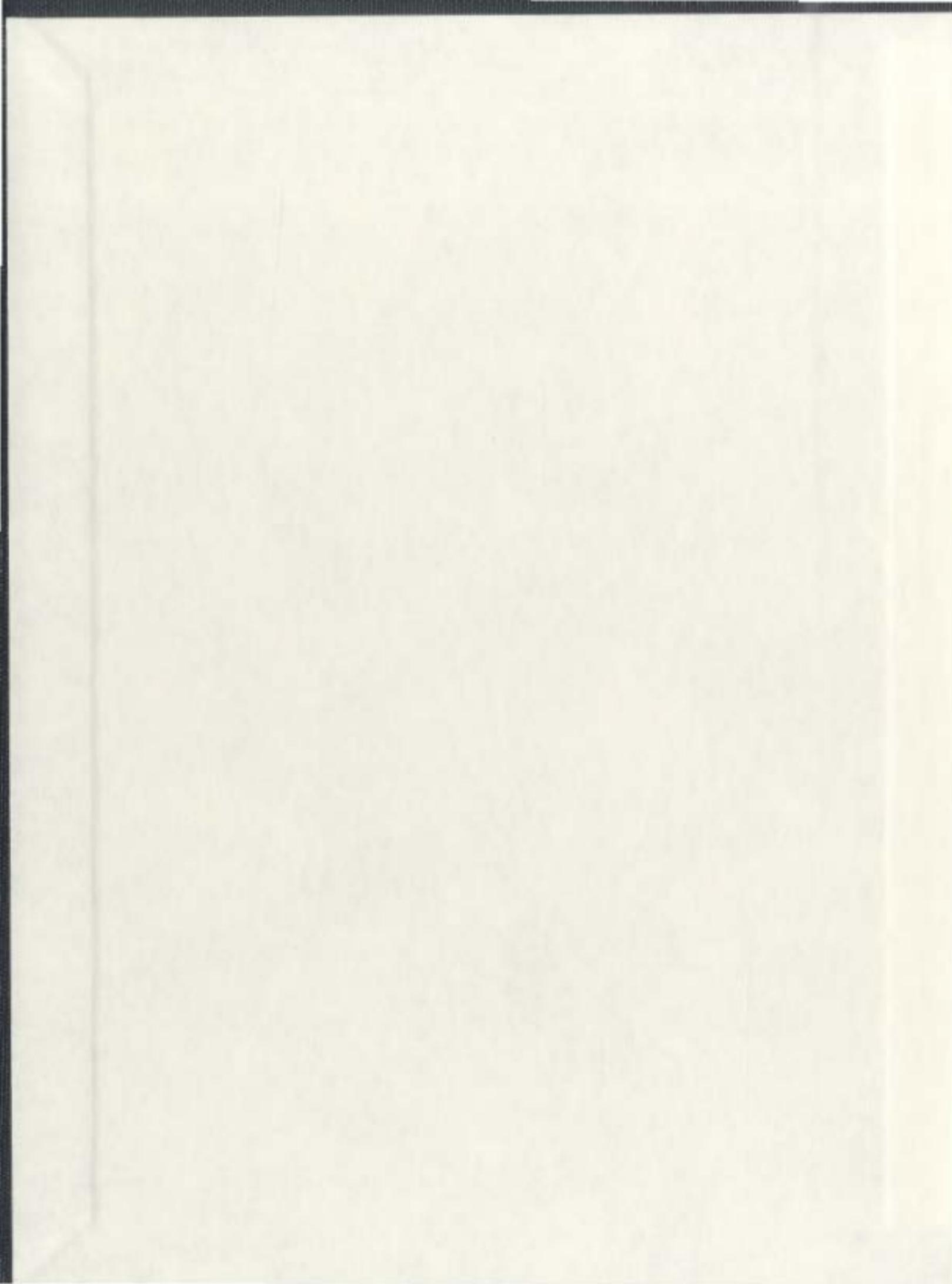


ANTIOXIDANT PHENOLICS OF DIFFERENT  
BARLEY (*Hordeum vulgare* L.) CULTIVARS

WAI SOORIYA MUDIYANSELAGE TERRENCE MADHUJITH





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**ANTIOXIDANT PHENOLICS OF DIFFERENT BARLEY  
(*Hordeum vulgare* L.) CULTIVARS**

By

**©WAISOORIYA MUDIYANSELAGE TERRENCE MADHUJITH,  
B.Sc., M.Sc.**

A thesis submitted to the School of Graduate Studies in partial fulfilment  
of the requirements for the degree of  
Doctor of Philosophy

