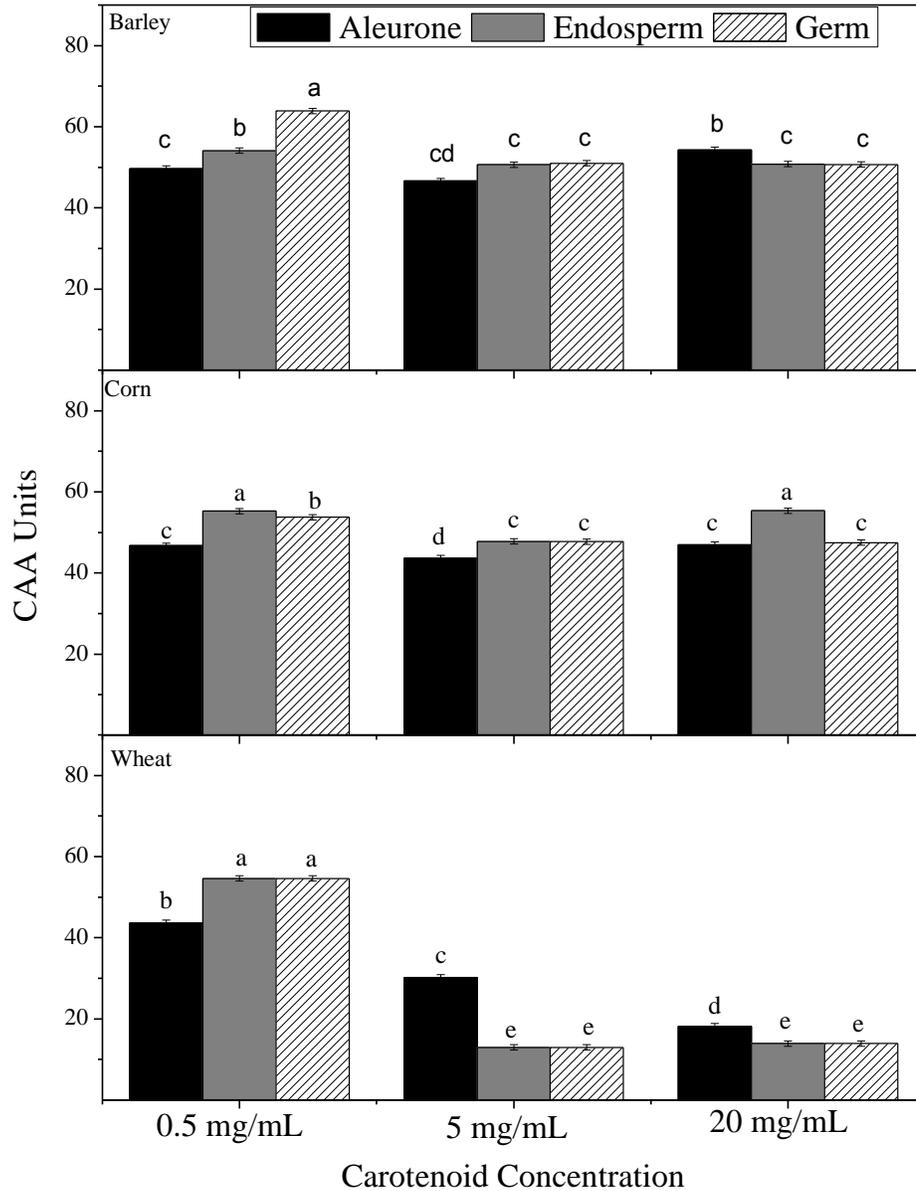


Figure 4.9 CAA values of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, $n = 8$) using FHs 74 Int cell line. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

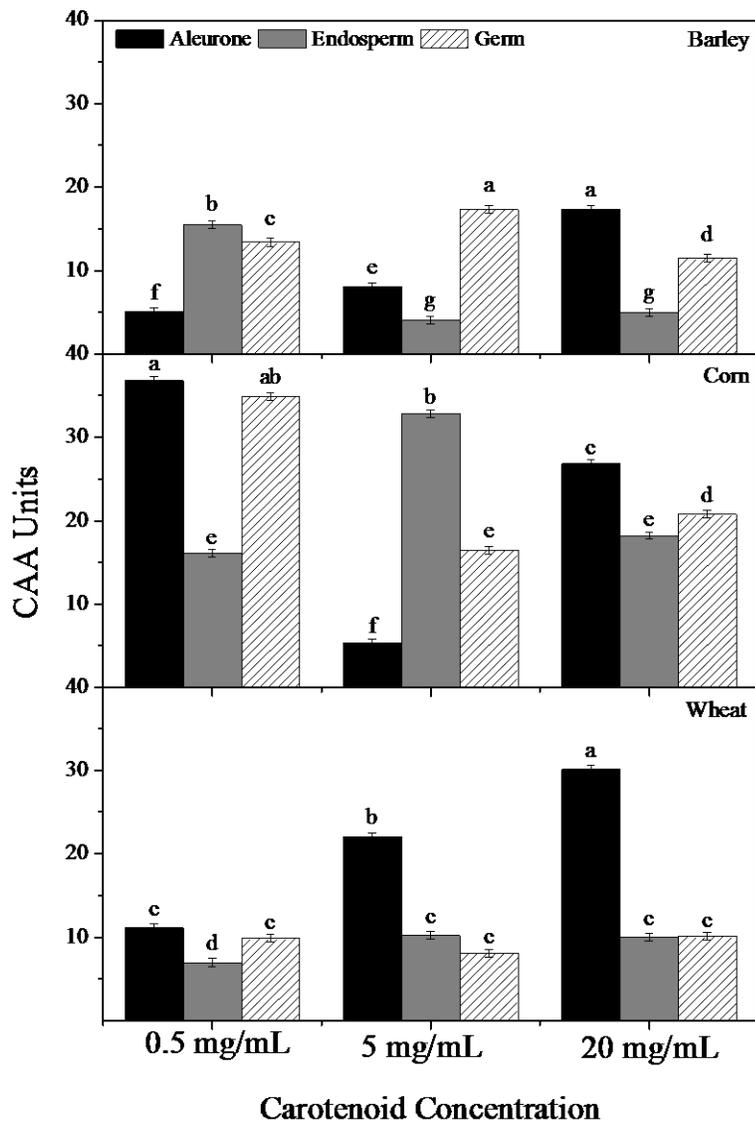


Figure 4.10 CAA values of aleurone, germ, and endosperm fractions of barley, corn and wheat at different concentrations, 0.5, 5, and 20 mg/mL (mean \pm standard error, n = 8) using HT-29. Different letters indicate significant differences ($p < 0.05$, Tukey-Kramer's multiple range test) among fractions for each cereal.

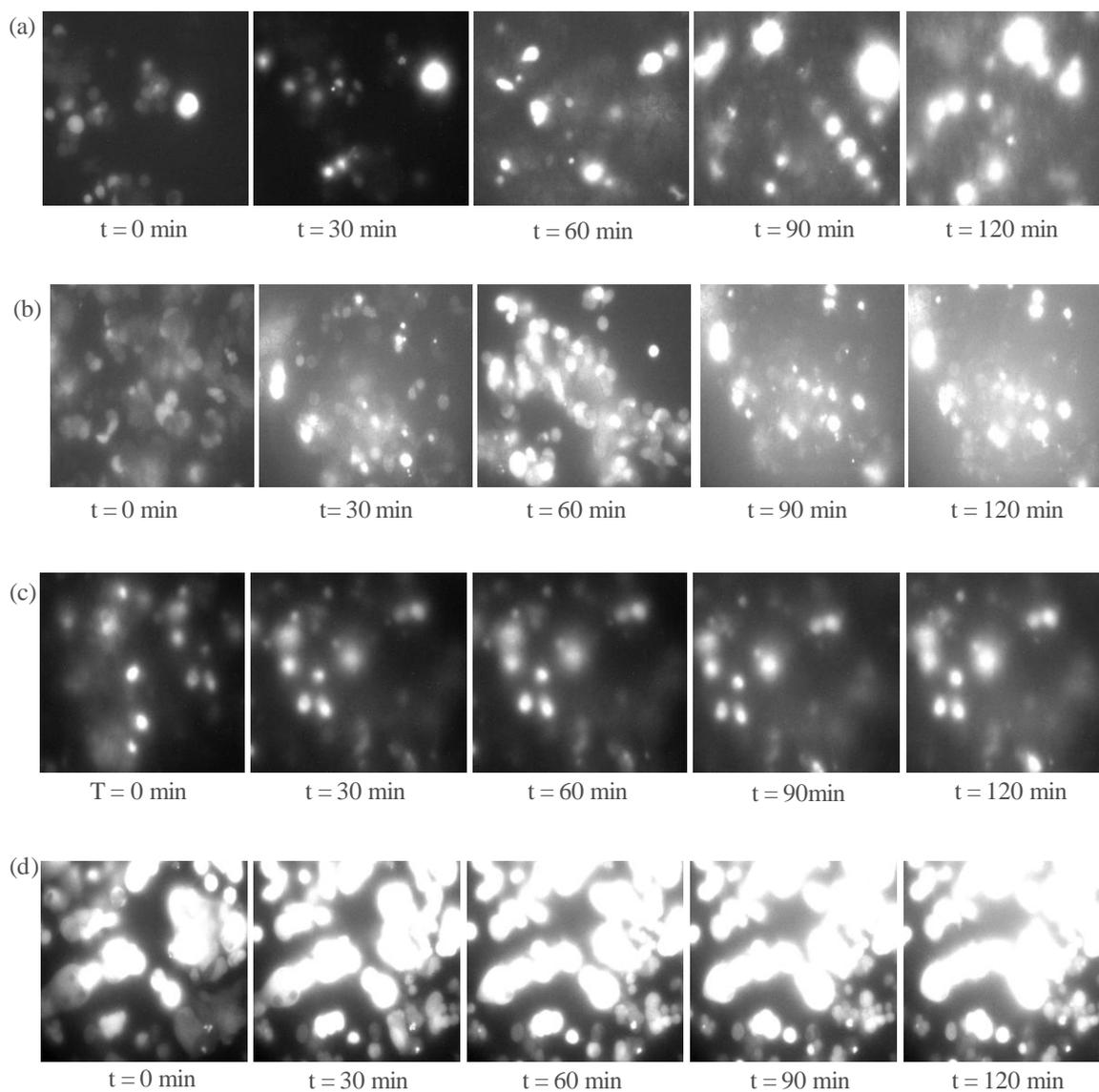


Figure 4.11 Fluorescence intensity of AAPH-induced oxidation with aleurone (a), endosperm (b), germ (c) and without (d) addition of corn fractions (5 mg/mL) for cellular antioxidant activity assay at different incubation time in HT-29 cell line.

4.4.5 Aleurone, Endosperm and Germ fractions of Corn and Antioxidant Gene

Regulation in HT-29 Intestinal Cells

Based on the TCC, lutein and zeaxanthin levels, cytoprotective activity and CAA results, HT-29 were treated with 20 mg/mL of aleurone, endosperm and germ fractions of corn to assess abundance of oxidative stress-related genes. Extracted RNA was determined spectrophotometrically at 260/280 nm with NanoDrop UV-Vis 2000 (NanoDrop Technologies, Inc) to be $A_{260/280} = 1.91$. Of the 84 antioxidant pathway genes included in this oxidative stress and antioxidants defense array, the expression of 28 genes was altered by corn aleurone, endosperm and germ treatments ($P < 0.05$) (**Figure 4.12**). However, only myoglobin (MB), neutrophil cytosolic factor 1 (NCF1) and NADPH oxidase, EF-hand calcium binding domain 5 (NOX5) showed 2-fold upregulation and 2-fold down regulation of chemokine (C-C motif) ligand 5 (CCL5), BCL2-associated athanogene 2 (BAG) and solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11 (SLC7A11) in different fractions (**Table 4.3**). However, P -values did not indicate statistical significance for NOX5 and CCL5 in all fractions, NCF1 in endosperm and SCL7A11 in aleurone and germ. The microarray antioxidant enzymes are enzymes that catalyse the reduction of hydroperoxides and other organic hydroperoxides. The upregulation of 28 genes therefore represents a cell-protective mechanism by which intestinal cells manage oxidative stress elicited by exposure to aleurone, germ and endosperm fractions.

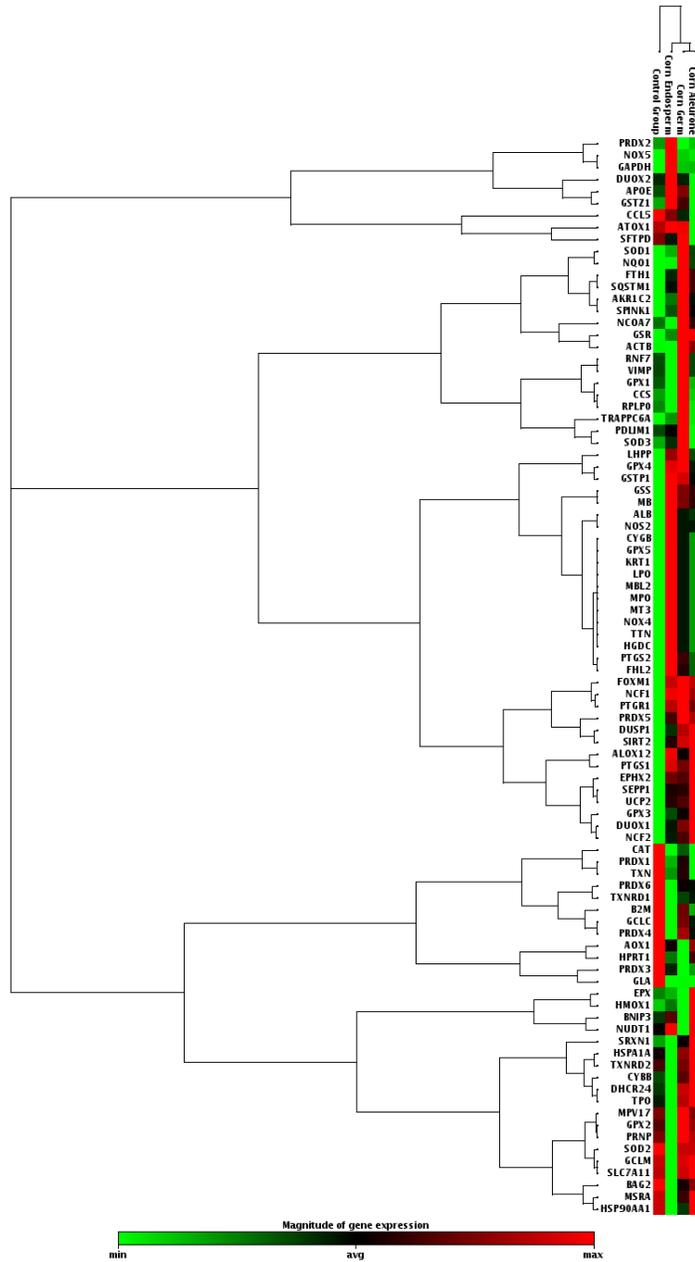


Figure 4.12 Clustergram or heatmap analysis: The clustergram or heatmap represents expression levels of 84 genes in four conditions (control, endosperm, germ and aleurone of corn) relative to an average of five housekeeping genes. Each horizontal line in the heatmap represents a single gene, and the columns represent groups. Samples clustered according to the condition. Red represents high expression, while green represents low expression.

Table 4.3 Effect of aleurone, germ, and endosperm fractions of corn on human oxidative stress gene expression in HT-29 cell line

Symbol	Description	Fold Change			P-value		
		Endo	Germ	Aleu	Endo	Germ	Aleu
CCL5	Chemokine (C-C motif) ligand 5	1.01	-2.36	-2.30	0.947	0.120	0.080
BAG2	BCL2-associated athanogene 2	-2.21	0.76	0.79	0.001	0.014	0.034
SLC7A11	Solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11	-2.06	1.10	1.07	0.003	0.501	0.514
NOX5	NADPH oxidase, EF-hand calcium binding domain 5	2.33	1.22	1.57	0.126	0.375	0.070
MB	Myoglobin	2.12	1.78	1.70	0.017	0.022	0.015
NCF1	Neutrophil cytosolic factor 1	2.02	2.92	2.63	0.236	0.019	0.034

List of differentially expressed genes. Fold change represents the average of DNA expression level in HT-29 cells treated with aleurone, germ, and endosperm fractions of corn. n=3 in each condition. Endo = Endosperm; Aleu = Aleurone.

4.5 Conclusion

Using MTT assay, it was found that carotenoid extracts were not cytotoxic in Caco-2, HT-29 and FHs 74 Int cell lines. The antioxidant capacity of carotenoid extracts of cereal grain fractions were demonstrated to be effective in suppressing AAPH-initiated intracellular oxidation. The carotenoid extracts were also shown to protect Caco-2, HT-29 and FHs 74 Int cell lines against AAPH- and H₂O₂-induced cytotoxicity. The protective effect of carotenoids extracts against AAPH- and H₂O₂-induced cytotoxicity was, however dependent on cell line tested and the concentration of the extract used to pre-treat the cells. Moreover, of the 84 antioxidant pathway genes included in microarray array analysis (HT-29 cells), the expressions of 28 genes were enhanced. Our findings suggest that carotenoids

of germ, aleurone, and endosperm fractions improved antioxidant capacity and thus have the potential to mitigate oxidative stress.

