Barley and Flax Hull Ingredients as Functional Foods

by

MEILI HAO

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In Partial Fulfillment of the Requirements of the Degree of

MASTER OF SCIENCE

Department of Food Science

University of Manitoba

Winnipeg, Manitoba

Copyright © 2010 by Meili Hao

I hereby declare that I am the sole author of this thesis.

I authorize the University of Manitoba to lend this thesis to other institutions or individuals for the purpose of scholarly research.

Meili Hao

I further authorize the University of Manitoba to reproduce this thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Meili Hao

The University of Manitoba requires the signature of all persons using or photocopying this thesis. Please sign below and give address and date.

ACKOWLEDGEMENTS

I owe my deepest gratitute to my advisor, Dr. Trust Beta, whose encouragement, guidance and support enabled me to complete my Master degree. Without her effort, I wouldn't have completed my research and this thesis would't have been possible, so to her, I offer my never ending appreciation for not only acceptting me as her student and giving me this great opportunity to do my Master degree in Canada, but also for providing all the tools neccessary to complete this degree. As well, I would like to show my sincere thanks to my committee members, Dr. Susan Arntfield and Dr. Karmin O, who unselfishly provided their expertise on many other aspects of my project. The financial support from the Canada Research Chairs (CRC) Secretariat, Canada Foundation for Innovation (CFI) -New Opportunities Fund and CFI-Leaders Opportunity Funds are greatly appreciated.

I would like to offer my special thanks to Dr.William Diehl-Jones, from Faculty of Nursing, for his time, generosity and willingness to help me conduct cell culture work in his lab. I could have certainly not attained the knowledge of cell culture without his excellent teaching and professional suggestions. I am grateful to all those who helped me complete the research, espeically all the assistance I have received from Wan Yeun (Allison) Ser, Patricia Kenyon from Food Science department, as well as Allistair Carrothers from Faculty of Nursing.

To my good friends, Karen, Rhanissa,Yulia, An Da, Lini, Yang, Xiaoran, Ronielle and my office mates, Mussarat and Kavita, as well as many other graduate students in Food Science, I appreciate our friendship and all your supports which made

me strong and not lonely in a foreign country. You were always there for me, when I got twists and turns in my life. I would like to say a big thank-you for being such a wonderful part of my life. I would also like to thank the whole department of food science, including all the professors and administritive staff who make this department as our “home” to grow, learn and play.

To my family and friends back home, I am absolutely indebted to my parents for giving me life in the first place, for raising me up and for supporting me to pursue my own dreams at all phases of my life. I couldn’t thank enough for your unconditional love and unwavering support. I also owe a huge thank-you to my brother, who always make me laugh when we were little, and who also sacrificed a lot to support all the way of my journey, even when it’s gone too far from home, and far beyond boundaries of language and geography.

ABSTRACT

The purpose of the research was to investigate the potential for converting agricultural by-products, barley hull and flaxseed hull as well as their co-extract, into value-added functional food ingredients.

Four varieties of barley hull and 3 types of flaxseed hull were hydrolyzed in calcium hydroxide solution in a water bath at 70℃ for 4 hrs with shaking. The major phenolic compounds in barley hull, flaxseed hull and their co-extracts were identified by reversed phase high performance liquid chromatography (HPLC) coupled with photodiode array detection (PAD) and quadrupole - time of flight (Q-TOF) mass spectrometry (LC-MS). Ferulic acid, *p*-coumaric acid, vanillic acid and vanillin, and four ferulate dehydrodimers were detected in barley hull and their co-extracts. Quantitative analysis was conducted on the phenolic acids using the available standards. However, the phenolic compounds in flaxseed were found to be distinct from that of barley hull. Large amounts of secoisolariciresinol diglucoside (SDG), ferulic acid glucoside (FeAG), p-coumaric acid glucoside (CouAG) were found in flaxseed hull with minor content of caffeic acid glucoside (CAG) and flavonoids herbacitin glucoside (HDG), whereas the phytochemical profile of the co-extract was enriched by combining major phenolic compounds from both barley hull and flaxseed hull.

The antioxidant activity of barley hull, flaxseed hull as well as their co-extract was evaluated using DPPH radical scavenging assay while total phenolic content was measured using the Folin-Ciocalteau method. After screening using chemical assays, the representative barley hull extract, flaxseed hull extract as well as their co-extract were

tested for their intracellular antioxidant activity and the antiproliferative activity in PC-3 human prostate cancer cells. Both chemical assays and the cell culture assays indicated that barley and flaxseed hull had strong antioxidant activity and antiproliferative activity. Although the co-extract exhibited the strong antioxidant activity in the chemicals assay, it behaved differently in the cell culture assay, which may be attributed to the chemical and biological properties of the major phenolics in the co-extract.

Following evaluation of the antioxidant activity and anticancer effect of barley hull extract, flaxseed hull extract as well as their co-extract, each type of extract was incorporated into Chinese steamed bread (CSB). The phytochemical profile of CSB was enriched by incorporating barley hull extract, flaxseed hull extract as well as their co-extract, which resulted in a significant enhancement in the antioxidant activity evaluated by DPPH and ORAC.

Therefore, barley hull, flaxseed hull and their co-extract are suggested as promising sources of functional food ingredients.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAPH	2,2'-azobis(2-amidinopropane hydrochloride
ABAP	2,2′-Azo-bis-amidinopropane
ABTS	2,2-azino-bis- 3-ethyl-benzothiazoline-6-sulfonic acid
ALA	Alpha-linolenic acid
Bcl2	B-cell lymphoma 2
CAG	caffeic acid glucoside
CouAG	p-coumaric acid glucoside
CSB	Chinese Steamed Bread
CVD	Cardiovascular disease
DCF	dichrolofluorescein
DCFH	dichlorofluorescin
DCFH-DA	Dichlorofluorescin diacetate
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGCG	Epigallocatechin gallate
END	Enterodiol
ENL	Enterolactone
ER-	Estrogen Recepter
FAO	Food and Agriculture Organization
FeAG	ferulic acid glucoside
FRAP	Ferric Reducing Antioxidant Power
HBSS	Hank’s Buddered Salt Solution

HDG	flavonoids herbacitin glucoside
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human Epidermal growth factor Receptor 2
IFT	Institute of Food Technologiests
IGF-1R	Insulin-like growth factor 1 receptor
LDL	Low Density Lipoprotein
MDA	Malondialdehyde
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
OD	Optical density
ORAC	Oxygen Radical Absorbance Capacity
PS2	Presenilun 2
ROS	Reactive Oxygen Species
SDG	secoisolariciresinol diglucoside
SECO	secoisolariciresinol
UV-B	Ultraviolet-B

LIST OF APPENDICES

Chapter 1: Introduction

Today, the functions of foods are not intended to only satisfy hunger and to provide basic nutrients for humans but also to prevent nutrition-related diseases and improve the state of well-being of the consumers. Functional foods and nutraceuticals fit into this niche market as they are regarded as nutrients that provide unique beneficial effects through reducing the risk of chronic disease, above and beyond their basic nutritional functions (Ho et al., 2006).

In the 1980s, Japanese researchers, who studied the relationships between nutrition, sensory satisfaction, fortification and modulation of physiological systems, first promoted the concept of functional food for food products fortified with special constituents that possess advantageous physiological effects (Sir ó et al., 2008). Although "functional food" is used commonly around the world, there is no formal definition for this group of foods. According to the Bureau of Nutritional Sciences, of the Food Directorate of Health Canada, a functional food is similar in appearance to a conventional food, is consumed as part of a usual diet, and it is demonstrated to have physiological benefits or reduce the risk of chronic disease beyong basic nutritional functions (Health Canada, 1998).

Given the advances in understanding the rerlationship between food, physiological function and disease, consumers are beginning to accept that, to a significant part, food has control over their health (Mollet and Rowland, 2002). Therefore the demand for functional food development and marketing was boosted over the decades. In order to meet this growing demand, government, industries, and researchers have to

look for new sources of functional foods with potential disease prevention attributes. Flaxseed incorporation into the diet is particularly attractive from the perspective of development of foods with specific health advantages.

Multiple-benefit products in the functional food industry are becoming more common. Conversion of barley hull from a low profit waste into a value-added functional food ingredient is a big challenge and the possibility of co-extraction of barley hull and flax hull to produce a better functional food ingredient needs further investigation.

The rationale and justification of this study has been established on the above literature review. The biological effect of phenolic compounds from barley hull has never been studied. An extract from barley hull fiber extract has been studied in a cell culture model with reference to phytate but not the phenolic compounds (Kennefick and Cashman, 2000). Unlike wheat and oat, barley and its fractions, especially the hull, are less studied for their antioxidant potential. Although the volume of studies on antioxidant capacity of barley grain *in vitro* is growing, there is a lack of information on barley hull antioxidant capacity both *in vitro* and *in vivo*. In the literature, phenolic compounds are concentrated in the hull which provides antioxidant defense system to protect the seed from oxidative stress (Lee et al., 2003); however, the detailed phytochemical profile of barley hull is yet to be completed. Moreover, the antioxidant capacity of barley hull extract has never been studied in cell culture or in animal models. On the other hand, flax hull is the only type of hull which can be directly added into the foods (Hall et al., 2005) and is available on the market. However, no studies have been reported involving the combination of flax hull with other type of hulls or by-products, such as cereal bran or hull. Human and animal models can be expensive and time-consuming, whereas a cell

culture model allows for rapid, inexpensive screenings (Liu and Finley, 2005). Therefore, the proposed study will extract the phenolic compounds from barley hull, flax hull, and their co-extract as potential functional food ingredients, identify the individual phenolic compounds and evaluate their antioxidant activity using chemical assays and cell culture models.

Hypothesis: The combination of barley hull and flax hull might produce a new functional food ingredient with high antioxidant and antiproliferative effect through interaction.

Objectives:

The main objective of the study is to investigate the potential of barley hull extract, flax hull extract and their co-extracts as functional food ingredients. The specific objectives of the study include:

1. To investigate the phytochemical profile as well as their antioxidant capacity *in vitro* in barley hull extract, flax hull extract, and their co-extract.
2. To investigate the effect of incorporating the above extracts into a food system.
3. To investigate the antioxidant and anticancer effects of the extracts using cell culture model.

Chapter 2: Literature Review

2.1 Barley Production & Utilization

Barley (*Hordeum vulgare vulgare L.*) is an ancient and important cereal grain, which has been domesticated primarily as a feed and malting grain (Baik et al., 2008). The world production of barley is over 130 million tonnes annually, ranking fifth among all crops in dry matter production around the world today (FAO, 2007). North America grows approximately 14% of the world annual production of barley (Kim & Dale, 2004). Canada is the third largest producer of barley in the world, the crop being grown mainly in the Prairie Provinces (Alberta, Manitoba, and Saskatchewan). During 2008-2009 crop year, annual production of barley in Canada was 11.78 M mt constituting 7.7 M mt used for feed, waste and docage, and only 0.157 M mt used for food and industrial use (Statistic Canada 2010). Furthermore, as one of the most genetically diverse cereal grain, barley can be classified as spring or winter types, two-row or six-row, hulled or hulless by presence or absence of hull tightly adhereing to the grain, and malting or feed by end-use type, normal, waxy, or high amylose starch types, high lysine, high β-glucan, and proanthocyanidin-free by composition (Baik et al., 2008).

The anatomical structure of barley kernel is presented in Figure 1. It is comprised of the caryopsis and the enclosing hull or husk formed from the lemma and palea. Recently, about two-third of the barley crop has been used for feed, about one-third for malting and about 2% for food directly (Baik et al., 2008).

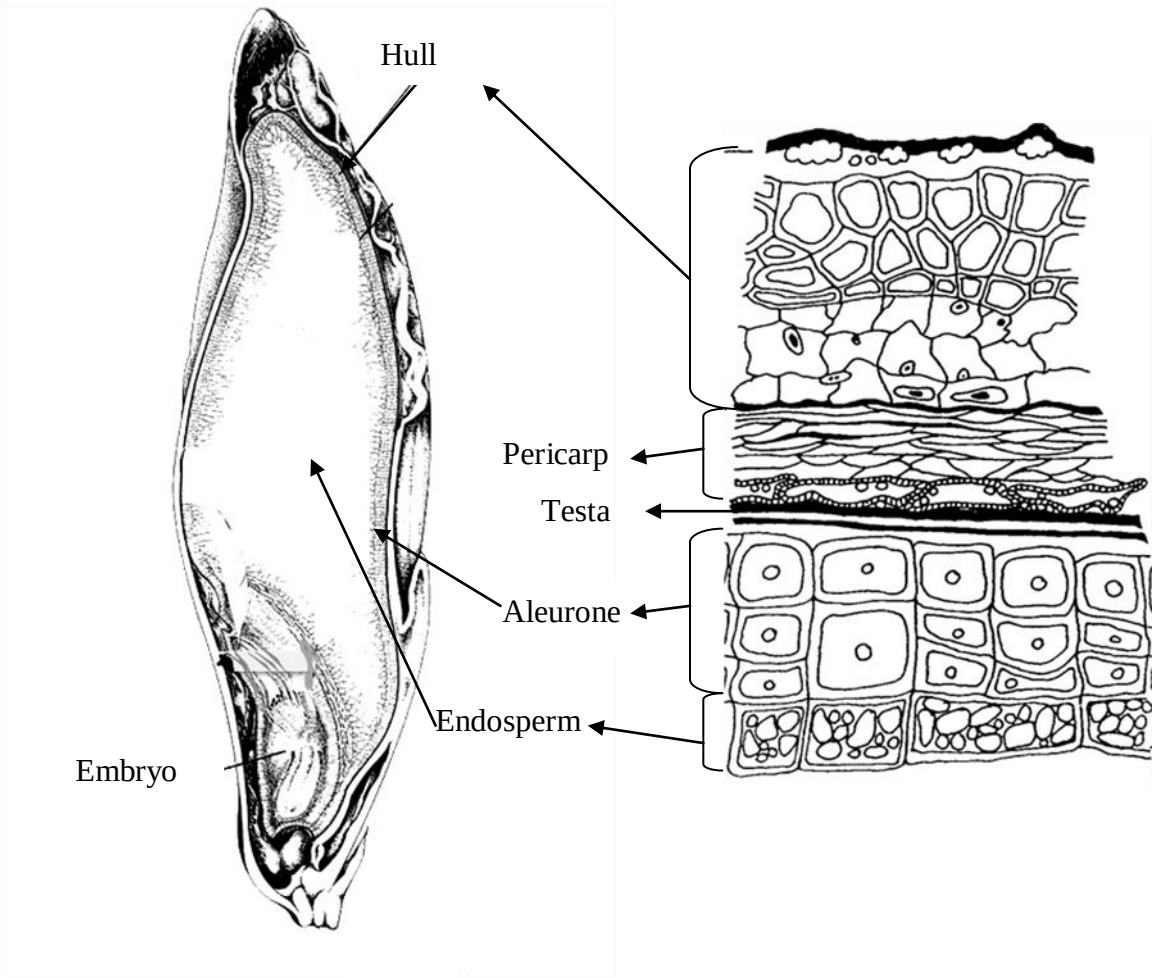


Figure 1. Anatomy of barley grain

In the brewing industry, brewer's spent grain, the most abundant by-product, mainly consists of the hull of the original barley grains, obtained after wort preparation (Mussatto et al., 2006). Due to the relatively low or no cost and abundant availability, as well as potential nutritional and functional values, there is growing attention paid to its conversion into value-added products. The chemical composition and potential utilization of brewer's spent grain, chiefly made of barley hull, are discussed below.

2.1.1 Barley Hull Characteristics & Potential Utilization

Brewers' spent grain, mainly consisting of barley hull, is a lignocellulosic material containing about 17% cellulose, 28% non-cellulosic polysaccharides, chiefly arabinoxylans, and 28% lignin (Mussatto et al., 2006). Currently, the main application is limited to animal feeding or simply as landfill, however, several potential applications have been suggested, such as food additive due to high content of protein and fiber, substrate in cultivation of mushrooms and actinobacteria, as a source of value-added products like ferulic acid and p-coumaric acids, xylose, arobinose, or as a raw material for xylitol and arabitol production (Mussatto et al., 2006; Mussatto et al., 2007, Özvural et al., 2009). Due to its high protein and fiber content, brewers' spent grain is too granular for direct addition in food and must be converted to flour (Hassona, 1993; Miranda et al., 1994a, b; Öztürk et al., 2002). Huige (1994) and Townsley (1979) successfully incorporated brewers' spent grain flour into a number of bakery products, including breads, muffins, cookies, mixed grain cereals, fruit and vegetable loaves, cakes, waffles, pancake, tortillas, snacks, doughnuts and brownies. Since brewers' spent grain is produced during malting and brewing, and may contain not only barley hull but also pericarp, seed coat layers as well as the residues from malted barley and wheat, rice or maize added during mashing (Mussatto et al., 2006), the chemical composition may differ from that of barley hull itself. Intensive research has been conducted on how to utilize brewers' spent grain integrally, however, there is limited information regarding pure barley hull. Therefore, it is necessary to investigate potential application of barley hull.

Similar to brewers' spent grains, barley hull is an agricultural by-product mainly used as a carbohydrate source for feed supplement or for manufacturing of glucose or ethanol (Moldes et al., 2002). Barley hull is composed of 23.0% cellulose, 32.7% hemicelluloses, 21.4% lignin, 1.6% acetyl groups, and 21.3% other (Moldes et al.,2002; Höije et al., 2005). Barley hull also contains protein, uronic acid and acetyl groups (Garrote et al., 2004). As xylan is the most abundant hemicellulose polymer in barley hull, it can be hydrolyzed into xylose and further fermented to produce xylitol. Xylitol, a low caloric pentitol with sweetening power and anticariogenic properties, is suitable as sugar substitute for diabetics (Parajó et al., 1997; Cruz et al., 2000). The utilization of barley hull as feed supplement is limited due to its low digestibility while combustion of hull material is difficult and not practical due to its high ash content, which results in mineral depositions in boilers (Cruz et al., 2007; Garrote et al., 2008). On the other hand, it is very expensive to transport barley hull to the disposal areas owing to its low density (Mahmudi, 2005; Searcyl et al., 2007; Garrote et al., 2008). Current interest in barley hull remains as substrate for saccharification and fermentation. Kim et al. (2008) treated the barley hull with aqueous ammonia for extended periods of time at 30-75 ℃ and used enzymatic saccharification to evaluate the potential of pre-treated hull for bioconversion to fuel ethanol and/or for use as a ruminant feed component with enhanced digestibility. In order to produce substrate for fermentation media, phenolic compounds have to be removed, because of their inhibitory effect on bacterial growth (Schwald et al., 1988). However, there is lack of detailed knowledge on the phenolic compounds found in barley hull, and a systematic study of its antioxidant capacity is urgently required.

2.1.2 Phenolic Compounds in Barley Hull

Due to the high content of lignin, phenolic acids can be isolated from barley hull (Cruz et al., 2001; Mussatto et al., 2006). Bartolomé et al. (1997, 2003) reported that ferulic acids and p-coumaric acids are the most abundant phenolic acids in brewers' spent grain. It has been known for decades that p-coumaric acid is the dominant phenolic in barley hull since it forms linkages with lignin (Higuchi et al., 1967). Salomonsson et al. (1980) indicated that *p*-coumaric acid was present in the lowest amount in the center of the barley kernel and rapidly increased toward the outer layers, particularly the lignified hull (Maillard and Berset 1995).

Non-isothermal treatment of barley hull in aqueous media can selectively degrade their hemicellulose fraction, and release araban, xylan, oligosaccharides, monosaccharides, furfural, furfural-degradation products, acetyl groups, acetic acid and phenolic compounds (Garrote et al., 2004; 2008). Garrote et al. (2008) treated barley hull with water at high temperature (185-260 ^{o}C) for autohydrolysis and then extracted phenolic acids by ethyl acetate (Table 1).

Table 1. Phenolic compounds present in liquors from isothermal autohydrolysis of barley husks at 216 ℃ (Garrote et al., 2008)

Compound	Concentration in original liquors (g/L)	Concentration after saponificaiton (g/L)
3,4-Dihydroxybenzaldehyde	0.098	0.141
4-Hydroxybenzaldehyde	0.123	0.284
Benzoic acid	3.428	2.783
Coumaric acid	0.134	0.226
Ferulic acid	0.066	0.179
Gallic acid	0.564	0.409
Vanillic acid	0.010	0.018
Vanillin	0.056	0.054

The identified phenolic compounds accounted for 3.3–3.6 g/100 g barley husks (dwb) (Garrote et al., 2008). Benzoic and cinnamic acids are the major phenolics obtained following depolymerisation of the acid-soluble lignin fraction (Garrote et al., 2008). The antioxidant capacity of ethyl acetate soluble extracts was measured in terms of EC_{50}, which was found in the range 0.7–0.9 g/L (Garrote et al., 2008). Cruz et al. (2007) detected ferulic acid and p-coumaric acid as the main phenolic compounds present in barley hull, and the levels of these two acids varied under different pH conditions. The extraction yield of ethyl acetate-soluble compound was 3.13g/100g (dwb) at pH=12.8 and more than three times at pH=3 (9.99/100g). Ferulic acid and p-coumaric acid were present at levels of 2.5% (w/w) and 3% (w/w) of the extracts, respectively (Cruz et al., 2007).

Similar to ferulic acid being the predominant phenolic acid in wheat, p-coumaric acid was found to be the main phenolic acid in the bran fraction of black barley lines (Siebenhandl et al., 2007). The bran fraction of black barley samples consisted of both pericarp and hulls (lemma and palea), thus, researchers associated the high content of p-coumaric acid with the hulls, and ferulic acid with the pericarp (Siebenhandl et al., 2007). The main monomeric phenolic compounds obtained from barley straw in decreasing order of abundance by alkaline hydrolysis are vanillin, syringaldehyde, p-coumaric acid, p-hydroxybenzaldehyde, ferulic acid, syringic acid, p-hydroxybenzoic acid, vanillic acid and acetovanillone (Sun et al., 2001 & 2002).

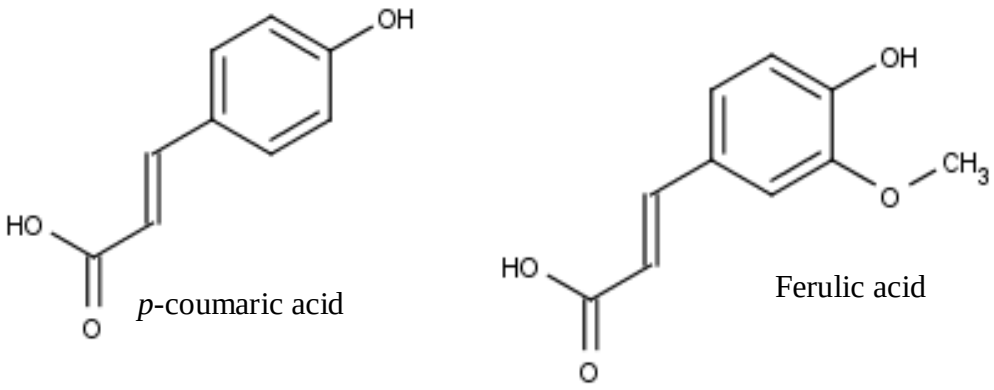
Compared to barley hull, whole grain barley contains a much wider range of compounds with potential antioxidant effects such as benzoic and cinnamic acid derivatives, proanthocyanidins, quinines, flavonols, chalcone, flavones, flavanones, and amino phenolic compounds (Hernanz et al., 2001). Barley grains contain phenolic compounds ranging from 0.2 to 0.4% (Bendelow and LaBerge, 1979). The phenolic content of grains, expressed as catechin equivalents, has been reported to be 24.3 μg/mg for barley, 17.6 μg/mg for oats, 10.2 μg/mg for wheat, and 8.9 μg/mg for rye (Zienliński & Kozlwaka, 2000). The free phenolics in barley grain are composed of flavonoids (mainly proanthocyanidins (PAs), anthocyanins and catechins) whereas the majority of bound phenolics are phenolic acids (Von Wettstein et al., 1985; Andreasen et al., 2001). These compounds are mainly concentrated in the hull, testa, and aleurone (Nordkvist et al., 1984, Jende-Strid, 1993; Goupy et al., 1999). Anthocyanin levels in the hull, pericarp and/or aleurone layer determine the grain color, thus, the grain colour of barley can vary from light yellow to purple, violet, blue and black (Baik et al., 2008). Blue aleurone

barley has five times more anthocyanin content than white/yellow aleurone barley, and two times more than that of weakly blue aleurone barley (Baxter & O'Farrell, 1987). According to Abdel-Aal & Hucl (1999), blue aleurone barley had an anthocyanin content of 174-291 µg/g. The flavonol content in barley ranged from 10.9 to 66 µg rutin equivalent/g (Goupy et al., 1999). Quinde-Axtell and Baik, (2006) quantified caffeic (15 to 36 µg/g), p-coumaric (hull-less genotypes: 4-21 µg/g, hulled genotypes: 23-68 µg/g), and ferulic acids (301 to 567 µg/g) in 11 barley genotypes, which is in agreement with previous studies (Hernanz et al.,2001; Zupfer et al.,1998). However, the p-coumaric acid content was significantly lower in hull-less genotypes (4-21 µg/g) as compared to hulled genotypes (23-68 µg/g) (Quinde-Axtell et al., 2006), which is in agreement with earlier reports that the aleurone layer is high in ferulic acid, while p-coumaric acid is concentrated in the hull (Nordkvist et al.,1984). Both FA and p-CA are associated with cell wall constituents because they are ester-linked to them, especially to arabinoxylans and lignin (Holtekjølen et al., 2006). Quinde-Axtell et al., (2006) observed significant differences in phenolic acid profiles among genotypes. They concluded that hulless barley had a lower total phenolic acid content as compared to hulled barley, mainly due to the low p-coumaric acid content of hulless barley (Quinde-Axtell et al., 2006).

2.1.3 Health Benefits of Ferulic acid & p-coumaric acid

Accounting for one third of dietary phenolic compounds excluding flavonoids, daily consumption of phenolic acids is estimated to range from 25 mg-1 g depending on a person's diet (fruit, vegetables, grains, teas, coffees, spices) (Clifford, 1999).

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, Fig 2.) and p-coumaric acid (trans-4-hydroxycinnamic acid) are ubiquitous phenolic compounds in cereal grain, fruits and vegetables and other plant tissues. Phenolics are the products of secondary metabolism in plants, providing essential functions in the reproduction and the growth of the plants, acting as defense mechanisms against pathogens, parasites, and predators, as well as contributing to the color of plants (Liu, 2004).



p-coumaric acid

Ferulic acid

Figure 2. Chemical stucture of p-coumaric acid and ferulic acid

In spite of their well-documented antioxidant activity, there is accumulating chemical, biochemical, clinical and epidimiological evidence supporting the chemoprotective effects of phenolic compounds. Generally, phenolics are considered as potential therapeutic agents against a wide range of ailments including neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunction, inflammatory diseases and in aging (Soobrattee et al., 2005). Chemically, ferulic acid and p-coumaric acid have a phenolic nucleus and an extended side chain (Fig.2) which readily forms a resonance-stabilized phenoxy radical and enables them to scavenge free radicals and prevent oxidative stress (Graf,1992). Ferulic acid can be absorbed, metabolized and distributed in

rodents and humans, before it is finally excreted in urine as 3-hydroxyphenyl and 3-methoxy-4-hydroxy phenyl derivatives of phenyl propionic acid, hydracrylic acid and glycine conjugates (Srinivasan et al., 2007). Due to the strong free radical scavenging capacity, ferulic acid may be beneficial in prevention and/or treatment of disorders linked to oxidative stress, including Alzheimer's disease (Jin et al., 2005), cancer (Chang et al.,2006), atherosclerosis (Dinis et al., 2002), inflammatory diseases (Murakami et al.,2002). Sodium ferulate, a salt of ferulic acid, is used for treatment of cardiovascular and cerebrovascular disease in China (Wang and Ou-yang, 2005). p-coumaric acid (Fig.2) is another phenolic acid of great interest due to its chemoprotective and antioxidative effect (Mussatto et al., 2007). Abdel-Wahab et al. (2003) reported that p-coumaric acid protected rat's heart against doxorubicin-induced oxidative stress, and suggested that p-coumaric acid can be used an adjuvant therapy in cancer management. A wide spectrum of biological effect of p-coumaric acid has been investigated in animal studies, including inhibition of LDL oxidation (Zang et al., 2000), reduction in oxidative damage to DNA (Guglielmi et al., 2003) and inhibition of platelet aggredation (Luceri et al., 2007). The antioxidant activity of phenolic acids against hydroxyl and peroxyl radical oxidation were investigated in synaptosomal and neuronal cell culture system *in vitro*, whereas ferulic acid showed far more potential than vanillic, coumaric, and cinnamic acid in attenuation of protein oxidation, lipid peroxidation, and ROS production (Kanski et al., 2002). p-Coumaric acid exerts antioxidant activity both *in vitro* and *in vivo*, and shows a protective effect from UV-B-induced oxidative damage in rabbit corneal-derived (SIRC) cells (Lodovici et al., 2003).The paharmacological actions of phenolic antioxidants is mainly attributed to their antioxidant activity, their free radical scavenging and chelation

of redox active metal ions, modulation of gene expression and interaction with the cell signaling pathways (Soobrattee et al., 2005).

2.2 Flaxseed Production & Utilization

Flax (*Linum usitatisimum* L.) is one of the ancient crops cultivated as early as 6000 B.C. in the earliest agrarian societies in the Tigris and Euphrates valleys in Mesopotamia (Oates, 1979). Nowadays, flax is grown as either an oil crop or as a fiber crop (Touré et al., 2010). Canada plays a dominate role as the world's largest flax producer, contributing about 40% of total world production and 75% of world export (Oomah and Mazza, 1998). It is mainly grown in the prairies (Manitoba, Saskatchewan, and Alberta). From 2008 to 2009, the annual production of flaxseed in Canada was 861 kt, of which 627 kt was exported, and only 181 kt was used domestically (Agriculture and Agri-Food Canada, 2010). Flaxseed provides essential nutrients, such as protein (30-35%), oil (30-45%) including omega-3 fatty acids, carbohydrates (30-35%), fiber (10%), as well as complex phenolic compounds know as lignans (Bhatty, 1995). The lignan component of particular interest in flaxseed is secoisolariciresinol diglucoside (SDG) due to its abundance in flaxseed and its health benefits related to its estrogen-like actions in animals and humans (Mitchell, 2001). Flaxseed is one of the most concentrated sources of the lignan precursor SDG and contains 75-800 times the amount found in other foods (Thompson et al., 1991). However, limiting factors for flaxseed consumption are the high content of glucoside (100-300 mg hydrogen cyanide/kg seed) and cadmuim ((294-1543 μg/kg) (Rosling, 1993; Oomah et al., 2007). The daily intake of flaxseed should be limited to 10-20 g whole flaxseed with regard to the levels of cyanogenic glucosides

(Strandås, 2008). A high content of mucilage (2%) in flaxseed also restrict the daily intake to approximately 45 g due to a laxative effect in humans (Clark et al., 2001)

2.2.1 Flaxseed Hull

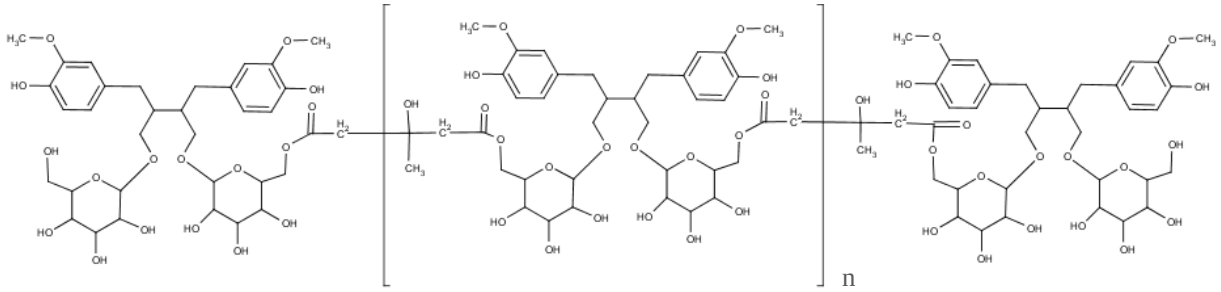
Flaxseed hull and mucilage constitute about 40% of the total seed (Oomah et al., 1996). The hull consists of an outer, true hull, which is tough and fibrous, with no oil and protein, and an inner soft hull containing some oil and protein (Oomah et al., 1996). Because much of the fiber is in the hull, efforts are directed towards removing the maximum amount of hull from the seed and meal and simultaneously obtaining a pure hull fraction with physiological properties (Oomah and Mazza, 1998).

As a byproduct of flaxseed oil extraction, flaxseed meal is generally obtained by cleaning, flaking, cooking and pressing of the seed followed by solvent extraction and solvent removal steps (Oomah and Mazza, 1997). The defatted meal mainly consisting of the hull (38% on dry weight basis) (Agriculture and Agri-Food Canada, 1997) is normally underutilized as food or discarded.

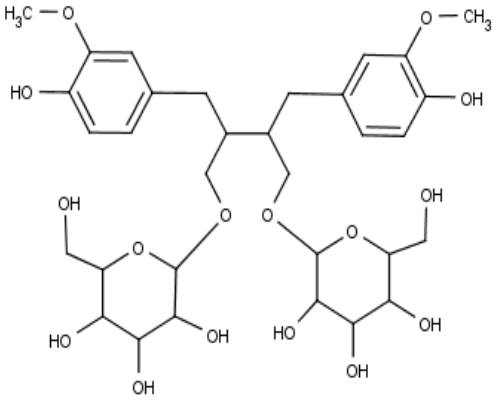
Removal of the seed coat (hull) from flaxseed has proven difficult due to a layer of endosperm tissue adhering to the hull. The exact location of SDG in flaxseed has never been established (Wiesenborn et al., 2003). A negative correlation has been found between SDG content and oil content in different fractions of dehulled flaxseed, indicating that SDG might be found in the hull (Madhusudhan et al., 2000; Wiesenborn et al., 2003).

2.2.2 Bioactive Compounds in Flaxseed Hull

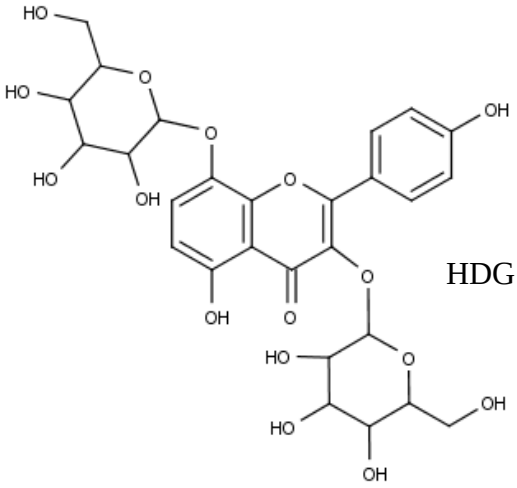
Recently, it is believed that flaxseed hulls are an excellent source of lignans, which belong to the group of phytoestrogens (Hallund et al., 2006). The main lignan in flaxseed is SDG, which varies in content between 6 and 29 mg/g in the defatted flaxseed powder (Eliasson et al., 2003; Beejmohum et al., 2007; Charlet et al., 2002; Johnsson et al., 2002). The formation of SDG takes place in the outer layer of the seed (Hano et al., 2006). Therefore, the concentration of SDG found in flaxseed hulls is higher than that of whole seeds (Madhusudhan et al., 2000). In flaxseed hull, lignans are present in an oligomeric structure ((Kamal-Eldin et al., 2001), which is referred to as the lignan macromolecule (Fig.3). The backbone of the lignan macromolecule is SDG which is esterified to hydroxyl-methyl-glutaric acid (Kamal-Eldin et al., 2001). Recently, it has been reported that p-coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) and flavonoids herbacitin glucoside (HDG), caffeic acid glucoside constitute part of the lignan macromolecule (Struijs et al., 2007, Struijs et al., 2008). They can be released after alkali treatment of the flaxseed extract containing lignan macromolecule (Johnsson et al., 2002, Westcott and Muir, 1996). Struijs et al. (2007) obtained 7.5% (w/w) of extraction yield from flaxseed hull, and 0.2% (w/w) HDG in flaxseed hulls is found.