Department of Biochemistry  
Memorial University of Newfoundland

September, 2007

**ST. JOHN'S**

**NEWFOUNDLAND & LABRADOR**

**CANADA**

**THIS WORK IS DEDICATED TO  
MY LOVING PARENTS, WIFE, AND TWO SONS**

## ABSTRACT

The extracts from six barley cultivars (*Hordeum vulgare* L.) grown in the Canadian prairies, namely AC Metcalfe, Falcon, Phoenix, Tercel, Tyto, and Peregrine, were evaluated for their potential antioxidant, antiradical, and antiproliferative efficacies by various chemical and biological methods. Antioxidative compounds were solvent-extracted after optimizing extraction parameters (solvent composition, extraction temperature, and time) using response surface methodology (RSM). In addition, the distribution of antioxidative constituents in the barley grain was investigated by separately pearling two barley cultivars (Falcon and AC Metcalfe) into seven fractions (F1-F7) in a layer-wise fashion up to 50% of the kernel weight. Antioxidative efficacy of the layers and the remaining kernel (pearled grain) was evaluated using chemical and biological methods. Furthermore, phenolic compounds present in the six barley samples were separated into free, soluble conjugates, and insoluble-bound fractions using alkaline hydrolysis and the antioxidative potential of each of the fractions was also investigated.

The optimum conditions for extraction of antioxidative components from barley extracts were 80% aqueous methanol, 60°C, and 40 min as determined by RSM. Total phenolic content (TPC) of whole kernel extracts ranged from 13.58 to 22.93 and 0.81 to 1.38 mg ferulic acid equivalents per gram lyophilizate and defatted material, respectively. The order of TPC was Peregrine > AC Metcalfe > Falcon > Tyto > Phoenix > Tercel. Total antioxidant capacity (TAC), as measured by Trolox equivalent antioxidant capacity (TEAC), ranged from 3.74 to 6.82  $\mu\text{mol}$  Trolox equivalents per gram defatted material. Antioxidant and antiradical efficacies of barley extracts were evaluated using different assays including 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide, oxygen radical absorbance capacity (ORAC<sub>FL</sub>), hydroxyl radical averting capacity (HORAC<sub>FL</sub>), and photochemiluminescence (PCL). All six barley extracts showed significant antioxidant and antiradical activities although the order of their activity changed from assay to assay. Barley extracts exhibited substantial metal chelation activity as measured by 2,2'-bipyridyl competition assay. Evaluating the whole barley extracts using a number of model systems such as  $\alpha$ -carotene/linoleate, bulk stripped corn oil as well as accelerated

oxidation study, using Rancimat<sup>®</sup>, further revealed that the whole barley extract possessed strong antioxidant activity. Whole grain extracts exhibited strong inhibitory effects against copper-induced human LDL cholesterol oxidation as well as peroxy and hydroxyl radical-induced DNA double strand scission. Barley extracts also exhibited substantial antiproliferative effect against growth of Caco-2 human colorectal adenocarcinoma cells in a concentration-dependent manner.

In the second phase of the study involving fractionation, it was revealed that antioxidative constituents were mainly located in the outer 9% (w/w) of the kernel. TPC of Falcon and AC Metcalfe fractions ranged from 0.51 to 6.26 and 0.17 to 4.16 as ferulic acid equivalents per gram defatted material. TAC, as measured by TEAC, varied from 0.45 to 60 and 0.69 to 56  $\mu\text{mol}$  Trolox equivalents per gram defatted material, respectively. Antioxidant efficacy gradually diminished from the outer layers to the inner layers. In general, antioxidant efficacy determined by other methods followed a similar trend to that of TAC.

In the last phase of the study involving hydrolysis of phenolic compounds, it was revealed that a greater proportion of antioxidative compounds were present in insoluble-bound form followed by soluble conjugate form. It was further revealed that the antioxidant and antiradical efficacies of insoluble bound phenolic compounds were the highest followed by soluble conjugates and free phenolics. The predominant phenolic acid detected in barley extracts was ferulic acid. The other phenolic acids detected were vanillic, caffeic, *p*-coumaric and sinapic acids.