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SECTION C: *IN VIVO* MODEL

CHAPTER 5

Dietary Corn Fractions Reduce Atherogenesis in Low Density Lipoprotein Receptor Knockout Mice

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5.1 Abstract

Accumulating evidence has suggested that intake of whole grains is a protective factor against pathogenesis of coronary artery disease. The exact mechanisms, however, are still not clearly understood. We hypothesized that adequate intake of corn fractions (aleurone, endosperm and germ) reduce atherosclerotic lesion development through alterations in lipid profile in LDL receptor deficient (LDLr-KO) mice. Four groups of male LDLr-KO mice were fed with the experimental diets supplemented with (treated) or without (control) 5% (w/w) of each of hand-separated corn fraction including aleurone, endosperm and germ for 10 weeks. All diets were supplemented with 0.06% (w/w) cholesterol. Supplementation of corn fractions did not change weight gain rate or food intake in LDLr-KO mice during the study. Consumption of aleurone and germ fractions significantly reduced the size of atherosclerotic lesions in the aortic roots of the mice. This was also accompanied by reduced hepatic fat deposits as compared to those in the control group. These effects were associated with significant reductions in plasma total LDL cholesterol concentrations as well as an increase in fecal cholesterol excretion. Furthermore, abdominal fat mass was significantly reduced by consumption of aleurone. Our findings support cholesterol lowering and anti-atherogenic effects of corn aleurone and germ fractions in this animal model. It seems reasonable to suggest that consumption of whole

grain containing these fractions in adequate amounts may lead to cardiovascular risk reduction.

5.2. Introduction

Atherosclerotic cardiovascular disease (CVD) is a major source of morbidity and mortality worldwide (Breslow, 1996; Stocker & Keaney Jr, 2004). Atherosclerosis is a complex process in which the lumen of a blood vessel becomes narrowed by cellular and extracellular substances to the point of obstruction. Atherosclerotic lesions tend to form at the branch points of arterial blood vessels and progress through three stages: the first stage being the fatty streak lesions, which is characterized by the presence of lipid-filled macrophages (foam cells) in the sub-endothelial space; the second stage is the fibrous plaque, which consists of a central lipid-rich area, mainly derived from necrotic foam cells, covered by a fibrous cap containing smooth muscle cells and collagen. In the final stage, these lesions get complicated through thrombus formation with deposition of fibrin and platelet (Breslow, 1996; Stocker & Keaney Jr, 2004). Elevated plasma LDL-cholesterol levels and LDL oxidation are among the well established risk factors for atherosclerotic CVD. Dietary modification has been suggested to play a crucial role in reduction of CVD risk (Kris-Etherton et al., 2002).

Several epidemiological studies have consistently reported that intake of whole grains is inversely associated with CVD and other chronic disorders (de Munter et al., 2007; Sahyoun et al., 2006; Jacobs & Gallaher, 2004; Liese et al., 2003; Steffen et al., 2003). Whole grains including wheat, corn, rice, barley, rye and oats are composed of different fractions, including endosperm at approximately 80-85%, bran at 12-18%, and

germ by 2-12% of the grain's weight (Pomeranz, 1988; Duffus & Cochrane, 1993; Watson, 2003; Bradolini & Hidalgo, 2012; Ndolo & Beta, 2013). The bran is a composite multilayer consisting of outer pericarp, inner pericarp, testa, hyaline layer and aleurone layer. The aleurone layer makes 5-8% of the kernel and 45-50% of the bran fraction. The aleurone layer is nutritionally rich (Pomeranz, 1988). The starchy endosperm fraction contains most of the protein and carbohydrates, while bran is rich in fiber, minerals, unsaturated fats, vitamins and other phytochemicals (Slavin, 2003; Holzmeister, 2002). The germ fraction is also rich in both macro and micro nutrients plus several phytochemicals such as carotenoids, flavonoids, phytosterols and policosanols (Pomeranz 1988; Pietrzak & Collins 1996; Nyströmet al., 2007; Eisenmenger & Dunford 2008; Hidalgo & Brandolini 2008; Ndolo & Beta 2013). A few studies have further suggested that the bran not the endosperm or germ of whole grains is the major component for disease prevention (de Munter et al., 2007; Jensen et al., 2004; Holzmeister, 2002). The content of phenolic acids in bran is 10-20% times higher than that of endosperm (Mattila et al., 2005; Ndolo & Beta 2014). This property has been suggested to be one of the disease protective mechanisms of bran (Jacobs et al., 2007; Jacobs & Gallaher, 2004).

Wheat and corn (*Zea mays* L.) are the most commonly consumed whole grains in North America. Corn has the highest total phenolic content and antioxidant activities among four main grains, namely wheat, corn, rye, and rice (Adom & Liu, 2002; Ndolo & Beta 2014). Thus, these natural products may have the potential to reduce the risk of oxidative stress-induced diseases. Increased intake of antioxidant rich foods has consistently been shown to beneficially impact CVD risk (Mukherjee, 2003). Shane et al

(1995) demonstrated that corn bran supplementation of low-fat controlled diet (20 g bran/day) lowers plasma total cholesterol in men with hypercholesterolemia.

In the present study, we tested the hypothesis that adequate intake of corn fractions (aleurone, endosperm and germ) reduce atherosclerotic lesion development most likely through alterations in lipid metabolism in LDL receptor deficient (LDLr-KO) mice. The LDLr-KO mouse model closely resembles the disease in humans and has been frequently used by our laboratory and others to test the impact of dietary agents on atherosclerosis (Surendiran et al., 2013; Kowala et al., 2000).

5.3. Materials and Methods

5.3.1 Corn Fractions Preparation

Dasca-flint corn was selected for this investigation as it had been previously characterized in terms of its phytochemical profiles (Ndolo & Beta, 2013; 2014). Corn (flint) was hand dissected to separate the aleurone, germ and starchy endosperm according to the procedure described by Stewart, Nield & Lott (1988) with slight modifications (Ndolo & Beta, 2013). The grains were brushed at the ends and germs were removed by a sharp scalpel. The degermed grains were soaked in 0.1% (v/v) sodium hypochlorite (NaClO) for 15-20 min to sterilize the surfaces and rinsed using sterile deionized water. The seeds were then placed in 10 cm petri dishes lined with 2 ashless filters, moistened with 10 mL of sterile deionized water. The petri dishes were wrapped in aluminum foil and kept at 20 °C for 48 h. The aleurone and endosperm were separated using a scalpel and stored at -20 °C. Samples were freeze dried thereafter and ground using a multi-use blade

grinder, model PCC 770 (Loblaws Inc. MO, Canada) to pass through a 0.5 mm sieve. The ground samples were stored at -20 °C before use.

5.3.2 Experimental Diets

The diets were designed based on AIN-93G Purified Rodent Diet (Dyets # 110700) with slight modification to meet the minimum requirement of AIN-93 diet specifications. All the diets were prepared in our laboratory using Mouse Diet 9F (LabDiet, St. Louis, MO, USA). The diets were stored in dark bags and closed containers in a cold room (0 °C to 4 °C) during the entire course of the study.

The mice were fed with one of the following diets for 10 weeks: (a) AIN-93G-based mouse chow supplemented with 0.06% (w/w) cholesterol and used as the control diet; the control diet was supplemented with (b) 5% (w/w) aleurone or (c) 5% (w/w) endosperm or (d) 5% (w/w) germ. The experimental diet composition is summarized in **Table 5.1**.

Table 5.1 The experimental diet composition (g/kg diet)

Ingredients	g/kg diet			
	Control	Aleurone	Endosperm	Germ
Protein	205	194.8	194.8	194.8
L-Cystine	3	2.85	2.85	2.85
Sucrose	100	95	95	95
Starch	381.9	362.9	362.9	362.9
Dyetrose	132	125.4	125.4	125.4
Fat	90	85.5	85.5	85.5
Cellulose	27	25.7	25.7	25.7
Mineral mix (AIN-93-MX)	48	45.6	45.6	45.6
Vitamin mix (AIN-93-VX)	10	9.5	9.5	9.5
Choline Bitartrate	2.5	2.4	2.4	2.4
Cholesterol	0.6	0.6	0.6	0.6
Aleurone	0	50	0	0
Endosperm	0	0	50	0
Germ	0	0	0	50

5.3.3 Animals and Experimental Design

Thirty-two male LDLr-KO mice (4 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in groups of two or three in conventional mouse cages in a room with controlled temperature (24 ± 1 °C) and a 12:12-h light-dark cycle. They had *ad libitum* access to food and water. The mice were maintained according to the Guide and Care and Use of Laboratory Animals approved by University of Manitoba Animal Care Committee (Winnipeg, MB, Canada). After consuming standard mouse chow for 1 week, blood samples were collected through jugular vein under light anesthesia after 4 hr fasting and baseline plasma total cholesterol was measured. The animals were divided into four groups (n = 8/group; control; aleurone; endosperm; germ) with similar mean plasma total cholesterol concentrations and body weight as previously described (Dupasquier et al., 2007; Yan, 2011). The length of the experiment was 10 weeks. The experimental diets were provided to the animals on a weekly schedule. The leftover food was discarded. The food was made as needed, and stored in dark bags, and closed containers in a cold room (at 4 °C) until used. During the experiment, we collected blood samples at 3 week intervals from the jugular vein in lightly anesthetized animals. Body weight and food intake were recorded every week. Fecal samples were collected during the last week of the study. Collected fecal materials were stored at -80 °C.

At the end of the experiment, mice were sacrificed using carbon dioxide followed by collection of final blood samples through cardiac puncture, per our standard procedures (Dupasquier et al., 2007; Yan, 2011). Various internal tissues including heart, aorta, spleen, abdominal fat, liver, and kidneys were collected, weighed and stored at -80 °C until

analysis. Parts of the tissues were fixed in 10% buffered formalin and sectioned for histological examinations. The experimental design is summarized in **Fig 5.1** below.

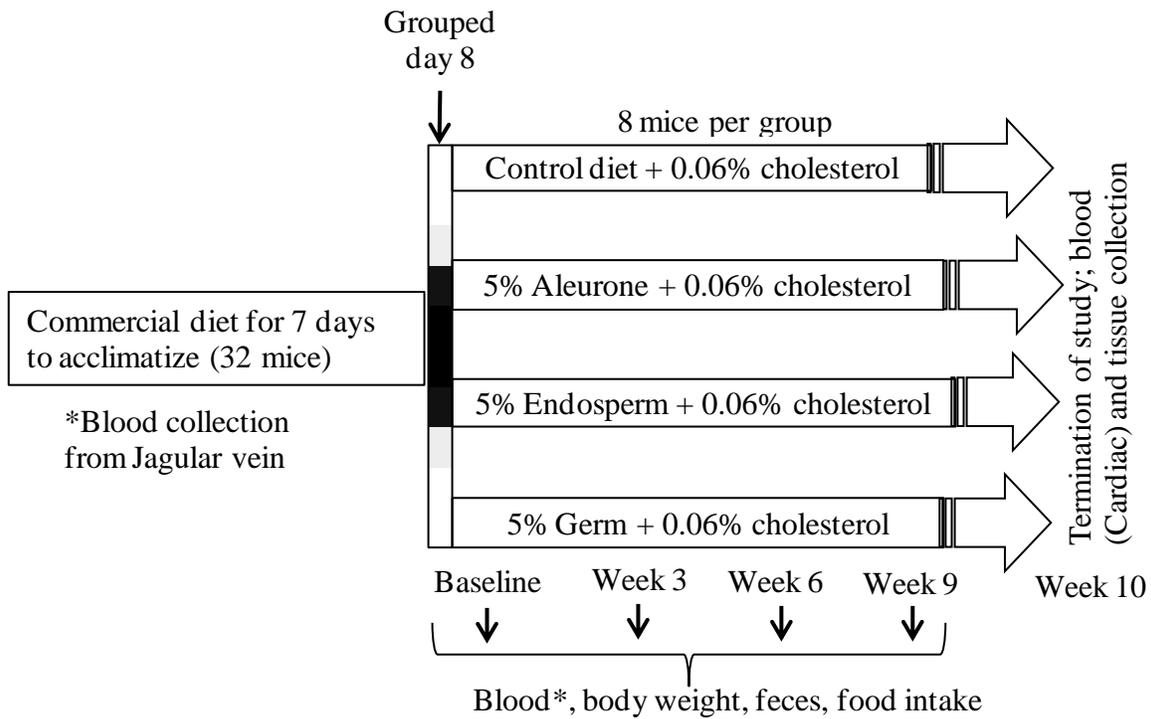


Figure 5.1 Design of the experiments. Approximately 160 μ L blood samples at week 0 (baseline) and at 3 week intervals were collected from the jugular vein in lightly anesthetized animals. Body weight and food intake were recorded every week. Fecal samples were collected every three week.

5.3.4 Ethics

Animal protocols and schedules, including animal housing, care, diets and animal experimental procedures, data collection and personnel, were approved by the Ethic

Committee for Animal Care on the use of animals in research at the University of Manitoba, Winnipeg, Manitoba, Canada.

5.3.5 Blood Collections

All mice were fasted for approximately 4 hr prior to blood collection during the study. Approximately a maximum of 0.6% of mice circulating blood volume (~150 μ L) was collected by certified animal technicians at the R. O. Burrell Lab, St Boniface Albrechtsen Research Centre. Blood samples were drawn through the jugular vein of lightly anesthetized animals during the study (at baseline, week 3, 6 and 9) using heparinized syringes. At the end of the study (week 10), mice were euthanized using carbon dioxide gas and final blood samples were collected via cardiac puncture. The blood samples were kept on ice during blood collection and thereafter centrifuged for 15 min at 4 °C using Centrifuge 5804 R (Eppendorf Canada, Mississauga, ON, Canada) to obtain plasma samples. Aliquots of plasma were stored at -20 °C until analysis.

5.3.6 Plasma Lipids

Plasma was used for determination of total cholesterol (TC) and triglyceride (TG) concentrations using standard enzymatic kits (Diagnostic Chemicals Limited, Canada, Charlottetown, PEI, Canada). The principle behind TC enzymatic assay is the simultaneous production of hydrogen peroxide (H_2O_2) with cholest-4-en-3-one as a result of hydrolysis and oxidation of cholesterol esters. In the presence of peroxidase, H_2O_2 reacts with 4-aminoantipyrine and phenol to yield a chromogen. The intensity of the color produced is proportional to TC concentration of the sample (Allain et al., 1974). Briefly, 250 μ L of TC

reagent was added to 3 μL of each standard, control and plasma samples pre-pipetted in a microplate. The readings were taken at 500 nm using microplate reader (Autoreader EL311, BioTek instruments, Winooski, VT, USA) after 20 min of incubation at room temperature. For TG assay, plasma TG are hydrolyzed, phosphorylated and oxidized to yield H_2O_2 . Hydrogen peroxide is coupled with p-chlorophenol and 4-aminoantipyrine to give rise to a red quinoneimine complex. The intensity of the color is proportional to TG concentration in the sample (McGowan et al., 1983). The procedure of plasma TG determination is similar to that of the plasma TC assay, except 300 μL of reagent was added to each 5 μL of standards and plasma samples. All samples were measured in duplicate to obtain mean values.

Plasma high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels were determined by standard enzymatic kit (LDL and HDL Ultra Sekisui Diagnostics, LLC, Framingham, MA, USA) according to manufacturers' instructions. The HDL method is based on accelerating the reaction of cholesterol oxidase with non-HDL unesterified cholesterol and dissolving HDL selectivity using a specific detergent. In the first reagent, non-HDL unesterified cholesterol is subjected to an enzyme reaction and the peroxide generated is consumed by a peroxidase reaction with N,N-bis(4-sulphobutyl)-m-toluidine-disodium (DSBmT) yielding colorless product. The second reagent consists of a detergent capable of solubilizing HDL specifically, cholesterol esterase and chromogen to develop color for quantitative determination of HDL-C. Briefly, 300 μL of HDL reagent 1 was added to 3 μL of each standard, control and plasma samples pre-pipetted in a microplate reader and incubated at 37 $^\circ\text{C}$ using heating block (IsoTemp. 125D, Fisher Scientific, Pittsburgh, PA, USA) for 5 min. Then 100 μL of reagent 2 was

added and incubated again at 37 °C for 5 min. The readings were taken at 630 nm using a microplate reader (Autoreader EL311, BioTek instruments, Winooski, VT, USA). The LDL method is based on accelerating the reaction of cholesterol oxidase with non-LDL unesterified cholesterol and dissolving LDL selectivity using a specific detergent. Reagent 1 solubilizes only the non LDL lipoprotein particles. The cholesterol released is consumed by cholesterol esterase and cholesterol oxidase in a non color forming reaction. Reagent 2 solubilizes the remaining LDL particles and a chromogenic coupler allows for color formation. The enzyme reaction with LDL-C in the presence of the coupler produces color that is proportional to the amount of LDL cholesterol present in the sample. Briefly, 300 µL of LDL reagent 1 was added to 3 µL of each standard, control and plasma samples pre-pipetted in a microplate reader and incubated at 37 °C using a heating block (IsoTemp. 125D, Fisher Scientific, Pittsburgh, PA, USA) for 5 min. Then 100 µL of reagent 2 was added and incubated at 37 °C for 10 min. The readings were taken at 546 nm using a microplate reader (Autoreader EL311, BioTek instruments, Winooski, VT, USA). Plasma very-low density lipoprotein cholesterol (VLDL-C) levels were calculated according to the Friedewald formula with some modifications ($VLDL-C = TG/5$) (Friedewald et al., 1972). Also HDL/LDL ratio was calculated.