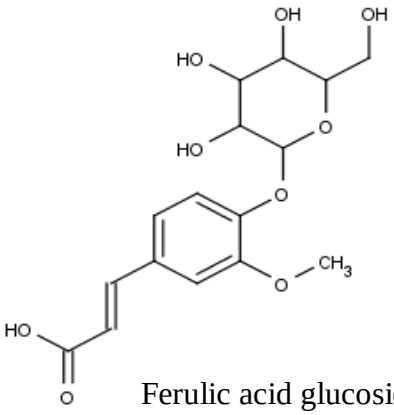
SDG-HMGA oligomers



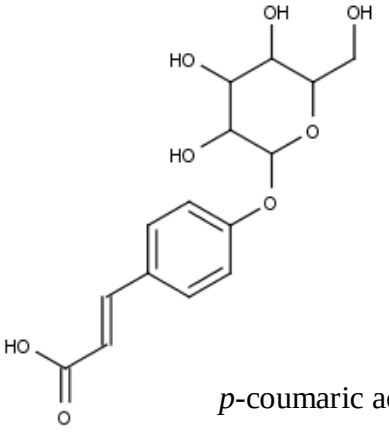
SDG



HDG



Ferulic acid glucoside



p-coumaric acid glucoside

Figure 3. Structure of the pheolic glucosides in the flaxseed phenolic complex; secoisolariciresinol diglucoside 3-hydroxy-3-methyl glutaric acid oligomers (SDG-HMGA oligomers (average n=3), secoisolariciresinol diglucoside (SGD), p-coumaric acid glucoside, ferulic acid glucoside, and herbacetin diglucoside (HDG).

Flaxseed contains 8-10 g/kg of total phenolic acids that include 5 g/kg of esterified phenolic acids, and 3-5 g/kg of etherified phenolic acids (Oomah et al., 1995). In the whole seed, the content of SDG and phenolic acid glucosides varies significantly. Eliasson et al. (2003) detected, on dry matter basis, that different samples of flaxseeds varied considerably in their content of (+)-SDG (11.9–25.9 mg/g), (-)-SDG (2.2–5.0 mg/g), p-coumaric acid glucoside (1.2–8.5 mg/g), and ferulic acid glucoside (1.6–5.0 mg/g). Other lignans present in flaxseed are matairesinol (MAT) (Liggins et al., 2000), isolariciresinol (isoLARI) (Meagher et al., 1999), pinoresinol (PINO) (Meagher et al., 1999), and lariciresinol (LARI) (Sicilia et al., 2003). Other phenolic compounds reported in flaxseed, which might contribute to the health effects ascribed to flaxseeds, are hydroxycinnamic acids including p-coumaric acid (Klosterman et al., 1955), ferulic acid, sinapic acid, caffeic acid (Dabrowski and Sosulski, 1984) and their glucosides, as well as the flavonoids herbacetin diglucoside (HDG) and kaempferol diglucoside (KDG) (Qiu et al., 1999).

2.2.3 Health Benefits of Flaxseed Lignan

A broad range of health benefits of flaxseed lignan is being revealed by growing numbers of cell culture studies, animal models as well as human clinical trials. It is believed that the beneficial effects of flaxseed on certain diseases can be partially attributed to SDG. The demonstration of clinical activity associated with the consumption of flaxseed has led the U.S. National Cancer Institude to target flax as one of the six plant materials for study as cancer-preventive foods (Caragay, 1992).

2.2.3.1 Antioxidant Activity

Recently, SDG has been reported to have antioxidant activity *in vitro* comparable to ferulic acid but higher than α-tocopherol (Srandas et al. 2008). The antioxidant activity of the flaxseed lignans and metabolites exerts protective effect against AAPH-induced or DPPH-induced oxidation (Hosseinian et al., 2006). Prasad (2000) also indicated that the antioxidant activities of SDG, secoisolariciresinol (SECO), enterodiol (END), and enterolactone (ENL) contributed in reduction of hypercholesterolemia, atherosclerosis, and diabetes. The antioxidant activity of flaxseed *in vivo* was measured by monitoring hepatic enzymes (catalase, SOD, and peroxidase) in weanling albino rats (Rajesha et al., 2006). In the flax lignan complex- (34-38% SDG, 15-21% cinnamic acid and 9-11% hydroxymethylglutaric acid by weight) treated hypercholesterolemic rabbits, serum malondialdehyde (a measure of lipid oxidation) level was decreased by 35%, aortic malondialdehyde was decreased by 58% (Prasad, 2005). It is been suggested that the beneficial effects of SDG in cancer and lupus nepheritis showed that these beneficial effects could be due to the ability of SDG to scavenge OH radicals (Prasad 1997).

2.2.3.2 Anticancer Effect

Flaxseed lignans could be a significant part of a treatment regimen for cancer based on the large numbers of animal studies as well as small scale human clinical trials. In the past two decades, researchers demonstrated the protecitve effect of flaxseed and its components against breast cancer using animal studies with induced cancer. Research evidence has shown that SDG prevents / inhibits mammary carcinogenesis in rats (Wei et al., 1995; Serraino & Thompson, 1991, 1992). Rajesha et al. (2006) reported that

weanling alibo rats fed with flaxseed supplemented diet for 14 days restored the hepatic marker enzymes like catalase, SOD, peroxidase after intoxication by carbon tetrachloride. Saarinen et al. (2008a) determined the accesibility and accumulation of lignans to breast cancer tissue after oral administration of tritium labeled dietary SDG (^{3}H-SDG) to athymic mice bearing MCF-7 tumors. The authors indicated that the accessibility of lignans to tumor tissue suggests that part of the anticancer effect of lignans may be due to their direct local effects on the breast cancer tissues (Saarinen et al., 2008a). The long term effect of flaxseed alone and in combination with soy protein were determined on the established estrogen responsive human breast cancer MCF-7 xenografts in ovariectomized athymic nude mice (Saarinen et al., 2006). Flaxseed supplimentation attenuated the tumor size similar to that of control, and also reduced the stimulating effect of soy protein on the growth of estrogen responsive MCF-7 cancers in ovariectomized mice during a 25-week study (Saarinen et al., 2006). Other observations also include reduced tumor occurrence and size at the initiation and promotion stages of carcinogenesis (Serraino & Thompson, 1992), reduced metastasis at the late progress stage of estrogen receptor negative carcinogenesis (Dabrosin et al., 2002) in flaxseed treated rats or mice. The beneficial effects of SDG or flaxseed combined with tamoxifen (a selective estrogen recepter modulator, clinically used in the treatment of breast cancer) reduced the tumor growth by reducing cell proliferation, expression of genes, and protein involed in the entrogen receptor-negative(ER-) and growth factor-mediated signalling pathways with flaxseed oil having the greatest effect in increasing apoptosis compared with tamoxifen treatment alone (Saggar et al., 2010a). However, SDG showed greater effect in the reduction of tumor growth compared to flaxseed oil (rich in alpha-linolenic

acid) treatment primarily through tumor cell proliferation, the reduction of presenilun 2 (PS2), B-cell lymphoma 2 (Bcl2), as well as insulin-like growth factor 1 receptor (IGF-1R) mRNA expression rather than increasing apoptosis (Saggar et al., 2010b). The mixture of SDG and flaxseed oil resulted in a significant interaction, in which the effect of this combination was different than the effect of the administration of either component alone, suggesting that SDG and flaxseed oil antagonize each other's effect (Saggar et al., 2010a,b). Consumption of 25g flaxseed in a muffin formulation reduced the tumor cell proliferation and human epidermal growth factor receptor 2 (HER2) index and increased apoptosis in a randomized, placebo controlled clinical trial in postmenopausal women with newly diagnosed breast cancer (Thompson et al., 2005). Chen et al. (2009) demonstrated that flaxseed and pure SDG had a similar effect in reducing tumor growth and in mechanisms of action, including downregulating ER- and growth factor mediated cell signalling, whereas flaxseed hull did not significantly reduce those biomarkers tested in the study. The authors pointed out several limitations of this study, which included unequal SDG concentration in flaxseed and in the hull, as well as lack of phytochemical analysis in the hull, particularly those with antinutritional effects, which may interact with the beneficial effect of SDG (Chen et al., 2009). The biological effects of other lignans, besides SDG, are also investigated. The mice treated with lignan schisandrin B exhibited increased activity of the antioxidant enzymes when compared to carbon tetrachloride treated mice and control group mice (Kitts et al., 1999). The lignan genistein has been shown to be a potential antioxidant *in vitro* (Ip et al., 1995). The action of lariciresinol, a dietary lignan, on hormone responsive mammary cancer in rats has been studied in a similar model used in the study conducted by Saarinen et al. (2006) and

Power et al. (2007). The results showed that lariciresinol enhanced tumor cell apoptosis and increased estrogen receptor beta expression in human MCF-7 breast cancer xenografts in athymic rats (Saarinen et al., 2008b).

On the other hand, accumulating evidence from clinical trials, animal models and cell culture studies allow us to underline specific anticancer mechanisms of lignans on growth and development of prostate cancer (Chen et al., 2009). Lin et al. (2001) demonstrated that enterodiol and enterolactone significantly decreased the cell viability in 3 human prostate cancer cell lines (LNCap [hormone sensitive], PC-3 and DU-145 [hormone insensitive]). Furthermore, enterolactone was found to induce apoptosis in LNCap cells via a mitochondrial-mediated, caspase-dependent pathway (Chen et al., 2007). A new finding about enterolactone against prostate cancer has been revealed by Chen et al., (2009), suggesting that enterolactone suppresses proliferation and migration of prostate cancer cells at nutritionally relevant concentrations (20-60 umol/L), at least partially through inhibition of IGF-1R signalling. McCann et al. (2008) observed that enterolactone inhibited the LNCap human prostate cancer cell proliferation at a concentration of 60 uM through altered expression of cell cycle associated genes.

Other than breast cancer, dietary flaxseed significantly decreased tumor multiplicity and size in Apc^{Min} mice bearing intestinal tumor (Bommareddy et al., 2009). According to Qu et al. (2005), lignans were involved in antitumor activity of wheat bran human colonic cancer SW480 cells. The combination of two metabolites of lignan, enterolactone and enterodiol respectively, caused more severe inhibition of SW480 cells (Qu et al., 2005).

2.2.3.3 Protective Effect of SDG in Other Diseases

Flaxseed lignans may also protect against cardiovascular disease (CVD) and diabetes by reducing lipid and gluose concentrations, lowering blood pressure, and decreasing oxidative stress and inflammmation (Adolphe et al., 2010). The cardioprotective effect of flaxseed is mainly attributed to the lingnan complex or SDG which can slow the progression of atherosclerosis, reduce the ischemia-reperfused myocardial infarct size, and reduce the oxidative stress in aorta in hyerchlesterolemic animal models (Prasad, 1999; Penumathsa et al., 2007). Furthermore, Prasad (2000) reported that SDG prevented the development of diabetes mellitus in diabetic prone BioBreeding rats by decreasing serum and pancreatic malondialdehyde (MDA) and increasing antioxidant reserve. Flaxseed and SDG are also suggested to have beneficial effects on renal function in animal models or in humans (Hall et al., 1993; Clark et al., 2001; Velasquez et al., 2003)

Therefore, the incorporation of lignans in food and in animal diet has great advantages, helping in the inhibition of diseases and the promotion of health.

2.2.4 Flaxseed Hull Potential as a Functional Food Source

In the United States, flaxseed (FS) and flaxseed meal (FLM, partially defatted FS) have found market acceptability as a component in some cereals, specialty breads, cookies, and salad dressings (Carter 1993; Nesbitt and Thompson 1997). According to Mintel's Global New Products Databese (GNPD), in 2005 and 2006, respectively, 72 and 75 new products were launched in the United States that listed flax or flaxseed as an ingredient (Flax Canada, 2007).

Flaxseed or flax hull are now considered as functional food ingredients which can be added to produce bread (Menteş et al., 2008; Pohjanheimo et al., 2006), macaroni (Hall et al., 2005), pasta (Manthey et al., 2008), and muffins (Ramcharitar et al., 2005). Many other functional food additives have been included in bread formulation to increase its diversity, nutrition, or product appeal (Fan et al., 2007). Flaxseed hull, which contains up to 5% SDG, is now being commercialized as an SDG concentrate (Oomah and Sitter, 2009).

It is still a big challenge for researchers to develop novel functional foods containing natural nutrients which can provide multiple health benefits.

2.3 Extraction & Hydrolysis

A specific extraction methodology to obtain the majority of phytochemicals in cereals has not yet been established. Due to its ease of operation and high recovery of target compounds, solvent extraction is the most common method to extract phytochemicals from cereal grain or from other plant tissues. With advances in knowledge about the phytochemicals in cereal grains, it has been recognized that more than 90% of phytochemicals are present in bound form, mainly ester-linked to polymers in the plant cell wall (Andreasen et al., 2000; Garcia-Conesa et al., 1997; Adom and Liu, 2002). Recently, alkaline hydrolysis, acid hydrolysis, or enzymatic digestion (Adom et al., 2002; Adom et al.2003; Bonoli et al., 2004; Mpofu et al. 2006; Zhao et al., 2006; Siebenhandl et al., 2007; Qiu et al. 2009, 2010) became popular for releasing the bound phenolic in cereal grains.

2.3.1 Solvent Extraction

For analytical purposes, simple organic solvents such as methanol, ethanol, acetone are often applied to extract phytochemicals from cereals and cereal hulls (Ramarathnam et al., 1988; Zhao et al., 2006; Li et al., 2005). Triticale bran and straw were also extracted by ethyl acetate after hydrolysis with 2 M NaOH (Hosseinian & Mazza, 2009);

The first article reporting a glucosidic phenolic complex being released from the flaxseed matrix by organic extraction using dioxane/ethanol was published by Klosterman & Smith (1954). The phenolic complex in defatted flax is extracted using more polar solvents such as dioxane/ethanol, aqueous ethanol or methanol in combination with heat and mixing (Johnsson et al., 2000; Westcott & Muir, 1996; Muir & Westcott, 2000). Traditionally, extraction of SDG from flaxseed is achieved sequentially using solid-liquid extraction with alcohol (methanol or ethanol) or alcohol-water mixtures such as aqueous ethanol, followed by alkaline hydrolysis (Eliasson et al., 2003). Struijs et al. (2007) extracted lignan macromolecule from defatted hulls by a three-step sequential extraction with 63% aqueous ethanol, followed by sodium hydroxide hydrolysis to liberate SDG and other phenolic components. Other organic solvent mixtures, for example, 1,4-dioxane with 95% ethanol (1:1 v/v) are also used to isolate SDG from flaxseed (Johnsson et al., 2000; Strandas et al., 2008). Although solvent extraction is easy to operate, it is necessary to extract the sample several times to fully recover the target compounds, which makes the extraction time relatively long, ranging from 4 hrs to 2 days (Johnsson et al., 2000; Meagher et al., 1999). On the other hand, the following still has to be addressed, that organic solvent extraction of phytochemicals is not desirable for food

ingredient development, and also is considered not friendly to the environment given the production of significant amounts of chemical waste.

2.3.2 Alkaline Hydrolysis

Alkaline hydrolysis has become the primary method used for extracting phytochemicals from cereal grains or other plants (Sun et al., 2001). Cruz et al. (2007) illustrated that part of the phenolic fraction of the barley husk can be dissolved in ethyl acetate after the alkaline treatment with NaOH. Mussatto et al. (2007) described the extraction of ferulic and p-coumaric acid from brewer's spent grain using 2% NaOH concentration at 120 ℃ for 90 min. Garrote et al. (2008) recovered phenolic acids using ethyl acetate after non-isothermal (190-229 ℃) autohydrolysis.

Furthermore, alkaline hydrolysis in water or methanol is used to break ester-linkages to release SDG, p-coumaric acid glucoside, and ferulic acid glucoside or their methyl esters from the phenolic complex (Johnsson et al., 2000, 2002). Eliasson et al. (2003) developed a simple, fast and reliable method for the quantitative preparation of SDG in flaxseed meal by using direct alkaline hydrolysis without the alcoholic extraction step. Optimal conditions for direct alkaline hydrolysis were established as 1 M NaOH at 20 ℃ for one hour of hydrolysis and polysaccharides and protein were precipitated using 60% aqueous ethanol (Standas, 2008). Yuan et al., (2008) suggested that the alkaline hydrolysis of flax hull process might be divided into two stages: (a) the release of SDG and the methyl esters of p-coumaric acid and ferulic acid glucosides and (b) the release of p-coumaric acid and ferulic acid glucosides from their respective precursors. Methanolic alkaline hydrolysis produces methyl esters of p-coumaric acid and ferulic acid glucoside,

whereas direct alkaline hydrolysis results in the immediate production of p-coumaric acid and ferulic acid glucoside (Yuan et al., 2008). The same authors also investigated the hydrolysis kinetics of SDG oligomers from flaxseed, and suggested that hot alkaline solution can completely transform SDG oligomers into SDG.

Preliminary results indicated that high concentration of alkaline solution, such as 2M or 4M NaOH is not suitable for barley hull. This is due to the fact that the color of the extract after freeze drying is much darker than that of calcium hydroxide hydrolyzed sample. Dark colour is a physical parameter generally unfavourable for further food application. Another concern is that such a high content of sodium hydroxide needs a high amount of HCl to neutralize thereby producing a high salt content which makes the extract chalky and hard to remove from the solution.

2.3.3 Acid Hydrolysis

Unlike alkaline hydrolysis, acid hydrolysis in combination with heat breaks ester and ether linkages to release glucose residues and obtain SECO (Mazur et al., 1996; Charlet et al., 2002). SECO can be further dehydrated to yield anhydrosecoisolariciresinol (Anhydroseco, also called Shonanin) depending on the acid concentration (Standas, 2008). Meagher et al., (1999) isolated lignans from flaxseed meal with alcoholic solvent system followed by acid hydrolysis to release the aglycons. However, acid hydrolysis can be destructive during prolonged heating periods or when too high hydrochloric acid concentrations are used and therefore should not be applied if quantitative results or high quantity of SECO are needed ((Kraushofer & Sontag, 2002; Li et al., 2008).

2.3.4 Other Methods

Lee et al., (2003) demonstrated that far-infrared radiation (FIR) increased the amounts of active compounds in methanolic extracts of rice hull. The authors suggested that FIR radiation with its simple process would be more effective in releasing antioxidant compounds from agricultural by-products such as rice hull at an industrial scale (Lee et al., 2003).

Recently, the phenolic complex was isolated from whole flaxseed by subcritical water extraction at high temperature in combination with high pressure (Cacace & Mazza, 2006). Subcritical water extraction, also known as pressurized low polarity water extraction (or pressurized hot water extraction, superheated water extraction), decreases the dielectric constant of water and provides similar properties to ethanol or methanol. Recovery of SDG, p-coumaric acid glucoside, and ferulic acid glucoside was 80% after subcritical water extractions at 140-160 ℃ and 5.2 Pa. In another study, microwave-assisted extraction was used to quantify SDG, p-coumaric acid and ferulic acid glucosides in flaxseed and was found to shorten the time of extraction and hydrolysis of traditionally used methods (Beejmohun et al., 2007). Renouard et al. (2010) improved the extraction of SDG from flaxseed hull and whole seeds using an enzymatic step assisted with cellulase that allowed better yield as compared to β-glucosidase.

2.4 Assessment of Antioxidant Activity Using Chemical Assays & Biological Techniques

2.4.1 Chemical Assays for Evaluation of Antioxidant Activity

The most frequently used methods to measure the *in vitro* antioxidant potential of cereals and their fractions include the oxygen radical absorbance capacity (ORAC), the 2,2-azino-bis- 3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), and the ferric reducing antioxidant power (FRAP) assays (Fardet et al., 2008). However, the reactions involved in these assays are different and basically classified into two types, which are hydrogen atom transfer reaction based assay and single electron transfer reaction based assay (Huang et al., 2005). The major antioxidant capacity assays *in vitro* are listed in Table 2.

Table 2. *In vitro* antioxidant capacity assays (Huang et al., 2005)

Hydrogen atom transfer reaction based assays	ORAC (oxygen radical absorbance capacity) Inhibition of linoleic acid oxidation Inhibition of low density lipoprotein (LDL) oxidation Total radical trapping antioxidant parameter Crocin bleaching assay
Electron-transfer (ET) reaction based assays	Trolox equivalent antioxidant capacity Ferric ion reducing antioxidant parameter DPPH reduction capacity Total phenols assay by Folin-Ciocalteu reagent

Total phenolic content, ORAC, DPPH scavenging assays are widely used to evaluate the antioxidant capacity of fruits (Sun et al., 2002), vegetables (Chu et al., 2002), and cereals (Li et al., 2007; Bellido & Beta, 2009; Zhao et al., 2006) worldwide by researchers.

2.4.1.1 Total Phenolic Content

The total phenolic content assay, measured by Folin-Ciocalteu reagent, was initially intended for the analysis of proteins taking advantage of the reagent's activity toward protein tyrosine (containing a phenol group) residue (Folin and Ciocalteu, 1927).

The mechanism behind this assay relies on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be monitored spectrophotometrically at 750-765 nm (Magaldaes et al., 2008). The total phenolic content assay is convenient, simple, and reproducible. Furthermore, literature indicates an excellent linear correlation between the "total phenolic content" and "the antioxidant activity" evaluated by ET-based assays; therefore, it has become a routine assay in studying phenolic antioxidants (Huang et al., 2005).

2.4.1.2 ORAC

ORAC is one of the most popular and best standardized chemical antioxidant methods (Ou et al., 2001; Prior et al., 2005). ORAC measures antioxidant inhibition of peroxyl-radical-induced oxidations and reflects classical radical chain-breaking antioxidant activity by hydrogen atom transfer (Ou et al., 2001).

As shown in Figure 4, as the peroxyl radical-antioxidant reaction progresses, the fluorescent probe is consumed and the fluorescence intensity decreases. In the presence of antioxidant, the fluorescence decay is inhibited. The protective effect of an antioxidant is measured by calculating the net area under the kinetic curve (AUC) ($AUC_{antioxidant}$-AUC_{blank}) and expressed as Trolox equivalent (Huang et al., 2005).

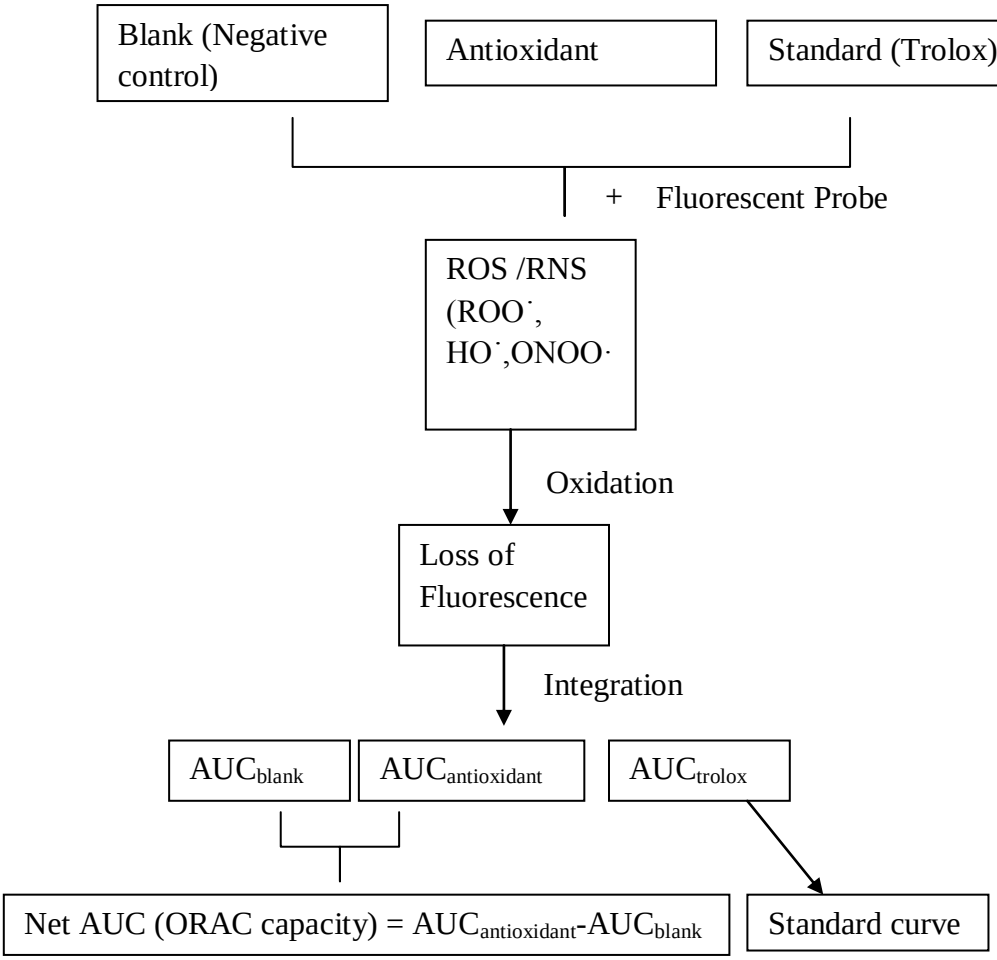


Figure 4. Principle of ORAC assay

The antioxidant capacity is quantified by recording the fluorescence decay of red-phycoerythrin (R-PE) or β-phycoerythrin (β-PE) in the presence of an antioxidant (Fig. 4). The improved ORAC assay employs a nonprotein fluorescein probe (FL) (3′, 6′-dihydroxyspiro [isobenzofuran-1[3H], 9′ [9H]-xanthen]-3-one) to overcome the limitations of β-PE (Ou et al., 2001). The advantage of ORAC over other methods is that it combines both inhibition time and inhibition percentage of free radical action by antioxidants and expresses the results as ORAC units or Trolox equivalents (Cao et al., 1995). The ORAC assay has provided substantial information regarding the antioxidant capacity of fruits and vegetables, dietary supplements, wines, juices, and nutraceuticals, and additionally, this assay also has been used to evaluate the total antioxidant status in biological systems, such as tissues and plasma (Sánchez-Moreno, 2002). Therefore, there is a broad range of applications of ORAC in academia and the food and supplement industry as a method of choice to quantify antioxidant capacity (Huang et al., 2005).

2.4.1.3 DPPH

DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•). This widely used method was first introduced by Brand-Williams et al. (1995). In this assay, the antioxidant activity is proportional to the disappearance of DPPH• in the test samples. The principle behind this assay was suggested to involve hydrogen atom transfer; however, the reaction in fact behaves like an electron transfer reaction ((MacDonald-Wicks et al. 2006). The unpaired electron is delocalized over the entire DPPH molecule, causing a deep violet colour and preventing dimerization which would normally occur in

the case of other free radicals. When this odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants, the absorbance of DPPH at 515 to 517 nm decreases. As a result, the violet colour turns to yellow colour (Ozcelik et al., 2003). The disappearance of DPPH• is proportional to the antioxidant effect in test samples. The DPPH assay is a valid, easy, accurate, sensitive and economical as well as reproducible method to evaluate scavenging activity of antioxidants of fruits and vegetanles, juice or extract (Singh and Singh, 2008). Recently, DPPH assay is quite popular and almost 90% of antioxidant studies use this assay combined with other assays (Moon et al., 2009).

2.4.2 Cell Culture

Direct assessment of antioxidant ability *in vitro* using chemicals assays is only a step to screen the compounds with higher antioxidant potential *in vivo*. If a compound is a poor antioxidant *in vitro*, it is unlikely to be any better antioxidant *in vivo* (Cadenas and Packer, 2001). Likewise, a strong *in vitro* antioxidant potential does not necessarily imply a strong antioxidant effect *in vivo*, particularly if the phytochemical is only slightly bioavailable. It is difficult to predict the *in vivo* efficiency of an antioxidant or cereal product based solely on *in vitro* measurements, especially the chemical assays. Therefore, as an intermediate testing method, cell culture models become popular for evaluation of the biological effect of a functional food ingredient due to the relatively low cost compared to the full clinical trials and gives more profound information beyond the chemical test. Cell culture assays are often used to investigate the cellular effects of

reactive species and antioxidants. The potential cell culture models for antioxidant and anticancer research are summarised as listed in Table 3.

Table 3. Potential cell culture models for antioxidant screening

Cell culture models	Biomarkers	References
Antioxidant		
Cell-based antioxidant protection assay in an erythrocyte model (CAP-e)	Similar to ORAC, scavenging oxygen radicals	Honzel et al.(2008)
Cellular antioxidant activity (CAA) assay	Inhibition of peroxyl radical induced oxidation	Wolfe and Liu (2007), Wolfe et al., (2008)
Anti-inflammatory		
Reactive oxygen species formation in Polymorphonuclear cells (ROS PMN) assay	Inhibition of ROS formation	Honzel et al.,(2008); Jensen et al., (2008)
Anticancer		Liu and Finley, 2005
Antiproliferation	Inhibition of proliferation	Liu and Finley, 2005
Caco-2 colon cancer cells		Liu and Finley, 2005; Mertens-Talcott et al.,(2006)
HT-29 colon cancer cells		Yi et al.,(2005)
HepG2 liver cancer cells		Liu and Finley, 2005
MCF-7 breast cancer cells		Liu and Finley, 2005
Cell cycle arrest	G1 arrest, G1/S ratio	Liu and Finley, 2005; Mertens-Talcott et al.,(2006)
Apoptosis	Induction/ inhibition of apoptosis	Liu and Finley, 2005; Mertens-Talcott et al.,(2006)

2.4.2.1 Cellular Antioxidant Activity Assay (CAA Assay)

With the first description of using dichlorofluorescin diacetate (DCFH-DA) as a fluoremetric assay for hydrogen peroxide quantification in cell-free system (Keston and Brandt, 1965), it became popular to use DCFH-DA as a probe to evaluate intracellular

oxidative stress. The original theory behind using DCFH-DA was that DCFH-DA was first activated by alkali removal of the diacetate moiety, and oxidized by hydrogen peroxide or peroxidase to form fluorescent dichrolofluorescein (DCF), and the fluorescence measurements were proportional to the concentration of hydrogen peroxide (LeBel et al., 1992).

This method has been modified by Bass et al. (1983) from cell-free system to cell culture to measure the respiratory burst H_2O_2 in phobol myristate acetate (PMA)-stimulated polymorphonuclear leukocytes. The mechanism of DCFH-DA oxidation in cell culture has been proposed as follows. Nonpolar DCFH-DA diffuses through the cell membrane, and once within the cell it is deacetylated by cellular esterases, forming nonfluorescent dichlorofluorescin (DCFH), which is trapped within the cell due to its more polar nature. In the presence of reactive oxygen species (ROS), DCFH oxidize to highly fluorescent DCF, a polar fluorescent compound that is also trapped with the cell. Recently, Wolfe et al. (2007) developed the so-called cellular antioxidant activity (CAA) assay based on the same principle as mentioned above, to evaluate the antioxidant activity of dietary supplements, phytochemicals, and foods.

As depicted in Figure 5, cells are pretreated with antioxidant compounds or fruit extracts and DCFH-DA. The antioxidants bind to the cell membrane and/or pass through the membrane to enter the cell. DCFH-DA diffuses into the cell where cellular esterases cleave the diacetate moiety to form the more polar DCFH, which is trapped within the cell. Cells are treated with ABAP, which is able to diffuse into cells. ABAP spontaneously decomposes to form peroxyl radicals. These peroxyl radicals attack the cell membrane to produce more radicals and oxidize the intracellular DCFH to the

fluorescent DCF. Antioxidants prevent oxidation of DCFH and membrane lipids and reduce the formation of DCF.

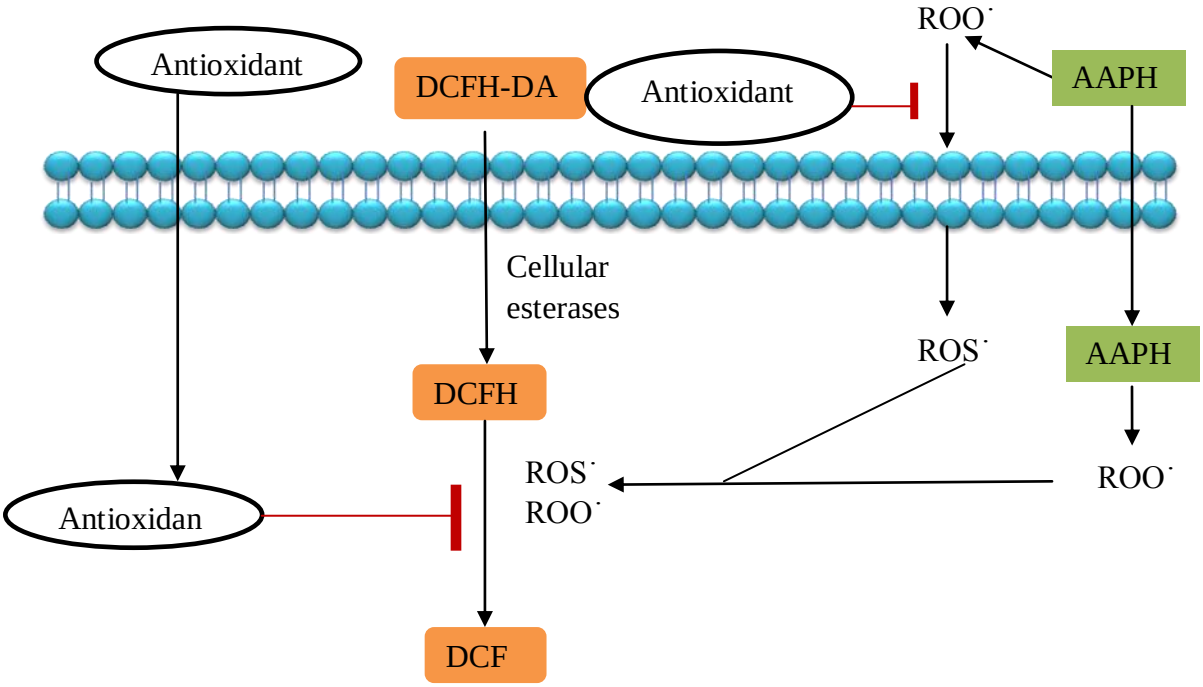


Figure 5. Method and proposed principles of the CAA assay

The decrease in cellular fluorescence when compared to the control cells indicates the antioxidant capacity. This assay is a more biologically relevant method than the popular chemical antioxidant activity assays because it accounts for some aspects of uptake, metabolism, and location of antioxidant compounds within cells (Wolfe et al., 2007). After developing this method, Wolfe et al. (2008) investigated the structure-activity relationship of flavonoid in the CAA assay, and indicated that ORAC values for flavonoids were not related to their CAA values. However, the authors believed that knowledge of structure-activity relationship in the CAA assay may be helpful in assessing potential *in vivo* antioxidant activity of flavonoids (Wolfe et al., 2008). Twenty-five common fruits consumed in the United States were evaluated for their antioxidant

activity using CAA assay (Wolfe et al., 2008). CAA assay was also applied to assess the antioxidant activities of phytochemicals purified from apple peel (He and Liu, 2008). Song et al., (2010) quantified the antioxidant acivity of 27 common vegetables consumed in the United States via CAA assay. The correlations between CAA assay and chemical assays vary. Wolfe et al. (2008) found that CAA values of 25 common fruits were significantly associated with total phenolic content and ORAC values, however, the correlation coefficients were much lower between CAA and ORAC values than between CAA and total phenolic content. Nevertheless, evaluation of the antioxidant activity of the test samples in cell culture is an important step in screening for potential bioactivity and it is more biologically representative than data obtained from chemical assays. It is believed that the CAA assay shows great improvement over the chemical assays, because it involves some aspects of uptake, metabolism, and location of antioxidant compounds (Wolfe et al., 2007, 2008).