5.3.7 Hepatic and Fecal lipids

Fecal and liver tissue lipids extractions were performed according to Folch et al. (1957) with slight modification (Surendiran et al., 2013). About 0.5 g of fecal samples were weighed and soaked overnight in 5 mL of 0.025% calcium chloride ($CaCl_2$). The mixture was then vortexed using a mini vortexer (VWR, Mississauga, ON, Canada) for 30 sec at

high speed to homogenize the softened fecal samples. For liver lipids extraction, approximately 0.15 g of liver tissue was homogenized in 5 mL of CaCl₂ for 3 min using tissue-tearor (BioSpec Product Inc, Bartlesville, OK, USA). Both fecal and liver extractions were followed by lipid extraction using 25 mL chloroform: methanol (2:1, v/v) solvent and vortexing for 1 min. The samples were centrifuged at 2000 rpm for 10 min at 4 °C using Centrifuge 5804 R (Eppendorf Canada, Mississauga, ON, Canada) to get three layers of which the bottom layer is the lipids. The collected lipids were dried at 55 °C on a heating block (IsoTemp. 125D, Fisher Scientific, Pittsburgh, PA, USA) using an evaporating unit (Pierce model: 18780 REACTI-VAP evaporating unit, Rockford, IL, USA) and nitrogen gas (N₂) and stored at -20 °C until analysis. For TC and TG, the samples were reconstituted with ethanol. Total cholesterol and TG concentrations in the fecal and liver samples were analyzed as described in section 5.3.6. Gas chromatography (GC) was used for determination of fecal sterol derivatives. .

5.3.7.1 Derivatization of Fecal Sterols

Samples were reconstituted with 1 mL of chloroform:methanol (2:1). Then 100 µL of the reconstituted sample was dried at 55 °C on a heating block (IsoTemp. 125D, Fisher Scientific, Pittsburgh, PA, USA) using an evaporating unit (Pierce model: 18780 REACTI-VAP evaporating unit, Rockford, IL, USA) and N₂, followed by addition of 200 µL of 1,1,1,3,3,3-hexamethyldisilane + trimethylchlorosilane + pyridine, chlorotrimethylsilane, hexamethyldisilazane, sylon[™] HTP, trimethylchlorosilane, trimethylsilyl chloride (HMDS+TMCS+Pyridine, 3:1:9 (Sylon[™] HTP)). The mixture was heated at 55 °C using a

heating block (IsoTemp. 125D, Fisher Scientific, Pittsburgh, PA, USA) for 1 hr. The mixture was dried down by nitrogen gas. Then reconstituted with 300 μ L of hexane, vortex and let the mixture sit for 15 min and centrifuge at 3000 rpm for 1 min at 4 $^{\circ}$ C using Centrifuge 5804 R, and transfer the supernatant to the new labeled GC tubes. The samples were kept at -20 $^{\circ}$ C until GC-MS analysis. The samples were sent to Department of Human Nutritional Science for GC-MS fecal sterols analysis.

5.3.7.2. GC-MS Analysis

Fecal sterol GC-MS analysis was performed according to Wu et al. (2009) with some modifications. The analysis of fecal sterol derivatives was carried out on an Agilent Series 6890 GC system (2X) coupled to a 5973 MS detector. The GC was fitted with a HP-5 MS capillary column (30 m \times 0.25 mm I.D., 0.25 μ m). Another HP-5 MS capillary column (20 m \times 0.25 mm I.D., 0.25 μ m) was used as a guard column. Helium was used as the carrier gas at 1.0 mL/min. The GC oven temperature programme was as follows: 70 $^{\circ}$ C for 2 min; then 20 $^{\circ}$ C/min to 270 $^{\circ}$ C, held for 1 min and finally 5 $^{\circ}$ C/min to 300 $^{\circ}$ C, held for 13 min. Temperatures of 150 and 230 $^{\circ}$ C were set as the GC-MS quadropole and the MS source temperature, respectively. Electron impact (EI) mass spectra were obtained at acceleration energy of 70 eV. A 2.0 μ L aliquot of extract was injected in the splitless mode. Data acquisition was performed in the full scan mode (in the range m/z 35-550) to confirm the retention times of analytes and in selected ion monitoring (SIM) mode for quantification. A dwell time of 100 ms was selected and the filament delay time was set as 15 min.

5.3.8 Heart and Liver Histology

Specimens of liver tissues and the base part of hearts containing aortic roots were collected at sacrifice and fixed in 10% buffered formalin. The formalin-fixed aorta and liver tissue were dehydrated with a series of gradual increasing alcohol concentration as follows; 70%, 80%, 95% and 100% (each for 1 hr) to completely remove water. After drying, the samples were cleaned twice with 100% xylene for 1 hr to completely remove the alcohol and were left in an open space to completely evaporate xylene. The dried tissues were placed in TRUFLOW™ tissue cassettes (Fisher Scientific, Ottawa, ON, Canada) and embedded by submerging in 100% pre-melted paraffin wax and incubated for 3 hrs at 70 °C using a Barnstead electrothermal paraffin section mounting bath (Thermo Scientific, Conroe, TX, USA). The samples were then removed from cassettes and molded into tissue blocks using metal mold and fresh pre-melted paraffin wax and were placed on ice to obtain tissue blocks. In order to see the tissue in detail down to a single cell, tissue blocks were sectioned at 5 µm using Shandon Finesse 325 microtome (Thermo Scientific Inc, Philadelphia, PA, USA). As soon as a good section was obtained from the microtome, the section was placed into a water bath (60 °C) and was fished onto the labeled microscope slide. Six sections were cut from different levels of the aortic sinuses of each mouse and the liver tissue. The slides were air dried overnight to allow good binding of the paraffin wax and to ensure good staining. In order to stain the sections, dried slides were incubated at 65 °C on a heating block (IsoTemp. 125D, Fisher Scientific, Pittsburgh, PA, USA) for 20 min to melt the paraffin wax followed by submerging in 100% xylene for 10 min to remove the paraffin, then 95% alcohol and 100% alcohol (each for 10 min) to remove xylene. Thereafter, rinsed several times (~ 5 times) with water to ensure there is no alcohol residue remaining. The

slides were stained with haematoxylin (nucleus stain) for 10 min and rinsed with water (~ 5 times), then with 2% sodium bicarbonate (NaHCO_3) and followed by rinsing immediately with water. The slides were then stained with alcoholic eosin for 30 s and rinsed with water followed by submerging in 100% alcohol, then 95% alcohol and then 100% xylene (each for 5 min) and lastly permanent mounting of the slides with permount and dry overnight. The six sections representing the whole aortic root length and liver sectioned were used for morphological and morphometrical analysis of atherosclerotic lesions (Moghadasian et al., 1997), using light microscopy techniques and Image ProPlus digitizing system, respectively. Atherosclerotic lesions were identified and quantified using imaging software. The lesion area can be determined at specific distances from the aortic sinus at 40-50 μm intervals, depending on the thickness of each section. A plot of these area measurements versus distance gives a profile of the atherosclerotic lesion. The area under the curve represents an estimate of the total atherosclerotic lesions.

5.3.9 Statistical Analysis

We had 8 mice in each group in order to have sufficient biological sample including plasma for various biochemical analyses. $N=7$ is the minimum requirement for statistical analysis to detect significant differences among the groups at $P < 0.05$ and a power of 80%. Data were analyzed as complete randomized design (CRD)-factorial plus control using one-way analysis of variance (ANOVA) with GLIMMIX Procedure of SAS, release 9.3 (SAS 2014; SAS Institute). Sample means were compared using Tukey-Kramer multiple

comparison procedure and data was considered significantly different at $P < 0.05$. All results are presented as mean \pm standard error.

5.4. Results and Discussion

5.4.1 Food Intake, Body Weight and Abdominal fat

The present work has characterized the effects of dietary corn fractions on cardiovascular risk factors in an atherosclerotic animal model. The mean food intake between groups was not statistically significant in the experimental groups ($P=0.354$, **Appendix III**). A steady body weight gain was observed in all the mice during the experimental feeding period (**Fig. 5.2**). Moreover, final body weight of the mice in this study did not significantly differ among the groups ($P=0.3634$; **Table 5.2**). This is in agreement with our previous studies showing no significant body weight changes in apolipoprotein E-knockout (apo E-KO) mice fed wheat or corn bran (Zhao et al., 2009) and LDLr-KO mice fed wild rice (Surendiran et al., 2013), and Chinese brown rice (Unpublished data).

Consumption of aleurone significantly reduced abdominal fat mass. Zhang et al (2009) reported that replacing white rice and processed wheat starch with wild rice as a chief carbohydrate source in a high fat cholesterol rats lowered body weight and abdominal fat mass, despite similar dietary energy intake among the experimental groups. Our results showed no statistically significant differences in tissue weights including heart, kidney, liver and spleen among the experimental groups.

Table 5.2 Effect of diets on final body weight and tissue weight in mice

	Weight (g)			
	Control	Aleurone	Endosperm	Germ
Final body weight	28.0±1.3	25.4±0.9	27.2±1.0	26.5±0.6
Liver	1.4±0.1	1.3±0.1	1.5±0.1	1.3±0.1
Kidney	0.36±0.02	0.35±0.02	0.36±0.01	0.37±0.01
Spleen	0.078±0.004	0.072±0.004	0.080±0.005	0.077±0.002
Heart	0.20±0.02	0.17±0.01	0.18±0.01	0.16±0.02
Abdominal Fat	0.59±0.09 ^a	0.36±0.02 ^b	0.39±0.05 ^{ab}	0.45±0.03 ^{ab}

Values are mean±standard error for each treatment groups, $n=8$. *Different letters* in each row indicates that the values differ significantly at $P < 0.05$ (Tukey-Kramer's multiple range tests).

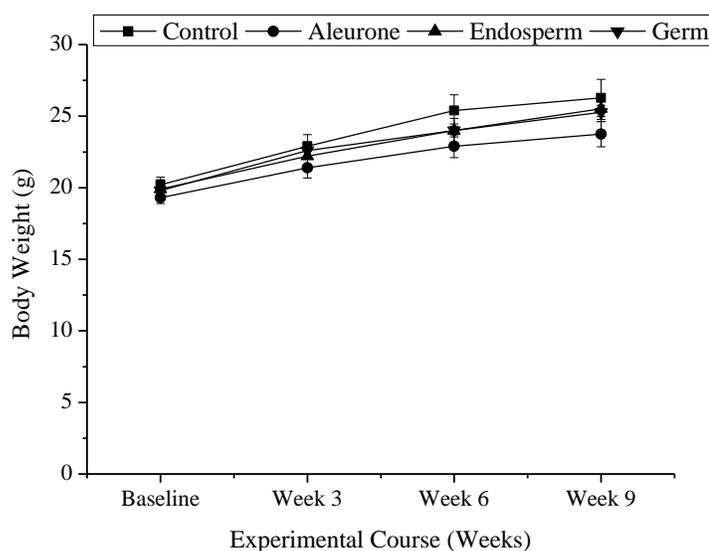


Figure 5.2 Effects of the experimental diets on body weights of LDLr-KO mice during the experimental course in all groups, $n=8$.

5.4.2 Plasma and Fecal Lipid Profile

The relationship between serum cholesterol concentrations and the risk of cardiovascular disease has been reported. In this study, introduction of an atherogenic diet containing 0.06% w/w cholesterol induced a prominent increase in plasma cholesterol levels in all groups of animals. However, addition of corn fractions to the diets reduced the levels of plasma cholesterol by various degrees. Consumption of aleurone and germ fractions significantly (**Fig. 5.3**) reduced the cholesterol-raising effect of the atherogenic diet from week 6 to the end of the study. However, the levels of TG remained similar among all treatment groups during the study course (**Fig. 5.4**).

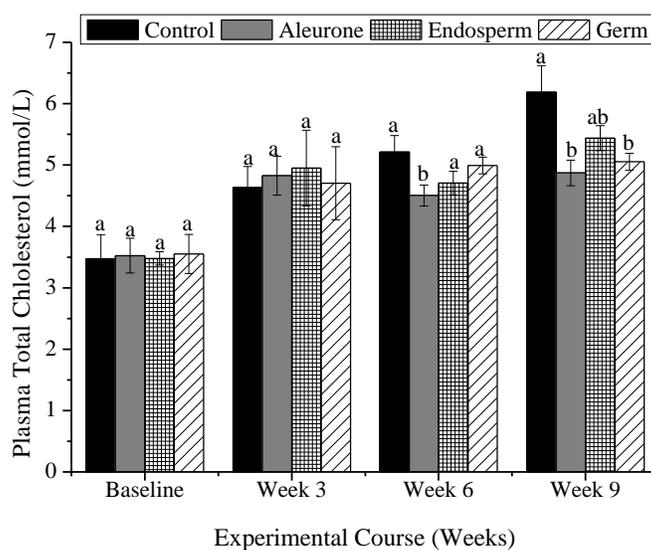


Figure 5.3 Effects of diets on plasma total cholesterol levels in LDLr-KO mice during the course of experimental treatment. Values are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups.

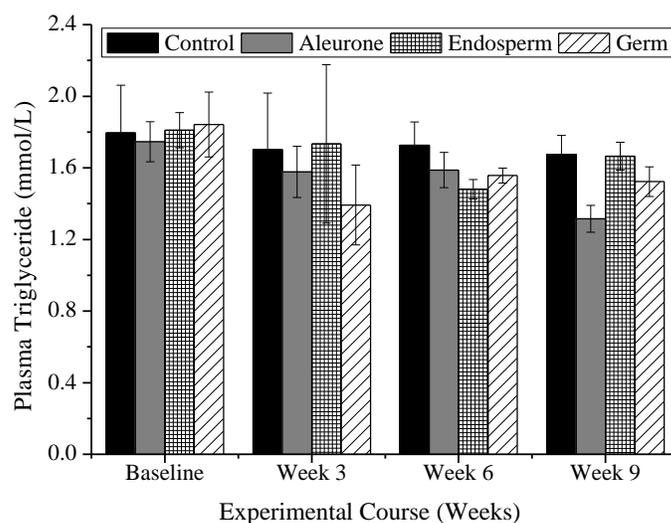


Figure 5.4 Effect of diets on plasma triglyceride levels in LDLr-KO mice during the course of experimental treatment. Values are mean \pm SE, $n=8$. No significant differences at $P < 0.05$ (Tukey-Kramer's multiple range tests) among the groups

Elevated plasma concentrations of LDL are a potent risk factor for atherosclerosis (Andersson et al., 2010). The LDLr-KO mice have elevated LDL-C levels, and the high cholesterol diet produced a further increase in these values, which was significantly inhibited by the aleurone fraction ($P = 0.041$) and germ fraction ($P = 0.031$, **Fig. 5.5**). On the other hand the levels of VLDL and HDL-cholesterol were comparable among all the experimental groups (**Fig. 5.5**). Comparisons of the LDL:HDL cholesterol ratios among groups were as follows: control 4.5 ± 0.4 ; aleurone 4.1 ± 0.7 ; endosperm 4.8 ± 0.6 and germ 3.0 ± 0.2 . Germ-fraction treated mice had the lowest LDL:HDL cholesterol ratio when compared to the aleurone, endosperm and control groups.

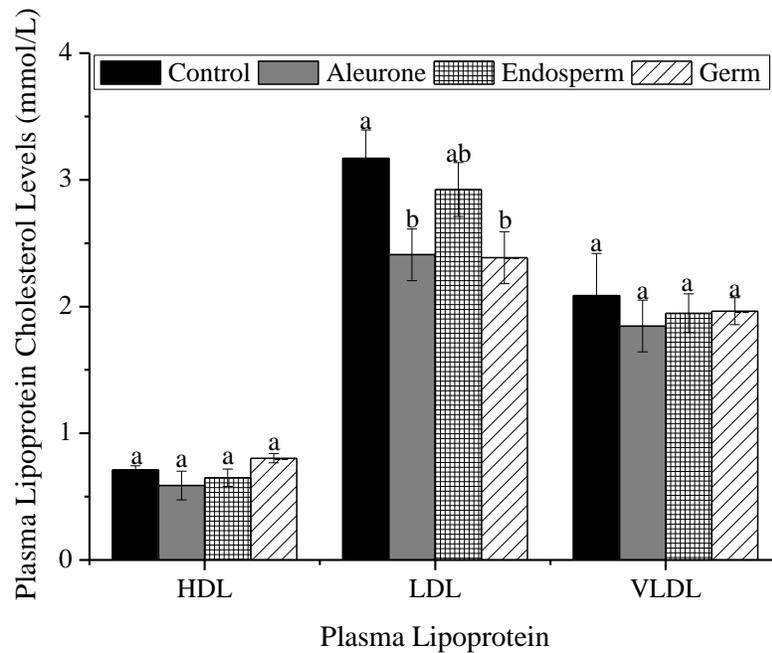


Figure 5.5 Effects of diets on plasma lipoprotein levels in LDLr-KO mice after 10 weeks of treatment. Values are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups

A few studies have suggested that the bran but not the starchy endosperm or germ of whole grain is the major component for disease prevention (de Munter et al., 2007; Steffen et al., 2003; Jensen et al., 2004). The content of phytochemicals including phenolic acids in the bran is 10-20 times higher than that in the starchy endosperm (Mattila et al., 2005; Ndolo & Beta 2014). This property has been suggested to be one of the disease protective mechanisms of bran (Jacob & Gallaher, 2004; Jacobs et al., 2007). Conflicting results on association between grain brans and lipid profile have been published in randomized clinical trials. Shane et al. (1996) showed that corn bran supplementation of low-fat

controlled diet lowers plasma TC, TG and VLDL-C in men with hypercholesterolemia; however, such effects were not found in wheat bran. Anderson et al (1994) showed that oat bran but not wheat bran (6 g in 100 g diet) significantly lowered liver cholesterol in rats. Oat bran contains more soluble fiber, whereas wheat bran contains significant amounts of insoluble fiber. Mongeau (1994) found that supplementation of diets with 14% w/w hard red wheat bran did lower plasma TC in rats. Zhao et al. (2009) reported that 18 weeks consumption of corn bran and wheat bran did not significantly alter lipoprotein profiles, nor reduce the atherosclerotic lesion development in apo E-KO mice.