2.4.2.2 Cell Proliferation Assay

Based on a great deal of research evidence on the proliferation of cancer cells, phenolic phytochemicals are believed as potential cancer chemopreventive agents.

The questions are why cancer cells can be so invasive and metastasic, and how phytochemicals such as phenolic compounds inhibit cancer cell proliferation. To elucidate the mechanism of phytochemicals, a basic understanding is needed of what stimulates cancer cell proliferation. Researchers believe that cancer cells, particularly those that are highly invasive or metastatic, may require a certain level of oxidative stress to maintain a balance between undergoing either proliferation or apoptosis (Kong et al.,

2000; Loo, 2003; Halliwell, 2007). The apparent reliance of cancer cells on the basal H_2O_2-induced oxidative stress for their vitality provides a logical approach to inhibit their proliferation with antioxidants that scavenge H_2O_2 in theory (Loo, 2003). As shown in Fig. 6, oxidative stress in cancer cells due to constitutive high production of tolerable amounts of H_2O_2 overactivate the mitogen-activated protein kinase (MAPK) signaling pathway, resulting in constant activation of redox-sensitive transcription factors and responsive genes that promote cancer cell viability (Loo, 2003; Hail and Lotan, 2009). Phenolic phytochemicals having antioxidant activity could scavenge the H_2O_2, thereby blocking MAPK signalling, or inhibiting the activation of redox-sensitive transcription factors and genes that are responsible for cancer cell poliferation (Loo, 2003; Yoon et al., 2007).

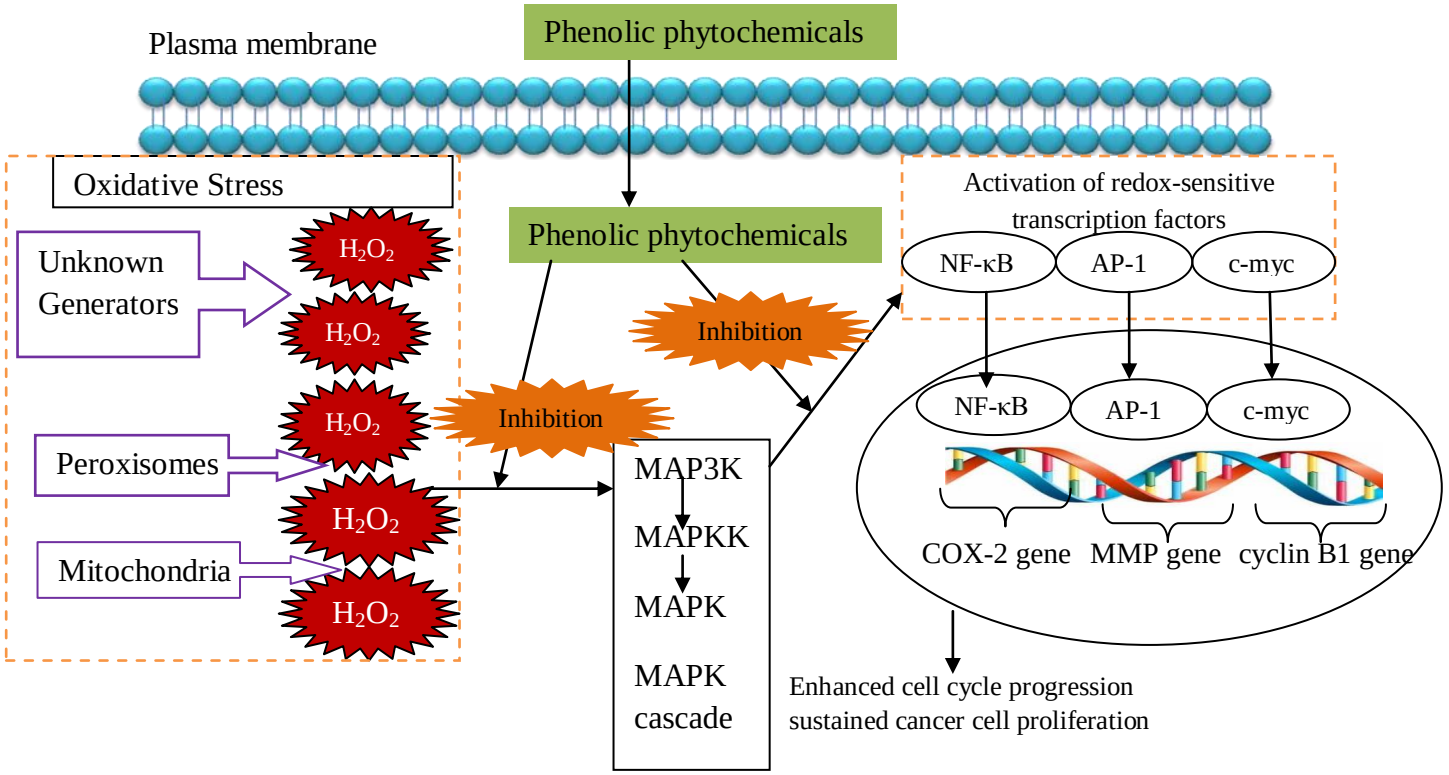


Figure 6. Putative role of H_2O_2 in the proliferation of cancers an potential impact of phenolic phytochemicals (adapted from Loo et al., 2003)

On the other hand, some phytochemicals can paradoxically induce oxidative stress under certain experimental conditions, although they unequivocally have antioxidant activity, for example epigallocatechin gallate (EGCG), quercetin, and gallic acid (Long and Clement, 2000). From this point of view, phytochemicals such as phenolic compounds induce intolerable oxidative stress in cancer cells which cause oxidative damage to DNA in conjuction with activation of the MAPK cascade that result in cell cycle arrest and /or further apoptosis (Loo, 2003).

The prooxidant effect of phytochemical on normal cells is questionable. However, it is believed that cancer cells are more susceptible to being killed by anticancer drugs

perhaps because they are already near a threshold for tolerating ROS compared to normal cells (Loo, 2003). Hileman et al. (2004) also mentioned that cancer cells in general are more active than normal cells in the production of O_2^-, are under intrinsic oxidative stress, and thus are more vulnerable to be damaged by ROS-generating agents.

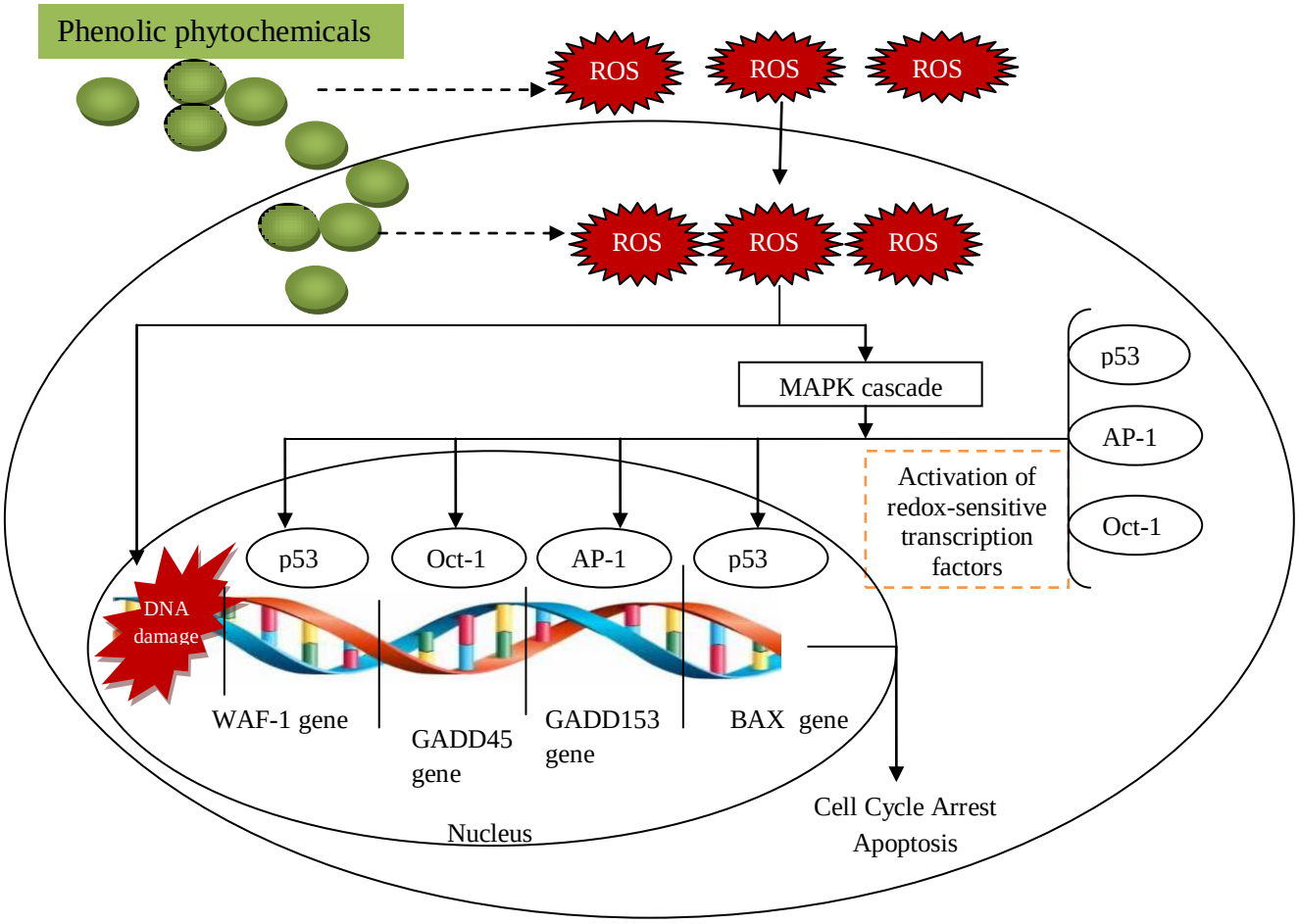


Figure 7. Concept of how phenolic phytochemicals and isothiocyanates inhibit cancer cell proliferation by inducing the formation of intolerable amounts of ROS (adapted from Loo et al., 2003)

Therefore, it is still unclear whether phenolic phytochemicals inhibit cancer cell proliferation using just their antioxidant activity alone or their prooxidant power alone.

For testing the anticancer or anti-proliferation activity of a phytochemical, the immortalized cell lines, such as tumor cell lines are the most commonly used cell types which provide consistent and straightforward evaluation (Honzel et al., 2008). Kim et al. (2007) applied 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-dye reduction assay against human colon cancer cells to evaluate the cytotoxic and antitumor activity of momilactone B extracted from rice hulls. Phenolic acid fraction extracted from blueberries showed much lower anti-proliferation effect on two colon cancer cell lines, HT-29 and Caco-2 with 50% of inhibition at around 1000 μg/mL, compared to anthocyanin fraction with IC_{50} of 15-50 μg/mL (Yi et al., 2005). A related technique used in the previous study is the MTT cell proliferation assay to detect the anti-proliferation effect. The antiproliferative activities of common vegetables (Chu et al., 2002; Yang et al.,2004) and fruits (Sun et al., 2002; Liu et al., 2002; Meyers et al., 2003; He & Liu, 2006 & 2008) were studied *in vitro* using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay) to determine the cell proliferation of HepG2 human liver cancer cells, Caco-2 human colon cancer cells as well as MCF-7 human breast cancer cells. Dong et al. (2007) determined the antiproliferative activities of isolated compounds from black bean seed coat against Caco-2 human colon cancer cells, HepG2 human liver cancer cells, and MCF-7 human breast cancer cells using colorimetric MTS assay. MTS-based colorimetric cell proliferation assay using soluble CellTiter96® Aqueous One reagent from Promega Corporation was optimized for quantitative determination of IL-15 dependent CTLL-2 cell proliferation activity (Soman et al., 2009).

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (a) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96® AQueous One Solution Reagent consists of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The mechanism of this assay is based on the bioreduction of this novel MTS tetrazolium compound by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge and Tan, 1993). According to the technical protocol supplied by Promega, assays are performed by adding a small amount of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 1–4 hours and then recording the absorbance at 490nm using a 96-well plate reader (Cory et al., 1991; Riss and Moravec, 1992). The quantity of formazan product as measured by the absorbance at 490nm is directly proportional to the number of living cells in culture. The main features of this assay are easy-to-use, fast, and convenient as well as being safe.

2.5 Incorporation of Functional Food Ingredients into Various Foods

A new trend in the functional food industry is marketing the novel functional foods containing natural nutrients with multiple-benefits (Teratanavat and Hooker, 2006). Incorporation of functional food ingredients into various foods can not only increase the nutritional value and the health benefits, but also improve food quality and safety. Zhang et al. (2007) illustrates that bamboo leaves extract, which is rich in flavonoids, could

significantly reduce acrylamide formation in fried chicken wings and yet still retain the original flavour and odour of the fried products.

Chapter 3: Co-extraction of Barley Hull and Flaxseed Hull and Identification of the Major Phenolic Compounds

3.1 Abstract

To investigate the advantages of barley hull and flaxseed hull co-extracts as functional food ingredient, the major phenolic compounds from both hulls were identified by reversed phase high performance liquid chromatography (HPLC) coupled with photodiode array detection (PAD) and quadrupole - time of flight (Q-TOF) mass spectrometry (LC-MS). The extraction yields of alkaline hydrolysis of barley hull, flaxseed hull, and their co-extracts ranged from 9.14 to 10.66%, 30.81 to 37.80% and 25.33 to 32.00% respectively. The total phenolic content significantly varied among different varieties of flaxseed hull (3), barley hull (4) as well as their co-extracts. Four phenolic acids were identified and quantified in the barley hull and in the co-extracts with ferulic acid and *p*-coumaric acid as the major constituents. The three samples of flaxseed hull varied significantly ($p<0.05$) in their content of secoisolariciresinol diglucoside (SDG) (16.38-33.92 mg/g), ferulic acid glucoside (35.68-49.22 mg/g), coumaric acid glucoside (5.07-15.23 mg/g). The phytochemical profiles of the co-extracts were improved by combining the major phenolic compounds from each type of hull. The co-extracts exhibited significantly higher DPPH radical scavenging capacity than individual barley hull extracts and reached the level of individual flaxseed hull extracts. Therefore,

the co-extraction of barley hull with flaxseed hull is a potential approach to convert barley hull into value-added functional food ingredients.

3.2 Introduction

Barley hull is an agricultural by-product, accounting up to 15-20% of the dry weight of the grain (Bhatty, 1993; Palmer and Bathgate, 1976); however, it is mainly regarded as a low profit by-product (Bhatty, 1993). The world production of barley is over 130 million tonnes annually, ranking fifth among all crops in dry matter production around the world today (FAO, 2007). North America grows approximately 14% of the world annual production of barley (Kim & Dale, 2004). Thus, a huge mass of barley hull is being produced every year without proper utilization. The utilization of barley hull as a feed supplement is limited due to its low digestibility (Cruz et al., 2007). Combustion of this material is difficult and not practical due to its high ash content which results in mineral depositions in boilers (Garrote et al., 2008). On the other hand, it is very expensive to transport barley hull to the disposal areas owing to its low density (Mahmudi, 2005; Searcyl et al., 2007; Garrote et al., 2008). Current interest in barley hull remains as a substrate for saccharification and fermentation. However, there is lack of information regarding the details on the phenolic compounds found in barley hull, and a systematic study of its antioxidant capacity is strongly recommended.

Flaxseed is the the richest source of mammalian lignan precursors, with levels 100 to 800 times higher than those in 66 other plant foods in the vegetarian diet (Thompson et al., 1991).

Flaxseed hulls are an excellent source of lignans, which belong to the group of phytoestrogens (Hallund et al., 2006). The formation of SDG takes place in the outer layer of the seed (Hano et al., 2006) and forms an oligomeric structure ((Kamal-Eldin et al., 2001), which is referred to as the lignan macromolecule. Recently, it has been reported that p-coumaric acid glucoside (CouAG), ferulic acid glucoside (FeAG), caffeic acid glucoside (CAG) and the flavonoid herbacetin glucoside (HDG) are suggested to be part of the lignan macromolecule, since they can be released after alkali treatment of a flaxseed extract containing the lignan macromolecule (Johnsson et al., 2002; Struijs et al., 2007; Struijs et al., 2008; Westcott and Muir, 1996). Other lignans present in flaxseed are matairesinol (MAT) (Liggins et al., 2000), isolariciresinol (isoLARI) (Meagher et al., 1999), pinoresinol (PINO) (Meagher et al., 1999), and lariciresinol (LARI) (Sicilia et al., 2003). Other phenolic compounds which may contribute to the health effects ascribed to flaxseeds are hydroxycinnamic acids like p-coumaric acid (Klosterman et al., 1955), ferulic acid, sinapic acid, caffeic acid (Dabrowski and Sosulski, 1984) and their glucosides, as well as the flavonoid kaempferol diglucoside (KDG) (Qiu et al., 1999).

Furthermore, lignans act as strong antioxidants comparable to ferulic acid and better than vitamin E (Prasad, 2000). They have been reported to lower the risk of cardiovascular diseases (Lucas et al., 2004). SDG, the major lignan in flax hull is converted into the mammalian lignans enterodiol and enterolactone, which have several beneficial health effects, such as inhibiting the development of hormone-related type of cancers including breast cancer and prostate cancer (Boccardo et al., 2004; Chen et al., 2002; Chen and Thompson, 2003; Thompson et al., 1996a, b), and non-hormone related colon cancer (Sung et al., 1998).

It is well known that the phenolic compounds are concentrated in the hull, and provide antioxidant defence system to protect the seed from oxidative stress (Lee et al., 2003). The majority of phenolic acids are linked through ester, ether, or acetal bonds either to the plant cell wall components, such as arabinoxylans and lignin, or to other compounds (Holtekjølen et al., 2006; Garrote et al., 2008), thus even mild autohydrolysis treatments may result in cleavage of bonds yielding free phenolics (Garrote et al., 2008).

To illustrate the potential advantages of barley hull and flax hull co-extracts as functional food ingredients, the main objective of the study was to evaluate the antioxidant capacity of barley hull, flax hull, and their co-extracts *in vitro* and to identify the major phenolic compounds in the extracts.

3.3 Materials and Methods

3.3.1 Samples

Four varieties of hulled barley, Peru3 (Peruvian line from CYMMIT collection), Peru16 (Peruvian line from CYMMIT collection), EX116 (Juton mutant line from World Collection), and EX83 (Mutant line derived from Moncalm barley) respectively, were used in the present study. Flaxseed hull samples (F, FG, PF) generously donated by Frutarom (Belgium, NV), Pizzey Milling (Angusville, MB, Canada) and Polar Foods (Fisher Branch, MB, and Canada) were also used. Barley hulls were separated from the grain by a grain polisher (Kett Electric Laboratory, Japan). Both barley hull and flax hull were ground using a coffee grinder.

3.3.2 Chemicals

Calcium hydroxide was purchased from Fisher Scientific (Fair Lawn, NJ). Folin-Ciocalteu reagent, DPPH, and ferulic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). HPLC grade methanol, MS grade water, acetonitrile, and acetic acid were used in LC-MS analysis. All of the HPLC grade and MS grade solvents were also purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

3.3.3 Extraction

Five grams of ground barley hull or flaxseed hull as well as their combination (1:1 wt/wt) and 1.25 g of calcium hydroxide were added to 250 mL of deionized water. The mixture was then heated in a water bath at 70˚C for 4 hours and stirred once every 10 minutes. The mixture was centrifuged at 2600 ×g (Sorvall instruments RC5C, MANDEL, Guelph, Ontario, Canada) for 25 minutes and then the supernatant fluid was decanted from the residue. The supernatant fluid was filtered using Whatman filter paper no. 4. The filtered product was neutralized with 1 N phosphoric acid. The product was filtered again using Whatman filter paper no. 4 to remove gel. The gel from sample was collected in containers and stored in a refrigerator. The remaining filtrate was concentrated to a volume of 65 mL using a rotary evaporator. The concentrate was then filtered again using a Whatman filter paper no. 4 to remove any impurities. The concentrated fractions were then stored in the freezer and freeze-dried after. Duplicate extractions were done per variety of flax or barley hull or their combinations.

3.3.4 TPC

The TPC of crude extracts was determined using the Folin–Ciocalteau reagent according to modified procedures (Beta et al., 2005; Gao et al., 2002; Mpofu et al., 2006; Li et al., 2007). Briefly, 200 μL of the 10-fold diluted crude extracts was reacted with 1.8 mLof freshly made 10-fold diluted Folin-Ciocalteu reagent. The mixture was then neutralized with 1.8 mL of sodium carbonate (60 g/L). The absorbance was measured at 725 nm after 90 min of reaction at room temperature. Ferulic acid was used as the standard. Results of duplicate determinations were expressed as milligrams of ferulic acid equivalents (FAE) per gram of sample (dry weight basis).

3.3.5 DPPH Radical Scavenging Capacity Assay

DPPH radical scavenging capacity assay was carried out according to Brand-Williams et al. (1995) and Yu et al. (2002) and Li et al. (2007) with some modifications. Briefly, 200 μL of crude extract (or fraction) was added to 3.8 mL of 60 μM DPPH radical solution, which was freshly made in 100% methonal. After 60 min of incubation at room temperature, the absorbance at 515 nm was measured. Centrifugation was applied if any precipitate was observed before measuring the absorbance. DPPH free radical scavenging activities of crude extracts were expressed as milligram of Trolox equivalents (TE) per g of sample (dry weight basis) using a standard curve of Trolox.

3.3.6 LC/MS/MS

3.3.6.1 Phenolic Acid Analysis

The chromatographic separation was carried out according to Qiu et al. (2010) using an HPLC (Waters 2695) equipped with a photodiode array detector (PAD) (Waters 996) and autosampler (Waters 717 plus) coupled with a quadrupole time-of-flight mass spectrometer (Q-TOF MS) (Waters Corp, Milford, MA) . The analytical column was a 150 mm × 4.6 mm, 5 μm RP 18 column (Gemini, Phenomenex, USA). The mobile phase consisted of A (0.1% acetic acid in high-purity water) and B (0.1% acetic acid in methanol). A 75 min-linear gradient was programmed as follows: 0-7 min, 15-20% B; 7-8 min, 20-15% B; 8-20 min, 15% B; 20-21 min,15-24%B; 21-33 min, 24 % B; 33-34 min, 24-13% B; 34-36 min, 13% B; 36-37 min, 13-20 % B;37-45 min, 20 % B; 45-46 min, 20-42% B; 46-62 min, 42 % B; 62-63 min, 42-100 % B; 63-68min, 100 % B; 68-69 min, 100-15% B; 69-75 min, 15 % B, with a flow rate of 0.7 mL/min. The injection volume of the autosamler was 10-μL sample solution. The Q-TOF MS was calibrated with sodium iodide for the negative mode through the mass range of 100-1500. Full mass spectra were recorded in negative mode by using the capillary voltage of 1.2 kV and cone voltage of 45V. The flow rate of desolvation gas (N2) and cone gas (N2) were 900 L/h and 50 L/h, respectively. The desolvation temperature and the source temperature were set at 350 ℃ and 150 ℃, respectively. The MS/ MS spectra were acquired by using collision energy of 20 V for phenolic acid dimmers, SDG, FeAG, and CouAG.

3.3.6.2 Lignan Analysis

This LC-MS/MS analysis was based on the method described by Qiu et al. (2009) with modification. Using the same equipment as above, the mobile phase was changed to A (0.1% acetic acid in high-purity water) and C (0.1% acetic acid in acetonitrile). A 35 min-linear gradient was programmed as follows: 0-5 min, 10-15% C; 5-20 min, 15-40% C; 20-25 min, 40% C; 25-30 min,40-10%C; 30-35 min, 10 % C; The injection volume was 10-µL sample solution and the flow rate was 0.5 mL/min. Q-TOF MS operating conditions of the instrument were the same as the phenolic acid method. This analysis was used for identification and quantification of SDG, CouAG and FeAG.

3.3.7 Semi-preparative Reverse Phase HPLC

To purify FeAG and CouAG, the crude extracts of flax hull (F) was fractionated on a Sephadex LH-20 column (50× 1.5 cm) and eluted by 50% methanol. The eluate was further separated on a Waters semi-preparative reverse phase HPLC, which is equipped with a waters 2489 UV/Vis Detector and waters 600 controller and a XBridge™ Prep C18 column (particle size 5µm, 10x25mm) (Waters, Ireland). The mobile phase consisted of A (0.1% acetic acid in high-purity water) and C (0.1% acetic acid in acetonitrile). A 30 min-linear gradient was programmed as follows: 0-5 min, 10-15% C; 5-10 min, 15-20% C; 20-25 min, 20-40% C; 25-30 min, 40-10%C. The injection volume was 1 mL sample solution and the flow rate was 3 mL/min. Fractions was collected based on the response at 280 nm. Appropriate fractions were pooled and lyophilized after evaporation of acetonitrile. The purity of each fraction was determined on analytical HPLC described as above in lingan analysis, using an area normalization method (Ma et al., 2009) as below.

$$Inon = \frac{\sum_{i=1}^{n} AiFi}{AcF_C + \sum_{i=1}^{n} AiFi} = \frac{A1F1 + A2F2}{Ac + A1F1 + A2F2} = \frac{A1 + A2}{Ac + A1 + A2}$$

Where I_{non} is the sum of non-volatile impurities; *Ai* and *Fi* are the peak area and response factor of impurity *I*, respectively*;* A_c and F_c is the peak area and response factor of CouAG or FeAG. The response factor *Fi* was considered to be equal to *Fc* to simplify calculation. The fractions were lyophilized after evaporation of acetonitrile, thus, the volatile impurities and moisture content was considered as 0. Therefore, Purity= (1-*I*non) ×100%.

3.3.8 Statistical Analysis

The results were reported as mean ±standard deviation (SD) (n=2). Data were analyzed by the general linear models (GLM) and one-way analysis of variance (ANOVA) using SAS software (SAS Institute Inc., Cary, NC).). Tukey's test was applied to assess the significant differences in the antioxidant activity. Quantitative results were expressed on a dry weight basis (dwb).

3.4 Results and Discussion

3.4.1 Extraction Yield of Barley Hull, Flaxseed Hull and Their Co-extracts

A specific extraction methodology to obtain majority of phytochemicals in cereals has not yet been established. With advances in knowledge about the phytochemicals in cereal grain, it has been recognized that more than 90% of phytochemicals are present in bound form, mainly ester-linked to polymers in the plant cell wall (Andreasen et al., 2000; Garcia-Conesa et al., 1997; Adom and Liu, 2002). Alkaline hydrolysis becomes the

primary method used for extracting phytochemicals from cereal grain or other plants (Sun et al., 2001; Li et al., 2007). However, the preliminary research showed that high concentration of alkaline solution, such as 2M or 4M of NaOH is not suitable for barley hull (data not shown). The latter renders the color of the freeze-dried extracts much darker than that of a calcium hydroxide hydrolyzed sample making them less favourable for further food application. Another concern is that a high concentration of sodium hydroxide requires high amounts of HCl for neutralization thereby producing high salt content which make the extract chalky and hard to remove from the solution. Thus, calcium hydroxide, a milder alkaline, was used for hydrolysis in this study.

The extraction yields of crude alkaline hydrolysate of barley hull, flaxseed hull and their co-extracts are listed in Table 4. Due to the fibrous nature of barley hull, only 9.14% to 10.66% (wt/wt) yield was obtained, whereas flaxseed hulls gave 3 to 4 times higher extraction yield. The co-extracts of barley hull and flaxseed hull (1:1 wt/wt) varied from 27.35% to 33.54% among different combinations, about 3 times higher than individual barley hull extraction yield and comparable to yield of individual flaxseed hull extracts.

Table 4. Extraction yield of crude alkaline hydrolysate

Class	Sample	Extraction yield (%)*	Tukey Grouping
Barley Hull	EX116	10.66 ±0.44	e
	EX83	10.64 ±0.85	e
	P16	9.14 ±0.61	e
	P3	9.44 ±0.94	e
Flaxseed Hull	F	33.87 ±0.8	b
	PF	33.36 ±1.26	b
	FG	40.93 ±6.29	a
Barley Hull + Flaxseed Hull	EX116+FG	29.12 ±2.53	bc
	EX116+F	32.83 ±0.84	b
	EX116+PF	28.84 ±2.17	bc
	EX83+FG	29.76 ±1.86	bc
	EX83+PF	27.35 ±0.55	c
	EX83+F	31.94 ±1.6	b
	P16+F	30.97 ±1.38	bc
	P16+PF	31.39 ±0.6	b
	P16+FG	27.92 ±1.73	c
	P3+F	31.17 ±0.79	b
	P3+PF	33.54 ±1.01	b
	P3+FG	31.67 ±2.8	b

*Data are expressed as mean ±standard deviation (n=2) on dry weight basis;

The extraction yield obtained for the co-extracts were higher than the expected extraction yield when barley hull and flaxseed hull were co-extracted in a ratio of 1:1 wt/wt. This may be attributed to the responses of barley and flaxseed hull matrix to the alkaline hydrolysis.

3.4.2 Total Phenolic Content

The total phenolic contents of crude alkaline hydrolysates, expressed as mg of ferulic acid equivalent/g of sample, are shown in Table 5. There were significant differences in TPC among individual barley hull, flaxseed hull and their co-extracts. Among four varieties of barley hull, EX83 and Peru16 showed significantly higher TPC than EX116 and Peru3. Flaxseed hulls, F and FG had two times higher TPC than that of

PF. The TPC values showed marked advantages of co-extraction of barley hull and flaxseed hull. Any barley hull co-extracted with F hull (1:1 wt/wt) exhibited 30.59 mg/g to 32.32 mg/g of TPCs which were comparable to the TPC of individual FG hull (31.07 mg/g) and F hull (33.99 mg/g).

Table 5. Total phenolic content (TPC) of crude alkaline hydrolysate

Class	Sample	TPC (FAE[1] mg/g)[2]
Barley Hull	EX116	7.1 ± 0.67^{b}
	EX83	10.71 ± 0.51^{a}
	P16	9.87 ± 0.72^{a}
	P3	6.14 ± 0.67^{b}
Flaxseed Hull	F	32.96 ± 1.45^{a}
	PF	15.38 ± 0.86^{b}
	FG	27.41 ± 0.71^{c}
Barley Hull+Flaxseed Hull	EX116+F	32.32 ± 0.87^{a}
	EX116+FG	21.11 ± 0.10^{cd}
	EX116+PF	13.8 ± 0.09^{e}
	EX83+F	30.59 ± 0.87^{a}
	EX83+FG	24.39 ± 0.17^{bc}
	EX83+PF	18.69 ± 0.32^{de}
	P16+F	31.21 ± 1.34^{a}
	P16+FG	22.15 ± 0.62^{bcd}
	P16+PF	14.27 ± 0.98^{e}
	P3+F	31 ± 1.64^{a}
	P3+FG	29.72 ± 3.07^{ab}
	P3+PF	19.04 ± 0.001^{de}

[1]Ferulic acid equivalent.

[2] Data are expressed as mean+standard deviation (n=2) on dry weight basis; significant differences are found in each class of sample, and values marked by the same letter are not significantly different (Tukey Grouping at $P<0.05$).

Compared to the individual barley hull extract, flaxseed hull PF significantly increased the TPC. Although flaxseed hull PF enhanced the TPC of barley hull extract, the TPC were significantly lower than that of the co-extracts containing flaxseed hull F and FG. Therefore, flaxseed hull F and FG are considered to have more advantages over

than PF. Higher TPC value is generally regarded as an indication of higher antioxidant capacity *in vitro* (Li et al., 2007), thus, the co-extraction of barley hull and flaxseed hull, especially with F, may remarkably increase the antioxidant capacity of individual barley hull.

3.4.3 DPPH Radical Scavenging Capacity

DPPH scavenging assays is widely used to evaluate the *in vitro* antioxidant capacity of fruits (Sun et al., 2002), vegetable (Chu et al., 2002), and cereal (Li et al., 2007; Bellido & Beta, 2009; Zhao et al., 2006) by researchers worldwide. The DPPH radical scavenging capacities of barley hull, flaxseed hull and their co-extracts, expressed as mg of Trolox equivalent/g of sample, are shown in Fig.8.

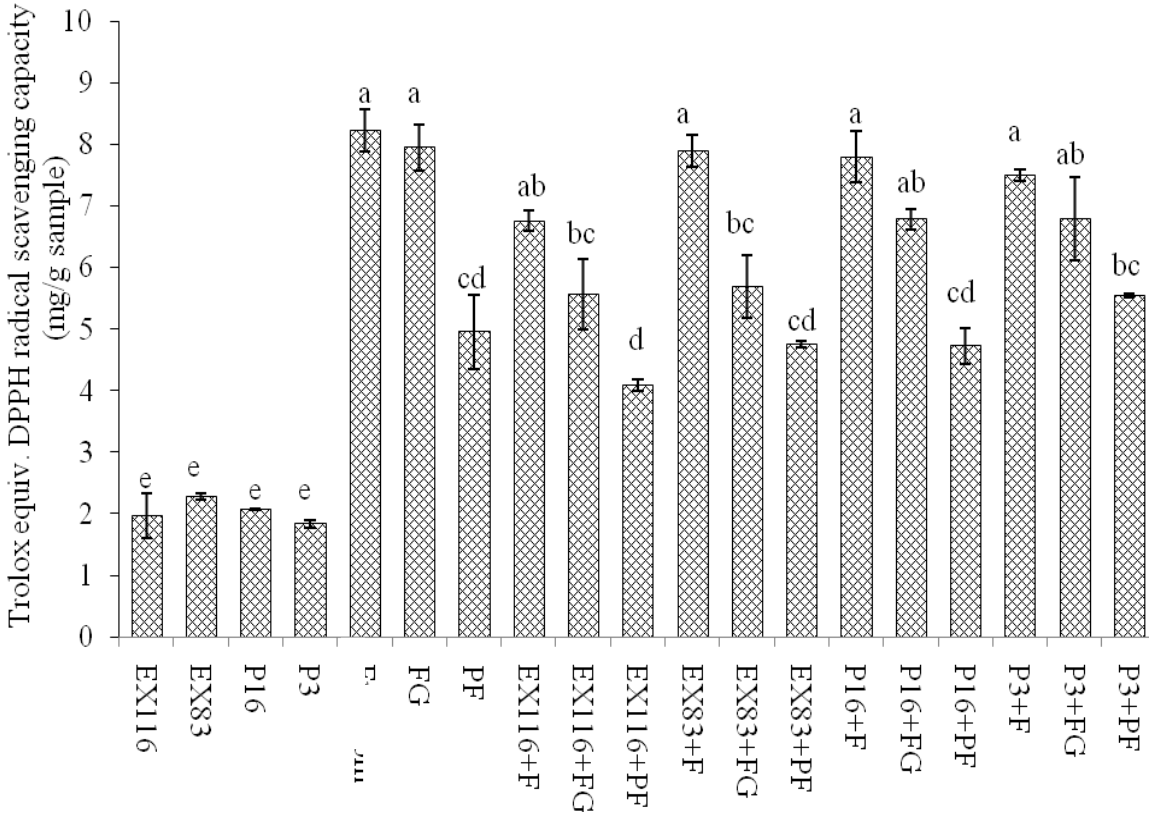


Figure 8. DPPH free radical scavenging capacity of cr.ude alkaline hydrolysate estimated as Trolox equiv. (mg/g dw sample).