Whole grains containing bran provide good amounts of dietary fiber and many micronutrients including potassium, magnesium, iron, zinc, thiamin, niacin, folate, vitamin E, lignin, and phenolic antioxidants (Truswell, 2002). Most of these nutrients have been shown to reduce coronary artery disease (CAD) risk factors such as hypercholesterolemia, hypertension, diabetes, insulin resistance, hyperhomocysteinaemia, and lipid oxidation (Slavin, 2003). However, the contents of these potential functional nutrients in bran vary largely with the type and variety of the grain. In this study, we report that consumption of germ fraction was associated with increased fecal cholesterol excretion and reduced total cholesterol levels as compared to those in the control group ($P= 0.043$, **Fig. 5.6**). Additionally, germ-treated animals had significantly increased fecal TG levels ($P= 0.015$, **Fig. 5.7**) and, significantly reduced campesterol in feces (**Table 5.3**, $P= 0.01$). Surprisingly, consumption of germ fraction led to significantly increased hepatic cholesterol contents ($P= 0.013$, **Fig. 5.8**); while hepatic TG contents were significantly increased in all treated groups as compared to the control group ($P= 0.0001$, **Fig. 5.8**). The reasons for increased hepatic levels of lipids are not known. The major route of sterol elimination from the body

is by excretion of unabsorbed cholesterol and bile acids as fecal, neutral and acidic sterols (Wang, 2007). Moreover, increased bile acid secretion was found to be inversely correlated with elevated cholesterol levels and the prevalence of coronary artery disease (Charach et al., 2012). Our results showed a significant reduction in campesterol. Campesterols are used as surrogate markers of cholesterol absorption as their levels have been shown to correlate with cholesterol absorption measured using the gold standard radio and stable isotopic methods (Miettinen et al., 1990; Tilvis & Miettinen, 1986). Therefore, reduced campesterol indicate inhibition of cholesterol absorption by corn fractions.

Table 5.3 Effects of experimental diets on fecal sterol excretion

	mg of Sterol per g Feces				P-Value
	Control	5% Aleurone	5% Endosperm	5% Germ	
5 α -Cholestane	4.2 \pm 0.7	4.3 \pm 0.2	3.7 \pm 1.3	4.3 \pm 0.4	0.93
Cholesterol	42.2 \pm 1.6	38.9 \pm 3.0	39.1 \pm 5.6	45.0 \pm 3.8	0.66
Demosterol	21.0 \pm 14.1	36.1 \pm 2.07	42.8 \pm 1.1	35.4 \pm 5.1	0.25
Campesterol	38.6 \pm 11.4 ^a	13.0 \pm 1.6 ^b	12.7 \pm 3.3 ^b	17.0 \pm 1.2 ^b	0.01
Stigmasterol	4.6 \pm 2.0	2.1 \pm 0.3	2.6 \pm 0.7	3.9 \pm 0.3	0.12
β -Sitosterol	28.6 \pm 3.4	26.6 \pm 4.5	30.3 \pm 5.2	36.1 \pm 2.0	0.40

Values are mean \pm standard error for each treatment groups, $n=8$. *Different letters* in each row indicates that the values differ significantly at $P < 0.05$ (Tukey-Kramer's multiple range tests).

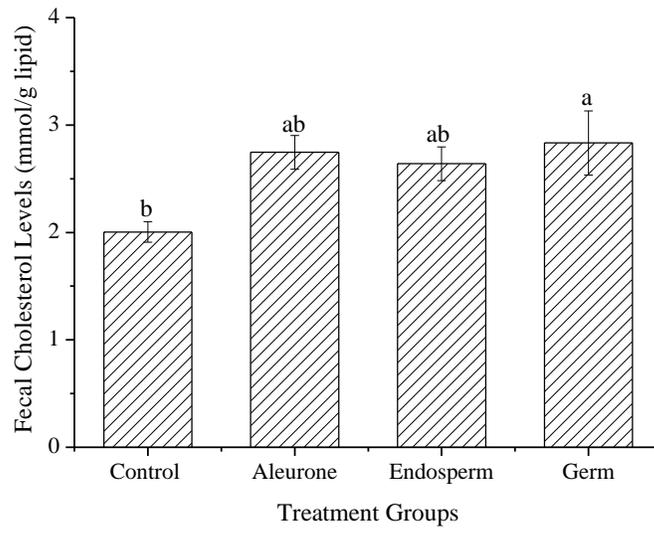


Figure 5.6 Effect of diets on fecal cholesterol levels in LDLr-KO mice after 10 weeks of treatment. Values are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups

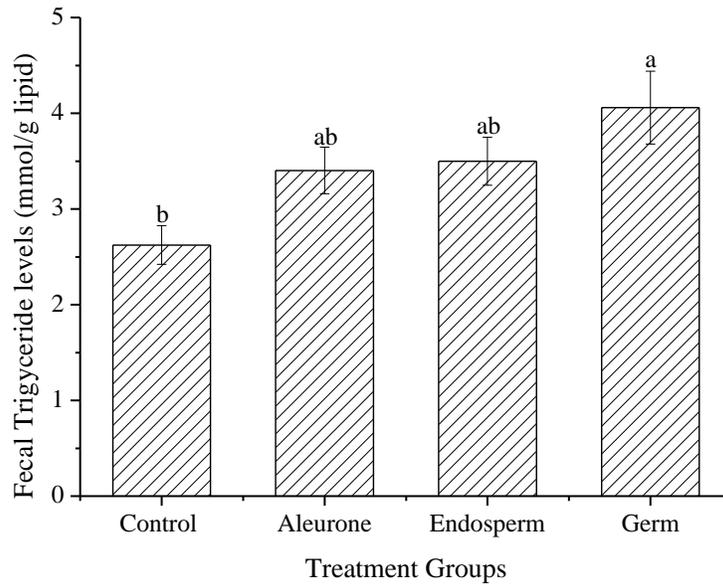


Figure 5.7 Effect of diets on fecal triglyceride levels in LDLr-KO mice after 10 weeks of treatment. Values are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups

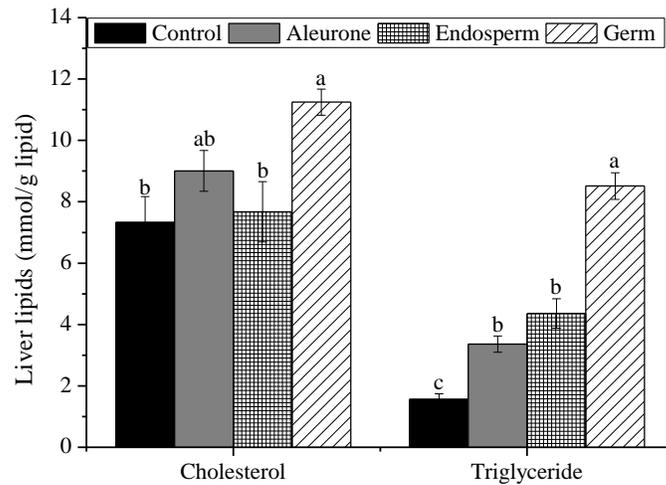


Figure 5.8 Effect of diets on liver cholesterol and triglyceride levels in LDLr-KO mice after 10 weeks of treatment. Values are mean± SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups

5.4.3 Heart and Liver Histology

Accumulation of fatty substances and cholesterol is likely to be a major cause of endothelial dysfunction, an early stage of atherosclerosis development (Ross, 1999). Total cholesterol/HDL-C and LDL-C/HDL-C ratios are used to predict the risk of ischemic heart disease (Lemieux et al., 2001). Results of prospective studies (Assmann et al., 1998; Manninen et al., 1992) have suggested that a high LDL-C/HDL-C ratio combined with hypertriglyceridemia is a major risk factor for coronary artery disease. Corn is richer in phytochemicals than other main grains, namely wheat, corn, rye, oats, and rice (Adom & Liu, 2002; Ndolo & Beta, 2013; 2014). This study shows advanced atherosclerotic lesions in the aortic root of the control mice, while corresponding sections from mice treated with

corn fractions showed a limited extent and severity of lesion involvement (**Figs. 5.9a** and **5.9b**). Representative photomicrographs of aortic roots showing various degrees of atherosclerotic lesions are presented in **Fig. 5.9**. The atherosclerotic lesion area was significantly different among the experimental groups ($P=0.003$). Germ- ($P =0.049$) and aleurone- ($P =0.002$) treated mice had the smallest atherosclerotic lesion area in their aortic root as compared with other groups. Previously, our laboratory showed no significant reduction in atherosclerotic lesions in apo E-KO mice fed corn bran or wheat bran over an 18 weeks study (Zhao et al., 2009). However, we previously reported significant reductions in atherosclerotic lesion development in LDLr-KO mice fed wild rice for 24 weeks (Surendiran et al., 2013).

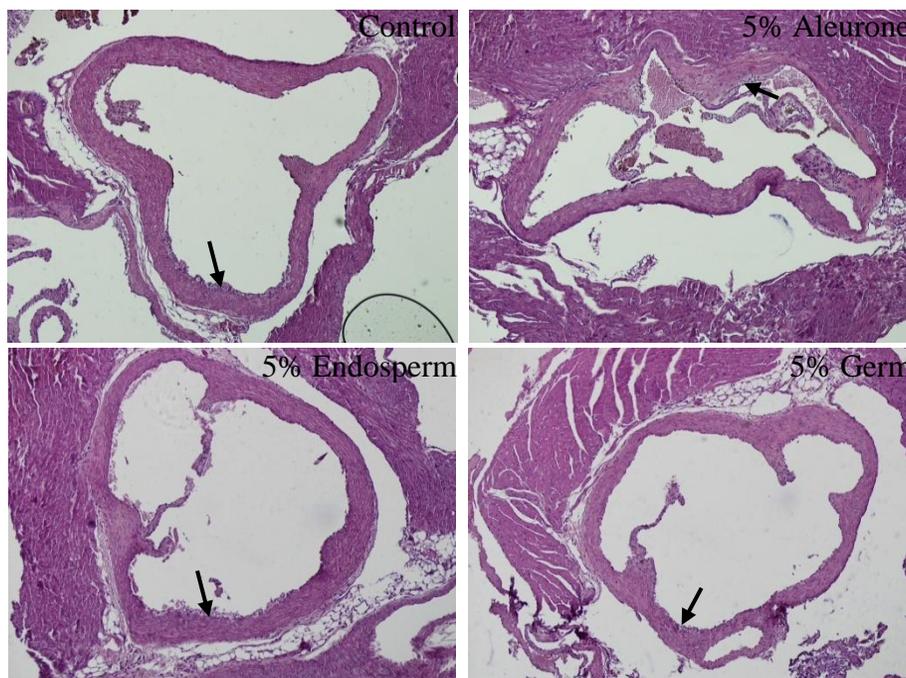


Figure 5.9a Effects of diets on early atherosclerotic lesion development in the aortic roots LDLr KO mice after 10 weeks of feeding trial. Arrows show fatty streak formation

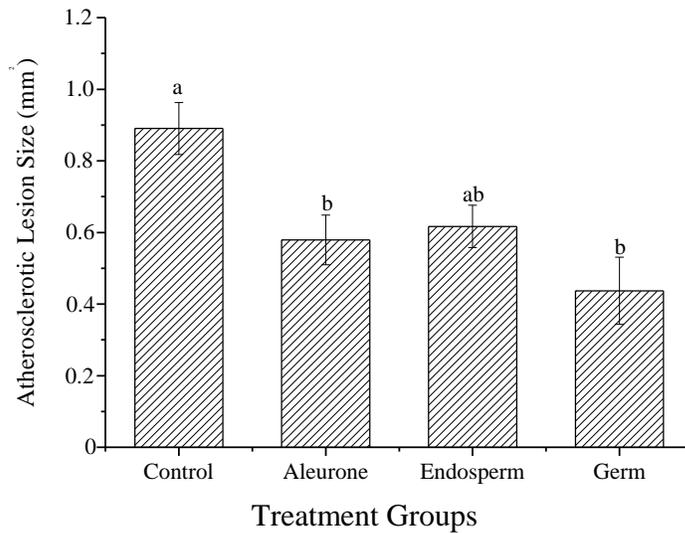


Figure 5.9b Effects of diets on atherosclerotic lesion size in the aortic roots of LDLr-KO mice after 10 weeks of feeding trial. Data are mean \pm SE, $n=6$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups

Histological examinations of the sections of the liver tissues are shown in **Figure 5.10a** and **5.10b**. Samples from corn fraction-treated animals showed lower degrees of fatty liver compared to the control group. Reducing atherosclerotic lesion area in aortic roots and fatty changes in the corn fraction-treated mice may be related to the inhibition of intestinal cholesterol absorption. The reduction of plasma cholesterol in corn fraction-treated animals most likely resulted in the absence of cholesterol clefts in the attenuated atherosclerotic lesions of the treated animals.

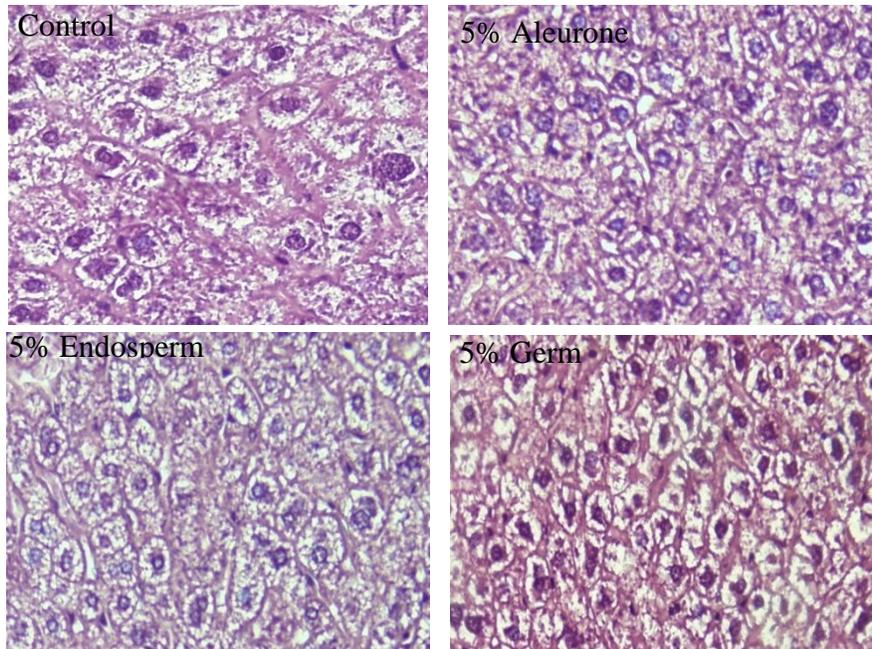


Figure 5.10a Effects of diets liver morphology in LDLr-KO mice after 10 weeks of feeding trial. As compared to the control group, the germ-treated liver tissue shows lesser apparent fatty changes

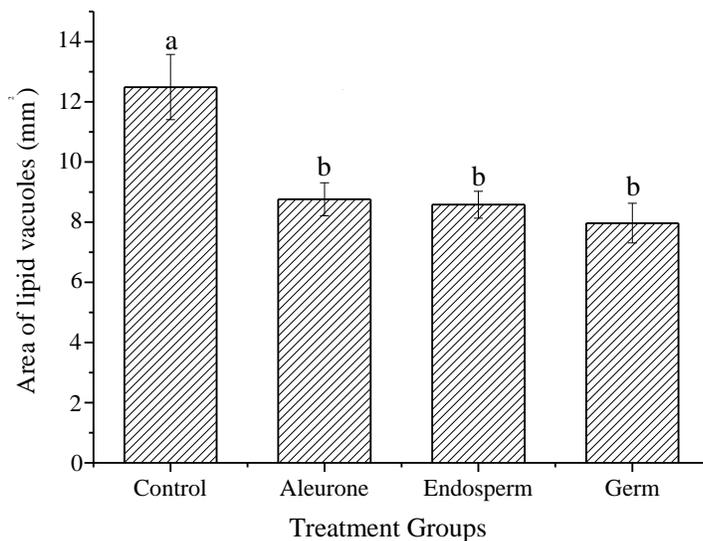


Figure 5.10b Effects of diets on apparent fatty changes after 10 weeks of feeding on the liver of LDLr-KO mice. Data are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups

5.0 Conclusion

In conclusion, we hereby report that incorporation of germ and aleurone in animal diets had anti-atherogenic properties in LDLr-KO mice. Potential mechanisms for such effects may include beneficial alterations in lipoprotein metabolism. A significant reduction in plasma total and LDL cholesterol concentrations was observed. This effect was accompanied by a significant increase in fecal cholesterol excretion. Our study suggests that consumption of germ and aleurone fractions has desirable protective roles on improving cardiovascular health in LDLr-KO mice. However, further studies are necessary

to investigate the exact mechanisms of action plus potential clinical outcome of such dietary approach in patients with cardiovascular risk factors.