Similar trends were observed in DPPH radical scavenging capacity as in TPC of barley hull, flaxseed hull and their co-extracts. For flax hulls, F and FG hulls had the highest DPPH radical scavenging capacity (8.23 & 7.95 Trolox equiv. mg/g respectively), which was more than four times higher than that of barley hull. Furthermore, barley hulls co-extracted with flax hulls, F and FG had significantly higher DPPH radical scavenging capacity than those of barley hulls co-extracted with PF hull. There were no significant differences found among different varieties of barley hulls in terms of DPPH radical scavenging capacity, while flax hull, PF had significantly lower

DPPH radical scavenging activity than the other flax hulls. Any variety of barley hulls co-extracted with flax hulls, F and FG showed promising advantages over individual extracts of barley hull, whose DPPH radical scavenging capacity had been elevated to as high as flax hulls, F and FG. Flaxseed hull PF also elevated the DPPH radical scavenging capacity of the co-extracts compared to individual barley hull extracts. Therefore, co-extraction of low-cost barley hull with flaxseed hull appears to be an excellent way to get a new functional food ingredient with high antioxidant activity comparable to expensive individual flaxseed hull. Barley hull and flaxseed hull are very distinct in their phenolic profiles. The enhancement of DPPH radical scavenging capacity of barley hull co-extracted with flaxseed hull (1:1 wt/wt) may be attributed to the synergy of the phenolic compounds from both hulls.

3.4.4 Identification and Characterization of Phenolic Compounds in Barley Hull, Flaxseed Hull and Their Co-extracts

3.4.4.1 Barley Hull

In the present study, the identification of monomeric phenolic acids in the samples were achieved by comparing retention times, UV maximum absorption and MS spectra with standards (Table 6). The HPLC chromatogram of EX83, flax hull (F) and their co-extracts are displayed in Fig. 9 along with the phenolic acid standards for comparative purposes.

Vanillic acid, vanillin (aldehyde form of vanillic acid), p-coumaric acid and ferulic acids with some other unknown compounds were detected in all four varieties of barley hulls (Fig. 9).

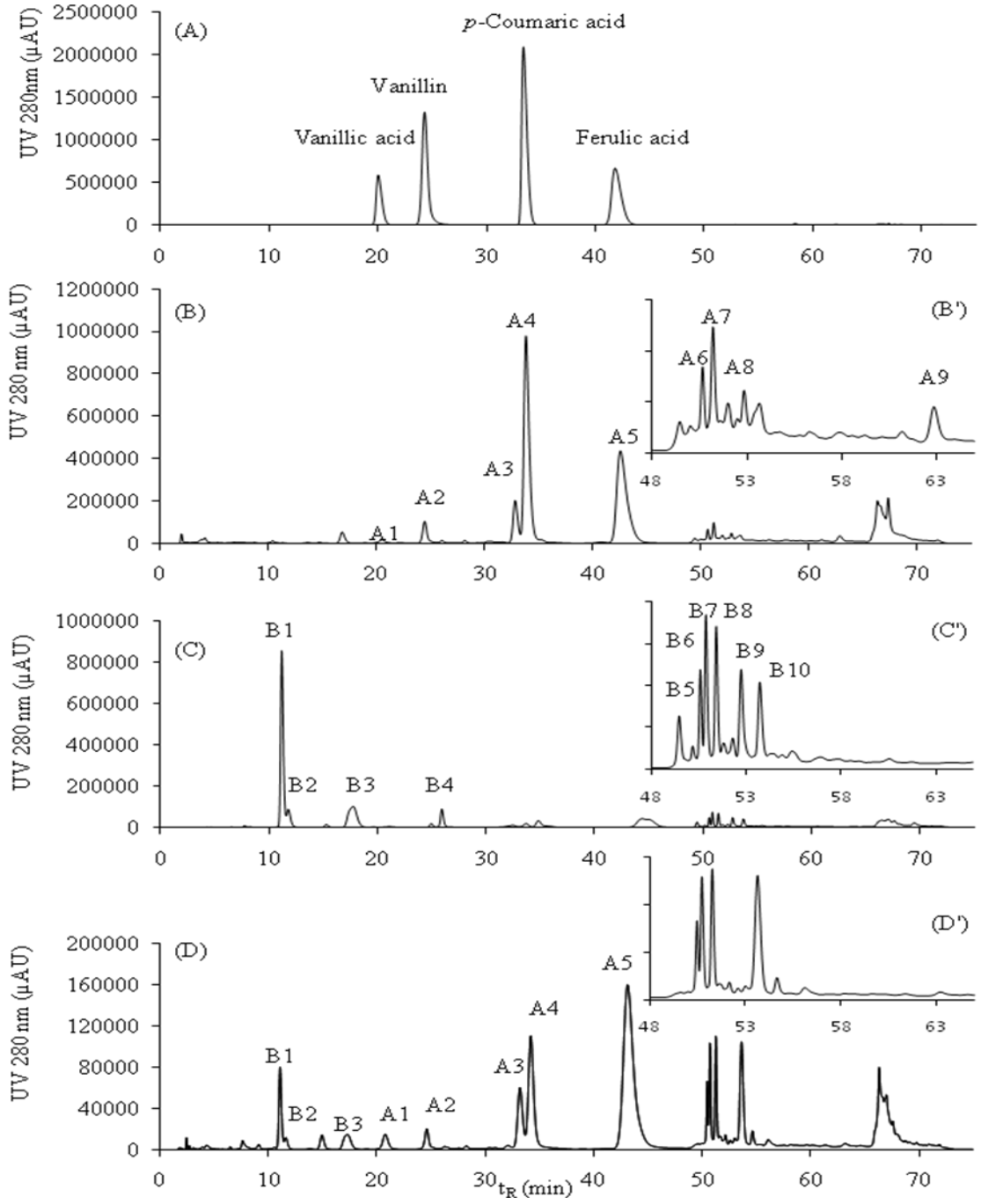


Figure 9. HPLC/UV Chromatogram at 280nm of (A) phenolic acid standards (B) EX83, (C) flaxseed hull F, (D) EX83+flaxseed hull F after alkaline hydrolysis. (B'), (C'), (D') Highlight of LC chromatogram (48-58min) of EX83 barley, flaxseed hull F, EX83+F, respectively

Since the monomeric phenolic acids are quite small molecules, the collision energy was set to zero to monitor their parent ions. All the peaks with their retention time, m/z ratio in negative mode, as well as possible peak annotation are shown in Table 6. Garrote et al., (2008) reported a number of phenolic compounds in barley hull treated with isothermal autohydrolysis (185-260^{o}C), such as benzoic acid, coumaric acid, ferulic acid, gallic acid, vanillic acid and vanillin,3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde.

Table 6. Phenolic compounds identified in barley hull (EX83) by LC-MS/MS in negative ion mode

Peak No	Retension time(min)	[M-H]-	MS/MS	Peak Annotation
A1	21.28	167	n/a	Vanillic acid
A2	24.97	151	n/a	Vanillin
A3	33.63	327	163,119	Coumaric acid dimer
A4	34.78	163	n/a	p-Coumaric acid
A5	44.15	193	n/a	Ferulic acid
A6	50.65	385	341,282,193	Ferulic acid dimmer
A7	51.24	385	282,165,193	Ferulic acid dimer
A8	53.85	385	341,193,165,134	Ferulic acid dimmer
A9	62.78	385	341, 282,193	Ferulic acid dimer

[n/a] MS/MS data were not acquired for these small molecules.

However, in the present study, benzoic acid, gallic acid, 3,4-dihydroxybenzaldehyde, and 4-hydroxybenzaldehyde were not detected.

Beside the phenolic acid, four ferulate dehydrodimers were detected in all 4 varieties of barley hulls (Fig.10).

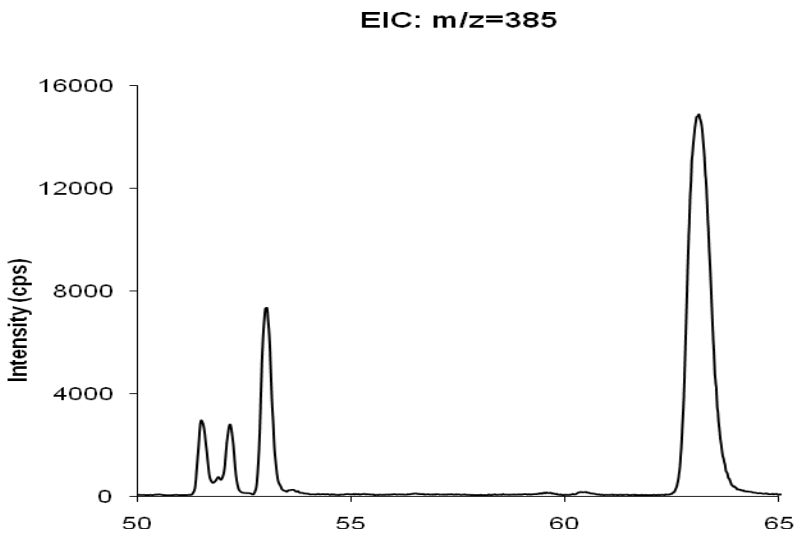


Figure 10. Extracted ion chromatogram (EIC) of deprotonated molecules with m/z=385 in balrey hull (EX83) extract.

The MS/MS data were acquired by setting collision energy to 20 V for phenolic acid dimers. Their retention time, m/z value, and MS/MS products ion m/z values are listed in Table 6. In the literature, four ferulate dehydrodimer isomers were reported previously in 11 barley varieties and in their hull and outer layer fraction (Hernanz et al., 2001). They are (E,E)-4,4'-dihydroxy-3,5'-dimethoxy-β,3'-bicinnamic acid (8,5'-diFA open form), (E,E)-4,4'-dihydroxy-5,5'- dimethoxy-3,3'-bicinnamic acid (5,5'-diFA), (Z)-β-{4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxycinnamic acid (8-O-4'-diFA), and trans-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid (8,5'-diFA dehydrobenzofuran form), among which 8-O-4'-diFA was the most abundant, ranging from 73 to 118 μg/g dry weight (Hernanz et al., 2001). Furthermore, Qiu et al. (2009) detected four different

ferulate dihydrodimers in 8,5'-diFA , 5,5'-diFA, 8-O-4'-diFA, and 8-8' diFA form in wild rice using the same LC-MS/MS method as described in the present study.

Compared to ferulic acid dimers, peak A3 in Fig.9 (B) was assigned to coumaric acid dimer, which is rarely reported. In tandem mass (MS/MS) spectrum, the parent ion m/z=327 was fragmented into two product ions at m/z=163 and m/z=119 by losing 163 Da and 44Da mass. Therefore, peak A3 was confirmed as coumaric acid dimer.

The retention time, m/z value, and MS/MS products ion m/z values and tentative peak annotation of the major phenolic compounds in flaxseed hull extract determined by the phenolic acid method were listed in Table 7. Peak B9 and B10 both gave m/z= 389 $[M-H]^-$, and fragmented into 341 and 193, by losing 44Da and 196Da molecular mass. Therefore, the compounds corresponding to peak B9 and B10 were proposed to be ferulic acid derivatives.

3.4.4.2 Flaxseed Hull

SDG, CouAG, FeAG as well as CAG and HDG were eluted using both the phenolic acid method and lignan method. However, SDG and HDG had poor response in the former method and were not very well separated (Fig. 9). Therefore, the lignan method was applied specifically to identify SDG and HDG in flaxseed hull.

In the phenolic acid method, peak B1, B2 and B3 were assigned to CouAG, CAG and FeAG respectively, which are parts of the lignan macromolecule as reported previously (Struijs et al., 2008). The m/z-ratio and MS/MS data in negative mode (Table 7 & Fig. 12) match the reported values (Struijs et al., 2008).

Table 7. Phenolic compounds identified in flaxseed hull (Frutarom) by LC-MS/MS in negative ion mode

Peak NO.	Retension time (min)	[M-H]-	MS/MS	Peak annotation
B1	11.18	325	163	CouAG
B2	11.91	341	179	CAG
B3	17.8	325,341,355	163,179,193	CouAG, CAG, FeAG
B4	25.93	355	193	FeAG
B5	49.53	327	163,	Di-coumaric acid
B6	50.62	523	361,343	SECO
B7	50.92	625, 685	463,301,	SDG, HDG
B8	51.47	341	179	CAG
B9	52.81	389	341,193,165,134	Ferulic acid derivatives
B10	53.82	389	193,165,150,134	Ferulic acid derivatives

Identification and characterization of SDG in flaxseed hull was based on the UV spectra and MS/MS spectra compared with the standards (Fig.11& Fig.12).

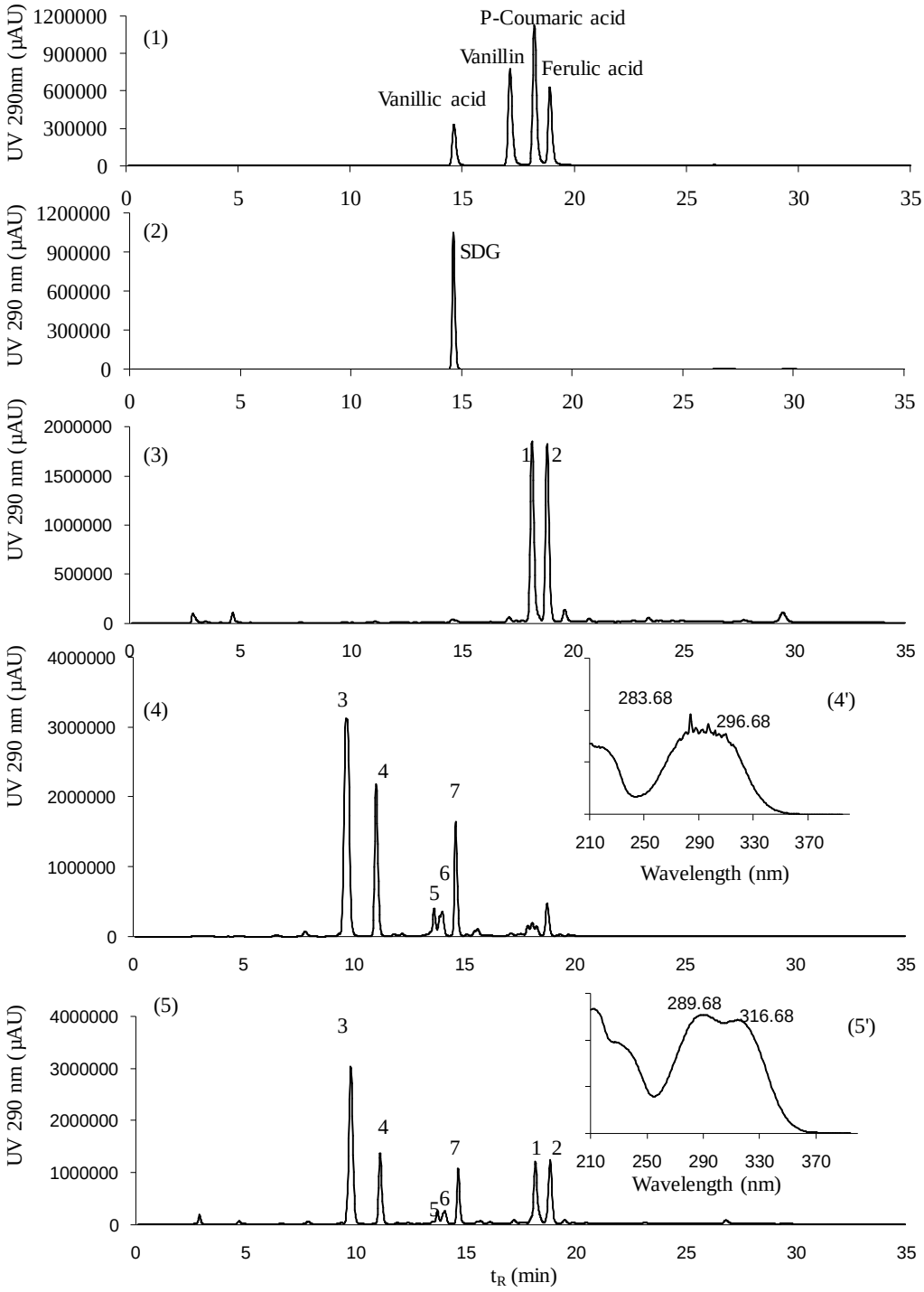


Figure 11. HPLC/UV Chromatograms of (1) phenolic acid standards; (2) SDG standards; (3) EX83; (4) flax hull F; (5) EX83+ flax hull F after alkaline hydrolysis. (4'), (5') Maximum UV absorbance wavelength of CouAG and FeAG.

Peak 7 in Fig.11 was assigned to SDG which gave m/z of 685 $[M-H]^-$, and fragmented into 523$[M-H-162]^-$, 361$[M-H-2\times162]^-$, 343$[M-H-2\times162-18]^-$ in MS/MS analysis. The fragmentation pathway for those fragment ions can be depicted as SDG→SMG (Secoisolariciresinol Monoglucoside) → SECO (Secoisolariciresinol) → ANSECO (Ansecoisolariciresinol), by losing one glucose molecule at a time, and a water molecule further (Fig. 12). Peak 6 was assigned to herbacetin 3, 8-O-diglucoside, which had m/z of 625 $[M-H]^-$ in negative mode MS analysis. The fragment ions in MS/MS fragmentation are 463 $[M-H-162]^-$ (herbacetin monoglucoside), and 301 $[M-H-2\times162]^-$ (Herbacetin) (Fig. 12). In fast atom bombardment (FAB)-MS positive mode, the m/z of herbacetin 3, 8-O-glucoside is 627, two fragment ions are 465 $[M+H-162]^+$, and 303$[M+H-2\times162]^+$ (Qiu et al., 1999). The sugar moiety in herbacetin 3, 8-O-diglucoside has been identified to be glucose as it gave the same thin layer chromatography Rf value and colored spot as reference glucose (Qiu et al., 1999).

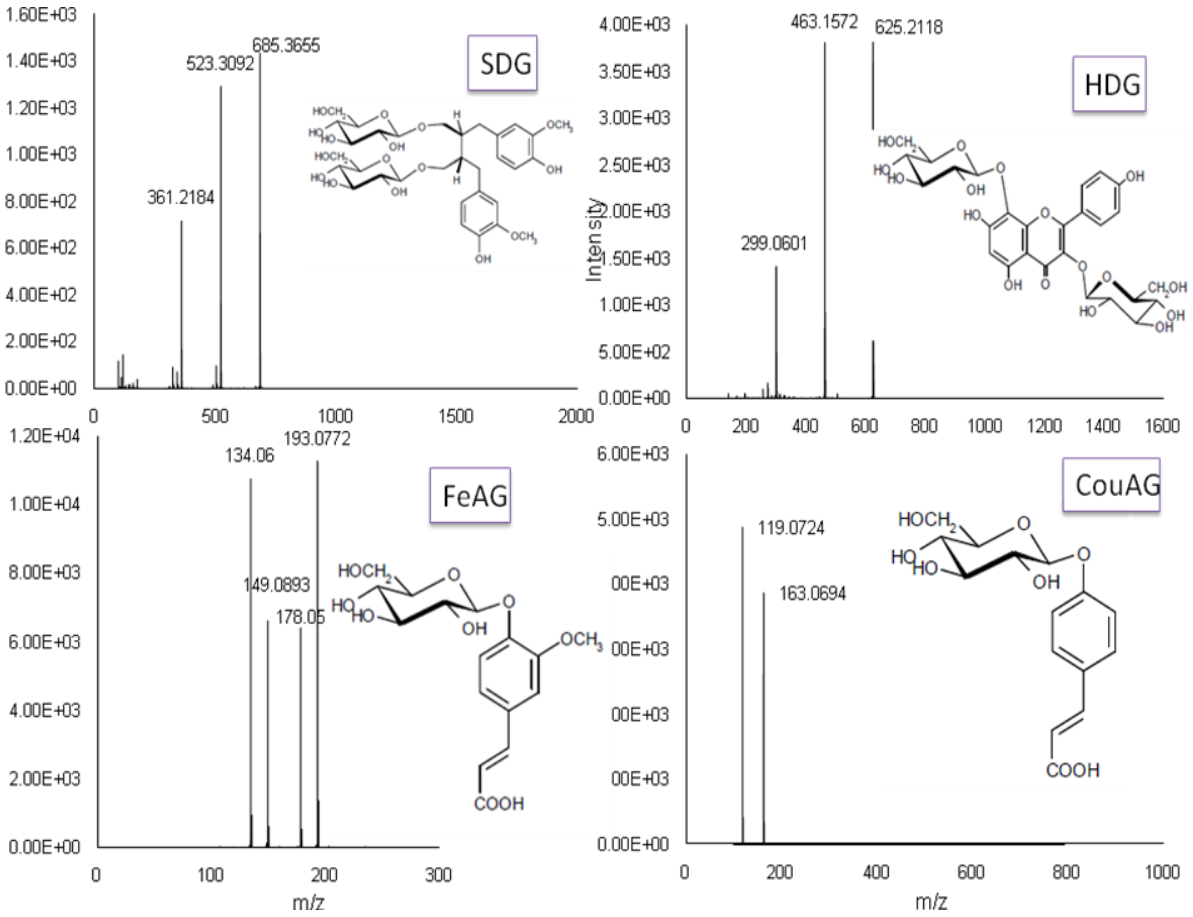


Figure. 12 MS/MS spectrum of peak 7 (SDG), peak 6 (HDG), peak 3 (FeAG), and peak 4 (CouAG) from Figure 11.

3.4.4.3 Barley Hull & Flaxseed Hull Co-extracts

Compared to individual extracts of barley hull or flaxseed hull, the chromatographic profile of the co-extracts was enriched, combining the phytochemicals from two distinct types of hull materials (Fig. 9 & Fig.11). However, the proportion of all phytochemicals was altered and several peaks appeared to be overlapping. From 48 min to 58 min, there were only 4 relatively big peaks in the co-extracts (Fig. 9 (D')). The

retention time, m/z-ratio and MS/MS data in negative mode as well as tentative peak assignments are listed in Table 8. Due to two different types of hull containing different phenolic compounds, the co-extracts were a mixture which was more complicated than individual extracts. Using the same chromatographic method, the peaks which appeared from 48 to 58 min were overlapping, and gave very complicated mass spectra, which make it very difficult to identify specific compounds corresponding to those peaks. However, the proportional changes of major phenolics are discussed in the following section.

Table 8. Peak identification of co-extracts (EX83+flax hull) by LC-MS/MS in negative ion mode

Peak NO.	Retention time (min)	[M-H]-	MS/MS	Peak annotation
B1	11.18	325	163	CouAG
B2	11.91	341	179	Caffeic acid glucoside(CAG)
B3	17.8	355	193, 134	FeAG
A1	21.28	167	n/a	Vanillic acid
A2	24.97	151		vanillin
A3	33.63	327	n/a	Coumaric acid dimmer
A4	34.78	163	n/a	p-Coumaric acid
A5	44.15	193	n/a	Ferulic acid
B6	50.48	523	n/a	SDG, HDG
A6+ B7	50.73	341,357,685,625	n/a	n
A7+ B9	51.28	193, 341, 389, 411,824	n/a	n
A8+B10	53.67	327, 341, 389	n/a	n

[n/a] MS/MS data were not acquired for these small molecules.

[n] overlapping peak without specific peak annotation.

3.4.5 Quantification of Major Phenolic Compounds in Barley hull, Flaxseed Hull and Their Co-extract

As shown in Fig.9, the major phenolic compounds in barley hull were vanillic acid, vanillin, p-coumaric acid, ferulic acid, which were not found in flaxseed hull with the exception of ferulic acid. The quantitative measurement was assessed at 280 nm wavelength. Table 9 showed that ferulic acid and *p*-coumaric acids were the most abundant phenolic acids in all four varieties of barley hull. The highest amount of ferulic acid was found in EX83 (2279 μg/g) and P16 (2260 μg/g), which also had relatively higher *p*-coumaric acid than EX116 and P3. Besides these two major compounds, smaller amounts of vanillic acid and vanillin were detected in all four varieties of barley hull. According to one of the only two studies on barley hull phenolic compounds, ferulic acid and *p*-coumaric acid were present at levels of 2.5% (w/w) and 3% (w/w) of the ethyl acetate extracts respectively (Cruz et al., 2007), which indicates that the amount of *p*-coumaric acid is slightly higher than ferulic acid. However, Garrote et al., 2008 detected much higher amount of benzoic acid and gallic acid than that of *p*-coumaric acid and ferulic acid, but the amount of individual phenolic compounds were reported in the unit of g/L, which is very difficult to compare with. The type of coumaric acid isomer was not specified.

Table 9. Phenolic acids content in barley hull extracts and in Barley hull+Flaxseed hull (1:1 wt/wt) co-extracts*

Class	Sample	Vanillic acid (µg/g)	Vanillin (µg/g)	p-Coumaric acid (µg/g)	Ferulic acid (µg/g)
Barley hull	EX116	26.88 ±6.91^{b}	116.59 ±2.13^{a}	1189.10 ±66.73^{a}	1751.16 ±50.35^{b}
	EX83	65.83 ±6.02^{a}	173.51 ±0.78^{a}	1408.80 ±102.09^{a}	2279.03 ±11.87^{a}
	P16	58.58 ±3.70^{a}	157.24 ±2.06^{b}	1365.11 ±6.88^{a}	2260.29 ±53.70^{a}
	P3	14.63 ±0.26^{b}	51.59 ±4.39^{b}	1176.79 ±80.93^{a}	1945.97 ±61.28^{b}
Barley hull + Flax hull Co-extracts	EX116+F	40.23 ±12.73^{a}	120.05 ±17.52^{a}	723.06 ±7.74^{abc}	1358.73 ±6.81^{abc}
	EX116+FG	15.72 ±5.04^{c}	97.96 ±4.56^{ab}	651.66 ±4.39^{bc}	1165.06 ±8.19^{edf}
	EX116+PF	25.97 ±0.18^{b}	36.82 ±1.83^{d}	420.99 ±26.12^{e}	1142.91 ±47.65^{edf}
	EX83+F	56.67 ±0.70^{a}	112.44 ±10.47^{a}	811.13 ±70.17^{a}	1406.74 ±7.71^{ab}
	EX83+FG	36.78 ±6.02^{b}	120.13 ±11.28^{a}	666.45 ±22.28^{bc}	1314.37 ±11.12^{abcd}
	EX83+PF	8.58 ±0.74^{c}	33.29 ±0.38^{d}	143.71 ±2.21^{f}	924.02 ±13.46^{g}
	P16+F	13.76 ±11.75^{b}	40.76 ±10.47^{d}	740.83 ±16.40^{ab}	1476.47 ±10.13^{a}
	P16+FG	36.41 ±7.15^{a}	71.33 ±0.07^{bc}	670.64 ±32.63^{bc}	1228.22 ±14.54^{cdef}
	P16+PF	39.38 ±36.81^{a}	120.79 ±12.22^{a}	601.76 ±85.27^{bcd}	1088.17 ±117.71^{fg}
	P3+F	9.31 ±4.44^{c}	44.15 ±5.70^{dc}	480.80 ±5.61^{de}	1368.18 ±1.99^{abc}
	P3+FG	36.67 ±1.52^{a}	114.28 ±0.70^{a}	583.92 ±8.34b^{cd}	1350.97 ±63.22^{abc}
	P3+PF	27.86 ±11.56^{b}	29.44 ±3.46^{d}	484.22 ±6.31^{de}	1298.52 ±15.34^{bcde}

*Data are expressed as mean+standard deviation (n=2) on dry weight basis. EX116, EX83 P16 & P3 represent barley and F, FG, PF represents flaxseed hulls.

In whole barley grain, ferulic acid is the most abundant phenolic acid with concentrations ranging from 359-624 µg/g dry weight, followed by p-coumaric acid level ranging from 79-260µg/g dry weight basis in 11 malting and feed barley varieties (Hernanz et al., 2001). Zupfer et al. (1998) found concentrations of ferulic acid ranging from 343 to 580 µg/g dry weights in 18 different malting and feed barleys. Andersson et al. (2008) reported that the total ferulic acid and p-coumaric acid content ranged from 149-413 µg/g and 5.25 to 115.5 µg/g (total of free, conjugated, and bound form) in 10 varieties of barleys, the findings of which are in agreement with previous studies (Hernanz et al., 2001). Other phenolic acid, such as vanillic, syringic, sinapic, and caffeic acid have been found in small amounts (Andersson et al., 2008; Hernanz et al., 2001). Thus, it can be concluded that the phenolic acids are concentrated in the barley hull rather than in the endosperm. The barley husk and outer layers contains about 77.7 to 82.3% of the total ferulic acid, 78.0 to 86.3% of p-coumaric acid and 79.2 to 86.8% of ferulic acid dehydrodimers of the total amount in barley grain (Hernanz et al., 2001). Hernanz et al. (2001) also reported that brewer's spent grain, mainly cell wall materials, exhibits 5-fold higher levels of ferulic acid (1867-1948 µg/g), p-coumaric acid (565-794 µg/g) and ferulic acid dehydrodimers than the unprocessed barley grains. In the present study, ferulic acid was slightly higher than p-coumaric acid, which contradicts the literature reporting that p-coumaric acid is the most abundant in the hull. The possible explanation might be the bran layer contamination of the hulls during the separation, which contributed to the higher ferulic acid content; however, it is hard to estimate this contamination level. The separation of hull from the grain by a grain polisher depends on the time and the variety of barley. Some barley hulls are attached to the endosperm very

tightly, which makes it very hard to get pure hull without bran; but, some are very loose, making it easy to remove and therefore less bran contamination. Another possible explanation for finding different levels of individual phenolic acids is also attributed to different chemicals and treatment of the barley hull, which affect the way chemical bonds break and release these monomeric phenolic compounds.

In the present study, the SDG detected in the flaxseed hull all refers to (+)-SDG. The content of SDG varied from 16.38 mg/g to 33.92 mg/g of sample, with the highest content of SDG being detected in flax hull F. Flax hull PF had the lowest content of SDG content (16.38 mg/g), about only half of the SDG content in flax hull F (Table 10). Eliasson et al., (2003) presented that different samples of flaxseeds varied considerably in their content of (+)-SDG (11.9–25.9 mg/g), (-)-SDG (2.2–5.0 mg/g), p-coumaric acid glucoside (1.2–8.5 mg/g), and ferulic acid glucoside (1.6–5.0 mg/g). However, SDG content of flaxseed hull from direct alkaline hydrolysis has not been reported yet. Thus, this is the first time to report SDG content in flaxseed hull. The most frequently used approach is to extract lignan macromolecules and then hydrolyze into SDG, FeAG, CouAG and HDG.

Due to the lack of authentic standards, CouAG and FeAG were first quantified as equivalent of p-coumaric acid and ferulic acid.

Table 10. SDG, CouAG, FeAG content (quantified as p-coumaric acid and ferulic acid equivalent) in flaxseed hull extracts and in Barley hull+Flaxseed hull (1:1 wt/wt) co-extracts[1]

Sample	SDG (mg/g sample)	CouAG (mg/g)[2]	FeAG (mg/g)[2]
F	33.92 ± 0.63^{a}	4.17 ± 0.04^{a}	3.49 ± 0.04^{a}
FG	28.59 ± 1.03^{b}	3.80 ± 0.06^{b}	3.18 ± 0.11^{a}
PF	16.38 ± 0.21^{c}	2.97 ± 0.11^{c}	1.21 ± 0.01^{c}
EX116+F	19.85 ± 0.41^{a}	2.71 ± 0.01^{d}	1.57 ± 0.04^{b}
EX116+FG	15.46 ± 0.61^{c}	1.96 ± 0.09^{f}	1.50 ± 0.01^{bc}
EX116+PF	10.24 ± 0.27^{d}	1.66 ± 0.05^{g}	0.55 ± 0.02^{d}
EX83+F	18.89 ± 0.20^{b}	2.77 ± 0.02^{cd}	1.71 ± 0.02^{b}
EX83+FG	15.37 ± 0.45^{c}	2.20 ± 0.00^{e}	1.53 ± 0.02^{bc}
EX83+PF	9.94 ± 0.02^{d}	1.71 ± 0.12^{g}	0.55 ± 0.13^{d}
P16+F	19.24 ± 0.18^{ab}	2.78 ± 0.00^{cd}	1.68 ± 0.00^{b}
P16+FG	15.65 ± 0.49^{c}	2.20 ± 0.00^{e}	1.49 ± 0.06^{bc}
P16+PF	8.83 ± 0.31^{d}	1.67 ± 0.01^{g}	0.58 ± 0.00^{d}
P3+F	18.29 ± 0.20^{b}	2.68 ± 0.01^{d}	1.71 ± 0.01^{b}
P3+FG	15.64 ± 0.49^{c}	2.19 ± 0.01^{e}	1.51 ± 0.03^{bc}
P3+PF	9.53 ± 0.03^{d}	1.86 ± 0.00^{gf}	0.64 ± 0.00^{d}

[1]Data are expressed as mean+standard deviation (n=2) on dry weight basis;

[2]CouAG, FeAG were quantified as equivalent of p-Coumaric acid and ferulic acid.

The content of CouAG and FeAG varied in three types of flaxseed hulls and various co-extracts. As shown in Table 10, flax hull F had highest CouAG (4.17 mg/g) and FeAG (3.49 mg/g) content while the lowest CouAG and FeAG was found in PF.

However, CouAG and FeAG standards were purified on the reverse phase semi-prep HPLC. Using the area normalization method, the purity of CouAG and FeAG reached 67.77% and 90.24% respectively. Due to the similarity in structure, CouAG is always eluted with FeAG, which is the major impurities in CouAG, causing the low purity of CouAG. It also has to be addressed that the area normalization method for

purity determination is based on the hypothesis that the response factors for major component and impurities are the same, which might result in a systematic error of purity. After using these two purified fractions as standards to quantify the CouAG and FeAG, the amount of each dramatically changed from previous quantification using the ferulic acid and coumaric acid equivalent method.

Table 11. SDG, CouAG, FeAG content (quantified by CouAG & FeAG purified standard) in flaxseed hull extracts and in Barley hull+Flaxseed hull (1:1 wt/wt) co-extracts[1]

Sample	SDG (mg/g sample)	CouAG (mg/g)[2]	FeAG (mg/g)[2]
F	33.92 ±0.63[a]	49.22 ±0.41[a]	15.23 ±0.19[a]
FG	28.59 ±1.03[b]	45.11 ±0.72[b]	13.85 ±0.49[b]
PF	16.38 ±0.21[c]	35.68 ±1.23[c]	5.07 ±0.03[c]
EX116+F	19.85 ±0.41[a]	32.70 ±0.08[a]	6.67 ±0.20[abc]
EX116+FG	15.46 ±0.61[c]	24.20 ±1.01[c]	6.36 ±0.05[bc]
EX116+PF	10.24 ±0.27[d]	20.74 ±0.52[e]	2.12 ±0.09[d]
EX83+F	18.89 ±0.20[b]	33.36 ±0.17[a]	7.28 ±0.10[a]
EX83+FG	15.37 ±0.45[c]	26.92 ±0.006[b]	6.48 ±0.09[abc]
EX83+PF	9.94 ±0.02[d]	21.36 ±1.31d[e]	2.10 ±0.58[d]
P16+F	19.24 ±0.18[ab]	33.53 ±0.05[a]	7.16 ±0.02[ab]
P16+FG	15.65 ±0.49[c]	26.90 ±0.003[b]	6.29 ±0.25[c]
P16+PF	8.83 ±0.31[d]	20.90 ±0.15[e]	2.22 ±0.004[d]
P3+F	18.29 ±0.20[b]	32.39 ±0.09[a]	7.38 ±0.05[a]
P3+FG	15.64 ±0.49[c]	26.83 ±0.13[b]	6.38 ±0.12[bc]
P3+PF	9.53 ±0.03[d]	23.03 ±0.002[dc]	2.52 ±0.01[d]

[1]Data are expressed as mean+standard deviation (n=2) on dry weight basis; significant differences were found in each class of sample, and values marked by the same letter are not significantly different (Tukey Grouping at $P<0.05$).