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CHAPTER 6

Dietary Corn Fractions Attenuate Atherosclerosis in LDL Receptor Knockout Mice by Differentially Modifying Antioxidant Enzymes Activities and Oxidative Indicators

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6.1 Abstract

Protective effects of corn fractions (aleurone, endosperm and germ) against the development of atherosclerosis were examined in low density lipoprotein receptor knockout (LDLr-KO) mice. LDLr-KO mice were fed Mouse Diet-9F (1 control group) or Mouse Diet-9F diet supplemented with 5% (w/w) of each of hand-separated corn fractions (3 treated groups) for 10 weeks. All diets were supplemented with 0.06% (w/w) cholesterol. Consumption of corn fractions significantly increased liver antioxidant capacity as measured by 1,1-diphenyl-2-picrylhydrazyl-scavenging (DPPH) ($P<0.0003$) and differentially modified the activities of plasma catalase ($P<0.01$) and glutathione peroxidase ($P<0.0001$) while the activity of superoxide dismutase ($P<0.01$) was decreased as compared with the control mice. Incorporation of corn fractions boosted the total carotenoid content (TCC), lutein and zeaxanthin of the diets. However, the concentrations of TCC, lutein and zeaxanthin were comparable in the liver and plasma samples among all groups. Therefore, dietary carotenoids of corn aleurone and germ fractions do not seem to affect the development of atherosclerosis in LDLr-KO mice.

6.2. Introduction

Wheat (*Triticum* spp) and corn (*Zea mays* L.) are the most common sources of whole grain in North America. Corn has the highest total phenolic content and antioxidant activities among four main grains, namely wheat, corn, rye, and rice (Adom & Liu, 2002; Ndolo & Beta, 2014). Thus, these natural products may have the potential to reduce the risk of oxidative stress-induced diseases. Increased intakes of antioxidant rich foods are consistently shown to reduce the risk for atherosclerotic cardiovascular disease (CVD) through their free radical scavenging ability (Kaliora et al., 2006; Mukherjee, 2003). Current findings support the hypothesis that oxidative stress plays an important role in the initiation and progression of atherosclerosis (Hulthe & Fagerberg, 2002; Stocker & Keaney Jr, 2004). Oxidized low density lipoprotein (Ox-LDL) is believed to generate a vicious cycle of inflammation oxidation within the intima and thus initiate atherosclerosis through foam cell formation (Bonomini et al., 2008; Stocker & Keaney Jr, 2004; Vogiatzi et al., 2009). This indicates that it is crucial to regulate oxidative stress and inhibit the inflammatory responses to prevent development of atherosclerosis.

Reactive oxygen species (ROS) produced *in vivo* at levels that cannot be adequately counteracted by endogenous antioxidant systems can lead to damage to lipids, proteins, carbohydrates and nucleic acids (Williams & Fisher, 2005). The oxidative modification of these molecules by toxic levels of ROS represents an extreme event that can lead to deleterious consequences such as loss of cell function, a process known as oxidative stress. Several studies have suggested that oxidative stress is one of the causative factors of atherosclerosis (Bonomini et al., 2008; Robert et al., 2005; Stocker and Keaney Jr, 2004; Vogiatzi et al., 2009; Williams & Fisher, 2005; Young & McEneny, 2001). To counteract

the oxidants, an important endogenous antioxidant system exists *in vivo*, which includes antioxidant compounds such as vitamin E and antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH) and *glutathione (GSH) reductase* (Scalbert & Williamson, 2000). The three major antioxidant enzymes in the cells are CAT, GPx and SOD (Lepelletier et al., 2013).

Atherosclerotic CVD is the major source of morbidity and mortality worldwide (Stocker and Keaney Jr., 2004; Breslow, 1996). It is characterized by the accumulation of fatty deposits in macrophages in large- and medium-sized arteries. Atherosclerosis lesions contain multiple oxidized lipids derived from lipids in low density lipoproteins (LDL) (Stocker and Keaney, 2004). The biological properties of the lipids in mildly oxidized LDL have been seen to be different from those induced by the lipid in highly oxidized LDL (Berliner et al., 1995). Once LDL is modified chemically, it is readily internalized by macrophages through a so called scavenger receptor pathway (Goldstein et al., 1979; Nagy et al., 1998). Exposure to vascular cells in a medium that contains transition metals also results in modification of LDL such that it serves as a ligand for the scavenger receptor pathway (Henriksen et al., 1981). It is now clear that one mechanism whereby cells *in vitro* render LDL as a substrate for the scavenger pathway is via oxidation of LDL particles and the resulting modification of apolipoprotein B-100 (Steinbrecher et al., 1984). These observations form the basis for the oxidative modification hypothesis of atherosclerosis, in which LDL transverses the sub-endothelial space of a lesion-prone arterial site. Oxidized LDL has also been shown to stimulate proliferation of smooth muscle cells (Stiko-Rahm et al., 1992) and to be immunogenic by eliciting the production of auto-antibodies (Salonen et

al., 1985) and the formation of immune complexes that can also facilitate macrophage internalization (Klimov et al., 1985).

Recently, we showed antioxidant capacity of corn fractions (aleurone, endosperm and germ) (Masisi et al., 2015) and its significant levels of phytochemicals (Ndolo & Beta 2014; Ndolo & Beta 2013). However, little is known about the antioxidant potential of corn fractions in experimental animals with atherosclerosis and dyslipidemia. We hypothesized that adequate intake of corn fractions reduces cardiovascular risks through alterations in antioxidant system in LDL receptor deficient (LDLr-KO) mice. The LDLr-KO mouse model closely resembles the disease in humans. It has been frequently used by our laboratory and others to test the impact of dietary agents on atherosclerosis (Surendiran et al., 2013; Kowala et al., 2000).

6.3. Materials and Methods

6.3.1 Corn Fractions Preparation

Corn (flint) was hand dissected to separate the aleurone, germ and endosperm as described in **Chapter 5**.

6.3.2 Experimental Diets

The diets were designed based on AIN-93G Purified Rodent Diet (Dyet # 110700) as described in **Chapter 5**.

6.3.3 Animals and Experimental Design

Blood plasma and tissues from male LDLr-KO mice as described in **Chapter 5** were used for this study. Thus the same animals tested in Chapter 5 were used, but different biochemical measures were taken for a related objective.

6.3.4 Biochemical analysis

6.3.4.1 Catalase, Glutathione Peroxidase and Superoxide Dismutase Assays

The activities of catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in plasma and liver tissue were analyzed using commercial assay kits (Cayman Chemical Company, Ann Arbor, MI, USA) per manufacturer's instructions.

The catalase method is based on the reaction of the enzyme with methanol in the presence of hydrogen peroxide (H_2O_2). The formaldehyde produced forms a bicyclic heterocycle with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole, which gives a purple color upon oxidation (Johansson & Borg, 1988). Briefly, 100 μ L of 100 mM of catalase assay buffer and 30 μ L of HPLC graded methanol were added into 20 μ L of each standard and sample in the microplate reader. The reaction was initiated by adding 20 μ L of 8.82 M of catalase hydrogen peroxide to all wells, and the 96 well plate was covered and incubated on a shaker for 20 min at room temperature. Then, 30 μ L of 10 M of catalase potassium hydroxide was added to each well to terminate the reaction, followed by 30 μ L of chromagen. The plate was covered and incubated for another 10 min. Lastly, 10 μ L of 0.5 M of catalase potassium periodate was added to each well and incubated for 5 min and the reading was taken at an absorbance of 540 nm using a microplate reader (Autoreader

EL311, BioTek instruments, Winooski, VT, USA). All samples were measured in duplicate to obtain mean values.

The GPx assay measures the activity indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione (GSSG), produced upon reduction of hydroperoxides by GPx, is recycled to its state by glutathione reductase (GR) and NADPH (reduced form of nicotinamide adenine dinucleotide phosphate). The oxidation of NADPH to NADP⁺ (Nicotinamide adenine dinucleotide phosphate) is accompanied by a decrease in absorbance at 340 nm. The reaction was initiated by adding 20 µL of cumene hydrogen peroxide to all wells consisting of 100 µL assay buffer, 50 µL of co-substrate mixture and 20 µL of blank, standard and sample. Absorbance was read at 340 nm using a microplate reader (Autoreader EL311, BioTek instruments, Winooski, VT, USA). All samples were measured in triplicate to obtain mean values.

The SOD assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Diluted radical detector (200 µL) was added into 10 µL of each standards and samples in the microplate reader. Then, 20 µL of diluted xanthine oxidase was added. The plate was covered and incubated on a shaker for 20 min at room temperature. Absorbance was read at 450 nm using a microplate reader (Autoreader EL311, BioTek instruments, Winooski, VT, USA). All samples were measured in duplicate to obtain mean values.

6.3.4.2 Determination of DPPH Radical Scavenging Activity

Determination of DPPH in liver tissue and plasma was performed according to the procedure by Brand-Williams et al. (1995) and Martinez et al. (2006) using a stable free-

radical DPPH, with some modifications. Concentration of DPPH (Sigma-Aldrich D9132) solution was 200 μM in methanol, prepared 1 h before use, and samples were read in polypropylene ninety-six-well plates. Plasma extracts were prepared by mixing 10 μL plasma and 40 μL methanol. The samples were then vortex mixed using a mini vortexer (VWR, Mississauga, ON, Canada) for 30 sec at high speed. The plasma extract was then centrifuged at 5000 rpm for 30 min at 4 $^{\circ}\text{C}$ using Centrifuge 5804 R (Eppendorf Canada, Mississauga, ON, Canada), and the supernatant was used to perform the DPPH analysis. Liver sample extracts were prepared by mixing 0.5 g and 1 mL methanol. The samples were then homogenized for 1 min using tissue-tearor (BioSpec Product Inc, Bartlesville, OK, USA). The liver extract was then centrifuged at 5000 rpm for 30 min at 4 $^{\circ}\text{C}$ using Centrifuge 5804 R (Eppendorf Canada, Mississauga, ON, Canada), and the supernatant was used to perform the DPPH analysis. Extracts (10 μL) were reacted with 290 μL of the DPPH radical solution for 30 min. The absorbance at 515 nm was measured against a blank of pure methanol using a microplate reader (Autoreader EL311, BioTek instruments, Winooski, VT, USA). The analysis was conducted in triplicate. Concentrations ranging from 100-800 μM were used for the trolox standard curve. Antioxidant activity was calculated as a % DPPH radical scavenging activity:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \left[\frac{A_{\text{sample}} - A_{\text{Control}}}{A_{\text{Control}} - A_{\text{blank}}}\right]\right) \times 100 \quad [1]$$

where A_{Control} is the absorbance of DPPH radical in methanol at 0 min, A_{sample} is the absorbance of DPPH radical for the sample extract or standard at 30 min. A calibration

curve of % DPPH decolorization obtained from different concentrations of trolox was used to quantify the antioxidant capacity of the extracts. Antioxidant capacity was expressed using Trolox equivalents (TE) per gram of extract.

6.3.4.3 Preparation of Biological Samples and Diets for Carotenoids Analysis

Carotenoids were extracted according to Yonekura et al. (2010) with some modifications. To determine the concentration of carotenoids, samples of plasma (100 µL) and tissues (60-70 mg) were saponified with 1000 µL of 4.5% KOH containing 9.5% butylated hydroxytoluene (BHT) in 95% ethanol in screw-capped tubes with argon headspace. After the 30 min reaction at 60 °C, 2 µL of water was added to each tube and carotenoids were extracted 3 times with 3 µL diethyl ether:hexane 2:1 (v:v). The supernatants were combined, dried under nitrogen gas, re-dissolved in methanol, and analyzed by microplate reader (Autoreader EL311, BioTek instruments, Winooski, VT, USA) at an absorbance of 450 nm for total carotenoids and reversed-phase HPLC for specific carotenoids. The HPLC quantification of carotenoids was carried out according to methods described by Ndolo and Beta (2013) and Masisi et al. (2015). Extraction of carotenoids in diets was performed according to Masisi et al. (2015) and concentrations of carotenoids were measured as above.

6.3.5 RNA Isolation

RNA from liver tissue was isolated using RNeasy mini kit (Qiagen Inc, Toronto, ON, Canada) according to the manufacturer's instructions. Purity of the extracted RNA was assessed by measuring the 260/280 nm absorbance ratio using a microplate reader

(Autoreader EL311, BioTek instruments, Winooski, VT, USA). The average 260/280 ratio of the extracted RNA samples was 1.94, thus confirming RNA purity of samples. Isolated RNA was stored at -80 °C until use.

6.3.6 Primer Design and Validation

Gene-specific primers were designed using the Primer3 program (<http://frodo.wi.mit.edu/>) (Massachusetts Institute of Technology, Cambridge, MA, USA) and NCBI program (<http://www.ncbi.nlm.nih.gov>) (National Center for Biotechnology Information, Bethesda, MD, USA). Primer design criteria included a base-pair length of 125 to 175 and a guanine-cytosine (GC) content of 40% to 60%. Designed primers were purchased from Invitrogen (Life Technologies Inc, Burlington, ON, USA). Primer pair sequences are provided in **Table 6.1**. Primer pairs for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and beta actin (β -actin) were used as reference genes.

Table 6.1 Primers pairs forward and reverse for antioxidant enzyme genes

Gene	Forward Primer	Reverse Primer
β -Actin	5'-gctgagagggaaatcgtgcgtgac-3'	5'-gcctcagggcacatcggaaccgctcg-3'
CAT	5'-ggctctcacatggctgcaaggga-3'	5'-tcaggaaacggcatcaaaagcatg-3'
GAPDH	5'-gaacggattggccgtattggcg-3'	5'-ctccacgacatactcagcaccggcc-3'
GPx1	5'-cccaacatctccagtatgtgtgct-3'	5'-gtcggacgtacttgagggaattcag-3'
GPX3	5'-ccgggccggggacaagagaagtct-3'	5'-ctccgagttctcgctggctctg-3'
SOD1	5'-cccggggaagcatggcgatgaaag-3'	5'-gcagtcacattgccaggtctcc-3'
SOD2	5'-cctacgtgaacaatctcaacgccac-3'	5'-gtcacgcttgatagcctccagcaac-3'
SOD3	5'-cctgaactcaccagagggaaga-3'	5'-gcctccagactgaaataggcctc'3'

6.3.7 Quantitative Real-Time Polymerase Chain Reaction Analysis

Real-time PCR analysis was performed using SensiFAST™ SYBR® Hi-Rox one step mix (FroggaBio Inc, Toronto, ON, Canada). Each 20 µL reaction contained 2X SensiFAST™ SYBR® Hi-Rox one step mix, 0.4 µmol/L gene specific forward and reverse primers, riboSafe RNase inhibitor, reverse transcription and 100 ng RNA. Polymerase chain reaction analysis was accomplished using the ABI 7500 standard Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following cycle conditions: 45 °C for 10 min followed by 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 sec, 60 °C for 10 seconds, and 72 °C for 5 sec. A melt curve analysis confirmed the amplification of a single cDNA product.

6.3.8 Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) with GLIMMIX Procedure of SAS, release 9.3 (SAS 2014; SAS Institute). Sample means were compared using Tukey-Kramer multiple comparison procedure and data was considered significantly different when $P < 0.05$. All results are presented as mean \pm standard error.

6.4. Results and Discussion

6.4.1 Plasma and Liver Enzymatic Antioxidant Activities

Oxidation of LDL particles is widely recognized as the key step in the initiation of atherosclerosis development (Andersson, 2009). Therefore, existence of sufficient amounts of various endogenous antioxidant compounds and enzyme activities are crucial to counteract the oxidative stress. In the present study, CAT, GPx and SOD activities, radical-

scavenging activity by the DPPH, the antioxidant enzymes and oxidative stress-related genes were determined to enable prediction or indication of oxidation level. Determinations of antioxidant enzymes CAT, GPx and SOD activities in blood are steady as compared to measurement of these activities in the liver or other tissues in which the cell contains nuclei and continuous synthesis of new protein (Actis-Goretta et al., 2004).

SOD is one of the first intracellular defences against reactive oxygen molecules (Adela et al., 2006). This enzyme causes a dismutation of the superoxide anion radical to H_2O_2 , which is further degraded by CAT and peroxidase actions (Stocker & Keaney Jr, 2004; Vasconcelos et al., 2007). In this study, consumption of corn fraction significantly reduced plasma SOD activity ($P<0.01$) compared with the control group, while hepatic SOD activity did not significantly differ among the groups (**Fig. 6.1**). These findings may suggest that the same amount of H_2O_2 was produced in the body fluids and tissues of mice fed or not fed corn fractions. Hosada et al. (2012) showed that lack of alteration in antioxidant enzymes could be due to a sufficient pool of non-enzymatic antioxidant substances probably in the defence system.

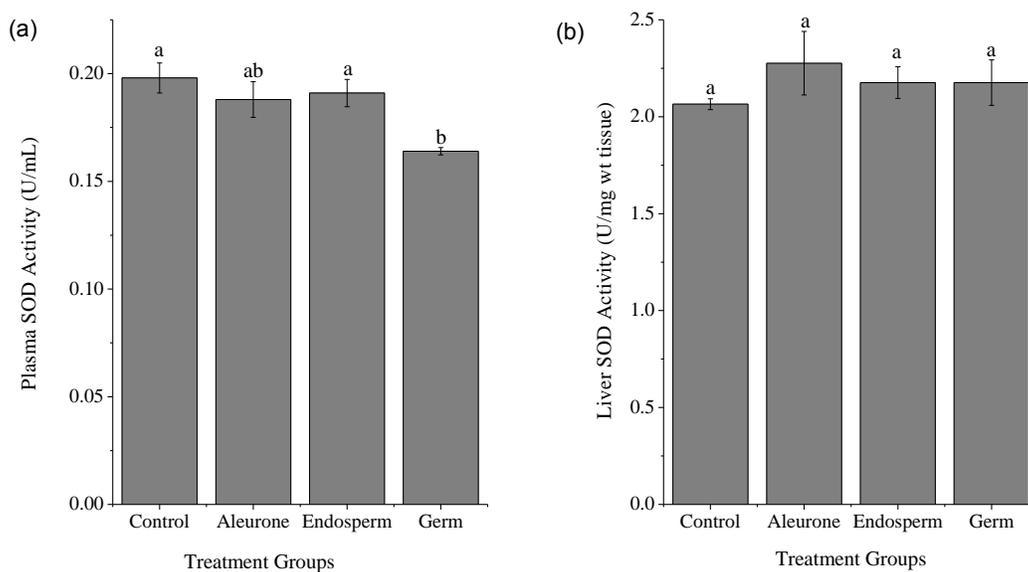


Figure 6.1 Activity of SOD in the (a) plasma and (b) liver of LDLr-KO mice fed experimental diets. Values are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups.