[2]CouAG, FeAG were quantified by the purified CouAG, FeAG as standard.

As shown in Table 11, the content of CouAG and FeAG significantly varied from 35.68-49.22 mg/g and 5.07-15.23 mg/g among flax hulls, F, FG and PF. In the co-extracts, the amount of CouAG and FeAG significantly differed according to the flaxseed

hull variety. The co-extracts with flax hull F had higher content of CouAG and FeAG than that of co-extracts with FG, which was also superior to the co-extracts with PF (Table 11).

The CouAG proportion was higher than that of FeAG as previously reported (Struijs et al., 2009). The latter explained that the different proportions may be attributed to the earlier formation of coumaric acid in the biosynthesis pathway followed by glucosylation and incorporation within lignan macromolecule without converting to ferulic acid and FeAG (Struijs et al., 2009). .

In the co-extracts of barley hull and flaxseed hull (1:1 wt/wt), p-coumaric acid content was enhanced by 15~ 22% in the EX116, EX83 with flax hull F combination. The barley hulls co-extracted with flax hull PF has no such advantages in the enhancement of p-coumaric acids. In the same way, the ferulic acid content was enhanced in all the co-extracts, from 23 % to 55%. The SDG was also released more on the basis of 50% of flaxseed hull, compared to the individual extracts of flaxseed hull. 17% of enhancement of SDG content was observed in the combination of Frutarom with EX116. In the co-extracts with F, CouAG was enhanced significantly and almost reached the level of CouAG in individual PF extracts, where similar increment of FeAG content in the co-extracts with F and FG were observed (Table 11). The co-extraction of barley hull and flaxseed hull enriched phytochemical profiles by combining the major phenolic compounds, and showed significantly higher TPC and DPPH radical scavenging activity than the individual barley hull extracts. Therefore, it indicated that the enrichment in the pytochemical profile corresponded to enhanced antioxidant activity.

3.5 Conclusion

In conclusion, mild alkaline hydrolysis using calcium hydroxide liberated four different phenolic acids in barley hull, and also extracted SDG, HDG, CouAG, and FeAG, making it possible to extract the barley hull and flaxseed hull together. Both qualitative and quantitative analyses were conducted on the major phenolic compounds in barley hull, flaxseed hull and their co-extracts. Compared to the individual barley hull extracts, the co-extracts of barley hull and flaxseed hull showed promising advantages in terms of TPC and DPPH radical scavenging activity. Thus, with enriched phytochemicals profile, the co-extraction of barley hull with flaxseed hull indicates potential approach to convert barley hull into value-added functional food ingredients. Further research is being conducted to assess the cellular antioxidant and antiproliferative activities of hull extracts on prostate cancer cells.

Chapter 4: Antioxidant and Antiproliferative Activities of Barley Hull, Flaxseed Hull and Their Co-extracts in Human Prostate Cancer Cells

4.1 Abstract

Barley hull is an agricultural by-product about which there is a paucity of information regarding the phenolic compounds while, on the other hand, flaxseed hull is an excellent source of lignans. Phytochemicals naturally occur as complex mixtures; however, little information is available regarding possible additive, synergistic, or antagonistic interactions among compounds. The present study is the first attempt to evaluate the antioxidant and antiproliferative activities of individual barley hull extracts, flaxseed hull extracts and their co-extracts in human prostate cancer cells *in vitro*. The cellular antioxidant activity assayed by a dichlorofluorescin (DCF) assay indicated that these three different extracts inhibited the AAPH – generated reactive oxygen species (ROS) oxidation in a dose-dependent manner when their concentrations ranged from 0.1 mg/ml to 1 mg/ml during a 60 min test. The MTS-based cell proliferation assay indicated that PC-3 prostate cancer cells were significantly inhibited in a dose dependent manner after exposure to all three extracts. However, there were no significant differences among the three types of extracts at a higher concentration level, at which they exhibited the same level of antiproliferative activity. The current findings imply that hull constituents have potential for use as ingredients in functional foods.

4.2 Introduction

Phytochemicals, the bioactive non-nutrient plant compounds derived from fruits, vegetables, whole grains and other plant foods, have been suggested to be associated with the reduced risk of major chronic diseases (Liu, 2003; 2004). Flaxseed hull is a rich source of lignan, especially secoisolariciresinol diglucoside (SDG), which belongs to the group of phytoestrogens (Hallund et al., 2006). Animal models and *in vitro* studies have presented that lignan have strong antioxidant (Prasad, 2000), and exhibit protective effects against hormone-related types of cancer such as breast cancer (Saarinen et al., 2006; 2008 a,b; Boccardo et al., 2004; Chen et al., 2002; Chen and Thompson, 2003) and also against non-hormone related colon cancer (Sung et al., 1998). The anticancer effect of flaxseed or pure SDG on breast cancer was investigated intensively alone or combined with soy, tamoxifen as well as flaxseed oil (Saarinen et al., 2006, Chen and Thompson, 2003, Saggar et al., 2010 a, b). In Canada, a rescent survay showed that more than 80% of all women with breast cancer use complementary and alternative medicine to supplement their medical treatment or to enhance their overall health, with flaxseed and soy comprising 12.4% and 5.1% of total complementary and altaernative products used, respectively (Boon et al., 2007).

Contrary to breast cancer in women, prostate cancer in men is the second most frequently diagnosed cancer worldwide, especially in western countries, and is one of the major causes of death in men (Chen et al., 2007; Teiten et al., 2010). However, limited knowledge is available regarding any interactions among lignans and other phenolic compounds in suppressing prostate cancer proliferation. A few studies reported the anticancer effects of lignan on prostate cancer, and most of them investigated the specific

mechanism of lignan metabolites, enterlactone, on growth and development of prostate cancer (Chen et al., 2009; McCann et al., 2008; Bylund et al., 2005; Hedelin et al., 2006; Lin et al.,2002,2001; Chen et al., 2007).

One of the most prophylactic methods to prevent the development of cancer is a long-term diet high in biologically active compounds, such as lignans (McCann et al., 2008), phenolic acids, and flavonoids. These phytochemicals are called chemopreventive agents which can reverse, inhibit, or prevent the development of cancer by inhibiting specific molecular steps in the carcinogenic pathway, such as regulation of cell proliferation, cell survival or cell death as well as angiogenesis, and development of metastasis (Teiten et al., 2010).

Recent research has shown that the complex mixture of phytochemicals in fruits, vegetables and grains provides a better protective effect on health than single phytochemicals through synergistic or additive effects (Chu et al., 2002; Yang & Liu, 2009). This may imply that a single antioxidant cannot replace the combination of natural phytochemicals in whole food in achieving health benefits. Antioxidant synergism has been observed with different compounds such as vitamins E and C (Scarpa et al., 1984), catechin and malvidin 3-glucoside (Rossetto et al., 2002), vitamin E and β-carotene (Palozza and Krinsky, 1992), flavonoids and urate (Filipe et al., 2001), and tea polyphenols and vitamin E (Zhou et al., 2000). Yang and Liu (2009) reported that the apple extracts plus quercetin 3-β-D-glucoside combination possessed a potent synergistic effect toward MCF-7 human breast cancer cell proliferation *in vitro*.

Limited knowledge is available regarding any interactions among phytochemicals in barley hull and flaxseed hull in inhibiting PC-3 cell proliferation. There is no direct evidence linked to synergistic, additive, or antagonistic effects on the inhibition of PC-3 prostate cancer cell proliferation by barley hull extract, flaxseed hull extract and their co-extracts. Thus, it is hypothesized that co-extraction of barley hull and flaxseed hull may compliment each other's limitation in bioactive components and produce a functional food ingredient with higher bioactivity than individually. Therefore, the objective for this study was to determine whether the barley hull extracts in combination with flaxseed hull extracts have additive and/or synergistic effects on PC-3 human prostate cancer cell proliferation and intracellular antioxidant activity.

4.3 Method and Material

4.3.1 Chemicals

5-(and-6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), and RPMI1640 media, penicillin/ streptomycin, Fetable bovine serum were purchased from Invitrogen (Carlsbad, CA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Sigma (Oakville, ON). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Oakville, ON). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) assay was purchased from Promega (Madison, WI).

4.3.2 Sample Preparation

Barley hulls (EX116, Juton mutant line from World Collection) were separated from the grain by a grain polisher (Kett Electric Laboratory, Japan). Both barley hull and flax hull (Frutarom from Belgium, NV) were ground using a coffee grinder.

Five grams of ground hull and 1.25 g of calcium hydroxide were added to 250 mL of deionized water. The mixture was then heated in a water bath at 70˚C for 4 hours and stirred once every 10 minutes. The mixture was centrifuged at 2600×g (Sorvall instruments RC5C, MANDEL, Guelph, Ontario, Canada) for 25 minutes and then the supernatant fluid was decanted from the residue. The supernatant fluid was filtered using Whatman filter paper no. 4. The filtered product was neutralized with 1 N phosphoric acid. The product was filtered again using Whatman filter paper no. 4 to remove gel. The remaining filtrate was concentrated to a volume of 65 mL using a rotary evaporator. The concentrated fractions were then stored in the freezer and freeze-dried after. The freeze dried extract was extracted again with 10 ml of 50% methanol for 2 hrs with ultra sound sonication, and the methanol was evaporated.

4.3.3 Cell Culture and Treatment

The human prostate carcinoma PC-3 cells from American Type Culture Collection (Manassas, VA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 10 mmol/L HEPES, 1% penicillin, and streptomycin. The cells were maintained at 37^{o}C in a 5% CO_2 humidified incubator.

4.3.4 Cytotoxicity Assay

The cytotoxicity of barley hull extract, flaxseed hull extract and their co-extracts at 20 mg/ml on confluent PC-3 human prostate cancer cells was tested by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) assay as described by Yang and Liu (2009) with minor modifications. 100 µL of cell suspension (with a concentration of 1×10^5/ml) was seeded in a 96-well plate. After 48 hrs of incubation, the culture media was aspirated and 100 µL of barley hull extract, flaxseed hull extract and their co-extract diluted by the culture media were added into appropriate wells except the control wells. Fresh media was added into the control wells. The cells were treated for 4 hrs, and 20 µL of MTS solution reagent was added to all except the background control wells. The absorbance at 490 nm were measured by Opsys MR 96-well plate reader (DYNEX Technologies, Chantilly, VA, USA) after 2 hrs incubation at 37^{o}C in a humidified, 5% CO_2 atmosphere. The cytotoxicity (percent) was determined by the corrected absorbance compared to that of control cultures. Data were reported a mean±SD for three sets of different plate replications.

4.3.5 Cellular Antioxidant Activity (CAA) Assay

The cellular antioxidant assay was conducted using the method of Wolfe and Liu (2008) with minor modifications. Human prostate carcinoma PC-3 cells were seeded at a density of 5×104/well on a 96-well microplate in 100 µL of growth medium/well. Twenty-four hours after seeding, the growth medium was removed. Then 100 µL of barley hull extracts, flaxseed hull and their co-extracts (at concentrations of 100µg/ml, 250µg/ml, 500µg/ml, 1000µg/ml) were added into appropriate wells and treated for 1hr.

At 1/2hr incubation time, 100 µL of 10 µM DCFH-DA solution was loaded to all wells. 50 µg of DCFH-DA was dissolved in 50 µL DMSO and further diluted in 10 mL growth media (DMSO content is 0.1% in the final solution), which produced a concentration of 10 µM of DCFH-DA solution. After 1hr incubation, the treatment solution was removed from each well, and 100 µL of 500 µM AAPH dissolved in Hank's Buddered Salt Solution (HBSS) was applied to the cells and the 96-well microplate was placed into a Fluoroskan Ascent FL plate-reader (ThermoLabsystems, Franklin, MA) at 37^{o}C. Emission at 527 nm was measured every 15 min for 1 h. The excitation wavelength was 485 nm. Each plate included eight control and blank wells; control wells contained cells treated with DCFH-DA and AAPH and blank wells contained cells treated with HBSS without AAPH. Data was obtained from three independent experiments. The ROS scavenging activity was expressed as the inhibition rate (%), which was calculated from the following formula:

ROS scavenging activity= $(f_{control} - f_{sample})/ f_{control} \times 100$.

Where $f_{control}$ represents the intensity of fluorescence of control, f_{sample} represent the intensity of fluorescence of sample.

4.3.6 Cell Proliferation Assay

The antiproliferative activities of barley hull extract, flaxseed hull extract and their co-extracts toward the growth of PC-3 human prostate cancer cells *in vitro* was measured by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) assay. 50 µL of different concentrations of extracts (10, 20, 30, 40 mg/ml) were added into the wells of a 96 well flat bottom plate; then 50 µL of cell suspension (at a

concentration of 1×10^5 cells/ml) was dispensed into half of the wells prepared above and 50 μL of growth media into another half as background control. The initial cell number was determined from a linear response curve between cell number and absorbance at 490 nm during 72 hrs of cell growth. The total volume in each well was 100 μL. Control cultures were maintained in the growth media and the blank wells contained 100 μL of growth medium with no cells. After 72 hrs of incubation, 20μL of MTS reagent was added into all the wells. The absorbance at 490 nm were measured by Opsys MR 96-well plate reader (DYNEX Technologies, Chantilly, VA, USA) after 3 hrs incubation at 37^oC in a humidified, 5% CO_2 atmosphere. The cell antiproliferation (percent) was determined by the corrected absorbance compared to that of control cultures. Data were reported as mean ±SD for three sets of different plate replications.

4.3.7 Statistical Analysis

Statistical analysis (ANOVA) was performed using SAS software (SAS Institute Inc., Cary, NC). The results were reported as mean ±Standard Deviation (SD). Tukey's test was applied to assess the significant differences with $p < 0.05$.

4.4 Results and Discussion

4.4.1 Cytotoxicity

The cytotoxicity of barley hull extract, flaxseed hull extract and their co-extract was tested in confluent PC-3 cells using the MTS-based assay.

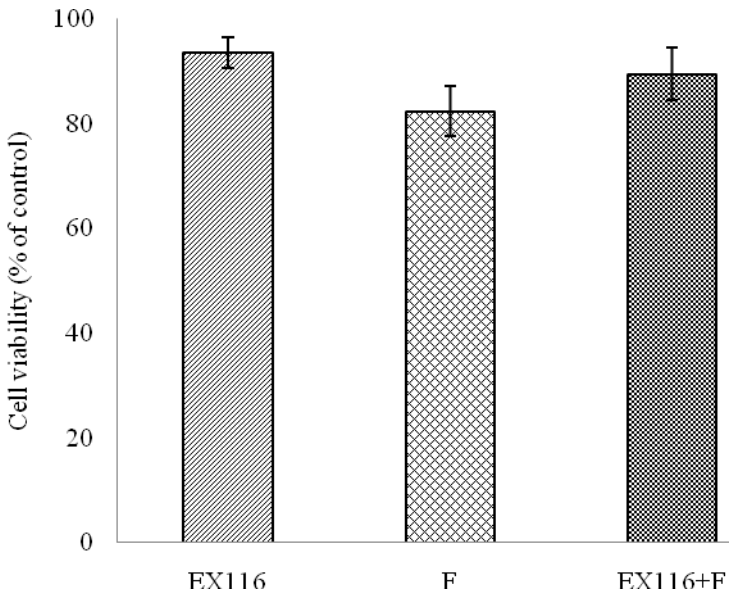


Figure 13. Cytotoxicity of barley hull (EX116) extract, flaxseed hull (F) extract and their co-extract at 20 mg/ml in confluent PC-3 prostate cancer cells (Data represent Mean±SD, n=3).

The cell viability was around 90% of the total cell treated with barley hull extract (EX116), flaxseed hull extracts (F), and barley-flaxseed co-extracts (EX116+F) (Fig. 13), therefore, these three types of extracts are considered as not cytotoxic toward PC-3 human prostate cancer cells.

4.4.2 Cellular Antioxidant Activity

CAA assay is a cell-based antioxidant activity assay which has better representation of the complexity of biological systems, involving cellular uptake, distribution, and the efficiency of protection against free radicals under physiological conditions (Wolfe et al., 2008). The principles of this method is that DCFH-DA can be easily taken up by the cells, deacetylated to DCFH, and then oxidized to fluorescent DCF by the reactive oxygen species (ROS) generated from AAPH (Wolfe et al., 2008). If

barley hull extracts, flaxseed hull extracts and their co-extracts can quench ROS and inhibit the generation of DCF, their relative intracellular antioxidant activity can be measured by the level of fluorescence, which is proportional to the level of oxidation. Thus, the fluorescence intensity increased dramatically with time of oxidation in the control (Dye with AAPH) (Fig.14).

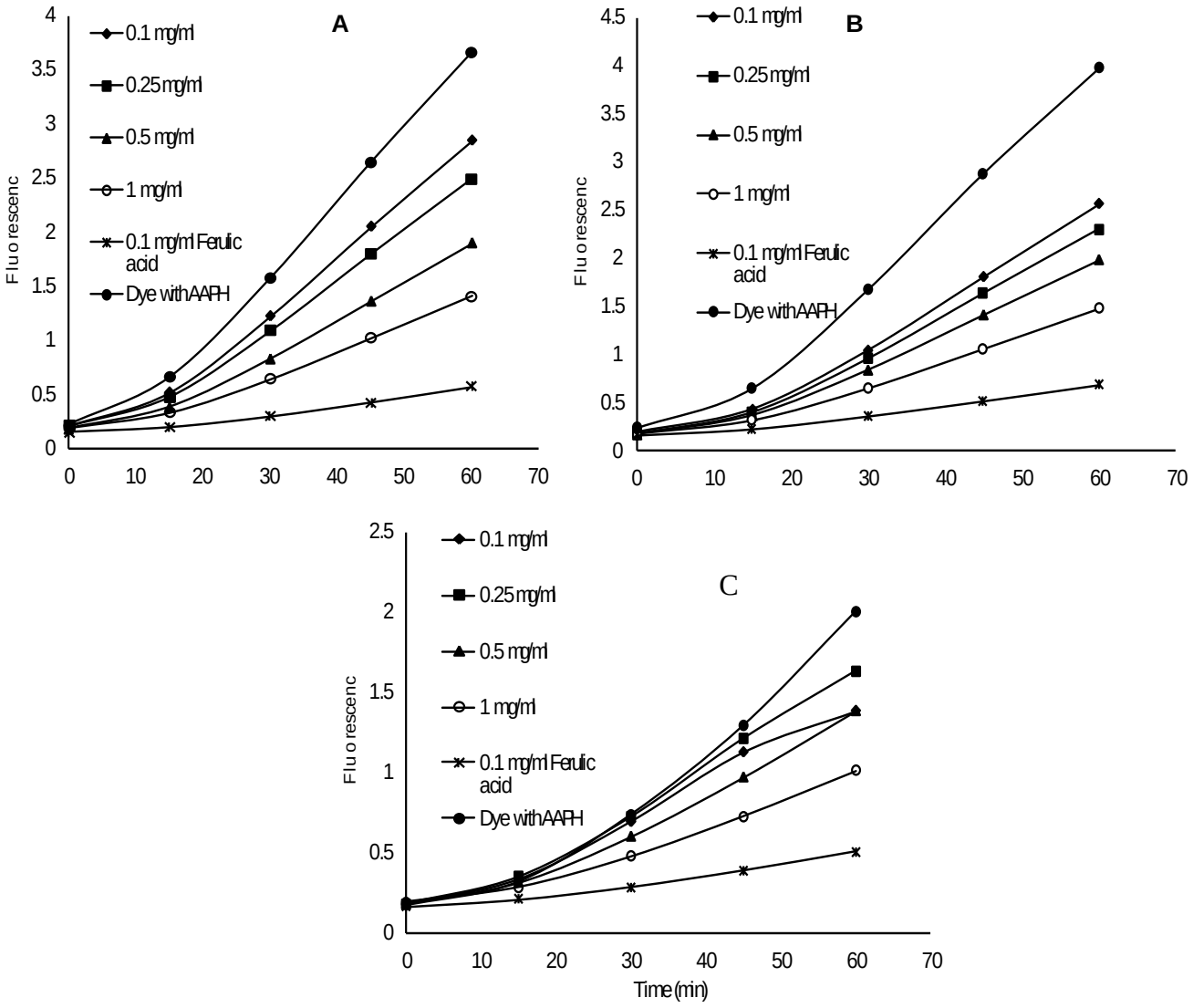


Figure 14. AAPH free radical induced oxidation of DCFH to DCF in PC-3 cells and the inhibition of oxidation by different concentrations of barley hull extracts (A), flaxseed hull extract (B) and their co-extracts (C) over time.

The increase in fluorescence from DCF formation was inhibited by barley hull extracts, flaxseed hull extracts and their co-extracts in dose dependent manner over 60min, as demonstrated by the lower fluorescence intensity in the curves in Figure14.

Ferulic acid was used as a positive control. The data points represent three separate sets of experiment. The fluorescence intensity for all dose treatments was corrected by subtracting the corresponding background fluorescence which may be produced by the treatment. The concentration tested in this assay ranged from 0.1 mg/ml to 1 mg/ml at the highest.

After the first 15 min, the curves in Figure 15 become plateau which indicate that the barley hull extracts (Fig.15A), flaxseed hull extracts (Fig.15B) and their co-extracts (Fig.15C) significantly inhibited the ROS-induced oxidation of DCFH to fluorescent DCF. At the highest concentration of treatment (1mg/ml), the relative intracellular antioxidant activities of barley hull, flaxseed hull, and their co-extracts at 60 min were 69%, 59% and 54%, respectively, among which the co-extracts exhibited the lowest cellular antioxidant activity. Barley hull extract inhibited 38% to 69% of fluorescence formation, while the flaxseed hulls extract inhibited 39% to 59% of fluorescence formation compared to the control. However, barley and flaxseed hull co-extracts behaved differently from individual hull extracts at the lower concentration. There was no protective effect at the beginning of the measurement, and the dose-dependent relationship was not clear. However, the inhibition rate of the co-extract on the fluorescence DCF reached 37% to 54.6% at 60 min.

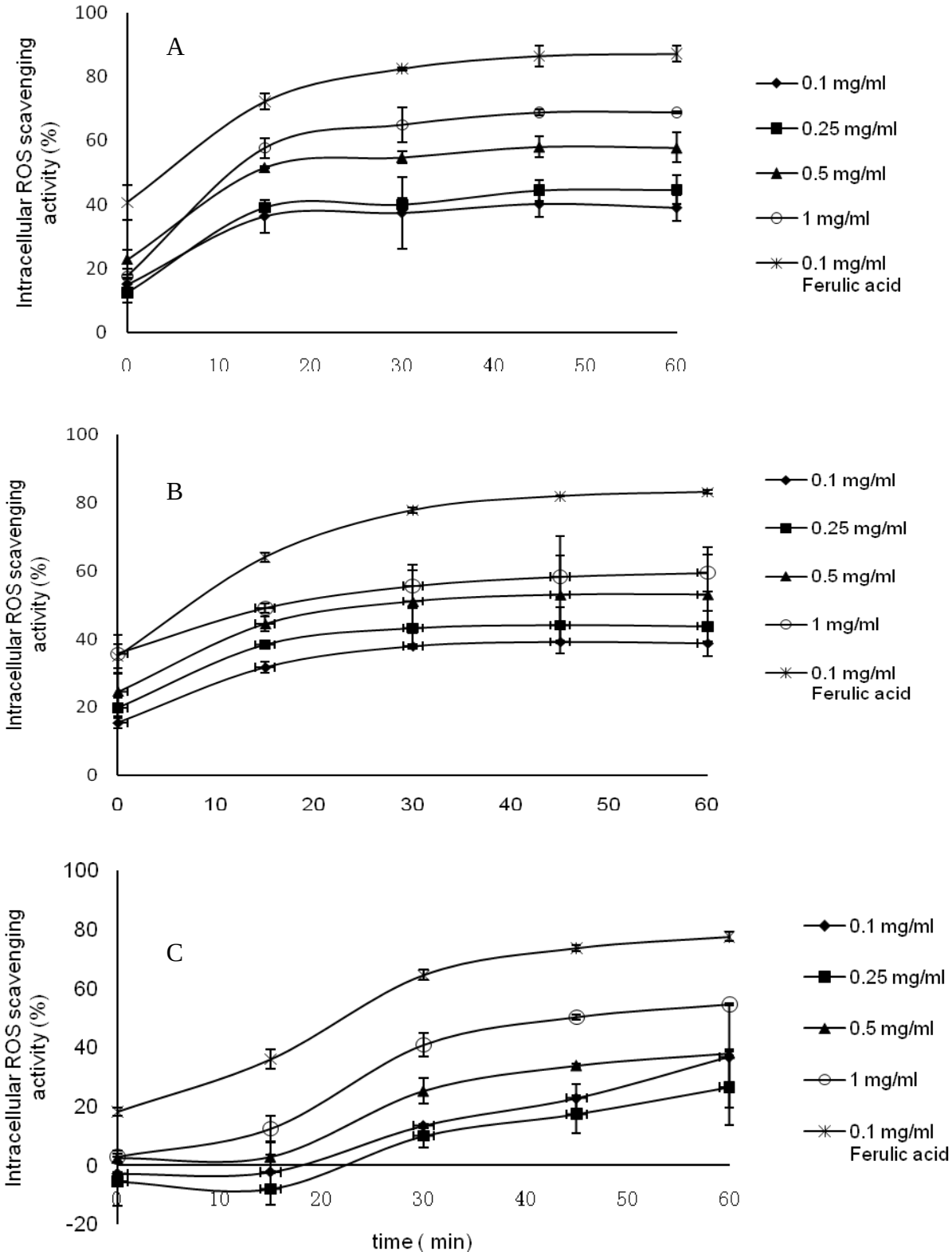


Figure 15. The relative intracellular ROS scavenging activity of different concentrations of barley hull extracts (A), flaxseed hull extracts (B) and their co-extracts (C) over time

4.4.3 Cell Antiproliferative Activity

The percentage cell proliferation was calculated by the corrected absorbance, subtracting the background absorbance of the treatment from the treated cell. The antiproliferative activities of barley hull extracts, flaxseed hull extracts and their co-extracts on PC-3 human prostate cancer cells are summarized in Figure 16. The proliferation of PC-3 cells was inhibited in a dose-dependent manner after exposure to the various concentrations of barley hull extracts, flaxseed hull extracts and their co-extracts. There was no significant difference found among the three types of extracts at the lowest concentration (5 mg/ml) and at the higher concentration (15 mg/ml and 20 mg/ml). More than 90% of the cell proliferation was inhibited by all three types of extracts at the highest concentration of 20 mg/mL.

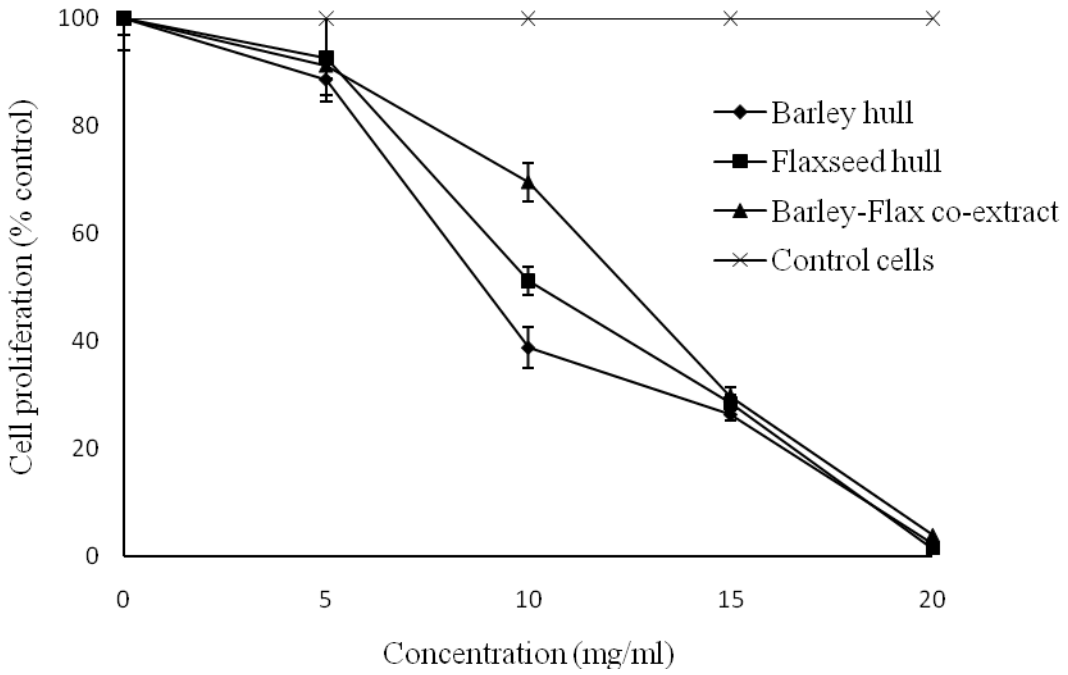


Figure 16. Effects of barley hull extracts, flaxseed hull extracts and their co-extracts on cell proliferation of PC-3 human prostate cancer cells

The EC_{50} represents the concentration corresponding to one-half the difference between the maximum and minimum absorbance values. Although the co-extracts exhibited lower inhibition rate on the PC-3 cell proliferation at 10 mg/mL, the EC_{50} of these three types of hull were not significantly different (Table 12).

Table 12. EC_{50} of barley hull extract, flaxseed hull extract and their co-extract

Sample	ED_{50} (mg/mL)
Barley hull	10.45[a]
Flax hull	11.00[a]
Barley +Flax hulls	11.54[a]

The antiproliferation activity of barley hull extract, flaxseed hull extract or their co-extracts had a consistent trend with barley having the highest intracellular antioxidant activity and antiproliferative activity, followed by flaxseed. The co-extract exhibited significantly lower antiproliferative activity at 10 mg/mL compared to the individual barley hull extracts and flaxseed hull extracts. This may be attributed to the chemical and biological properties of the phenolic compounds in the extracts, and the cell responses to these compounds. The major phenolic compound found in barley hull and flaxseed hull differ in molecular size, polarity and solubility, which may affect their bioavailability and distribution in different subcellular organelles, cells, tissues, and organs (Yang and Liu, 2009). This may explain why barley hull with its low total phenolic content and DPPH radical scavenging activity (Table 5 & Figure 8) still gave high intracellular antioxidant and antiproliferative activities in a cell culture model.

4.5 Conclusion

The present study demonstrates a potential functional food ingredient converted from barley hull and flaxseed hull with strong intracellular antioxidant and antiproliferative activities in PC-3 human prostate cancer cell model. The co-extraction of barley hull and flaxseed hull aimed to have a richer phytochemicals profile, combined from the individual hulls, since the health benefits of fruits and vegetables are likely due to the additive and synergistic effects of an array of phytochemicals, rather than to a single compound alone (Yang and Liu, 2009). Our findings have important implications for developing a novel value-added functional food ingredient from these agricultural by-products, such as barley hull and flaxseed hull. However, future studies are needed to elucidate the underlying mechanisms of the antiproliferative activity of these hull extracts, especially the co-extracts in prostate cancer cells.

Chapter 5: Phytochemical Profile and Antioxidant Activity of Chinese Steamed Bread Supplemented with Barley Hull Extract, Flaxseed Hull Extract and Their Co-extracts

5.1 Abstract

The phytochemical profile and antioxidant activity of Chinese steamed bread (CSB) containing barley hull extract, flaxseed hull extract as well as their co-extracts were investigated. HPLC and LC-MS/MS analyses showed that the phytochemical profile of CSB with the addition of barley hull extract was enriched in ferulic acid and p-coumaric acid. The flaxseed hull extract introduced new phenolic compounds including secoisolariciresinol diglucoside (SDG), ferulic acid glucoside (FeAG) and coumaric acid glucoside (CouAG) into the CSB. All the major phenolic compound originating from two different hulls were found in CSB to which barley-flaxseed hull co-extracts were added. In general, the antioxidant activity of CSB was significantly enhanced by incorporating barley hull extracts, flaxseed hull extracts and their co-extracts. The total phenolic content was improved by 83.1%, 138.3% and 70.3% when barley hull extract, flaxseed hull extract, and their co-extract respectively, were added. Flaxseed hull showed the highest enhancement in DPPH radical scavenging activity, followed by the co-extract, and then barley hull. However, the co-extracts exhibited the highest enhancement in ORAC of the CSB, followed by the flaxseed hull extract and barley hull. In conclusion, these findings indicate that barley hull extracts, flaxseed hull extracts and their co-extracts can be targeted for development of functional food ingredients.

5.2 Introduction

Functional foods are regarded as innovative and promising products which can provide additional health benefits beyond the basic nutrition. Although the functional foods have no formal definition, some groups define the primary category of functional foods as modified foods that claim to have been fortified with nutrients or enhanced with phytochemicals or botanicals to provide specific health benefits (IFT, 2005). Therefore, the addition of components with nutritional functional properties to food is the main procedure for the manufacture of functional food. However, the nutritional properties (e.g. bioavailability) of the health ingredients as well as the technological functional properties of all ingredients may change considerably by incorporation into the food product and during processing and preparation of food products. It is necessary to investigate the assumed beneficial properties of the potential ingredients such as phytochemicals. Peng et al. (2010) reported that grape seed extract, a well-known nutraceutical product with abundant content of catechin and proanthocyanidins, fortified the antioxidant activity of bread compared to the blank. Zhang et al. (2007) reported that addition of antioxidants from bamboo leaves containing high level of flavonoids was an effective way to reduce the formation of acrylamide in fried chicken wing. Furthermore, there is a growing interest in developing novel food products supplemented with natural antioxidants, which are derived from fruits, vegetables, whole grains, oilseeds as well as their by-products. Antioxidant effect of cherry fruits, citrus fruit by-products, grape seed extracts, rosemary, orange extract, kinnow rind, pomegranate rind and their seed powder extracts have benn researched for their use in all kinds of meat products (Britt et al.,

1998; Fernandez-Lopez et al., 2004; Brannan, 2008; Rojas and Brewer, 2008; Devatkal et al., 2010).

Flaxseed or flax meal (partially defatted flaxseed) are increasingly being used in cereal-based products, such as bread, muffins, bagels, cookies as well as other bakery products (Carter, 1993; Nesbitt and Thompson, 1997; Muir and Westcott, 2000; Menteş et al.,2008). Its growing popularity is due to the rich content of lignan, especially SDG, and omega 3-fatty acid as well as dietary fiber (Alhassane and Xu, 2010). However, flaxseed hull, which comprises about 40% of the seed are enriched in SDG compared to the cotyledons (Madhusudhan et al., 2000; Oomah and Mazza, 1997; Wiesenborn et al., 2003). Furthermore, it is shown that SDG is present in flaxseed hull in the form of lignan macromolecule with coumaric acid glucoside (CouAG), ferulic acid glucoside (FeAG) and herbacetin diglucoside (HDG) as part of the macromolecule structure (Struijs et al., 2007). On the other hand, barley hull is an agricultural by-product which comprises up to 15-20% of the grain on dry weight basis (Cruz et al., 2007). The major phenolic compounds identified in barley hull are ferulic acid, p-coumaric acid, syringic acid, 3,4-dihydroxybenzaldehyde, vanillic acid and vanillin (Cruz et al., 2007; Conde et al., 2008, Garrote et al., 2008). The utilization of barley hull as feed supplement is limited due to its low digestibility; and combustion of this material is difficult and not practical due to its high ash content, which results in mineral depositions in boilers (Cruz et al., 2007; Garrote et al., 2008). On the other hand, it is very expensive to transport barley hull to the disposal areas owing to its low density (Mahmudi, 2005; Searcyl et al., 2007; Garrote et al., 2008). Thus, conversion of barley hull from a low profit waste into a value-added functional food ingredient is a big challenge and the possibility of co-extraction of barley

hull and flax hull to produce a better functional food ingredient needs further investigation. Although flaxseed has been recently used in baked goods, studies regarding the incorporation of flaxseed hull and barley hull extracts appear to be limited.