Consumption of aleurone and germ fractions significantly increased plasma GPx activity ($P < 0.0001$) compared with the control group. Aleurone fraction hepatic GPx activity significantly decreased ($P < 0.04$) as compared with the control group and there were no significant differences among other groups (**Fig. 6.2**). The increased GPx activity in plasma may suggest that a proportion of 5% aleurone or germ fractions may provide enough antioxidants to improve the oxidative status of LDLr-KO mice.

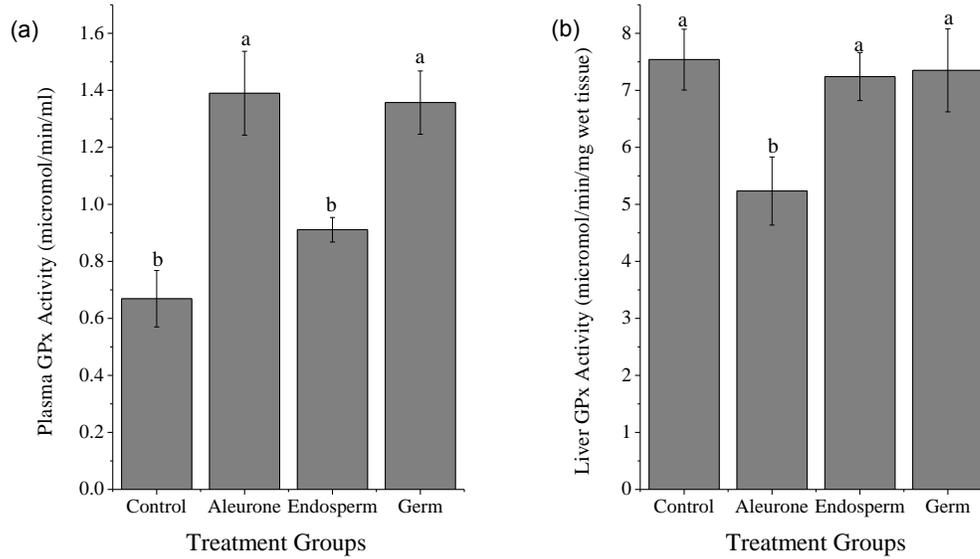


Figure 6.2 Activity of GPx in the (a) plasma and (b) liver of LDLr-KO mice fed experimental diets. Values are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups.

Consumption of corn fraction significantly increased plasma CAT ($P < 0.01$) compared with the control group. Germ fraction hepatic CAT activity significantly increased ($P < 0.04$) as compared with the control group and there were no significant differences among other groups (**Fig. 6.3**). The increased CAT activity in plasma and hepatic tissue (germ fraction) may suggest that a proportion of corn fraction may provide enough antioxidants to improve the oxidative status of LDLr-KO mice.

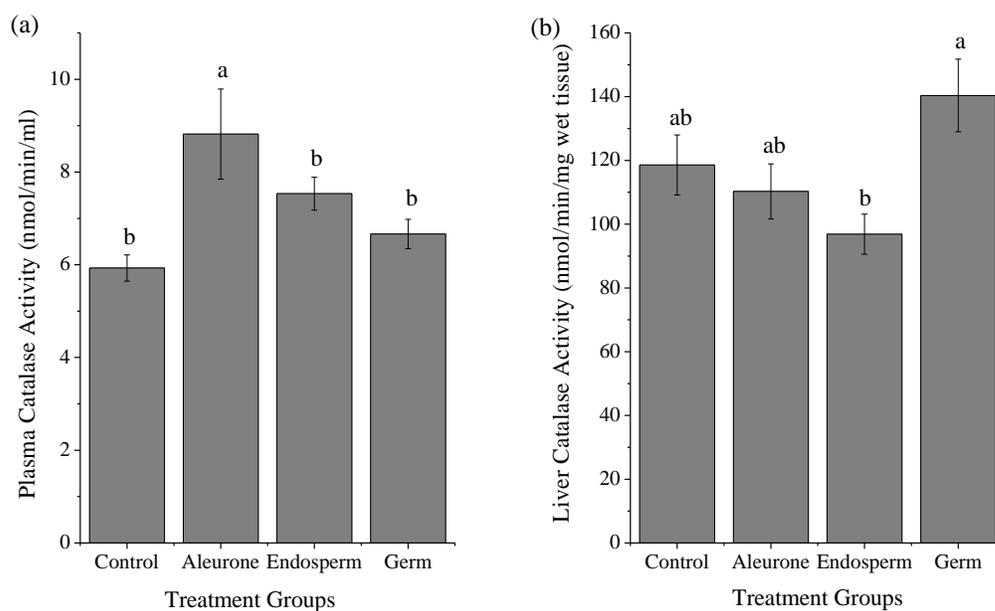


Figure 6.3 Activity of CAT in the (a) plasma and (b) liver of LDLr-KO mice fed experimental diets. Values are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups.

Similar responses were observed for both plasma CAT and GPx activities, which are enzymes degrading H_2O_2 . According to Celi (2010), when SOD activity increases the production of H_2O_2 , a protection from reactive oxygen substances would only be provided by a simultaneous increase in CAT and GPx activities and the availability of glutathione. However, consumption of corn fractions had no effect on CAT, GPx and SOD activity in liver of LDL-r-KO mice.

6.4.2 DPPH Antioxidant Activity

In this study, radical-scavenging activity was assessed by the DPPH assay, which measures modifications in antioxidant activity (Brand-Williams et al., 1995). Consumption of corn fractions suggested a better ability to quench DPPH free radicals by the germ ($P < 0.0001$) and aleurone ($P < 0.047$) than that of endosperm and control in the liver of LDLr-KO mice. However, plasma samples showed comparable DPPH radical activity among all the groups (Fig 6.4).

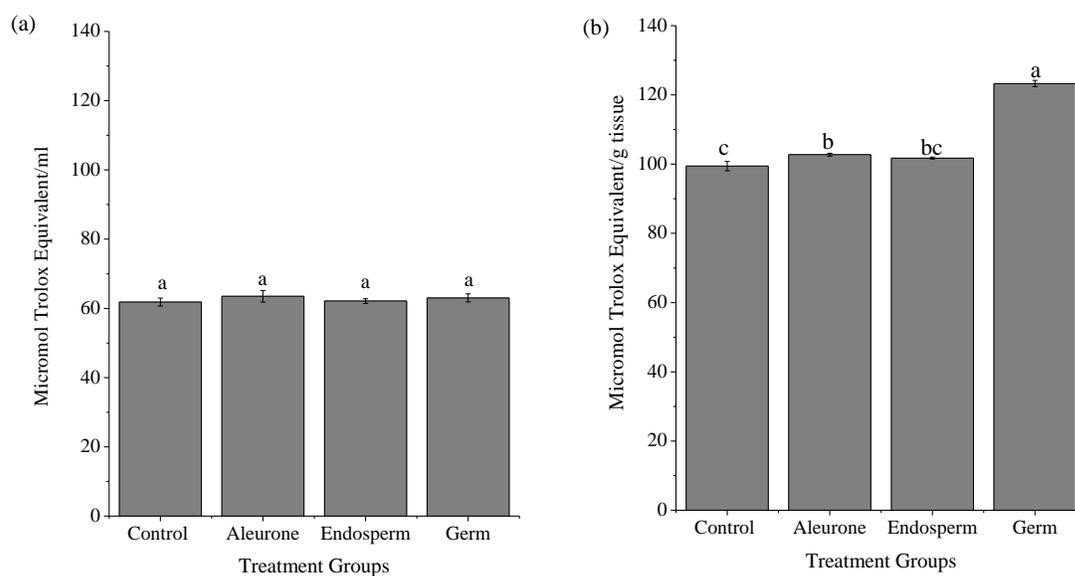


Figure 6.4 Effect of diets on (a) plasma and (b) liver oxidative stress indicator (2,2-diphenyl-1-picrylhydrazyl, DPPH) in LDLr-KO mice. Values are mean \pm SE, $n=8$. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's multiple range tests) among the groups.

6.4.3 Antioxidant Enzymes Genes

Figure 6.5 shows representative results for antioxidant enzyme genes. Consumption of corn fraction had no statistical significance on the fold change of *GPx1* and *SOD1* genes (**Figure 6.5**). The reasons for lack of treatment effect on *GPx1* and *SOD1* genes remain to be determined.

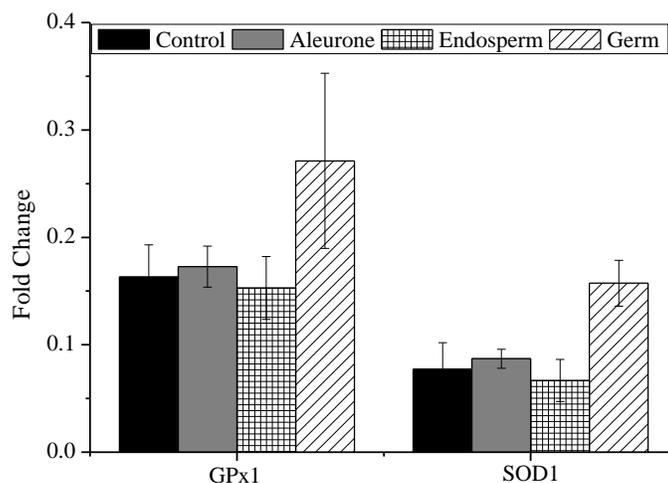


Figure 6.5 Effect of diets on expression of *GPx1* and *SOD1* antioxidant genes. Values are mean \pm SE, $n=8$.

6.4.3 Carotenoid Levels in Biological Samples and Diets

Carotenoids are consistently shown to have potent antioxidant properties that play an essential role in preventing oxidative stress-induced diseases, like cardiovascular disease (Fiedor & Burda, 2014; Mayne, 1996; Voutilainen, 2006). Recently our laboratory showed that carotenoid extracts of aleurone, germ, and endosperm fractions of barley, corn and wheat improve antioxidant capacity (Masisi et al., 2015). This is a follow up study but

using animals with atherosclerosis and dyslipidemia. **Table 6.2** shows total carotenoid content (TCC) expressed as xanthophyll equivalent (mg/kg) in experimental diets. In the present study, incorporation of corn fractions boosted the TCC of corn fraction diets, which was 5-18% more than that in the control diet (**Table 6.2**). There was an increase in TCC among the treated diets ($P<0.06$) as compared to the control diet. However, TCC was significantly highest in the germ fraction diet group (2.5 ± 0.07 mg/kg, $P<0.05$) compared to the other diets. Plasma TCC of the aleurone, endosperm and germ mice diet groups were 26%, 37% and 38%, respectively, higher than those of the control group. Similarly, liver TCC of the aleurone, endosperm and germ mice diet groups were 15%, 6% and 11% respectively, higher than those of control group. However, TCC was not significantly different among all the groups.

Carotenoid identification was accomplished by comparing the retention times (t_R) in the sample and those of external standards and the UV-visible absorption spectra from literature. Two peaks were identified for lutein and zeaxanthin, the primary carotenoids found in cereal grains and products (Fratianni et al., 2005; Panfili and Fratianni, 2004). Quantitative data were calculated from the linear calibration curves. Incorporating corn fractions boosted lutein and zeaxanthin in the experimental diets more than in the control diets (**Table 6.2**). There were significant increases for lutein ($P<0.004$) and zeaxanthin ($P<0.002$) among the treated diets as compared to the control diet. Consumption of the germ fraction diet significantly increased hepatic zeaxanthin levels as compared to the control diets. The effect was comparable in aleurone and endosperm fraction diets. Hepatic lutein levels were comparable among the groups. Plasma lutein and zeaxanthin levels were also comparable among all the groups (**Table 6.2**). Even though incorporation of corn

fractions boosted carotenoid levels in the diets, the liver and plasma carotenoid levels were not significantly affected.

Table 6.2 Concentration of carotenoids in diets, mouse plasma and liver at the end of 10 week supplementation with corn fractions.

		mg/kg	µg/kg	
		Total Carotenoids	Lutein	Zeaxanthin
Diet	Control	2.1±0.1 ^b	57.8±1.6 ^b	18.3±1.6 ^b
	Aleurone	2.2±0.1 ^b	381.6±30.5 ^a	232.0±6.0 ^a
	Endosperm	2.2±0.1 ^b	551.8±70.7 ^a	255.6±13.7 ^a
	Germ	2.5±0.1 ^a	492.2±28.9 ^a	282.7±36.3 ^a
Plasma	Control	1.2±0.3	68.9±6.5	60.2±5.5
	Aleurone	1.6±0.3	66.1±2.5	58.4±3.7
	Endosperm	1.7±0.7	55.6±5.7	59.7±3.5
	Germ	1.7±0.4	70.9±7.3	67.0±2.4
Liver	Control	2.9±0.3	17.6±0.8	19.6±1.8 ^{ab}
	Aleurone	3.3±0.1	21.4±1.0	19.8±0.3 ^{ab}
	Endosperm	3.0±0.1	19.3±0.6	18.8±0.8 ^b
	Germ	3.2±0.1	26.6±4.2	23.6±1.4 ^a

Values are mean±standard error for each treatment groups, $n=3$ for diet and $n=6$ for liver and plasma. Different letters in each column indicates that the values differ significantly at $P < 0.05$ (Tukey-Kramer's multiple range tests).

6.5 Conclusion

The results showed an important mitigation by the germ fraction collected from corn to reduce SOD enzyme activities in plasma, but not liver, and similar results were obtained with GPx and catalase. The analysis of gene expression for SOD and GPx, however, did not show a treatment effect that could be attributed to a specific fraction. Of interest was

the finding that the germ which contained a very high zeaxanthin content gave relatively high content in liver tissue of animals when fed the same fraction. However, there was no effect seen with total carotenoid or lutein content. Although germ and aleurone fractions did produce increases in antioxidant enzymes and capacity to quench DPPH, the fact that this could not be associated with a parallel greater deposition of carotenoids in tissues, led to the conclusion that the positive effects of these fractions could not be attributed specifically to carotenoid contents. Other bioactive components that could also exist in these fractions may have contributed to the positive antioxidant observations. The aleurone and germ contain various amounts of dietary fibers, carotenoids, vitamins, minerals, polyphenols, and other phytochemicals (Adom & Liu, 2002; Ndolo & Beta, 2014; Slavin, 2003). Future studies on measuring the degree of absorption, metabolism and distribution of carotenoid compounds from corn fractions are essential to evaluate the antioxidant properties of corn fractions. Furthermore, anti-atherogenic effects of aleurone, germ fractions and carotenoids alone, the combination of aleurone or germ fraction plus carotenoids can be tested in LDLr-KO mice. Any beneficial effects of corn fractions on the oxidative status of LDLr-KO mice could lead to a prophylactic strategy against atherosclerosis.

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CHAPTER 7

General Discussion and Conclusion

7.1 General Discussion

7.1.1 Overview

Oxidative stress is a complex, and potentially cytotoxic biological phenomenon. Oxidative stress relevancy to health is emphasized by its implication in over one-hundred pathologies (Djordjevic, 2004; Genestra, 2009). The lack of safe and efficacious therapies for management of oxidative stress related diseases is a pressing issue given that the incidence of the disorder is increasing worldwide (Djordjevic, 2004; Genestra, 2009; Loomba & Sanyal, 2013; Stocker & Keaney, 2004). Innovative studies are needed to identify novel functional food ingredients for oxidative stress management. Through the use of biochemical, *in vitro* and *in vivo* model systems, our studies investigated the antioxidant potential of diverse cereal grain fractions. To achieve the overall objectives, hand separated fractions (aleurone, germ and starchy endosperm) were used. The fractions were screened under bright field microscope to ensure purity, and were considered pure as compared to mechanically separated fractions. Our findings demonstrated i) differential antioxidant potential of the aleurone, starchy endosperm and germ fractions of barley, corn and wheat in both biochemical and *in vitro* models; that ii) aleurone and germ fractions supplementation significantly reduced the size of atherosclerotic lesions in the aortic roots as compared to those in the control group through significant reductions in plasma total and LDL cholesterol concentrations as well as an increase in fecal cholesterol excretion; that iii) the antiatherogenic effects of germ and aleurone fractions do not seem to be associated with

dietary carotenoids of these fractions in LDLr-KO mice. Aleurone and germ fractions might have therapeutic potential for managing oxidative stress and atherosclerosis.

7.1.2 Biochemical Model

Whole grain phytochemicals have recently been viewed as important in modulating oxidative stress related diseases. Among the phytochemicals, carotenoids analyzed in this research are known for their efficient antioxidant capacity (Fiedor & Burda, 2014). Cereals are a significant source of carotenoids with lutein and zeaxanthin being the most abundant carotenoids (Adom et al., 2003; Ndolo & Beta, 2013; Panfili et al., 2004). In this study, identification and quantification of carotenoids in the botanical fractions were accomplished by several techniques including mass spectrophotometry and HPLC, while antioxidant potential of carotenoid extracts of these botanical fractions were assessed by several assays including ABTS, DPPH and ORAC. The findings of this model have demonstrated that we can get better understanding on the carotenoid composition and distribution profiles of diverse types of cereal grains. Furthermore, we can understand antioxidant capacities of the carotenoid extracts of these botanical fractions in diverse cereals.

The first experimental chapter (the research) provided information on variation of carotenoids in fractions of barley, corn and wheat as evidenced by significant differences in concentrations and the distribution. The finding adds to literature about the compositional information of hand separated grain fractions of diverse cereal grains. The trend in concentrations of carotenoid content was different with barley, corn and wheat having highest concentration in germ fraction followed by aleurone and starchy endosperm. For

carotenoids, zeaxanthin was higher in endosperm of corn but was not detected and lower in endosperm of barley and wheat, respectively. These findings contribute to our understanding of why lutein has been reported as the abundant and dominant carotenoid of barley and wheat, while zeaxanthin has been reported as the dominant one for corn (Hentchel et al., 2002; Ndolo & Beta 2013; Okarter et al., 2010; Panfili et al., 2004).

In addition to further confirming the uneven distribution of carotenoids within the grain kernel, there was variation in antioxidant capacities of carotenoid extracts of the botanical fractions. The germ fraction showed better antioxidant capacities followed by aleurone and starchy endosperm as measured by ABTS, DPPH and ORAC. The effect correlates with carotenoid concentrations in these botanical fractions suggesting that carotenoids are responsible for antioxidant potential in the botanical fractions of carotenoid extracts. This research adds to literature about the antioxidant capacity of carotenoid extracts of aleurone, endosperm and germ fractions of barley, corn and wheat.

7.1.3 *In Vitro* Model

In this study, antioxidant capacities of carotenoid extracts of botanical fractions were assessed by commonly used human intestinal cell models including Caco-2, HT-29 and FHs Int 74 (Elisia et al., 2011; Etcheverry et al., 2012; Liu et al., 2004; Mahler et al., 2009; Parker, 1996). The findings of this model have demonstrated that we can get better understanding on the antioxidant capacities of the carotenoid extracts of these botanical fractions in diverse cereals. Carotenoids are lipophilic and are so called antioxidants because of their capacity to physically and chemically quench singlet oxygen ($^1\text{O}_2$), as well as potency to scavenge other reactive oxygen species (ROS) (Fiedor et al., 2005; Edge &

Truscott, 2010; Cvetkovic et al., 2013). Previous studies have successfully used dimethyl sulfoxide (DMSO) to process transepithelial transport of lipophilic drugs in Caco-2 cell culture (Demirbas & Stavchamsky, 2003; Krishna et al., 2001). In the present study, we used 1% DMSO as a carrier of carotenoid compounds.

To demonstrate cytoprotective of the carotenoid extracts in a biological system, cell lines were induced with AAPH and H₂O₂. AAPH is known as a peroxy radical generator (Terao & Niki, 1986), while H₂O₂ is known as a hydroxyl radical generator (Rada et al., 2008). The active peroxy and hydroxyl radicals attack various biological molecules, induce cellular damage, and eventually cause pathogenesis (Yokozawa et al., 2000). Therefore, an AAPH- and H₂O₂-induced study may be a promising *in vitro* assay for evaluating biological activity of antioxidant activity of carotenoid extracts of cereal grain fractions. Elisia and Kitts (2008) previously reported that AAPH-initiated loss of cell viability in Caco-2 is partially attributed to the inductive effect of AAPH on cell apoptosis. Under thermal decomposition, AAPH produces peroxy radicals in cultured cells. Accumulation of intracellular peroxy radicals increases lipid peroxidation, damage cell membrane integrity, and eventually inducing cell apoptosis (Kulkarni et al., 2008). Wijeratne et al. (2005) previously reported that H₂O₂-initiated loss of cell viability in Caco-2 is partially attributed to the inductive effect of H₂O₂ on membrane permeability. The biologically significant reaction of H₂O₂ is its spontaneous conversion, catalyzed by Fe²⁺ (Fenton reaction), to highly reactive hydroxyl radicals (HO·) that reacts instantaneously with any biological molecule from which it can abstract a hydrogen atom (Wijeratne et al., 2005). Therefore, in this study, cytoprotective and cellular antioxidant activities were employed to analyze the cellular antioxidant potential of carotenoid extracts. The cell model studies revealed the

protective function of carotenoid extracts of botanical fractions in cultured human intestinal cells. Although the extracts performed differently in defending against various free radicals in three distinct cell models, an efficient cytoprotective was observed in AAPH- and H₂O₂-induced cells. Cellular antioxidant activity revealed differential inhibition of oxidative stress in all the cell lines. Furthermore, our results showed carotenoid extracts regulated genes involved in the antioxidant defence system. These effects were speculated to be associated with carotenoid concentrations within the grain kernel and antioxidant activity of carotenoids. Taken together, this study demonstrated for the first time, that carotenoid extracts of aleurone, endosperm and germ of barley, corn and wheat effectively regulated oxidative stress in three intestinal cell lines. The antioxidant effects of aleurone, endosperm and germ in intestinal cells could contribute to a significant role in the regulation of oxidative stress and its related risks.

7.1.4 *In Vivo* Model

LDL oxidation is a major cause of injury to the endothelium and underlying smooth muscle cells, recruiting macrophage and resulting in foam cell formation, an early stage of atherosclerosis (Kato et al., 2009; Ross, 1999). Antioxidants that inhibit LDL oxidation are expected to have potential anti-atherogenic effects (Stocker, 1999). Whole grain fractions are good sources of phytochemical antioxidants, and therefore, the phytochemical antioxidants are thought to produce health benefits through their antioxidant properties (Jacobs & Gallaher, 2004; Masisi et al., 2015; Ndolo & Beta, 2013; Slavin, 2004). Since lipid metabolism and endogenous antioxidant system are important pathogenic mediators in atherosclerosis, this study investigated the effect of supplementation of corn fractions on

LDLr-KO mice. The LDL receptor removes cholesterol-rich intermediate density lipoprotein (IDL) and LDL from plasma and thereby regulates plasma cholesterol level (Brown & Goldstein, 1986). Genetic defects in the LDL receptor produce hypercholesterolemia in humans with familial hypercholesterolemia (Hobbs et al., 1992). A number of studies have reported that intake of whole grains reduces coronary artery risk factors such as hypercholesterolemia, hypertension, diabetes, insulin resistance, hyperhomocysteinaemia, and lipid oxidation (Anderson et al., 1994; de Munter et al., 2007; Jacobs Jr & Gallaher, 2004; Jensen et al 2004; Mongeau, 1994; Shane & Walker, 1995; Slavin, 2003; Xia et al., 2003; 2006). The mechanisms responsible for these observations remain unclear. Findings of this study showed that mice maintained on treatment diets for 10 weeks had insignificantly body weight changes as compared to the control mice. Histological analysis revealed reduced atherosclerotic lesion in the aortic roots and was consistent with significant reductions in total and LDL cholesterol concentration as well as increased fecal cholesterol excretion in mice consuming diet supplemented with corn aleurone and germ fractions. Moreover, corn aleurone and germ fractions supplementation maintained the activity of plasma enzymatic antioxidants catalase and GPx of LDLr-KO mice, and improved antioxidant capacity in the liver as measured by DPPH. In addition to these multifaceted antioxidant effects, histological analysis revealed that supplementation with aleurone and germ fractions improved the morphological integrity of the liver. Taken together, results from this study demonstrated for the first time that aleurone and germ fractions are anti-atherogenic, and mitigates atherosclerosis in LDLr-KO mice via cholesterol-lowering and improved antioxidant status.

Our investigation in 10 weeks LDLr-KO mice model confirms that lipid metabolism and endogenous antioxidant system are important therapeutic target in atherosclerosis. Results from this study suggest that aleurone and germ fractions may be therapeutically advantageous in managing atherosclerosis in LDLr-KO mice. Aleurone and germ of cereals are sources of bioactive compounds and antioxidants (Jacobs & Gallaher, 2004; Masisi et al., 2015; Ndolo & Beta, 2013; Slavin, 2004). Our previous biochemical and *in vitro* models have shown that the aleurone and germ possess antioxidant properties and inhibition of oxidative stress. However, the anti-atherogenic effects were not associated with dietary carotenoids of germ and aleurone fractions. Taken together, the sum of the evidence suggests that germ and aleurone fractions of corn may be important for the management of cardiovascular disease risk factors in LDLr-KO mice.

Finally, one of the particularly thought provoking findings in this study was the ability of germ and aleurone fractions to improve lipid profile, atherosclerotic lesion, antioxidant status and liver and heart histology in LDLr-KO mice without affecting body weight of these animals. Currently, gradual and sustained weight loss is one of the acceptable strategies to reduce the risk of CVDs. Our findings might therefore have significant implications since not only do they identify a potential therapeutic benefit of supplementation with germ and aleurone fractions, but they also suggest that atherosclerosis could be managed independent of weight loss.

7.1.5 Connecting Concepts and Themes

Even though these three studies differ in their outlined objectives and model systems, there are connecting concepts. A major commonality in all three models emphasizes the

antioxidant potential of the cereal grain fractions. Furthermore, these studies emphasize the underlying complexity by which oxidative stress is regulated in cells and tissues by the grain fractions. Our studies demonstrated that germ fractions have a better antioxidant capacity as compared to the aleurone and endosperm fractions. Antioxidant rich food therapies have often been chosen based on their efficiency to react with ROS. However, antioxidant effectiveness varies for different oxidants (Winterbourn, 2008). Thus, cereal grain fractions with differential scavenging properties were shown to effectively improve antioxidant capacity and regulate oxidative stress in these studies. The ability of cereal grain fractions to possess antioxidant capacity and to regulate oxidative stress indirectly by restoring balance between prooxidant and antioxidant systems, was likely an important property that facilitated their effectiveness in these studies.

7.1.6 Conclusion

Investigating the antioxidant potential and anti-atherogenic benefits of aleurone, endosperm and germ fractions of barley, corn and wheat has been a central theme throughout my studies. Increased intakes of antioxidant rich foods are consistently shown to reduce the risk oxidative stress related disease through their free radical scavenging ability to lower oxidative stress *in vitro* and *in vivo* studies (Kaliora et al., 2006; Mukherjee, 2003). Oxidative stress is a complex and dynamic phenomenon. The recognition of ROS and their biological role has shifted tremendously from the early theories that focused primarily on cytotoxicity. ROS are now understood to be regulated and to play a fundamental role in many aspects of cell physiology. There is also evidence showing that their dysregulation can contribute to pathology. Recently accumulating evidence has

suggested that intake of whole grains is a protective factor against pathogenesis of chronic diseases. This study demonstrates that aleurone, endosperm and germ fractions of barley, corn and wheat are source of carotenoids and in particular, lutein and zeaxanthin. These carotenoids are unevenly distributed within grain kernel and vary widely. Aleurone, endosperm and germ fractions exhibit considerable antioxidant capacity, which was evaluated in this study using biochemical model.

The aleurone, endosperm and germ carotenoid extracts exhibited effects against free radical-initiated intracellular oxidation and free radical-induced cytotoxicity. The suppression of AAPH-induced intracellular oxidation and, AAPH and H₂O₂ mediated cytotoxicity occurred in all different cell lines but was dependent on the concentration of carotenoid extracts used to treat the cells. The protective effect of carotenoid extracts varied in magnitude for various cell types and dependent on the severity of oxidative damage imposed to the cell. The germ carotenoid extract was shown to be more effective than the aleurone and endosperm at suppressing intracellular oxidation and free radical-induced capacity.

The results of the animal study have implications for the use of cereal grain fractions for management of cardiovascular disease risk. The current recommendations for reducing cardiovascular risk focus on lipid lowering (NCEP, 2002), with LDL cholesterol concentrations of <2.60 mmol/L. This recommendation is based on accumulating evidence from the literature showing relationships between cholesterol concentrations and CVD risks (Keys, 1997; Prospective Studies Collaboration et al., 2007; Verschuren et al., 1995). Epidemiological studies have suggested that consumption of whole grains or grain bran is a therapeutic option to cholesterol lowering. Our data showed dietary consumption of

aleurone and germ fractions had beneficial effects to the management of CVD risks through significantly reducing the size of atherosclerotic lesions in the aortic roots and, improving antioxidative status as compared to those in the control group. These beneficial effects do not seem to be associated with dietary carotenoids of aleurone and germ fractions. Overall, this study therefore elucidated potential mechanisms by which the antioxidant activity of hand separated aleurone, germ, and endosperm fractions of barley, corn and wheat may protect against free radical-induced cell injury and management of CVD risks. This study contributes to the body of research on antioxidant potential of whole grain and their fractions. Findings obtained also suggest that whole grain containing corn aleurone and germ fractions in adequate amounts may reduce cardiovascular disease risks. Moreover, our data may promote consumption of these fractions as functional food ingredient. Finally, the data obtained may suggest use of these fractions as potentially alternative functional food ingredients to the current commercialized wheat aleurone layer.

7.1.7 Recommendations for Future Studies

The findings of this research showed improved antioxidative status of aleurone and germ fractions of barley, corn and wheat, therefore, they might be the potentially alternative functional food ingredients to the current commercialized wheat aleurone layer; however, further research is needed to:

- 1) Focus on the mechanisms by which carotenoids prevent free radical mediated apoptosis, possibly through modulation of specific cell signalling pathway involved in cell death.

- 2) Determine if carotenoids modulate stress-induced cell signalling pathways.
- 3) Measure the degree of absorption, metabolism and distribution of phytochemicals, particularly carotenoid compounds, from corn fractions in animal tissues so as to evaluate the antioxidant properties of these fractions.
- 4) Determine whether the aleurone or germ fractions in combination with carotenoids have additive or synergistic effects on prevention of oxidative stress and atherosclerosis.
- 5) Encourage further studies on LDL oxidation and investigations of potential cardiovascular benefits of cereal grain fractions. The results taken together can further encourage examining the potential clinical outcome of these dietary fractions in patients with cardiovascular risk factors, and oxidative stress related diseases.

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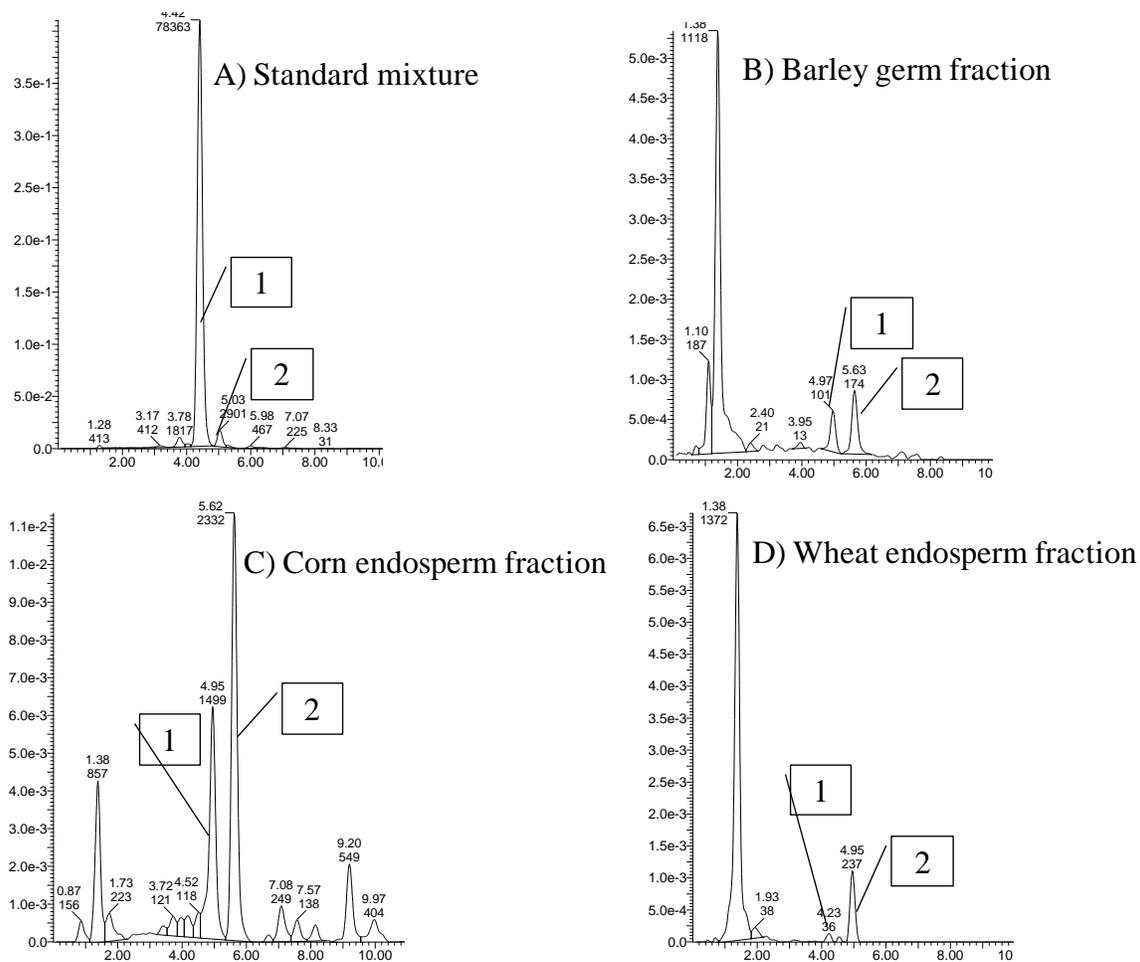
Appendices

Appendix I: Hand dissected fractions of barley and wheat



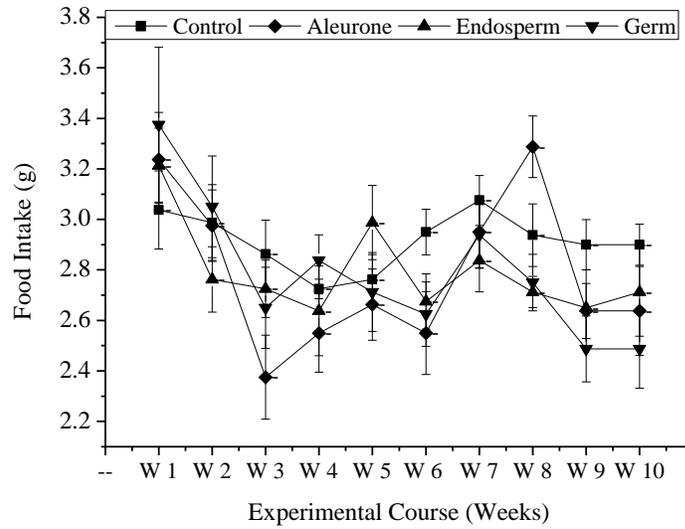
Supplementary Figure A. Raw hand dissected fraction (aleurone, endosperm and germ) of normal barley (A), MSU Wheat (B).