Thus, the objectives of the present study were to investigate the phytochemical profile as well as the antioxidant activity enhancement of Chinese steamed bread supplemented with barley hull extracts, flaxseed hull extracts, and their co-extracts.

5.3 Materials and Methods

5.3.1 Chemicals

Calcium hydroxide was purchased from Fishers Scientific (Fair Lawn, NJ). Folin-Ciocalteu reagent, DPPH, and phenolic acid standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). SDG standard was purchased from ChromaDex. HPLC grade methanol, MS grade water, acetonitrile, and acetic acid were used in LC-MS analysis. All of the HPLC grade and MS grade solvents were also purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

5.3.2 Preparation of Crude Extracts from Barley Hull, Flaxseed Hull and Their Mixture

Barley hull (EX116), flaxseed hull (F) and their mixture (1:1 wt/wt) were subjected to alkaline hydrolysis. Five grams of ground hulls and 1.25 g of calcium hydroxide were added to 250 mL of deionized water. The mixture was then heated in a water bath at 70˚C for 4 hours and stirred once every 10 minutes. The mixture was centrifuged at 2600×g (Sorvall instruments RC5C, MANDEL, Guelph, Ontario, Canada)

for 25 minutes and then the supernatant fluid was decanted from the residue. The supernatant fluid was filtered using Whatman filter paper no. 4. The filtered product was neutralized with 1 N phosphoric acid. The product was filtered again using Whatman filter paper no. 4 to remove gel. The gel from sample was collected in containers and stored in a refrigerator. The remaining filtrate was concentrated to a volume of 65 mL using a rotary evaporator. The concentrate was then filtered again using a Whatman filter paper no. 4 to remove any impurities. The concentrated fractions were then stored in the freezer and freeze-dried after. Freeze dried extracts were redissolved in 50% methoanl for further analysis.

5.3.3 Procedure for Laboratory Preparation of Chinese Steamed Bread (CSB)

The procedure for preparation of CSB was described by Jiang et al. (2010) with some modifications: 1 g of freeze-dried barley hull extract, flaxseed hull extract and their co-extract were added into regular white wheat flour (100 g), dehydrated yeast (0.8 g), and water (48 ml). After mixing and kneading to form the mixture into dough, the dough was sheeted 20 times. The dough was rounded and molded manually and proofed for 40 min at 38^{o}C and 85% relative humidity. After proofing, the dough was steamed for 19 min using a steam tray and boiling water.

5.3.4 CSB Extraction

Freeze-dried CSB was subjected to alkaline hydrolysis in the same way as described above in the crude extract preparation.

5.3.5 LC/MS/MS

The chromatographic separation was carried out based on the method described by Qiu et al. (2009) with modifications, using an HPLC (Waters 2695) equipped with a photodiode array detector (PAD) (Waters 996) and autosampler (Waters 717 plus) (Waters, Milford, MA). The analytical column was a 150 mm × 4.6 mm, 5 μm RP 18 column (Gemini, Phenomenex, USA). The mobile phase consisted of A (0.1% acetic acid in high-purity water) and C (0.1% acetic acid in acetonitrile). A 35 min-linear gradient was programmed as follows: 0-5 min, 10-15% C; 5-20 min, 15-40% C; 20-25 min, 40% C; 25-30 min,40-10%C; 30-35 min, 10 % C; The injection volume was 10-μL sample solution and the flow rate was 0.5 mL/min. The Q-TOF MS was calibrated with sodium iodide for the negative mode through the mass range of 100-1500. Full mass spectra were recorded in negative mode by using the capillary voltage of 1.2 kV and cone voltage of 45V. The flow rate of desolvation gas (N2) and cone gas (N2) were 900 L/h and 50 L/h, respectively. The desolvation temperature and the source temperature were set at 350 ℃ and 150 ℃, respectively. The MS/ MS spectra were acquired by using collision energy of 20 V.

5.3.6 Measurement of Total Phenolic Content (TPC)

The TPC of CSB extracts was determined using the Folin–Ciocalteau reagent with some modifications (Beta et al., 2005; Gao et al., 2002; Mpofu et al., 2006; Li et al., 2007). Briefly, 200 μL of the 10-fold diluted CSB extracts was reacted with 1.8 mLof freshly made 10-fold diluted Folin-Ciocalteu reagent. The mixture was then neutralized with 1.8 mL of sodium carbonate (60 g/L). The absorbance was measured at 725 nm after

90 min of reaction at room temperature. Ferulic acid (a concentration range of 0.0625 mM to 1 Mm) was used as the standard. Results were expressed as milligrams of ferulic acid equivalents (FAE) per gram of sample (dry weight basis).

5.3.7 Evaluation of DPPH radical scavenging capacity

DPPH radical scavenging capacity assay was carried out according to Brand-Williams et al. (1995) and Yu et al. (2002) and Li et al. (2007) with some modifications. Briefly, 200 µL of 100-fold diluted CSB extract was added to 3.8 mL of 60 µM DPPH radical solution, which was freshly made in 100% methanol. After 60 min of incubation at room temperature, the absorbance at 515 nm was measured. Trolox was used as standard with the concentration ranging from 0.1 µM to 1 mM of Trolox dissolved in 50% methanol. DPPH free radical scavenging activities of CSB extracts (duplicate extracts) were expressed as milligram of Trolox equivalents (TE) per g of sample (dry weight basis).

5.3.8 Determination of Oxygen Radical Absorbance Capacity (ORAC)

This assay was implemented as described by Qiu et al., (2009). A Precision 2000 automated microplate pipetting system (BIO-TEK Instruments, Inc., Winooski, VT) was used for plate-to-plate transfer of solutions. An FL_800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by software KC4 3.0 (version 29) was used with fluorescence filters for an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. First, 120 µL of fluorescence working solution was automatically transferred to a 96-well flat-bottom polystyrene microplate (Corning Inc., Corning, NY) and used as the substrate. Then 20 µL each of buffer solution (blank),

Trolox (standard control), appropriately diluted samples, and catechin (sample control) were added to the designated wells, respectively. After 20 min of incubation at 37 ℃, 60 μL of freshly made AAPH solution was added to each well to generate a peroxyl radical. The total reaction time was 50 min. The fluorescence of the reaction mixture was recorded every minute. The area under the fluorescence decay curve (AUC) was calculated according to the equation

$$AUC = 0.5+f_1/f_0 + f_i/f_0 + ... + f_{49}/f_0 + 0.5(f_{50}/f_0)$$

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i min. Final ORAC values were calculated from the Trolox standard curve and expressed as milligram of TE per 1g of extract (dry weight basis).

5.3.8 Statistical Analysis

Data were analyzed by the general linear models (GLM) and one-way analysis of variance (ANOVA) using SAS software (SAS Institute Inc., Cary, NC). Tukey's test was applied to assess the significant differences in the antioxidant activity. Quantitative results were expressed on a dry weight basis (dwb).

5.4 Results and Discussion

5.4.1 HPLC-MS/MS

Thermal treatment is one of the most popular ways of food processing. During heating, a complex array of chemcial reactions takes place, such as Maillard reaction, which produces some potential antioxidative compounds. However, some compounds may be destroyed during food processing. In order to avoid many complex reactions,

Chinese steamed bread was selected as target food product in the present study. It is vital to investigate the phytochemcial profiles of the food product after heat treatment, which can reveal the nature behind the additional health benefit of the ingredient added to the food, and also can demonstrate the alteration of the phenolic compounds due to the interaction with the fundamental food components. In the present study, the HPLC chromatogram showed that the phytochemcial profile of CSB was significantly improved by adding barley hull extracts, flaxseed hull extracts and their co-extracts compared to the control bread (Fig. 17). Corresponding peaks with retention time, m/z, and MS/MS fragment ion mass as well as the tentative annotation of identified phenolic compounds are listed in Table 13.

Table 13. Major phenolic compounds identified in steamed bread supplemented with EX116+F co-extracts at the level of 1g/100g flour.

Peak No.	Retention time (min)	[M-H]-	MS/MS	Peak Annotation
1	3.82	179	149	Caffeic acid
2	4.75	179	149	Caffeic acid
3	9.45	325	163,119	Coumaric acid glucoside
4	10.75	355	193	Ferulic acid glucoside
5	14.63	685,721	523,361	SDG
6	18.18	163	119	p-coumaric acid
7	18.92	193	178,134	Ferulic acid
8	19.83	193	178,134	Isoferulic acid

The extracted ion chromatogram (EIC), mass spectra, MS/MS spectra are shown in Figure 18. It was obvious that barley hull extract enhanced ferulic acid and p-coumaric

acid content without introducing a new compound to the CSB (Fig17. A & B). Muir and Westcott (2000) reported that 73-75% of the SDG was recovered when flax meal or aqueous alcohol extracts were incorporated into bread.

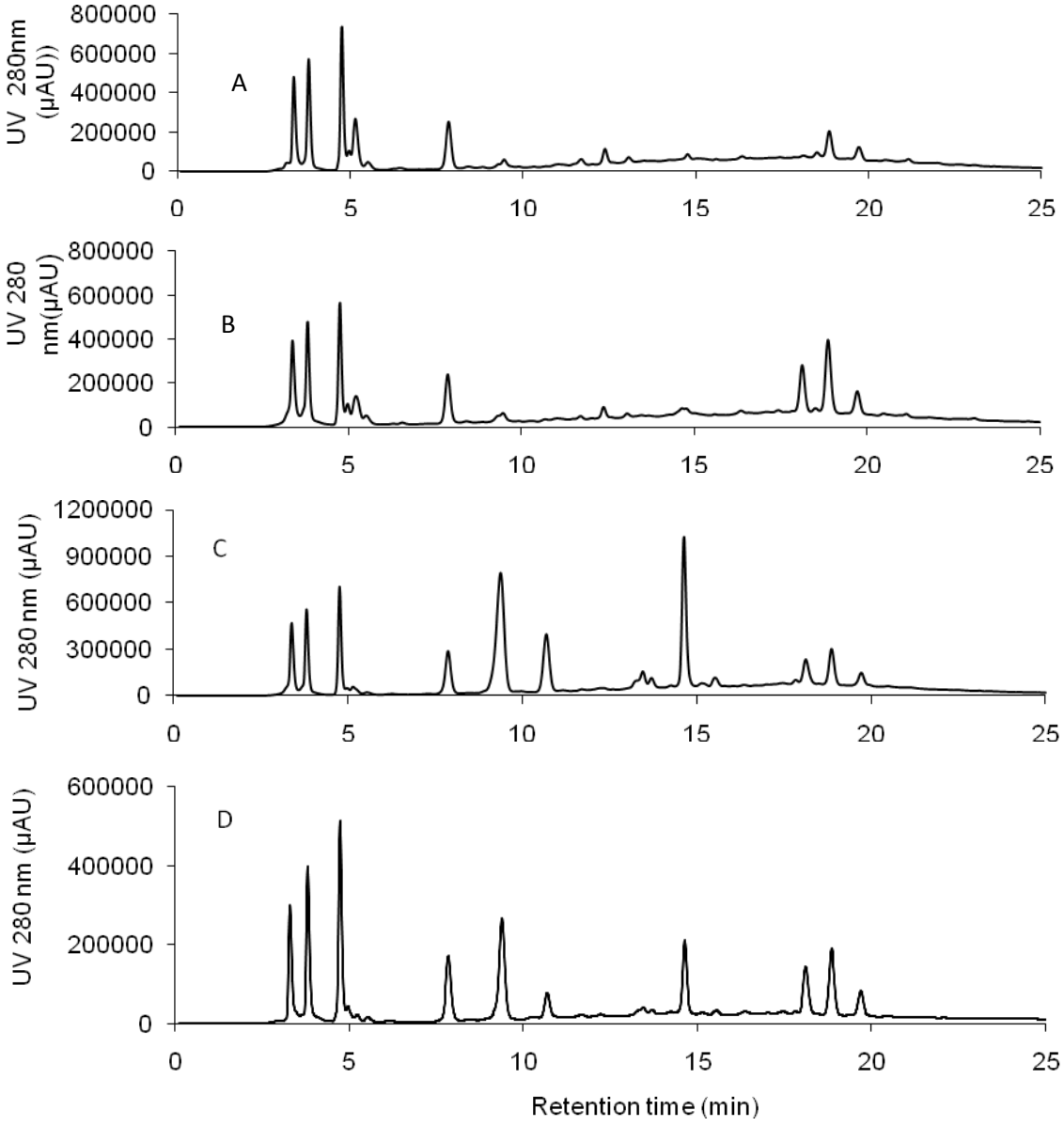


Figure 17. HPLC/UV chromatograms of Chinese steamed bread (CSB); (A) control bread; (B) CSB+Barley hull extract; (C) CSB+Flaxseed hull extrac; (D) CSB+ Barley-flaxseed hull co-extract.

The EIC, MS spectra, as well as MS/MS spextra confimed that there was no chemical alteration to the major phenolic compounds added into the bread after processing.

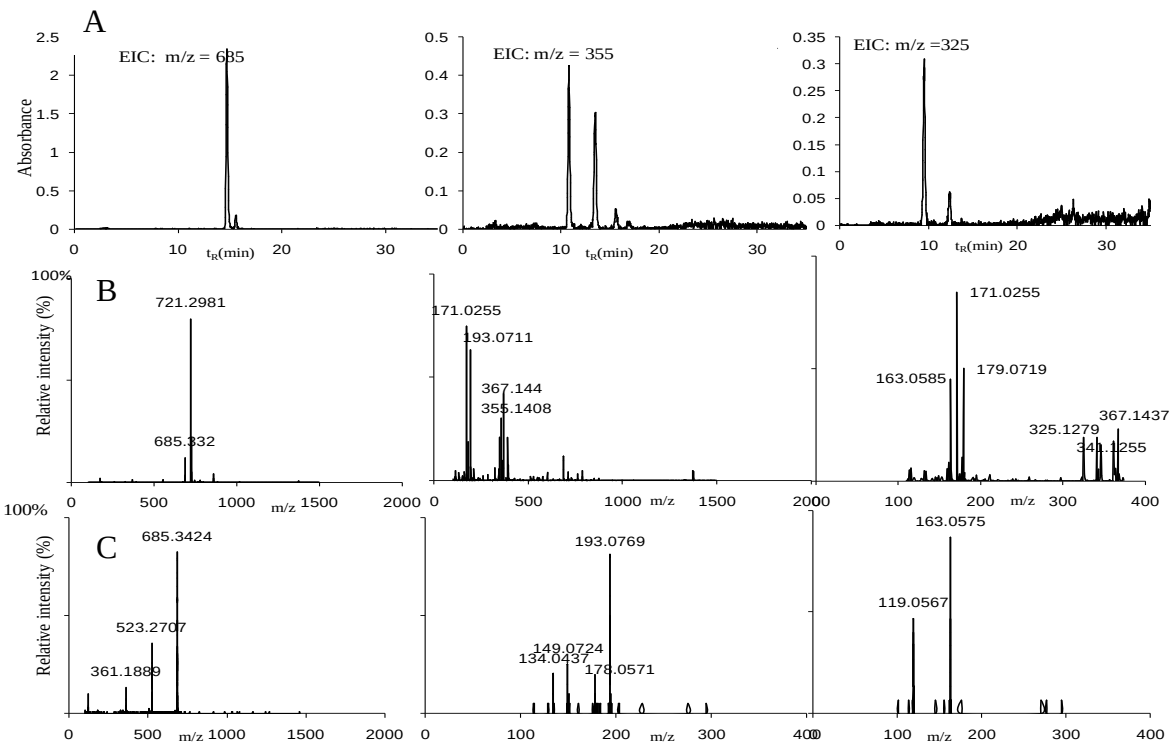


Figure 18. Extracted Ion Chromatogram (A), Mass spectra (B), MS/MS spectra (C) of SDG, FeAG, and CouAG detected in CSB supplemented with flaxseed hull extracts.

However, flaxseed hull extract introduced SDG, FeAG, and CouAG which were not found in the control bread (Fig 17.A&C). Moreover the phytochemcial profile of barley-flaxseed hull co-extract supplemented CSB contained all the major phenolic compounds originating from both barley hull and flaxseed hull compared to the control bread (Fig 17.A&D). Thus, this result indicated that the major phenolic compounds from

barley hull extracts, flaxseed hull extracts, and their co-extracts were still available without any alteration after adding into the CSB. Therefore, the antioxidant activity may be enhanced in the CSB supplemented with these extracts.

5.4.2 TPC

The total phenolic content of CSB with 1 g extract addition level was evaluated. TPC of CSB was significantly enhanced by incorporating barley hull extracts, flaxseed hull extracts and their co-extracts, with 83.12%, 138.34% and 99.36% enhancement respectively compared to the control bread (Table 14).

Table 14. Total phenolic content of CSB at 1g extract with 1g extract incorporation (mg ferulic acid equivalent/ 1 g extract)

Steamed Bread	Mean ±SD[a]	% Enhancement
Control	1.70 ±0.16[b]	
Barley hull	3.11 ±0.75[ab]	83.12
Flaxseed hull	4.05 ±0.31[a]	138.34
Barley-Flaxseed	3.39 ±0.21[ab]	99.36

[a]Data are expressed as mean+standard deviation (n=2) on dry weight basis;

Since barley hull and flaxseed hull have distinct profile of phenolic compounds, there was a huge difference in their total phenolic content, which resulted in more than 50% difference in the enhancement to the TPC of CSB. Moreover, individual flaxseed hull extracts significantly increased the TPC compare to the control bread. Although the TPC of bread supplemented with individual barley hull extract and barley-flaxseed hull

co-extract are not significantly different from the control bread, the supplementation of the two types of extract into the CSB may still have an enhancement in TPC to some extend.

5.4.3 DPPH

The DPPH radical scavenging activity of CSB incorporated with barley hull extract, flaxseed hull extract and their co-extract was evaluated at a level of 1 g-extract addition (Table 15).

Table 15. DPPH radical scavenging activity of CSB with 1g extracts incorporation (mg Trolox equivalent/ 1 g extract)

Steamed Bread	Mean ±SD[b]	% Enhancement
Control	6.75 ±1.82[b]	
Barley hull	11.25 ±2.79[a]	66.79
Flaxseed hull	12.87 ±2.02[a]	90.69
Barley+Flaxseed hulls	11.49 ±0.48[a]	70.30

[b]Data are expressed as mean+standard deviation (n=2) on dry weight basis;

Compared to the control bread, CSB with barley hull extracts had 66.79% of enhancement in the DPPH radical scavenging activity whereas the flaxseed hull extracts enhanced the DPPH radical scavenging activity of CSB by up to 90.69%. The co-extracts had an enhancement rate of 70.30%, which is higher than individual barley hull extracts, but lower than individual flaxseed hull extracts. The DPPH radical scavenging activity enhancement was consistent with that of TPC of CSB incorporated with barley hull extracts, flaxseed hull extracts and their co-extracts. All three different types of extracts

significantly increase the DPPH radical scavenging activity of CSB; however, there are no significant differences among them. The enhancement in DPPH radical scavenging activity must be related with the phenolic compounds profile of the bread supplemented with different types of extract.

5.4.4 ORAC

The ORAC values are shown in Table 16. The ORAC values are not significantly different between control bread and the CSB supplement with barley hull extract, flaxseed hull extract, and their co-extract. However, the enhancement percentage may be an indication of advantages for incorporation of these extracts into CSB. The control bread showed the lowest ORAC values (20.78 mg TE/ 1g extract), which was enhanced by 34.5% after adding 1 g of barley hull extract into 100 g of flour. On the other hand, incorporation of 1 g of individual flaxseed hull extract into CSB enhanced ORAC values up to 67.43% compared to the control bread, which indicated flaxseed hull extracts had stronger antioxidant activity than barley hull extracts. Thus, incorporation of barley-flaxseed hull co-extracts resulted in highest ORAC values (36.44 mg TE/ 1g extract) and highest enhancement rate (75.32%) compared to the control bread. The phenolic profiles of barley hull, flaxseed hull and their co-extracts may contribute to the enhanced oxygen radical absorbance capacity when added into the CSB.

Table 16. ORAC values of CSB with1g extract incorporation (mgTrolox equivalent/ 1 g extract)

Steamed Bread	Mean ±SD[c]	% Enhancement
Control	20.78 ±5.28[a]	
Barley hull	27.95 ±0.03[a]	34.50
Flaxseed hull	34.80 ±6.95[a]	67.43
Barley+Flaxseed hulls	36.44 ±14.01[a]	75.32

[c]Data are expressed as mean+standard deviation (n=4) on dry weight basis;

ORAC assay is a hydrogen atom transfer based reaction to assess the inhibition of peroxyl-radical-induced oxidations by classical radical chain-breaking antioxidants (Ou et al., 2001). The ORAC assay is suggested as a standard assay to assess the antioxidant capacity against peroxyl radicals due to the reaction condition (37 ℃, pH 7) which is the most relevant to human biology (Prior et al., 2005). On the other hand, the DPPH radical scavenging capacity assay is an electron transfer-based assay which can measure the capacity of an antioxidant in the reduction of DPPH generated organic nitrogen radicals (Huang et al., 2005). Due to the different mechanisms involved in ORAC and DPPH radical scavenging capacity assay, the results from these two assays on the CSB were different; however, the antioxidant capacity of CSB supplemented with barley hull extracts, flaxseed hull extracts as well as their co-extracts was impoved compare to the control bread.

5.5 Conclusions

Incorporation of barley hull extracts, flaxseed hull extracts, as well as their co-extracts into CSB can significantly enrich the phytochemical profile of the bread, and thereby increase the antioxidant activity. Although, the antioxidant activity evaluated by ORAC assay are not significantly enhanced at the level of incorporation (1g extract/ 100 g flour), the enhancement in TPC, DPPH radical scavenging activity, ORAC values may still give a indication of possible advantages of incorporation of these extracts. The incorporation of co-extracts has certain advantages, such as richer phytochemical profiles combining the major phenolic compounds from barley hull and flaxseed hull and higher DPPH radical scavenging activity in the CSB. This provides an opportunity to convert non-edible barley hull to value-added functional food ingredients by co-extracting with flaxseed hull. Thus, barley hull and flaxseed hull extracts can be a potential source of functional food ingredients. However, further evidence from animal studies or clinical trials is needed to demonstrate the safety and the potential health benefits of the barley hull and flaxseed hull extracts for food application. In addition, their effects on bread quality also have to be studied in the future.

Chapter 6: General Discussion, Conclusions and Future Research Opportunities

6.1 Discussion

The hull (outer layer) of cereal grain is generally rich in antioxidants likely protecting the seed from oxidative stresses such as those from ultra violet light and heat (Lee et al., 2003). Abundant amount of ferulic acid and p-coumaric acid were found in barley hull, with minor content of vanillic acid and vanillin. Besides the phenolic acid monomers, four ferulic acid dimers were detected in 4 varieties of barley hull tested in this research. However, there is no consistent data in the literature about the phenolic compounds in barley hull. Garrote et al. (2008) reported that gallic acid accounted for 70% of the identified compounds, which were benzoic acid, ferulic acid, coumaric acid, vanillic acid, vanillin, 3,4-dihydroxybenzaldehyde, and 4-hydroxybenzaldehyde. However, other studies reported ferulic acid and p-coumaric acid as the predominant constituents in barley hull (Cruz et al., 2007). Compared to the values reported for ferulic acid (359-624 μg/g) and p-coumaric acid (79-260 μg/g), ferulic acid and p-coumaric acid content found in this study were about 5 times higher than that, which is consistent with the result from brewer's spent grain (Hernanz et al., 2001), a residue remaining after extraction of wort in brewing industry.

Likewise, lignan formation takes place in the outer layer of the seed (Hano et al., 2006), acting as a defensive substance in plants (Gang et al., 1997) and making flaxseed hull the richest source of lignans. The lignan pinoresinol is formed when the plant is wounded and is therefore, toxic to microorganisms (Shahidi and Marian, 2004). Dinkova-Kostova et al. (1996) also reported that lignans play a protective role in plant growth and

in defense against predators owing to their antifungal and insecticidal properties. Lignans in flaxseed have been studied to a great extent; however, this is the first time reporting the amount of SDG, FeAG, and CouAG in flaxseed hull from direct alkaline hydrolysis. Struijs et al. (2009) once reported that SDG contributes 62% to the lignan macromolecule, while CouAG, FeAG, and HDG contribute 12.2, 9.0, and 5.7% by weight respectively. However, the extraction method is different from the method used in this study, and this weight percentage reported does not represent the extract content of those compounds in flaxseed hull as a whole. Furthermore, purification of FeAG and CouAG as standards is important for accurate quantification of FeAG and CouAG in the sample.

Challenging is the fact that the content and composition of barley hull and flaxseed hull may be significantly affected by the cultivar, year of harvest and growing location, the type of seed processing and types of analytical methods used (Westcott and Muir, 1996).

In chemical assays, flaxseed hull extract (F) as well as the co-extract containing barley and flax hulls had about 3 times higher TPC, and 4 times higher DPPH radical scavenging activity than the barley hull extract. The co-extracts reached the same level of TPC and DPPH radical scavenging activity with individual flaxseed hull extracts. However, barley hull extracts exhibited stronger intracellular antioxidant activity and antiproliferative activity than that of flaxseed hull extracts and their co-extracts at the same level of treatment concentration in PC-3 human prostate cancer cells. These results indicate that the anoxidant activity of barley hull and flaxseed hull extracts as well as their co-extracts is significantly correlated with their phenolic content, however, the

chemical assay can not truly reflect the intracellular antioxidant capacity due to differences in antioxidant solubility/bioavailability as well as metabolism involved in the cell culture.

Incorporation of barley hull and flaxseed hull extracts as well as their co-extract into the CSB significantly enriched the phytochemical profile, and increased the antioxidant activity as a result. HPLC-MS/MS analysis demonstrated that the phenolic compounds from the extract were not altered chemically after incorporating into CSB and processing, implying they retained their antioxidant activity after processing. However, DPPH and ORAC showed slight variations between the results. Compared to the control bread, flaxseed hull extracts introduced new phenolic compounds such as SDG, FeAG, and CouAG into CSB, while barley hull extracts enhanced the content of ferulic acid and p-coumaric acid. The incorporation of co-extracts resulted in a much lighter color effect with enhanced DPPH radical scavenging activity comparable to that of flaxseed hull extract, and higher enhancement in ORAC values than when flaxseed hull extract was incorporated (Appendix C) As known in the literature review, the various methods essentially measuring antioxidant activity of the samples may cause different results due to the use of unique radicals, involvement of several mechanisms, as well as extraction solvents, preparation methods and experimental conditions.

6.2 Conclusion

In conclusion, barley hull and flaxseed hull extracts as well as their co-extracts have great potential to be converted into functional food ingredients which can be incorporated into certain food products and enhance their phytochemical profile and increase the antioxidant activity. The co-extracts showed advantages in the chemical assays over the barley hull extracts, however, they did not show synergistic or additive effect in cell culture assays. Chemicals assays have no similarity to biological systems; therefore, they do not measure bioavailability, *in vivo* stability, retention of antioxidants by tissues, and reactivity in situ (Huang et al., 2005). Thus, the intracellular antioxidant activity and antiproliferative activity in PC-3 human prostate cells were evaluated to provide more valid confirmation on the biological effects of these extracts. Barley hull extracts showed the strongest intracellur antioxidant activity and antiproliferative activity in PC-3 cells dose-dependently, followed by flaxseed hull and the co-extracts. Overall, this is the first time reporting the phytochemical profile of the extract of barley hull and flaxseed hull as well as their mixture obtained using direct alkaline hydrolysis. It is also the first time that barley hull extracts have been subjected to both chemical and cell culture assays for their antioxidant activity as well as their anticancer effect. The present research also poses for the first time, the possibility to incorporate the barley hull and flaxseed hull extracts and their co-extracts into CSB to develop novel functional foods which provide multiple health benefits, such as antioxidant activity and anticancer effects.

6.3 Future Research Opportunities

Development of functional food ingredients based on phenolic compounds could provide excellent opportunities for converting these agricultural by-products, such as barley hull and flaxseed hull into value-added functional food ingredients. However, further research is still required in many areas in order to validate the food safety and quality aspects regarding barley hull and flaxseed hull extract as well as their co-extracts as functional food ingredients. Long term health benefits as well as safety issues should be tested using animal studies. The amount of incorporation of these extracts into certain food products has to be optimized without compromising food quality. Therefore, sensory analysis is needed. Also, the mechanism involved in PC-3 human prostate cancer cell antiproliferation has to be investigated to a greater extent including cellular uptake, cell cycle arrest, gene activation or expression.

References

Abdel-Aal, E.-S. M.; Hucl, P. 1999. A rapid method for quantifying total anthocyanins in blue aleurone and purple pericarps wheats. Cereal Chem., 76, 350-354

Abdel-Wahab, M.H., El-Mahdy, M.A., Abd-Ellah, M.F., Helal, G.K., Khalifa, F., Hamada, F.M.A. 2003. Influence of p-coumaric acid on doxorubicin-induced oxidative stress in rat's heart. Pharmacological Research, 48, 461–465

Adolphe, J.L., Whiting, S.J., Juurlink, B.H.J., Thorpe, L.U., Alcorn, J. 2010. Health effects with consumption of the flax lignan secoisolariciresinol diglucoside. British Journal of Nutrition, 103, 929–938

Adom, K. K., Liu, R. H. 2002. Antioxidant activity of grains. J. Agric. Food Chem. 50, 6182 6187

Adom, K. K., Sorrells, M. E., Liu, R. H. 2003. Phytochemical profiles and antioxidant activity of wheat varieties. J. Agric. Food Chem. 51, 7825–7834

Adom, K. K., Sorrells, M. E., Liu, R. H. 2005. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. J. Agric. Food Chem. 53, 2297–2306

Agriculture and Agri-Food Canada. 2010. Canada: Grains and Oilseeds (G&O) Outlook: 2010-11.http://www.agr.gc.ca/pol/mad-dam/index_e.php?s1=pubs&s2=go-co&s3=php&page=go-co_2010-05-05

Alhassane, T., and Xu, X.M. 2010. Flaxseed lignans: source, biosynthesis, metabolism, antioxidant activity, bio-active components, and health benefits. Comprehensive Reviews in Food Science and Food Safety. vol.9, 261-269

Andersson, A.A.M., Lampi, A.M., Nystrom, L., Piironen, V., Li, L., Ward, J.L., Gebruers, K., Courtin, C.M., Delcour, J.A., Boros, D., Fras, A., Dynkowska, W., Rakszegi, M., Bedo, Z., Shewry, P.R., Aman, P. 2008. Phytochemical and Dietary Fiber Components in Barley Varieties in the HEALTHGRAIN Diversity Screen. J. Agric. Food Chem., 56, 9767–9776

Andreasen, M. F., Christensen, L. P., Meyer, A. S., Hansen, Å. 2000. Ferulic acid dehydrodimers in rye (Secale cereale L.). J. Cereal Sci. 31, 303–307

Andreasen, M.F., Landbo, A.K., Christensen, L.P., Hansen, A., Meyer, A.S. 2001. Antioxidant Effects of Phenolic Rye (Secale cereale L.) Extracts, Monomeric Hydroxycinnamates, and Ferulic Acid Dehydrodimers on Human Low-Density LipoproteinsAgric. Food Chem. 49 (8), pp 4090–4096

Baik, B.K., Steven, E., Ullrich, E.S. 2008. Barley for food: Characteristics, improvement, and renewed interest. Journal of Cereal Science. 48, 233–242

Bartolom´e, B., Faulds, C.B., Williamson, G. 1997. Enzymic release of ferulic acid from barley spent grain. J. Cereal Sci. 25, 285–288

Bartolomé, B., Gómez-Cordovés, C., Sancho, A.I., Díez, N., Ferreira, P., Soliveri, J., Copa-Patiño, J.L. 2003. Growth and release of hydroxycinnamic acids from brewer's spent grain by Streptomyces avermitilis CECT 3339. Enzyme Microb. Technol. 32, 140–144.

Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C., and Thomas, M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. J. Zmmunol., 130, 1910-1917

Baxter, E. D., O'Farrell, D. D. 1987. Malting and brewing with barleys having blue aleurones. J. Inst. Brew., 93, 308-312

Beejmohun, V., Fliniaux, O ., Grand, E., Lamblin, F., Bensaddek L., Christen, P., Kovensky, J., Fliniaux, M.A., Mesnard, F. 2007. Microwave-assisted Extraction of the Main Phenolic Compounds in Flaxseed. Phytochem. Anal., 18, 275–282

Bellido, G., and Beta, T. 2009. Anthocyanins and ORAC values in pearled and milled purple, black and common barley. Journal of Agricultural and Food Chemistry, 57 (3), 1022–1028

Bendelow, V.M., LaBerge, D.E. 1979. Relationship among barley, malt and beer phenolics. Journal of the American Society of Brewing Chemists. 37, 89–90

Berridge, M.V. and Tan, A.S. 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch. Biochem. Biophys. 303, 474–82.

Beta, T., Nam, S., Dexter, J.E., Sapirstein, H.D. 2005. Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. Cereal Chemistry, 82 (4), 390-393

Bhatty, R.S. 1993. Nonmalting uses of barley. In: MacGregor, A.W., Bhatty, R.S. (Eds.), Barley: Chemistry and Technology. American Association of Cereal Chemists, St. Paul, MN, pp. 355–417

Bhatty, R.S. 1995. Nutrient composition of whole flaxseed and flaxseed meal. Pages 56-58 in Cunnane, S. and Thompson, L., (eds). Flaxseed in human nutrition. AOAC Press. Champain, IL.