Appendix II: HPLC chromatograms of carotenoids



Supplementary Figure B. HPLC profile (at 450 nm) of carotenoids separated from standard mixture (A); germ: normal barley (B) endosperm: DASC corn (C); endosperm: MSU wheat (D); 1, lutein; 2, zeaxanthin.

Appendix III: Average food intake measurements



Supplementary Figure C. Average food intake measurements (g) of LDLr-KO mice after 10 weeks of experimental diet treatment. The average food intake among the four groups was comparable throughout the study. Data are presented as mean \pm standard error (SE).

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Title: Antioxidant properties of diverse cereal grains: A review on in vitro and in vivo studies
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Publication: Food Chemistry
Publisher: Elsevier
Date: 1 April 2016
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Title: Carotenoids of Aleurone, Germ, and Endosperm Fractions of Barley, Corn and Wheat Differentially Inhibit Oxidative Stress
Author: Kabo Masisi, William L. Diehl-Jones, Joseph Gordon, et al
Publication: Journal of Agricultural and Food Chemistry
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Appendix VI: Ethics approval for study in chapter 5 and 6

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Schedule 1, Personnel

1 Personal Information

Complete a separate Schedule 1 for each person working with live animals.

Masisi	Kabo	Kabo
Surname	First Name	Name Normally Used
Graduate Student	masisik@myumanitoba.ca	
Academic Position	E-mail address (Must be a UM email address)	
Office phone number	Lab phone number	

2 Procedures

Indicate all procedures this person will perform on animals in **this protocol**. Please be specific, e.g. if euthanasia is being performed, indicate method for each species (if more than one species is being utilized in the protocol). (Click +/- to add or delete rows to the table)

Name of Procedure	Competency Level *	If novice, indicate name of expert team member **
Feeding	Novice	Khuong Le
Handling	Novice	Khuong Le
weighing	Novice	Khuong Le

* Competency Level Definitions (note: This should be determined by the principal investigator or the expert team member).

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a. Was ethics training obtained at the U of M

Yes

b. If YES, Veterinary Services will generate a training report and attach it with the protocol.

c. If NO, was ethics training obtained from another Canadian Institution

d. If YES to 'c' above, please complete the table below and provide documentation from the applicable institution. (Click +/- to add or delete rows to the table)

Institution	Year	Month

Note: Ethics training must have been received from a Canadian institution with CCAC GAP status.



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Research Ethics and Compliance
Office of the Vice-President (Research and International)

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208-194 Daloe Road
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Phone [REDACTED]
Fax [REDACTED]
veterinaryservices@umanitoba.ca

6 January 2015

TO: Dr. Mohammed Moghadasian
Department of Human Nutritional Sciences

[REDACTED] Dr. R. Madziak, Acting Chair, Bannatyne Campus Animal Care Committee

RE: Amendment to Protocol 13-053/1 (AC10879)

Please be advised that the amendment as documented in your Application for Amendment (attached), to the above noted protocol, has been approved.

MT/ck

Copy: Dr. R. Aitken, Director, R.O. Burrell Lab

Attach.

umanitoba.ca/research

AC10879

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Application For Amendment To Animal Use Protocol

NOTE: An amendment may be used for minor changes in numbers of animals; addition and/or deletion of species; and minor modifications to procedures on live animals. Changes requiring full protocol submission include substantial changes in procedures previously described in an active protocol, addition of new procedures not before described in an active protocol, large changes in the number of animals.

For more detailed information pertaining to amendment versus full protocol submission, see link to Guideline 002 http://umanitoba.ca/research/orec/animal_care/animalcare_compliance_guidelines.html

13-053/1	December 12, 2015	950-222464	(UM Project # can be found using My Research Tools) http://umanitoba.ca/research/ors/mri-fan.html
Protocol #	Expiry Date	UM Project #	

PRINCIPAL INVESTIGATOR AND EMERGENCY CONTACT

Principal Investigator:

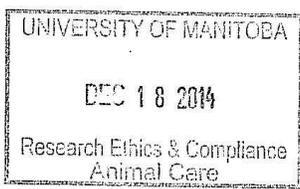
Moghadasian	Mohammed	MM		
Surname	First Name	Initial	Telephone	Fax
Professor	Human Nutrition Sciences	mmoghadasian@sbrc.ca		
Academic Appointment	Department/Faculty	Email Address		
351 Tache Avenue	Winnipeg	Manitoba	R2H2A6	
Office Address	City	Province	Postal Code	

An Investigation of Antioxidant Properties and Beneficial Lipids Profile Effects of the Northern Wild Rice, Chinese Brown Rice and Saskatoon Berry extract in Experimental Animals

Project Title

1 REASON FOR AMENDMENT REQUEST

- Personnel Changes: Addition or Deletion of Personnel, change in Competency Level and/or Change/Addition of duties/procedures assigned to existing personnel
- Transfer of Protocol to Different PI
- Change in Animal Numbers (addition/deletion)
- Change in Species, Strain, Line, genotype, age and/or sex of animals
- Change in anesthetics and/or analgesics
- Change in drugs or compounds given to animals
- Change in Procedures and/or Addition of New Procedure(s)
- Change in Funding and/or Title of Project
- Other (Please Indicate):



2 PERSONNEL CHANGES

(Indicate name of personnel, place an "x" in the applicable box in column and follow instructions given in column heading)
(Click +/- to add or delete rows to the table)

Name	Addition *	Deletion **	Change in Competency Level ***	Change/Addition of duties/procedures assigned to existing personnel ***
Kabo Maslsl	X			

* Submit a new Schedule 1; ** Nothing else required; *** Submit a revised Schedule 1

Note: Where protocols have an associated Schedule 10A Risk Assessment or SBRC Schedule 10, addition of personnel will require a Schedule 10B to be submitted with the amendment. http://umanitoba.ca/research/orec/animal_care/animalcare_forms.html

3 TRANSFER OF PROTOCOL TO DIFFERENT PRINCIPLE INVESTIGATOR (PI)

Indicate below the name of the PI now assigned to this protocol and the date this becomes effective. This PI assumes all responsibility for oversight of procedures, personnel, etc. Submit a Schedule 1 and 10B (if applicable) for the new PI.

Name of New PI

Effective date of transfer

Signature of New PI

4 CHANGE IN NUMBERS OR TYPES OF ANIMALS REQUIRED

a) Complete the table below if you are:

- removing a currently approved species, strain/line/genotype or age/weight group from the protocol
- adding a new species, strain/line/genotype or age/weight group to the protocol
- requesting an increase in numbers for an already approved species, strain/line/genotype or age/weight group on the protocol

Note: Requests for different age groups of the same species, strain/line/genotype should be made separately.
(example: Neonatal rats versus adult rats, weaned pigs versus feeder pigs, SCID mice versus CD1 mice)

Species (as per CCAC definition)	Strain/breed/Line/Genotype/Common Name	Sex	Age or Weight
Mice - older than 21 days	LDL Receptor Knock-out	Male only	4 weeks, 16 g
Number currently approved "0" if new	Additional number being requested	Number to be removed (if applicable)	Total
75	32	0	107

b) Below, justify the animals requested in the table. Address the following in your explanation:

- how the change in number or type of animals relates to the current objectives of the protocol;
- provide information on experimental and control groups (including number of animals per group) by briefly indicating what procedures all the requested animals will undergo. New procedures must also be stated in section 7.
- expected failure rates for the procedures and impact on number of animals requested;
- what statistical calculations were used to arrive at number requested;
- if using animals to provide tissues for in vitro work, give the expected product yield from each animal.

We would like to request approval for testing the antioxidant properties and lipid-modifying effects of aleurone, germ and endosperm fractions of corn in LDL-r-KO mice. Hence we will use 32 LDL receptor KO mice and divide them in 4 groups of 8 as following, Control, Aleurone fraction, Germ fraction and Endosperm fraction. The experimental course and every other procedure will remain as the original and approved amendments. We have almost consistently used 8 mice per group to be able to obtain statistically significant biochemical and histological data as well as allowing pooling samples when needed due to small sample size from the mice.

5 CHANGES IN ANESTHETIC AND/OR ANALGESIC DRUGS

a) Complete the following table

Additional/New Anesthetics or Analgesics Requested	Dose and route of administration

b) Indicate the reasons for the changes as it relates to protocol objectives, animal welfare improvement, etc.

6 CHANGES TO DRUGS OR OTHER COMPOUNDS GIVEN TO ANIMALS (TEST AGENTS, ANTIBIOTICS, ETC.)

a) Complete the following table

Additional Agents Requested	Dose and Route of administration
Aleurone, germ, and endosperm fractions of corn	5% (w/w) supplemented to the diet. Oral administration

b) Indicate the reasons for the changes as it relates to protocol objectives, animal welfare improvement, etc.

Dr. Trust beta from Department of Food Science has hypothesized that the aleurone, germ and endosperm fractions have different antioxidant capacity and lipid-lowering effects. She has included in her Canada Research Chair application in vivo studies for testing such beneficial effects of corn-derived agents in mice. Her Canada Research Chair application was funded. We have been collaborating with Dr. Beta over the past several years. Thus, we will collaborate on this study with her too. This study is in line with our original study in which we tested the antioxidant and cholesterol-lowering properties of wild rice. Thus, we will use the same animal model, namely LDL-r-KO mice to test the beneficial effects of these dietary agents.

c) Please specify any expected side effects that may result from each of these changes.

None

7 CHANGE IN PROCEDURES AND/OR ADDITION OF NEW PROCEDURES

Note: If major procedural changes are to be made in this project, a new protocol must be submitted. Consult the Clinical Veterinarians or the Chair of the ACC for help in deciding whether your changes are minor or major. Alternatively, see link to Guideline 002 http://umanitoba.ca/research/orec/animal_care/animalcare_compliance_guidelines.html

a) Describe and justify any procedure changes and/or additions of new procedures to the protocol. Indicate what current study objective this change aims to address.

None

8 CHANGE IN FUNDING/MERIT AND/OR TITLE OF PROJECT

Source/Agency	Status of Funding	Was the project described in this protocol (including animal use) included in the proposal that was approved for funding?	Status of Merit Review

Canada Research Chairs	awarded	Included	completed
------------------------	---------	----------	-----------

* If not included or if scientific merit review is required, please see the process for obtaining scientific merit review http://umanitoba.ca/research/orec/animal_care/animalcare_compliance_guidelines.html

Indicate revised project title if applicable.

9 SCHEDULES

Changes made to the protocol may result in the need to review and revise a number of existing Schedules or necessitate completing a new Schedule. On the list below, check off revised/new Schedules required and attach to this renewal. If you are unsure whether you need to submit a new/revised Schedule with this renewal, consult with the applicable Veterinarian for your institution. (Clinical Veterinarians at U of M, Dr. Randy Aitken at SBHRC)

	Yes	No
Schedule 1, Personnel Complete a Schedule 1 for all personnel as identified in Block 3 who are using live animals.	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Schedule 2, Anesthesia, Sedation, Chemical Restraint When anesthesia, sedation, and/or chemical restraint agents are being used. If anesthesia immediately precedes euthanasia, a Schedule 2 is not required.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 2B, Use of a Neuromuscular Blocking Agent (NMB)	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 3, Surgery When surgical procedures are being performed (both recovery and non-recovery).	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 4, Humane Endpoints Required for C, D or E category of invasiveness experiments. The schedule will ask you to provide a description of conditions that may cause distress/discomfort, how they will be identified and what will be done to alleviate them.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 5, Physical Restraint For restricted housing, e.g. metabolism crates/cages, or any restraint not normally part of regular husbandry practices and longer or more severe than normally required for examination, injection or a single blood collection in conscious animals. Completion of this schedule is not required for cattle restrained in a head gate/squeeze chute for surgical procedure.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 6, Nutrient and/or Diet Modifications For any alteration to the diet in which (a) specific nutrients are added or removed from the diet; (b) feedstuffs not normally fed are being used; (c) physical form of the diet is changed significantly from the usual form.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 7, Behavioural Experiments If the project involves behavioural manipulation, shock, negative reinforcement, punishment, removal of feed or water for behavioural reasons, predator/prey relationships, or sensory deprivation.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 8, Environmental Manipulation If the project involves environmental manipulation or imposes any potential adverse environmental effect. (Examples: changes in atmospheric gases, temperature, exposure to noxious gases, etc.)	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 9, Teaching When the main purpose of animal use is education, including courses, workshops, demonstrations, etc.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 10A, Risk Assessment (U of M) To be completed if any of the administered agents in the protocol are: used to create a disease model, classified as risk group 1 or higher, radioactive, classified as hazardous under WHMIS, drugs used in a manner not recommended by the manufacturer (off label use).	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 10, Risk Assessment (SBHRC) To be completed if any of the administered agents of the protocol meet the criteria described on the schedule instructions.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 11, Field Study Where animal use is in whole or in part conducted in the field and/or the project involves capture or release of animals in the wild.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 12, Common Procedures To provide more detail for common procedures including blood and/or tissue collection prior to euthanasia (including tail snips and ear punches), fecal and ingesta collections; individual marking; administration of compounds via injection, oral administration (gavage or via feed or water); catheter placement, physiological measurements such as blood pressure and ultrasound, etc., removal of all feed and/or water, indwelling osmotic pumps, etc. NOTE: Injectable anesthetic and euthanasia agents do not need to be listed here.	<input type="checkbox"/>	<input checked="" type="checkbox"/>

Schedule 13, Genetically Engineered Laboratory Animals (Including establishment of a breeding colony) If using any genetically modified animal including transgenic, knockout, knock-in, knock-down, etc. A Schedule 13 must be submitted for each genetically modified animal model.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 14, Offsite Housing If the project involves the use of animals on non-university property, excluding SBHRC and CancerCare Manitoba.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 15, Establishment and Maintenance of Breeding Colonies for Non-Genetically Manipulated Animals To provide details regarding the establishment and maintenance of an in-house breeding colony of laboratory animals which are neither "Livestock" nor genetically engineered (for example: rare species/strains which are not available commercially).	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Schedule 15B, Establishment and/or Maintenance of Livestock Breeding Herds or Flocks (dairy, swine, poultry) To provide more detail about herd/flock management personnel, animal information and numbers used and produced	<input type="checkbox"/>	<input checked="" type="checkbox"/>

DECLARATION

The signature of the principal investigator below indicates agreement to all terms and conditions applied to the original protocol and this amendment. No other changes can be made to this protocol without further approved amendments or submission and approval of a new protocol to cover them.

Principal Investigator

16/12/2014
Date

Protocol Approved By:

Acting _____
Chair, Animal Care Committee

Dec 22/14
Date

_____ *Dec 18/14*
Chief Veterinarian

Date

April 2014



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Schedule 1, Personnel

1 Personal Information

Complete a separate Schedule 1 for each person working with live animals.

Masisi	Kabo	Kabo
Surname	First Name	Name Normally Used
Graduate Student	masisik@myumanitoba.ca	
Academic Position	E-mail address (Must be a UM email address)	
Office phone number	Lab phone number	

2 Procedures

Indicate all procedures this person will perform on animals in this protocol. Please be specific, e.g. if euthanasia is being performed, indicate method for each species (if more than one species is being utilized in the protocol). (Click +/- to add or delete rows to the table)

Name of Procedure	Competency Level *	If novice, indicate name of expert team member **
Feeding	Novice	Khuong Le
Handling	Novice	Khuong Le
weighing	Novice	Khuong Le

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3 Ethics Training

- a. Was ethics training obtained at the U of M Yes
- b. If YES, Veterinary Services will generate a training report and attach it with the protocol.
- c. If NO, was ethics training obtained from another Canadian institution
- d. If YES to 'c' above, please complete the table below and provide documentation from the applicable institution.
(Click +/- to add or delete rows to the table)

Institution	Year	Month

Note: Ethics training must have been received from a Canadian institution with CCAC GAP status.

4 Wet Lab Training

a. Was wet lab training obtained at the U of M

b. If YES, Veterinary Services will generate a training report and attach it with the protocol.

c. If NO to 'a' above, list all wet labs pertaining to the species/procedures in this protocol which were obtained at a site other than the U of M and provide documentation. (Click +/- to add or delete rows to the table)

Wet Lab Name	Institution	
Country	Year	Month

April 2014

Schedule 1 -Training Report

Kabo Masisi

Food Science masisik@cc.umanitoba.ca

Skills Training

<u>Skill Description</u>	<u>Species</u>	<u>Certification Date</u>
IP Injection	Mice	November, 2014
IP Injection	Rats	November, 2014
Wet Lab: Introduction	Mice	November, 2014
Wet Lab: Introduction	Rats	November, 2014

Courses

<u>Skill Description</u>	<u>Species</u>	<u>Certification Date</u>
AUTC: Biomedical - Acute & Chronic		October, 2014

Friday, 19 December, 2014

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