Boccardo, F., Lunardi, G., Guglielmini, P., Parodi, M., Murialdo, R., Schettini, G., Rubagotti, A. 2004. Serum enterolactone levels and the risk of breast cancer in women with palpable cysts. Eur. J. Cancer 40, 84–89

Bommareddy, A., Zhang, X.Y., Schrader, D., Kaushik, R.S., Zeman, D., Matthees, D.P. and Dwivedi, C. 2009. Effects of Dietary Flaxseed on Intestinal Tumorigenesis in Apc^{Min} Mouse. Nutrition and Cancer. 61, 2,276-283

Bonoli, M., Marconi, E., Caboni, M. F. 2004. Free and bound phenolic compounds in barley (*Hordeum Vulgare L*) flours. Evaluation of extraction capability of different solvent mixtures and pressurized liquid methods by micellar electrokinetic chromatographiy and spectrophotometry. J. Chromatogr. A , 1057, 1–12

Boon, H.S., Olatunde, F., and Zick, S.M. 2007. Trends in complementary/alternative medicine use by breast cancer survivors: Comparing survey data from 1998 and 2005. BMC Women's Health,7:4

Brand-Williams, W., Cuvelier, M.E. and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. Lebensmittel Wissenschaft und Technologie, 28, 25–30

Brannan, R. G. 2008. Effect of grape seed extract on physicochemical properties of ground, salted chicken thigh meat during refrigerated storage at different relative humidity levels. Food Chemistry. 73, 36–38

Britt, C., Gomma, E. A., Gray, J. I., & Booren, A. M. 1998. Influence of cherry tissue on lipid oxidation and heterocyclic aromatic amine formation in ground beef patties. Journal of Agricultural and Food Chemistry. 46, 4891–4897

Bylund, A., Saarinen, N., Zhang, J.X., Bergh, A., Widmark, A., Johansson, A., Lundin, E., Adlercreutz, H., Hallmans, G., Stattin, P., Makela, S. 2005. Anticancer effects of a plant lignan 7-hydroxymatairesinol on a prostate cancer model in vivo. Exp Biol Med. 230, 217–23

Cacace, J.E., Mazza, G. 2006. Pressurized low polarity water extraction of lignans from whole flaxseed. Journal of Food Engineering, 77, 1087–1095

Cadenas, E. and Packer, L. 2001. Handbook of Antioxidant. Second edition, CRC Press. pp.5

Cao, G., Verdon, C., Wu, A., Wang, H., Prior, R.L. 1995. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. Clin. Chem. 41, 1738–1744

Caragay, A.B. 1992. Cancer-Preventive Foods and Ingredients. Food Technol. 46(4), 65-68

Carter, J. F. 1993. Potential of flaxseed and flaxseed oil in baked goods and other products in human nutrition. Cereal Foods World. 38, 753–9

Chang, C. J., Chiu, J. H., Tseng, L. M., Chang, C. H., Chien, T. M., Wu, C. W. 2006. Modulation of HER2 expression by ferulic acid on human breast cancer MCF7 cells. European Journal of Clinical Investigation, 36, 588–596

Charlet, S., Bensaddek, L., Raynaud, S., Gillet, F., Mesnard, F., Fliniaux, M. A. 2002. An HPLC procedure for the quantification of anhydrosecoisolariciresinol. Application to the evaluation of flax lignan content. Plant Physiol. Biochem. 40, 225–229

Chen, J.M., Stavro, P.M., Thompson, L.U. 2002. Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. Nutr. Cancer 43, 187–192

Chen, J.M., Thompson, L.U., 2003. Lignans and tamoxifen, alone or in combination, reduce human breast cancer cell adhesion, invasion and migration in vitro. Breast Cancer Res. Treat. 80, 163–170

Chen, L.H., Fang, J., Li, H., Demark-Wahnefried, W., Lin, X. 2007. Enterolactone induces apoptosis in human prostate carcinoma LNCaP cells via a mitochondrial-mediated, caspase-dependent pathway. Mol Cancer Ther., 6, 2581–2590

Chen, L.H., Fang, J., Sun, Z.J., Li, H.X., Wu,Y., Demark-Wahnefried, W., and Xu Lin, X. 2009. Enterolactone Inhibits Insulin-Like Growth Factor-1 Receptor Signaling in Human Prostatic Carcinoma PC-3 Cells. The Journal of Nutrition, 653-659

Chu Y.F., Sun, J., Wu,X.Z., and Liu, R.H. 2002. Antioxidant and Antiproliferative Activities of Common Vegetables. J. Agric. Food Chem. 50, 6910-6916

Clark, W.F., Kortas, C., Heidenheim, P., Garland, J., Spanner, E., Parbtani, A. 2001. Flaxseed in Lupus Nephritis: A two-year nonplacebo-controlled crossover study. Journal of the American College of Nutrition 20(2), 143-148

Clifford, M. N. 1999. Chlorogenic acids and other cinnamates - nature, occurrence, and dietary burden. J. Sci. Food Agric. 79, 362-372

Conde, E., Moure, A.,Dominguez, H., and Parajo, J.C. 2008. Fractionation of Antioxidants from Autohydrolysis of Barley Husks. J. Agric. Food Chem., 56, 10651–10659

Cory, A.H., Owen, T.C., Barltrop, J.A., Cory, J.G. 1991. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun. 3, 207–12.

Cruz, J.M., Domíınguez, J.M., Domínguez, H., Parajó, J.C. 2001. Antioxidant and antimicrobial effects of extracts from hydrolysates of lignocellulosic materials. J. Agric. Food Chem., 49, 2459–2464.

Cruz, J.M., Dom ńguez, J.M., Dom ńguez, H. & Parajó, J.C. 2000. Preparation of fermentation media from agricultural wastes and their bioconversion into xylitol. Food Biotechnology. 14 (1-2), 79-97

Cruz, J.M., Moldes, B.A., Bustos, G., Torrado, A., and Domíınguez, J.M. 2007. Integral utilisation of barley husk for the production of food additives. J Sci Food Agric. 87, 1000–1008

Czuchajowska, Z., Klamczynski, A., Paszczynska, B., Baik, B. K. 1998. Structure and functionality of barley starches. Cereal Chemistry. 75, 747–754

Dabrosin, C., Chen, J.M., Wang, L., Thompson, L.U. 2002. Flaxseed inhibits metastasis and decreases extracellular vascular endothelial growth factor in human breast cancer xenografts. Cancer Letters, 185 (1), 31-37

Dabrowski, K.J., Sosulski, F.W. 1984. Composition of free and hydrolysable phenolic acids in defatted flours of 10 oilseeds. J. Agric. Food Chem. 32, 128–130

Devatkal, S.K., Narsaiah, K., Borah, A. 2010. Anti-oxidant effect of extracts of kinnow rind, pomegranate rind and seed powders in cooked goat meat patties. Meat Science, 85, 155–159

Dinis, T.C., Santosa, C.L., & Almeida, L. M. 2002. The apoprotein is the preferential target for peroxynitrite-induced LDL damage protection by dietary phenolic acids. Free Radical Research, 36, 531–543

Dinkova-Kostova, A.T., Gang, D.R., Davin, L.B., Bedgar, D.L., Chu, A., Lewis, N.G. 1996. (+)-Pinoresinol/(+)-lariciresinol reductase from Forsythia intermedia - Protein purification, cDNA cloning, heterologous expression and comparison to isoflavone reductase. The Journal of Biological Chemistry, 271, 29473-29482

Dong, M., He, X.J., Liu, R.H. 2007. Phytochemicals of black bean seed coats: Isolation, structure elucidation, and their antiproliferative and antioxidative activities. J. Agric. Food Chem., 55, 6044-6051

Dvorakova, M., Moreira , M.M., Dostalek , P, Skulilova, Z., Guido, F.L., Barros, A.A. 2008. Characterization of monomeric and oligomeric flavan-3-ols from barley and malt by liquid chromatography–ultraviolet detection–electrospray ionization mass spectrometry. Journal of Chromatography. A, 1189, 398–405

Eliasson, C., Kamal-Eldin, A., Andersson, R., Åman, P. 2003. High performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction. J Chromatogr. A, 1012, 151–159.

Fan, L., Zhang, S., Yu, L. and Ma, L. 2007. Evaluation of antioxidant property and quality of breads containing Auricularia Auricula polysaccharide flour. Food Chemistry. 101, 1158–1163.

FAO. 2007. http//:www.fao.org.

Fardet, A., Rock, E., Rémésya,C. 2008. Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? Journal of Cereal Science. 48, 258-276

Fastnaught, C.E. 2001. Barley fibre. In: Cho, S., Dreher, M. (Eds.), Handbook of Dietary Fibre. Marcel Dekker, New York, pp. 519–542

Fastnaught, C.E., Berglund, P.T., Holm, E.T., Fox, G.J. 1996. Genetic and environmental variation in β-glucan content and quality parameters of barley for food. Crop Science. 36, 941–946

Fernandez-Lopez, J., Fernandez-Gines, J. M., Aleson-Carbonell, L., Sendra, E., Sayas-Barbera, E., & Perez-Alvarez, J. A. 2004. Application of functional citrus by products to meat products. Trends in Food Science and Technology. 15, 176–185

Ferrari, C. K. and Torres, E. A. 2003. Biochemical pharmacology of functional foods and prevention of chronic diseases of aging. Biomed Pharmacother. 57(5-6), 251-60

Filipe, P., Lanca, V., Silva, J. N., Morliere, P., Santus, R., Fernandes, A. 2001. Flavonoids and urate antioxidant interplay in plasma oxidative stress. Mol. Cell. Biochem. 221, 79–87

Flax Canada. 2007. Flax Heart Health - an April 2007 supplement to Prepared Foods magazine .

http://www.flaxcouncil.ca/english/pdf/Flax%20Supplement%20Heart%20Health.pdf

Folin, O., Ciocalteu, V. 1927. Tyrosine and tryptophan determinations proteins J. Biol. Chem., 73, 627

Gang, D.R., Fujita, M., Davin, L.B., Lewis, N.G. 1997. Lignans in cancer prevention/treatment and in plant defense. Plant Physiology, 114 (3), S1181-S1181

Gao, L., Wang, S., Oomah, B.D., and Mazza, G. 2002. Wheat quality: antioxidant activity of wheat millstreams. In: P. Ng and C.W. Wrigley, Editors, Wheat quality elucidation, AACC International, St. Paul, MN, 219–233

Garcia-Conesa, M. T.; Plumb, G. W.; Waldron, K. W.; Ralph, J.; Williamson, G. 1997. Ferulic acid dehydrodimers from wheat bran: Isolation, purification and antioxidant properties of 8-O-4′ diferulic acid. Redox Rep. 3, 319–323

Garrote, G., Cruz, J.M., Domínguez, H., Parajó, J.C. 2008. Non-isothermal autohydrolysis of barley husks: Product distribution and antioxidant activity of ethyl acetate soluble fractions. Journal of Food Engineering. 84,544–552

Garrote, G., Domínguez, H., & Parajó, J. C. 2004. Production of substituted oligosaccharides by hydrolytic processing of barley husks. Industrial and Engineering Chemistry Research. 43, 1608–1614

Goupy, P., Hugues, M., Boivin, P., Amoit, M. J. 1999. Antioxidant composition and activity of barley (Hordeum Vulgare) and malt extracts and of isolated phenolic compounds. J. Sci. Food Agric. 79, 1625-1634

Graf, E. 1992. Antioxidant potential of ferulic acid. Free Radic Biol Med. 13(4), 435–448

Guglielmi, F., Luceri, C., Giovannelli, L., Dolara, P., Lodovici, M. 2003. Effect of 4-coumaric and 3, 4-dihydroxybenzoic acid on oxidative DNA damage in rat colonic mucosa. Br. J. Nutr. 89, 581–587

Hail, N., Lotan,R. 2009. Cancer chemoprevention and mitochondria: Targeting apoptosis in transformed cells via the disruption of mitochondrial bioenergetics/redox state. Molecular Nutrition & Food Research. Volume: 53 Issue: 1 Pages: 49-67

Hall, A.V., Parbtani, A., Clark, W.F., Spanner, E., Keeney, M., Chin-Yee, I., Philbrick, D.J. & Holub, B.J. 1993. Abrogation of MRL/lpr lupus nephritis by dietary flaxseed. American Journal of Kidney Diseases. 22(2), 326-332

Hall, C.A. III, Manthey, F.A., Lee, R.E. and Niehaus, M. 2005. Stability of a-linolenic acid and secoisolariciresinol diglucoside in flaxseed-fortified macaroni. J. Food Chem. 70, C483–C489

Hall, C.A., Manthey, F.A., Lee, R.E., Niehaus, M. 2005. Stability of alpha-linolenic acid and secoisolariciresinol diglucoside in flaxseed-fortified macaroni. Journal of food science. 70 (8), C483-C489

Halliwell, B. 2007. Oxidative stress and cancer: Have we moved forward? Biochem. J. 2007, 401, 1–11

Hallund, J., Ravn-Haren, G., Bũgel, S., Tholstrup, T., Tetens, I. 2006. A lignan complex isolated from flaxseed does not affect plasma lipid concentrations or antioxidant capacity in healthy postmenopausal women. J. Nutr. 136, 112–116

Hano, C., Martin, I., Fliniaux, O., Legrand, B., Gutierrez, L., Arroo, R.R.J., Mesnard, F., Lamblin, F., Laine, E. 2006. Pinoresinol–lariciresinol reductase gene expression

and secoisolariciresinol diglucoside accumulation in developing flax (Linum usitatissimum) seeds. Planta 224, 1291–1301

Hassona, H.Z. 1993. High fibre bread containing brewer's spent grains and its effect on lipid metabolism in rats. Die Nahrung. 37, 576–582

He, X., Liu, R.H. 2008. Phytochemicals of Apple Peels: Isolation, Structure Elucidation, and Their Antiproliferative and Antioxidant Activities. J. Agric. Food Chem., 56, 9905–9910

He, X.J., Liu, R.H. 2006. Cranberry phytochemicals: Isolation, structure elucidation, and their antiproliferative and antioxidant activities. J. Agric. Food Chem., 54, 7069-7074

Health Canada, 1998. Nutraceuticals/Functional Foods AND Health Claims on Foods Final Policy. Therapeutic products programme and the food directorate of the health protection branche. http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/label-etiquet/nutra-funct_foods-nutra-fonct_aliment-eng.pdf

Hedelin, M., Klint, A., Chang, E.T., Bellocco, R., Johansson, J.E., Andersson, S.O., Heinonen, S.M., Adlercreutz, H., Adami, H.O. Gronberg, H., Balter, K.A. 2006. Dietary phytoestrogen, serum enterolactone and risk of prostate cancer: the cancer prostate Sweden study (Sweden). Cancer Causes Control. 17, 169–80

Hernanz, D., Nuez, V., Sancho, A. I., Faulds, C. B., Williamson, G., Bartolome´, B., Go´mez-Cordove´s, C. 2001. Hydrocinnamic acids and ferulic acid dehydrodimers in barley and processed barley. J. Agric. Food Chem., 49, 4884-4888

Higuchi, T., Ito, Y., Shimada, M., and Kawamura, I. 1967. Chemical properties of milled wood lignin grasses. Phytochemistry, 6, 1551–1556

Hileman, E.O., Liu, J.S., Albitar, M., Keating, M.J., Huang, P. 2004. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. Cancer Chemotherapy and Pharmacology Volume: 53 Issue: 3 Pages: 209-219

Ho, C.H.L. 2006. Pressurized low polarity water extraction of lignans, proteins and carbohydrates from flaxseed meal. M.Sc Thesis, University of Manitoba

Hoije, A., Grondahl, M., Tommeraas, K., Gatenholm, P. 2005. Isolation and characterization of physicochemical and material properties of arabinoxylans from barley husks. Carbohydr. Polym. 61, 266–275

Holtekjølen, A.K., Kinitz, C., Knutsen, S.H. 2006. Flavanol and Bound Phenolic Acid Contents in Different Barley Varieties J. Agric. Food Chem. 54, 2253-2260

Honzel, D., Carter, S.G., Redman, K.A., Schauss, A.G., Endres, J.R., Jensen, G.S. 2008. Comparison of chemical and cell-based antioxidant methods for evaluation of foods and natural products: Generating multifaceted data by parallel testing using erythrocytes and polymorphonuclear cells. J. Agric. Food Chem., 56, 8319–8325

Hosseinian, F.S., Muir, A.D., Westcott, N.D., Krol, E.S. 2006. Antioxidant capacity of flaxseed lignans in two model systems. Journal of the American Oil Chemists' Society, 83(10), 835-840

Hosseinian, S.F., Li, W.D., Beta,T. 2008. Measurement of anthocyanins and other phytochemicals in purple wheat. Food Chemistry. 109, 916–924

Hosseinian, S.F., Mazza, G. 2009. Triticale bran and straw: Potential new sources of phenolic acids, proanthocyanidins, and lignans. Journal of Functional Food, S1, 57 –64

Huang, D.J., Ou, B.X., Prior, R.L. 2005.The Chemistry behind Antioxidant Capacity Assays. J. Agric. Food Chem. 53, 1841-1856

Huige, N.J. 1994. Brewery by-products and effluents, in: Hardwick, W.A. (Ed.), Handbook of Brewing. Marcel Dekker, New York, pp. 501–550

IFT. 2005. "Functional Foods: Opportunities and Challenges" (accessed May 9, 2008), [available at http://members.ift.org/ IFT/Research/IFTExpertReports/functionalfoods_report.htm]

Ip, S. P., Poon, M. K. T., Wu, S. S. 1995. Effect of schisandrin B on hepatic glutathione antioxidant system in mice: Protection against carbon tetrachloride toxicity. Planta Med. 61, 398-401

Izydorczyk, M.S., Storsley, J., Labossiere, D., MacGregor, A.W., Rossnagel, B.G. 2000. Variation in total and soluble b-glucan content in hulless barley: effects of thermal, physical, and enzymic treatments. Journal of Agricultural and Food Chemistry, 48, 982–989

Jende-Strid, B. 1988. Analysis of proanthocyanidins and phenolic acids in barley, malt, hops and beer. In Modern Methods of Plant Analysis 7; Linskens, H. F., Jackson, J. F., Eds.; Springer: Berlin, Heidelberg, New York, 7,109-127

Jende-Strid, B. 1993. Genetic control of flavonoid biosynthesis in barley. Hereditas. 119, 187-204

Jensen, G. S. Cell-based antioxidant protection assay; U.S. provisional patent application 60/985,166

Jensen, G.S., Wu, X.L., Patterson, K.M., Barnes, J., Carter, S.G., Scherwitz, L., Beaman, R., Endres, J.R., Schauss, A.G. 2008. In vitro and in vivo antioxidant and anti-inflammatory capacities of an antioxidant-rich fruit and berry juice blend. Results of a pilot and randomized, double-blinded, placebo-controlled, crossover study. J. Agric. Food Chem., 56, 8326–8333

Jiang, Z.Q., Cong, Q.Q., Yan, Q.J., Kumar, N., Du, X.D. 2010. Characterisation of a thermostable xylanase from Chaetomium sp. and its application in Chinese steamed bread. Food Chemistry. 120, 457–462

Jin, Y., Yan, E.Z., Fan, Y., Zong, Z. H., Qi, Z. M., & Li, Z. 2005. Sodium ferulate prevents amyloid-beta-induced neurotoxicity through suppression of p38 MAPK and upregulation of ERK-1/2 and Akt/protein kinase B in rat hippocampus. Acta Pharmacologica Sinica, 26, 943–951

Johnsson, P., Kamal-Eldin, A., Lundgren, L.N., Aman, P. 2000. HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. J. Agric. Food Chem., 48, 5216-5219

Johnsson, P., Peerlkamp, N., Kamal-Eldin, A., Andersson, R. E., Lundgren, L. N., Åman, P. 2002. Polymeric fractions containing phenol glucosides in flaxseed. Food Chem., 76, 207–212

Kamal-Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L.N., Aman, P. 2001. An oligomer from flaxseed composed of

secoisolariciresinoldiglucoside and 3-hydroxy-3-methyl glutaric acid residues. Phytochemistry. 58, 587–590

Kanski, J., Aksenova, M., Stoyanova, A., Butterfield. D.A. 2002. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure–activity studies. Journal of Nutritional Biochemistry. 13, 273–281

Kennefick, S., Cashman, K.D. 2000. Inhibitory effect of wheat fibre extract on calcium absorption in Caco-2 cells: evidence for a role of associated phytate rather than fibre per se. Eur. J. Nutr., 39: 12–17

Kent, N.L., Barley, in Technology of Cereals, 3rd ed., Pergamon Press, Oxford, U.K., 1983, 154

Keston, A.S., Brandt, R. 1965. The fluorometric analysis of ultramicro quantities of hydrogen peroxide. Analytical Biochemistry, 11, 1-5

Kim, H.T., Taylor, F., & Hicks, B.K. 2008. Bioethanol production from barley hull using SAA (soaking in aqueous ammonia) pretreatment. Bioresource Technology. 99, 5694–5702

Kim, K.H., Tsao, R., Yang, R.W.S., & Cui, S.W. 2006. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chemistry, 95, 466–473

Kim, S.; Dale, B. E. 2004. Global Potential Bioethanol Production from Wasted Crops and Crop Residues. Biomass and Bioenergy, 26, 361-375

Kim, S.J., Park, H.R., Park, E., Lee, S.C. 2007. Cytotoxic and Antitumor Activity of Momilactone B from Rice Hulls. J. Agric. Food Chem, 55, 1702-1706

Kitts, D. D., Yuan, Y. V., Wijewicreme, A. N., Thompson, L. U. 1999. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. Mol. Cell. Biochem. 202, 91-100

Klosterman, H.J., Smith, F. 1954. The isolation of beta-hydroxy-betamethylglutaric acid from the seed of flax (Linum usitatissimum). J. Am. Chem. Soc. 76, 1229–1230

Klosterman, H.J., Smith, F., Clagett, C.O.1955. The Constitution of Linocinnamarin. Journal of The American Chemical Society, 77 (2), 420-421

Kong, Q., Beel, J. A., Lillehei, K. O. 2000. A threshold concept for cancer therapy, Med. Hypotheses. 55, 29–35

Kraushofer, T., & Sontag, G. 2002. Determination of some phenolic compounds in flax seed and nettle roots by HPLC with coulometric electrode array detection. European Food Research and Technology, 215 (6), 529–533

LeBel, C. P., Ishiropoulos, H., Bondy, S. C. 1992. Evaluation of the probe 2,7-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231

Lee, S.C., Kim, J.H., Jeong, Kim., D.R., Ha, J.U., Nam., K.C., Ahn, D.U. 2003. Effect of Far-Infrared Radiation on the Antioxidant Activity of Rice Hulls. J. Agric. Food Chem. 51, 4400-4403

Li, W., Pickard, M.D., and Beta, T. 2007. Effect of thermal processing on antioxidant properties of purple wheat bran. Food Chemistry, 104, 1080-1086

Li, W., Shan, F., Sun, S., Corke, H., Beta, T. 2005. Free radical scavenging properties and phenolic content of Chinese black grained wheat. J. Agric. Food Chem. 53, 8533–8536.

Li, X., Yuan, J. P., Xu, S. P., Wang, J. H., & Liu, X. 2008. Separation and determination of secoisolariciresinol diglucoside oligomers and their hydrolysates in the flaxseed extract by high-performance liquid chromatography. Journal of Chromatography. A, 1185(2), 223–232.

Liggins, J., Grimwood, R., Bingham, S.A. 2000. Extraction and quantification of lignan phytoestrogens in food and human samples. Anal.Biochem. 287, 102–109.

Lin, X., Gingrich, J.R., Bao, W., Li, J., Haroon, Z.A., Demark-Wahnefried, W. 2002. Effect of flaxseed supplementation on prostatic carcinoma in transgenic mice. Urology. 60, 919–24

Lin, X., Switzer, B.R., Demark-Wahnefried, W. 2001. Effect of mammalian lignans on the growth of prostate cancer cell lines. Anticancer Res. 21, 3995–9

Liu, M., Li, X.Q., Weber, C., Lee, C.Y., Brown, J., Liu, R.H. 2002. Antioxidant and anti proliferative activities of raspberries. J. Agric. Food Chem., 50, 2926-2930

Liu, R. H. 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. J. Nutr. 134, 3479S–3485S

Liu, R.H. 2003. Health benefits of fruits and vegetables are from additive and synergistic combination of phytochemicals. Am. J. Clin. Nutr. 78, 517S–520S

Liu, R.H., Finley, J. 2005. Potential cell culture models for antioxidant research. J. Agric. Food Chem., 53, 4311-4314

Lodovici, M., Raimondi, L., Guglielmi, F., Gemignani, S., & Dolara, P. 2003. Protection against ultraviolet B-induced oxidative DNA damage in rabbit corneal-derived cells (SIRC) by 4-coumaric acid. Toxicology. 184 (2–3), 141−147

Long, L.H., Clement, M.V. 2000. Halliwell B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)-epigallocatechin gallate, (-)-catechin, and quercetin to commonly used cell culture media. Biochem Biophys Res Commun, 273, 50–53

Loo, G. 2003. Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation (Review). Journal of Nutritional Biochemistry, 14, 64–73

Lucas, E.A., Lightfoot, S.A., Hammond, L.J., Devareddy, L., Khalil, D.A., Daggy, B.P., Smith, B.J., Westcott, N., Mocanu, V., Soung, D.Y., Arjmandj, B.H. 2004. Flaxseed reduces plasma cholesterol and atherosclerotic lesion formation in ovariectomized Golden Syrian hamsters. Artherosclerosis. 173, 223–229

Luceri, C., Giannini, L., Lodovici, M., Antonucci, E., Abbate, R., Masini, E., Dolara, P. 2007. p-Coumaric acid, a common dietary phenol, inhibits platelet activity in vitro and in vivo. Br J Nutr. 97, 458–463

Ma, K., Wang, H.F., Zhao, M., Xing, J.J. 2009. Purity determination and uncertainty evaluation of theophylline by mass balanced method, high performance liquid chromatography and differential scanning calorimetry. Anlytica Chinica Acta. 650, 227-233

MacDonald-Wicks, L.K., Wood, L.G., Garg, M.L. 2006. Methodology for the determination of biological antioxidant capacity in vitro: a review. J Sci Food Agric., 86 (13), 2046-2056

Madhusudhan, B., Wiesenborn, D., Schwarz, J., Tostenson, K., Gillespie, J. 2000. A dry mechanical method for concentrating the lignan secoisolariciresinol diglucoside in flaxseed. Food. Sci. Technol. 33, 268–275

Magalhaes, L.M., Segundo, M.A., Reis, S., Lima, J.L.F.C. 2008. Methodological aspects about in vitro evaluation of antioxidant properties. Anal. Chim. Acta., 613(1), 1-19

Mahmudi, H., Flynn, P.C., Checkel, D.M. 2005. Life cycle analysis of biomass transportation: trains vs. trucks, SAE Technical Papers, Doc. No: 2005-01-1551

Maillard MN, Berset C. 1995. Evolution of antioxidant activity during kilning: role of insoluble bound phenolic acids of barley and malt. J Agric Food Chem. 43,1789–93

Manthey, F. A., Sinha, S., Wolf-Hall,C., Hall, A. C. III. 2008. Effect of Flaxseed Flour and Packaging on Shelf Life of Refregerated Pasta. Journal of Food Processing and Preservation. 32, 75–87

McCann, M.J., Gill, C.I.R., Linton, T., Berrar, D., McGlynn, H., Rowland, I.R. 2008. Enterolactone restricts the proliferation of the LNCaP human prostate cancer cell line in vitro. Mol. Nutr. Food Res. 52, 567 – 580

Meagher, L.P., Beecher, G.R., Flanagan, V.P., Li, B.W. 1999. Isolation and characterization of the lignans, isolariciresinol and pinoresinol, in flaxseed meal. J. Agric. Food Chem. 47, 3173–3180

Mentes, O., Bakkalbasi, E., Ercan, R. 2008. Effect of the Use of Ground Flaxseed on Quality and Chemical Composition of Bread. Food Science and Techonology International, 14 (4), 299-306

Mertens-Talcott, S. U., Jilma-Stohlawetz, P., Rios, J., Hingorani, L., Derendorf, H. 2006. Absorption, metabolism, and antioxidant effects of pomegranate (*Punica granatum L.*) polyphenols after ingestion of a standardized extract in healthy human volunteers. J. Agric. Food Chem., 54, 8956–8961

Meyers, K.J., Watkins, C.B., Pritts, M.P., Liu, R.H. 2003. Antioxidant and Antiproliferative Activities of Strawberries. J. Agric. Food Chem., 51, 6887-6892

Miranda, M.Z., Grossmann, M.V.E., Nabeshima, E.H. 1994a. Utilization of brewers' spent grain for the production of snacks with fiber. 1. Physicochemical characteristics. Brazilian Archives of Biology and Technology 37, 483–493

Miranda, M.Z., Grossmann, M.V.E., Prudencioferreira, S.H., Nabeshima, E.H. 1994b. Utilization of brewer spent grain (BSG) for production of snacks with fiber. 2. Sensory analysis of snacks. Brazilian Archives of Biology and Technology, 37, 9–21

Mitchell, J.H. 2001. Phytoestrogen: involment in breast and prostate cancer. Pages 99-112 in : Handbook of nutraceuticals and functionalf foods. Wildman, R.E.C. (ed). CRC Press, Boca Raton, FL.

Moldes, B.A., Cruz, M.J., Dom ńgues, M.J., & Paraj ó,C. 2002. Production of a cellulosic substrate susceptible to enzymatic hydrolysis from prehydrolyzed barley husks. Agriculture and Food Science in Finland., 11, 51-58

Mollet, B., & Rowland, I. 2002. Functional foods: At the frontier between food and pharma. Current Opinion in Biotechnology. 13, 483–485

Mpofu, A., Sapirstein, H.D., Beta, T. 2006. Genotype and Environmental Variation in Phenolic Content, Phenolic Acid Composition, and Antioxidant Activity of Hard Spring Wheat. J. Agric. Food Chem., 54, 1265-1270

Muir, A.D., Westcott, N.D. 2000. Quantitation of the lignan secoisolariciresinol diglucoside in baked goods containing flax seed or flax meal. J. Agric. Food Chem. Volume: 48, Issue: 9, Pages: 4048-4052

Murakami, A., Nakamura, Y., Koshimizu, K., Takahashi, D., Matsumoto, K., Hagihara, K. 2002. FA15, a hydrophobic derivative of ferulic acid, suppresses inflammatory responses and skin tumor promotion: Comparison with ferulic acid. Cancer Letters, 180, 121–129

Mussatto, S.I., Dragone, G. & Roberto, I.C. 2006. Brewers' spent grain: generation, characteristics and potential applications. J Cereal Sci. 43, 1–14

Mussatto, S.I., Dragone, G. & Roberto, I.C. 2007. Ferulic and pcoumaric acids extraction by alkaline hydrolysis of brewer's spent grain. Ind Crops Prod. 25, 231–237

Nesbitt, P., Thompson, L.U. 1997. Lignans in homemade and commercial products containing flaxseed. Nutr. Cancer 29:222–227

Nordkvist, E., Salomonsson, A. C., Åman, P. 1984. Distribution of insoluble bound phenolic acids in barley grain. J. Sci. Food Agric., 35, 657-661

Özturk, S., Ö zboy, Ö., Cavidoglu, I., Köksel, H., 2002. Effects of brewers' spent grain on the quality and dietary fibre content of cookies. Journal of the Institute of Brewing 108, 23–27

Oates, J. 1979. Babylon. Thames and Hudson Ltd., London. ISBN 0-500-020957.

Oliver, M. H., Harrison, N. K., Bishop, J. E., Cole, P. J., Laurent, G. J. 1989. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J. Cell Sci. 92 (Part 3), 513–518

Oomah, B.D., Berekoff, B., Li-Chan, E.C.Y., Mazza, G., Kenaschuk, E. O., and Duguid, S.D. 2007. Cadmium-binding protein components of flaxseed: Influence of cultivar and location. Food Chemistry, 100, 318-325

Oomah, B.D., Kenaschuk, O.E., and Mazza. G. 1995. Phenolic Acids in Flaxseed J. Agric. Food Chem. 43 (8), 2016-2019

Oomah BD, Mazza G, Kenaschuk EO. 1996. Dehulling characteristics of flaxseed. Food Sci. Techonol., 29 (3), 245-250

Oomah, B.D., Mazza,G. 1997. Effect of dehulling on chemical composition and physical properties of flaxseed. Lebensm.-Wiss.u.-Technol., 30, 135-140

Oomah, B. D., Mazza, G. 1998a. Fractionation of flaxseed with a batch dehuller. Industrial Crops and Products, 9 (1), 19–27

Oomah, B.D., Mazza, G. 1998b. Flaxseed products for disease prevention. Pages 91-138 in Functional foods: biochemcial % processing aspects. Mazza, G. (Ed), Technolic Publishing Co, Inc. Lancaster, Pennsylvania.

Oomah, B.D., Sitter, L. 2009. Characteristics of flaxseed hull oil. Food Chemistry, 114 (2), 623-628

Ou, B., Woodill-Hampsch, M., Prior, R.L. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem. 49, 4619-4626

Ozcelik, B., Lee, J.H., Min, D.B. 2003. Effects of light, oxygen, and pH on the absorbance of 2,2-diphenyl-1-picrylhydrazyl (DPPH). J. Food Sci., 68 (2), 487-490

Özvural, E.B., Vural, H., Gökbulut, İ., Özbaş, Ö. Ö. 2009. Utilization of brewer's spent grain in the production of Frankfurters, International Journal of Food Science and Technology, 44, 1093–1099

Palmer, G.H. and Bathgate, G.N. 1976. Malting and brewing. Adv Cereal Sci Technol. 1:237–324

Palozza, P., Krinsky, N. I. 1992. β-Carotene and R-tocopherol are synergistic antioxidants. Arch. Biochem. Biophys., 297, 184–187

Panfili, G., Fratianni, A., Cruscio,D.T., Marconi, E. 2008. Tocol and β-lgucan levels in barley varieties and in pearling by-products. Food Chemistry 107, 84-91

Paraj ó, J.C., Dom ínguez, H. & Dom ínguez, J.M. 1997. Improved xylitol production with Debaryomyces hansenii Y-7426 from raw or detoxified wood hydrolyzate. Enzyme Microbiology Technology. 21, 18-24

Peng , X.F., Ma,J.Y., Cheng, K.W., Jiang, Y., Chen, F., Wang, M.F. 2010. The effects of grape seed extract fortification on the antioxidant activity and quality attributes of bread. Food Chemistry. 119, 49–53

Penumathsa, S.V., Koneru, S., Thirunavukkarasu, M., Zhan, L.J., Prasad, K., Maulik, N. 2007. Secoisolariciresinol diglucoside: relevance to angiogenesis and

cardioprotection against ischemia–reperfusion injury. J Pharmacol Exp Ther. 320, 951–959

Pohjanheimo, T.A., Hakala, M.A., Tahvonen, R.L., Salminen, S.J. and Kallio, H.P. 2006. Flaxseed in breadmaking: Effects on sensory quality, aging, and composition of bakery products. Journal of Food Science, 71, S343–348.

Power, K.A., Ward, Wendy E., Chen, Jian Min, Saarinen, Niina M. and Thompson, L. U. 2007. Flaxseed and Soy Protein Isolate, Alone and in Combination, Differ in their Effect on Bone Mass, Biomechanical Strength, and Uterus in Ovariectomized Nude Mice with MCF-7 Human Breast Tumor Xenografts, Journal of Toxicology and Environmental Health. Part A, 70, (22), 1888-1896

Prasad, K. 1997. Hydroxyl radical-scavenging property of secoisolariciresinol diglucoside (SDG) isolated from flaxseed. Mol Cell Biochem., 168, 117–123

Prasad, K. 1999. Reduction of serum cholesterol and hypercholesterolemic atherosclerosis in rabbits by secoisolariciresinol diglucoside (SDG) isolated from flaxseed. Circulation, 99, 1355–1362

Prasad, K. 2000. Oxidative stress as a mechanism of diabetes in diabetic BB prone rats: Effect of secoisolariciresinol diglucoside (SDG). Molecular and Cellular Biochemistry, 209, 89-96

Prasad, K. 2005. Effect of chronic administration of lignan complex isolated from flaxseed on the hemopoietic system. Molecular and Cellular Biochemistry, 270, 139–145

Prior, R.L., Wu, X., and Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements, J Agric Food Chem., 53, 4290–4302

Qiu, S.X., Lu, Z.Z., Luyengi, L., Lee, S.K., Pezzuto, J.M., Farnsworth, N.R., Thompson, L.U., Fong, H.H.S. 1999. Isolation and characterization of flaxseed (*Linum usitatissimum*) constituents. Pharm. Biol., 37, 1–7

Qiu, Y., Liu, Q., Beta, T. 2009. Antioxidant properties of commercial wild rice and analysis of soluble and insoluble phenolic acids. J Agric Food Chem., 57 (16)7543-7551

Qiu, Y., Liu, Q., Beta, T. 2010. Antioxidant properties of commercial wild rice and analysis of soluble and insoluble phenolic acids. Food Chemistry. 121 (1), 140-147

Qu, H.Y., Madl, R.L., Takemoto, D.J., Baybutt, R.C., Wang, W.Q. 2005. Lignans are involved in the antitumor activity of wheat bran in colon cancer SW480 cells. J. Nutr., 135, 598-602

Quinde-Axtell, Z., Baik, B.K. 2006. Phenolic compounds of barley grain and their implication in food product discoloration. J. Agric. Food Chem., 54 (26), 9978-9984

Rajesha, J., Murthy, K.N.C., Kumar, M.K., Madhusudhan, B. & Ravishankar, A.G. 2006. Antioxidant potentials of flaxseed by in vivo model. J. Agric. Food Chem., 54, 3794-3799

Ramarathnam, N., Osawa, T., Namiki, M., Kawakishi, S. 1988. Chemical Studies on Novel Rice Hull Antioxidant. 1. Isolation, Fractionation, and Partial Characterization. J. Agric. Food Chem., 36 (4), 732-737

Ramcharitar, A., Badrie, N., Mattfeldt-Beman, M., Matsuo, H., and Ridley, C. 2005. Consumer Acceptability of Muffins with Flaxseed (Linum usitatissimum). Journal of Food Science, 70 (7) 504-507

Ratnam, D. V., Ankola, D. D., Bhardwaj, V., Sahana, D. K., Ravi Kumar, M. N. V. 2006. Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective. J. Controlled Release, 113, 189-207

Renouard, S., Hano, C., Corbin, C., Fliniaux, O., Lopez, T., Montguillon, J., Barakzoy, E., Mesnard, F., Lamblin, F., Laine, E. 2010. Cellulase-assisted release of secoisolariciresinol from extracts of flax (Linum usitatissimum) hulls and whole seeds. Food Chemistry, 122, 679–687

Riss, T.L. and Moravec, R.A. 1992. Comparison of MTT, XTT, and a novel tetrazolium compound for MTS for in vitro proliferation and chemosensitivity assays. Mol. Biol. Cell (Suppl.), 3, 184a

Rojas, M. C., & Brewer, M. S. 2008. Effect of natural antioxidants on oxidative stability of frozen vacuum packaged beef and pork. Journal of Food Quality. 31,173–185

Rosling, H. 1993. Cyanide exposure from linseed. The Lancet, 341, 177

Rossetto, M., Vanzani, P., Mattivi, F., Lunelli, M., Scarpa, M., Rigo, A. 2002. Synergistic antioxidant effect of catechin and malvidin 3-glucoside on free radical-initiated peroxidation of linoleic acid in micelles. Arch. Biochem. Biophys., 408, 239–245

Saarinen, M.N., Power, K., Chen,J.M., and Thompson.L.U. 2006. Flaxseed attenuates the tumor growth stimulating effect of soy protein in ovariectomized athymic mice with MCF-7 human breast cancer xenografts Int. J. Cancer, 119, 925–931

Saarinen, M.N., Power, K.A., Chen, J.M., and Thompson, L.U. 2008a. Lignans are Accessible to Human Breast Cancer Xenografts in Athymic Mice. Nutrition and Cancer, 60 (2), 245-250

Saarinen, M.N., Wärri,A., Dings, R.P.M., Airio, M., Smeds, A.I., and Mäkelä S. 2008b. Dietary lariciresinol attenuates mammary tumor growth and reduces blood vessel density in human MCF-7 breast cancer xenografts and carcinogen-induced mammary tumors in rats. Int. J. Cancer, 123, 1196–1204

Saggar, J.K.; Chen, J.M.; Corey, P., Thompson, L.U. 2010a. Dietary flaxseed lignan or oil combined with tamoxifen treatment affects MCF-7 tumor growth through estrogen receptor- and growth factor-signaling pathways. Molecular nutrition & food research, 54 (3), 415-425

Saggar, J.K., Chen, J.M., Corey, P., Thompson, L.U. 2010b. The Effect of Secoisolariciresinol Diglucoside and Flaxseed Oil, Alone and in Combination, on MCF-7 Tumor Growth and Signaling Pathways. Nutrition and Cancer, 62 (4), 533-542

Salomonsson, A.C., Theander, O., Aman, P. 1980. Composition of normal and high-lysine barley. Swed J Agric Res 10:11–16

Sanchez-Moreno, C. 2002. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food science and technology international, 8 (3), 121-137

Scarpa, M., Rigo, A., Maiorino, M., Ursini, F., Gregolin, C. 1984. Formation of R-tocopherol radical and recycling of R-tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes: an electron paramagnetic resonance study. Biochim. Biophys. Acta., 801, 215–219

Schwald, W., Brownell, H.H., Saddler, J. 1988. Enzymatic hydrolysis of steam treated aspen wood: Influence of partial hemicellulose and lignin removal prior to pre-treatment. J. Wood Chem. Technol., 8 (4), 543-560

Searcyl, E., Flynn, P., Ghafoori, E., Kumar, A. 2007. The relative cost of biomass energy transport. Appl. Biochem. Biotech., 137–140 (1), 639-652

Serraino, M., Thompson, L. U. 1991. The effect of flaxseed supplementation on early risk markers for mammary carcinogenesis. Cancer Lett., 60, 135-42

Serraino, M., Thompson, L.U. 1992a. The Effect of Flaxseed Supplementation on the Initiation and Promotional Stages of Mammary Tumorigenesis. Nutrition and Cancer-an Iinternational Journal, 17(2), 153-159

Serraino, M.; Thompson, L. U.1992b. Flaxseed supplementation and early markers of colon carcinogenesis. Cancer Lett., 63, 159-165

Shahidi, F., and Naczk, M. 2004. Phenolics in food and nutraceuticals .chapter 1. Biosynthesis, classification, and nomenclature of phenolics in food and nutraceuticals. pp:10

Sicilia, T., Niemeyer, H.B., Honig, D.M., Metzler, M., 2003. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. J. Agric. Food Chem., 51, 1181–1188

Siebenhandl, S., Grausgruber, H., Pellegrini, N., Del Rio, D., Fogliano, V., Pernice, R., Berghofer, E. 2007. Phytochemical profile of main antioxidants in different fractions of purple and blue wheat, and black barley. J. Agric. Food Chem., 55 (21), 8541–8547

Singh, S., and Singh, R. P. 2008. In Vitro Methods of Assay of Antioxidants: An Overview, Food Reviews International, 24(4), 392-415

Siro, I., Kapolna, E., Kapolna, B., Lugasi, A. 2008. Functional food. Product development, marketing and consumer acceptance-A review. Appetite, 51, 456–467

Soman, G., Yang, X.Y., Jiang, H.G., Giardina, S., Vyas, V., Mitra, G., Yovandich, J., Creekmore, S.P., Waldmann, T.A., Quinones, O., Alvord, W.G. 2009. MTS dye based colorimetric CTLL-2 cell proliferation assay for product release and stability monitoring of Interleukin-15: Assay qualification, standardization and statistical analysis Journal of Immunological Methods, 348, 83–94

Song, W., Derito, C.M., Liu, M.K., He, X.J., Dong, M., Liu, R.H. 2010. Cellular Antioxidant Activity of Common Vegetables. Journal of Agricultural and Food Chemistry, 58(11), 6621-6629

Soobrattee, M.A., Neergheen, V.S., Luximon-Ramman, A., Aruoma, O.I., and Bahorun, T. 2005. Phenolics as potential antioxidant therapeutic agents : Mechanism and actions. Mutat. Res., 579, 200-213.

Srinivasan, M., Sudheer, A.R., Menon, V.P. 2007. Ferulic acid: Therapeutic potential through its antioxidant property. J. Clin. Biochem. Nutr., 40, 92-100

Statistic Canada, 2010. Canada: Grains and Oilseed (G &O) Outlook: 2010-11. http://www.agr.gc.ca/pol/mad-dam/pubs/go-co/pdf/go-co_2010-01 28_e.pdf?PHPSESSID=5e35a299574cb96c62940a26ac9df06d

Strandås, C., 2008. The Phenolic Complex in Flaxseed Analysis, structural features and bioactivity.

Strandas, C., Kamal-Eldin, A., Andersson, R., Aman, P. 2008. Composition and properties of flaxseed phenolic oligomers. Food Chemistry, 110, 106–112

Struijs, K., Vincken, J. P., Verhoef, R., van Oostveen-van Casteren, W. H. M., Voragen, A. G., Gruppen, H. 2007. The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls. Phytochemistry. 68, 1227–1235

Struijs, K., Vincken, J.P., Doeswijk, T.G., Voragen, A.G.J., Gruppen, H. 2009. The chain length of lignan macromolecule from flaxseed hulls is determined by the incorporation of coumaric acid glucosides and ferulic acid glucosides. Phytochemistry. 70, 262–269

Struijs, K., Vincken, J.P., Verhoef, R., Voragen, A.G.J., Gruppen, H. 2008. Hydroxycinnamic acids are ester-linked directly to glucosyl moeities within the lignan macromolecule from flaxseed hulls. Phytochemistry. 69, 1250–1260

Sun R.C., Sun, X.F. and Wen, J.L. 2001. Fractional and structural characterization of lignins isolated by alkali and alkaline peroxide from barley straw. J Agric Food Chem., 49, 5322–5330

Sun R.C., Sun, X.F., Fowler, P. and Tomkinson, J. 2002. Structural and physico-chemical characterization of lignins solubilized during alkaline peroxide treatment of barley straw. Eur. Polym, J. 38, 1399–1407

Sung, M.K., Lautens, M., Thompson, L.U. 1998. Mammalian lignans inhibit the growth of estrogen-independent human colon tumor cells. Anticancer Res. 18, 1405–1408

Teiten, M.H., Gaascht, F., Eifes, S., Dicato, M., Diederich, M. 2010. Chemopreventive potential of curcumin in prostate cancer. Genes Nutr. 5, 61–74

Teratanavat, R., Hooker, N.H. 2006. Consumer valuations and preference heterogeneity for a novel functional food. Journal of Food Science, 71 (7), S533-S541

Thompson, L.U., Chen, J.M., Li, T., Strasser-Weippl, K., Goss, P.E. 2005. Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. Clinical Cancer Research, 11 (10), 3828-3835

Thompson, L.U., Rickard, S.E., Orcheson, L.J., Seidl, M.M. 1996a. Flaxseed and its lignan and oil components reduce mammary tumor growth at a late stage of carcinogenesis. Carcinogenesis. 17, 1373–1376

Thompson, L.U., Robb, P., Serraino, M., Cheung, F. 1991. Mammalian lignan production from various foods. Nutr Cancer. 16, 43-53

Thompson, L.U., Seidl, M.M., Rickard, S.E., Orcheson, L.J., Fong, H.H.S. 1996. Antitumorigenic effect of a mammalian lignan precursor from flaxseed. Nutr. Cancer, 26, 159–165

Toure, A., Xu, X.M. 2010. Flaxseed Lignans: Source, Biosynthesis, Metabolism, Antioxidant Activity, Bio-Active Components, and Health Benefits. Comprehensive Reviews in Food Science and Food Safety, 9 (3), 261-269

Townsley, P.M. 1979. Preparation of commercial products from brewer's waste grain and trub. MBAA Technical Quarterly, 16, 130–134

Velasquez, M.T., Bhathena, S.J., Ranich, T., Schwartz, A.M., Kardon, D.E., Ali, A.A., Haudenschild, C.C. & Hansen, C.T. 2003. Dietary flaxseed meal reduces proteinuria and ameliorates nephropathy in an animal model of type II diabetes mellitus. Kidney International, 64(6), 2100-2107

von Wettstein, D., Nilan, R. A., Ahrenst-Larsen, B., Erdal, K., Ingversen, J., Jende-Strid, B., Kristiansen, K. N., Larsen, J., Outtrup, H., Ullrich, S. E. 1985. Proanthocyanidin-free barley for brewing: Progress in breeding for high yield and research tool in polyphenol chemistry. Master Brew. Assoc. Am. Technol. Q., 22, 41-52

Wang, B.H., & Ou-Yang, J.P. 2005. Pharmacological actions of sodium ferulate in cardiovascular system. Cardiovascular Drug Reviews, 23, 161–172

Wei, H., Bowen, R., Cai, Q., Barnes, S., Wang, Y. 1995. Antioxidant and antipromotional effects of the soybean isoflavone genistein. Proc. Soc. Exp. Biol. Med. 208, 124-130

Westcott, N.D., Muir, A.D. 1996. Process for extracting and purifyinglignans and cinnamic acid derivatives from flaxseed. International Patent WO 9630468

Wiesenborn, D., Tostenson, K. & Kangas, N. 2003. Continuous abrasive method for mechanically fractionating flaxseed. Journal of the American Oil Chemists' Society, 80(3), 295-300

Wolfe, K. L., Liu, R. H. 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. J. Agric. Food Chem., 55, 8896–8907

Wolfe, K. L., Liu, R. H. 2008. Structure-Activity Relationships of Flavonoids in the Cellular Antioxidant Activity Assay. J. Agric. Food Chem., 56, 8404–8411

Wolfe, K.L., Kang, X.M., He, X.J., Dong, M., Zhang, Q.Y., Liu, R.H. 2008 Cellular antioxidant activity of common fruits. J. Agric. Food Chem., 56(18), 8418-8426

Yang, J., Liu, R.H. 2009. Synergistic Effect of Apple Extracts and Quercetin 3-beta-D-Glucoside Combination on Antiproliferative Activity in MCF-7 Human Breast Cancer Cells in Vitro. J. Agric. Food Chem., 57, 8581–8586

Yang, J., Meyers, K.J., Van der Heide, J., Liu, R.H. 2004. Varietal Differences in Phenolic Content and Antioxidant and Antiproliferative Activities of Onions. J. Agric. Food Chem., 52, 6787-6793

Yang,J., Liu,R.H. 2009. Synergistic Effect of Apple Extracts and Quercetin 3-β-D-Glucoside Combination on Antiproliferative Activity in MCF-7 Human Breast Cancer Cells in Vitro. J. Agric. Food Chem. 57, 8581–8586

Yi, W.G., Fischer, J., Krewer, G., Akoh, C.C. 2005. Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. J. Agric. Food Chem., 53, 7320-7329

Yoon, H.; Liu, R. H. 2007. Effect of selected phytochemicals and apple extracts on NF-κB activation in human breast cancer MCF-7 Cells. J. Agric. Food Chem. 55, 3167–3173

Yu, L., Haley, S., Perret, J., Harris, M., Wilson, J., and Qian, M. 2002. Free radical scavenging properties of wheat extracts. Journal of Agricultural and Food Chemistry, 50, 1619–1624

Yuan, J.P., Li, X., Xu, S.P., Wang, J.H., Liu, X. 2008. Hydrolysis Kinetics of Secoisolariciresinol Diglucoside Oligomers from Flaxseed. J. Agric. Food Chem., 56, 10041–10047

Zang, L.Y., Cosma, G., Gardner, H., Shi, X.L., Castranova, V., Vallyathan, V. 2000. Effect of antioxidant protection by p-coumaric acid on oxidation low-density lipoprotein cholesterol. Am J Physiol Cell Physiol., 279, C954–C960

Zhang, Y., Xu, W.Z., Wu, X.Q., Zhang, X.L.,Zhang, Y. 2007. Addition of antioxidant from bamboo leaves as an effective way to reduce the formation of acrylamide in fried chicken wings. Food Additives and Contaminants, 24 (3), 242-251

Zhao, H. F, Dong,J .J., Lu, J., Chen, J., Li, Y., Shan,L. J., Lin, Y., Fan, W., and Gu. G. X. 2006. Effects of Extraction Solvent Mixtures on Antioxidant Activity Evaluation and Their Extraction Capacity and Selectivity for Free Phenolic Compounds in Barley (Hordeum vulgare L.). J. Agric. Food Chem., 54 (19), 7277-7286

Zhou, B., Jia, Z. S., Chen, Z. H., Yang, L., Wu, L. M., Liu, Z. L. 2000. Synergistic antioxidant effect of green tea polyphenols withR-tocopherol on free radical initiated peroxidation of linoleic acid in micelles. J. Chem. Soc., Perkin Trans., 2, 785–791

Zienliński, H., Kozłwska, H. 2000. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. J. Agric. Food Chem., 48, 2008-2016

Zupfer, J. M., Churchill, K. E., Rasmusson, D. C., Fulcher, R. G. 1998. Variation of ferulic acid concentration among diverse barley cultivars measured by HPLC and microspectrophotometry. J. Agric. Food Chem., 46, 1350-1354

APPENDIX A Moisture content of barley hull and flaxseed hull

	Sample	Moisture content (%)*
Barley hull	EX116	5.31 ±0.18
	EX83	5.63 ±0.11
	P16	5.41 ±0.11
	P3	5.49 ±1.77
Flaxseed hull	Frutarom	7.77 ±0.22
	FG	7.67 ±0.08
	PF	7.63 ±0.22

* Data represent (mean ±SD) (n=3).

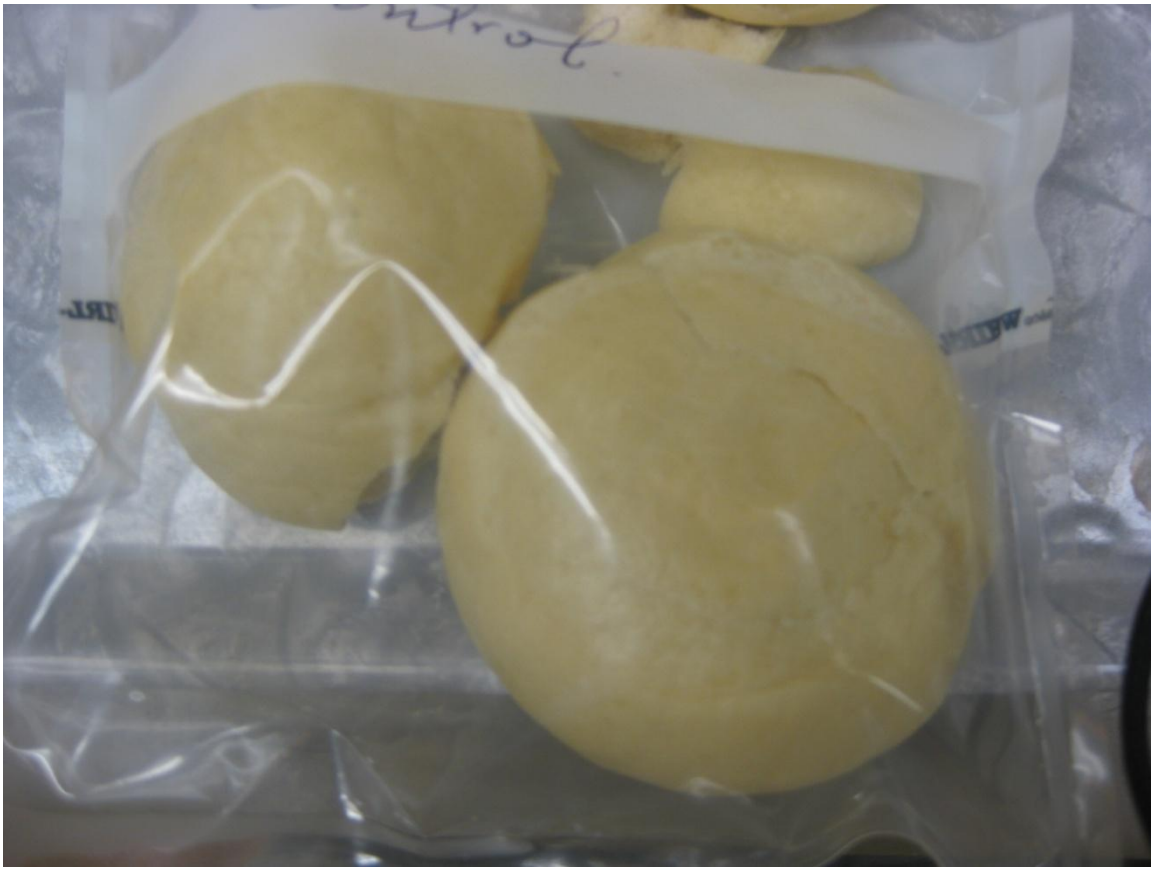
APPENDIX B Image of crude extracts of barley hull, flaxseed hull, and their co-extract

Barley hull extract

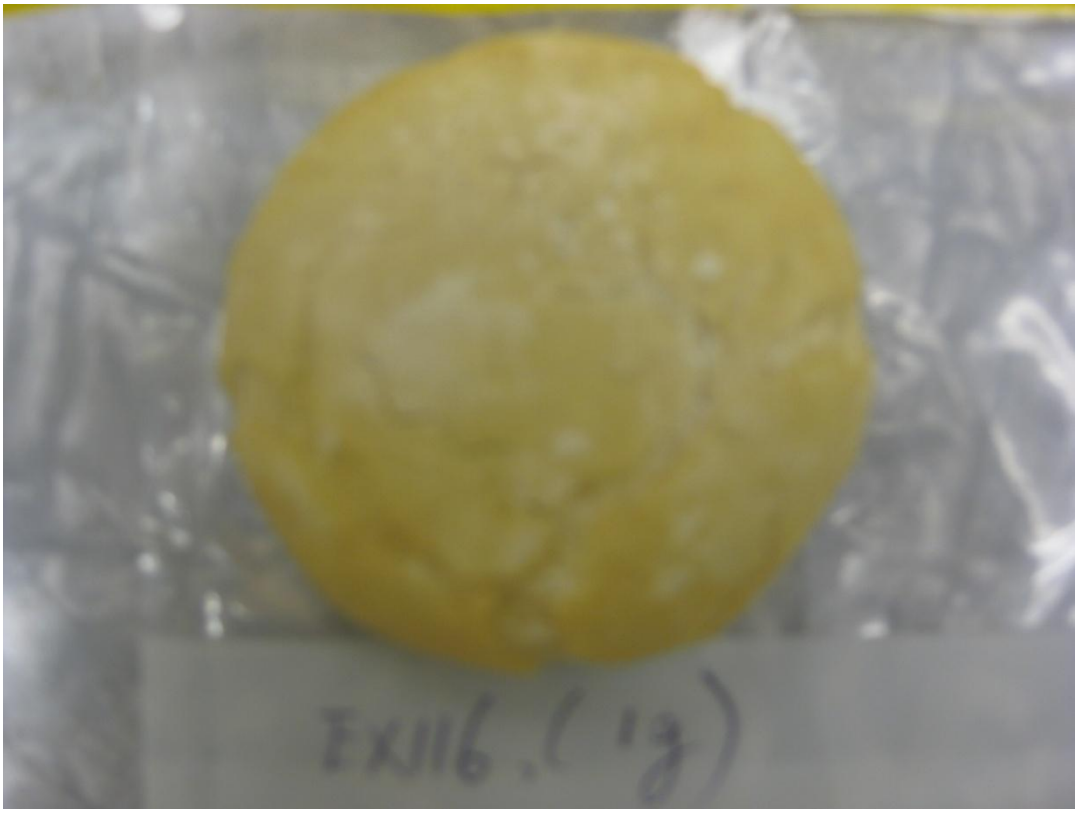
Flaxseed hull extract

Barley-flax hull co-extract

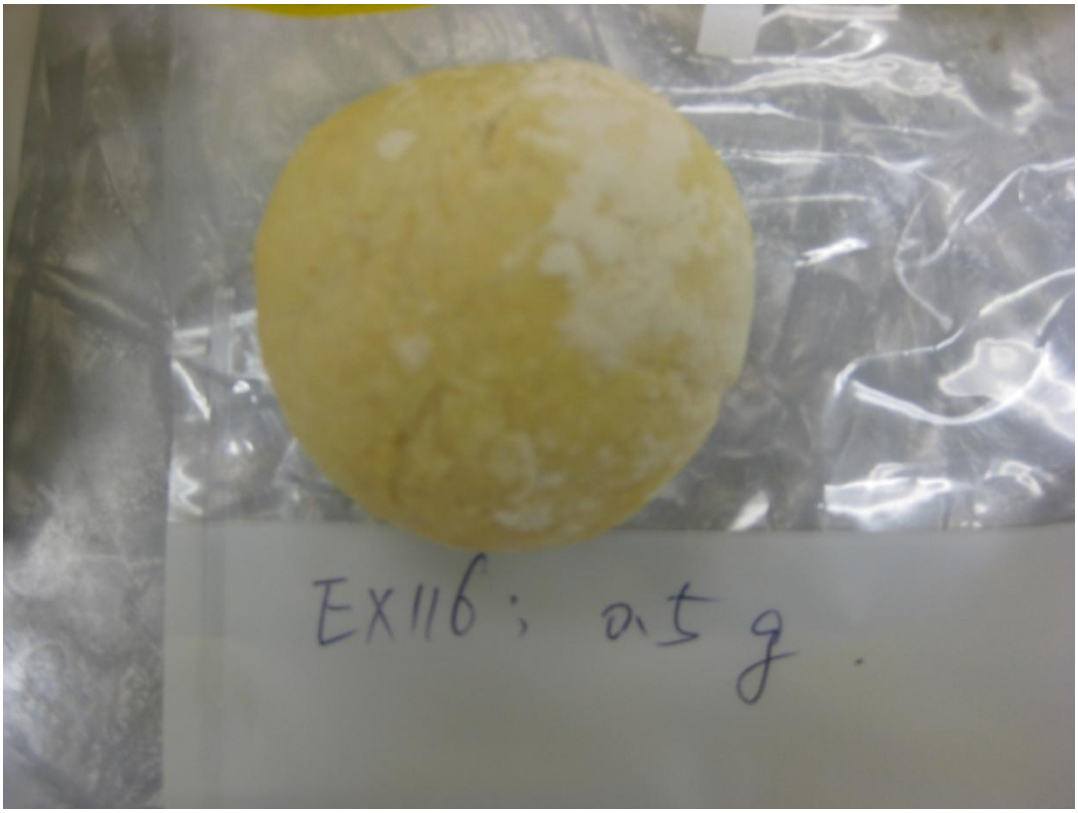
APPENDIX C Images of Freeze dried Chinese steamed bread supplemented with barley hull extract, flaxseed hull extract, and their co-extract



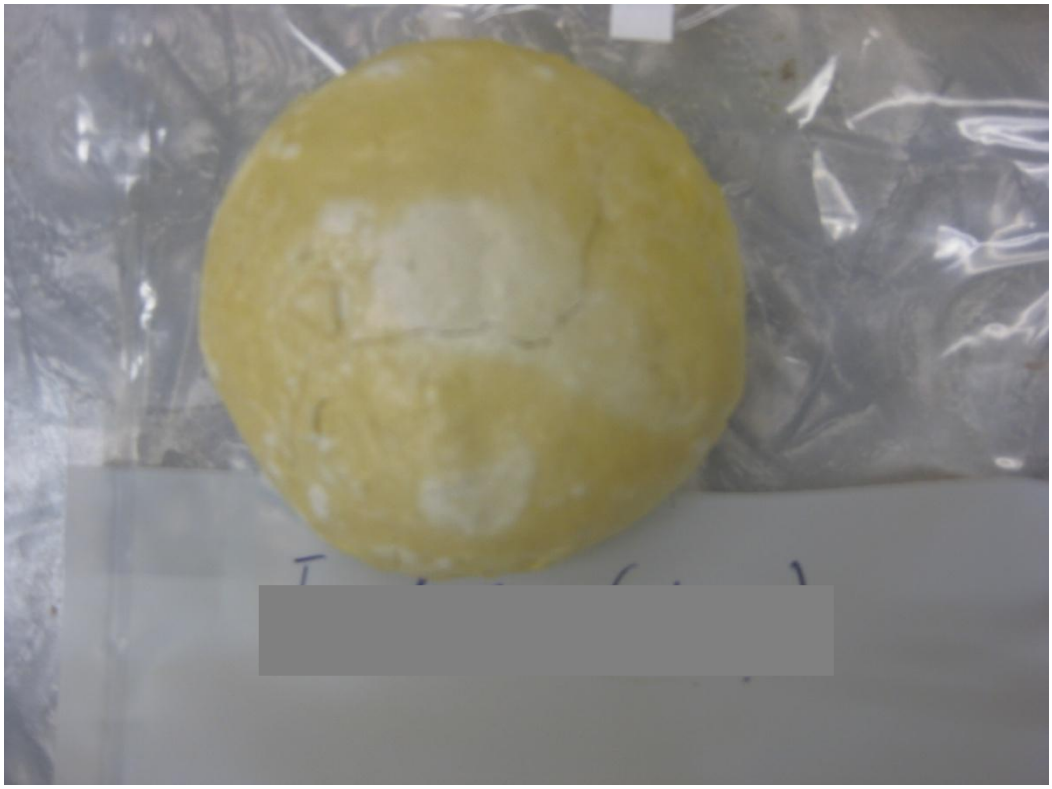
Control bread



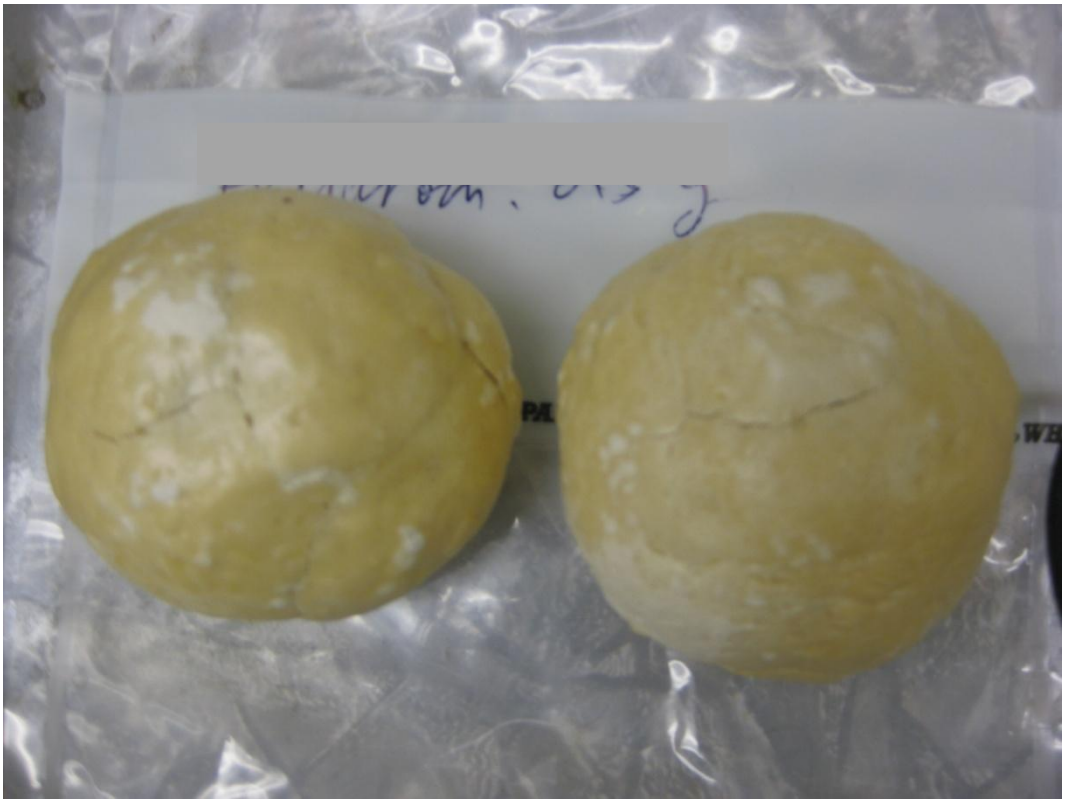
CSB+1 g Barley hull extract



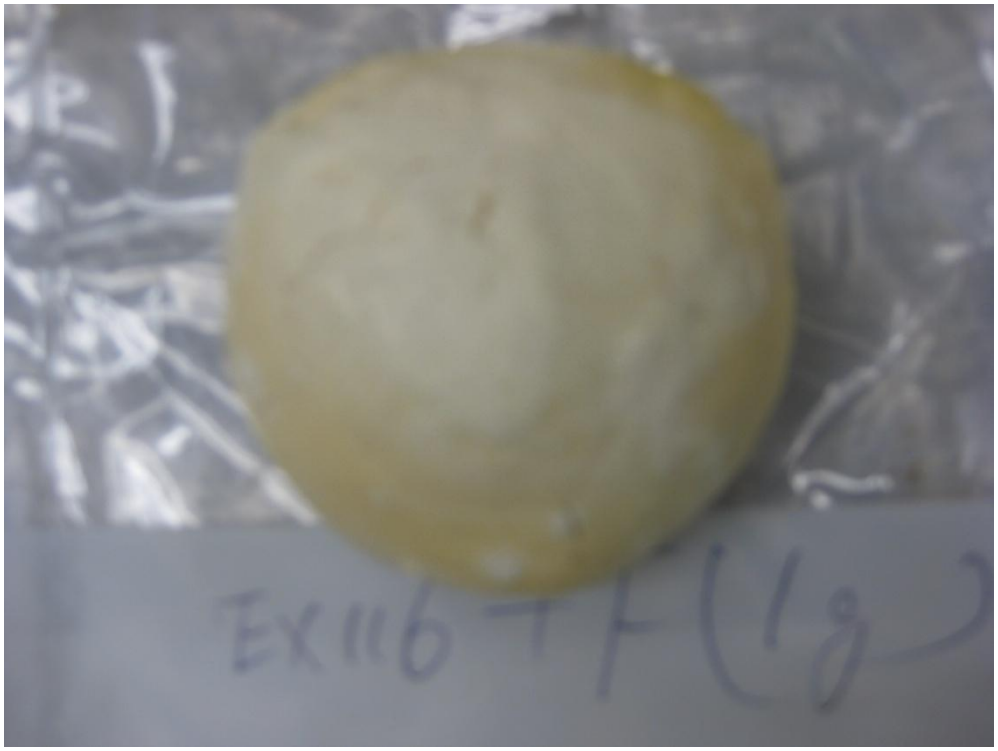
CSB+ 0.5 g Barley hull extract



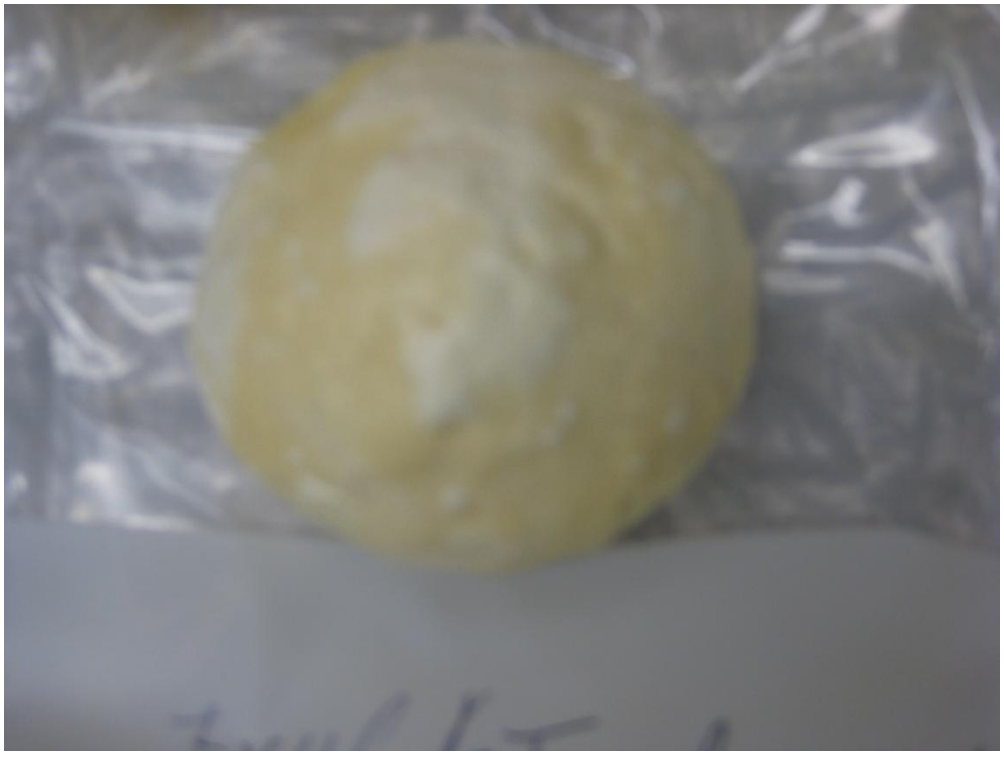
CSB+1 g Flaxseed hull extract



CSB + 0.5 g Flaxseed hull extract



CSB+1 g Barley+Flax hull co-extract



CSB+0.5 g Barley+Flax hull co-extract