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## LSIT OF ABBREVIATIONS

AAPH	2,2'-azobis-(2-methylpropionamidine)dihydrochloride
ABTS <sup>·-</sup>	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) radical anion
ABTS <sup>2-</sup>	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)
ANOVA	Analysis of variance
AOCS	American Oil Chemists' Society
AOAC	Association of Official Analytical Chemists'
AUC	Area under curve
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CHD	Coronary heart diseases
CNS	Central nervous system
CV	Coefficient of variation
CVD	Cardiovascular diseases
DG	Dodecyl gallate
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EPR	Electron paramagnetic resonance
Eq	Equivalentents
F1-F7	Pearled barley fractions
FBS	Foetal bovine serum
FDA	Food and <sup>D</sup> rug <sup>A</sup> dministration
FL	Fluorescence
GLM	General linear model

GSH	Reduced glutathione
GSHPX	Glutathione peroxidase
GSSG	Oxidized glutathione
hLDL	Human low density lipoprotein
HNE	4-hydroxynonenal
HPLC	High performance liquid chromatography
IP	Induction period
LDL	Low density lipoprotein cholesterol
LOX	Lipoxygenase
MA	Malonaldehyde
MFO	Mixed function oxidase
NADPH	Nicotinamide adinine diphosphate
$^1\text{O}_2$	Singlet oxygen
$\text{O}_2^{\bullet-}$	Superoxide radical anion
$\bullet\text{OH}$	Hydroxyl radical
ONOO	Peroxynitrite
ORAC <sub>FL</sub>	Oxygen radical absorbance capacity
PAL	Phenylalanine lyase
PBS	Phosphate buffered saline
PCL	Photochemiluminescence
PE	Phycoerythrins
PF	Protection factor
ppm	Parts per million
PUFA	Polyunsaturated fatty acids
$r^2$	Correlation coefficient
ROO $\bullet$	Peroxyl radical
ROS	Reactive oxygen species
RSM	Response surface methodology
SAS	Statistical analysis software

<sup>1</sup> Sen	Ground state sensitizer
<sup>1</sup> Sen*	Excited sensitizer
<sup>3</sup> Sen*	Excited triplet sensitizer
SHBG	Sex hormone binding globulins
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TBHQ	<i>tert</i> -butylhydroquinone
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
TPR	Tetrapyrroles
<sup>1</sup> TPR*	Excited tetrapyrroles
<sup>3</sup> TPR*	Excited triplet tetrapyrroles
USDA	United States Department of Agriculture
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase
X/XO	hydroxanthine/xanthine oxidase system

## CHAPTER 1

### INTRODUCTION

The importance of oxidation in foodstuff and biological systems is widely recognized. Lipid oxidation is of paramount importance to the food industry as oxidative deterioration leads to the development of undesirable quality attributes and generation of potential toxic substances. Oxidation in biological systems causes damage to biomolecules such as nucleoproteins and cell membranes, cellular organelles, and enzymes, triggering mutagenesis, carcinogenesis, circulatory disturbances and ageing, among others (Halliwell *et al.*, 1995a).

In both biological and food systems, the above-mentioned damages are primarily caused by oxygen free radicals and related species, collectively known as reactive oxygen species (ROS). Despite the fact that living organisms depend on biological oxidation as a source of energy, the action of oxygen is two-sided. Significant quantities of ROS are produced as by products of cellular metabolism in biological systems while the rest is produced by foreign matter metabolism, prostaglandin biosynthesis, and antimicrobial action of cells (Namiki, 1990). Aerobic organisms have evolved with enzyme systems that protect the living organisms from the effects of ROS (Aruoma, 1998). However, supplementing the natural defense with dietary antioxidants provides better protection, especially where these defense systems are not functioning adequately. Antioxidative potential of phytochemicals contained in plant materials is partly attributed to the phenolic compounds such as flavonoids, and phenolic acids.

The data from both experimental and epidemiological studies have indicated that fruits, vegetables, and grains contain a myriad of phytochemicals that can potentially

mitigate the occurrence of oxidative stress-related disease conditions (Adom and Liu, 2002). There is mounting interest in plant-derived bioactives, especially antioxidants, present in common food sources. Over the past decade or so there has been an explosion of research on natural antioxidants and other bioactives. Most of this work has focused on identifying antioxidative constituents from various sources and evaluating their health promotion potential. The disease prevention properties of fruits, vegetables and cereal grains are in part due to their polyphenolic content (Nichenametta *et al.*, 2006). Cereal grains contain unique phytochemicals that complement those of fruits and vegetables when consumed together. The importance of including whole grains in the daily diet in significant quantities has been emphasized in the latest North American Food Guides (USDA, 2005; Canada Food Guide, 2007).

The principal cereal crops grown and consumed in the world include wheat, rice, maize, barley, oat, rye, sorghum, and millet. Cereal grains provide approximately two thirds of the calorie requirement and protein intake of the world and even more in some African and Latin American countries (Penderson *et al.*, 1989). Canada is one of the largest barley producers in the world with production of 12.48 million metric tonnes (MMT) in the 2005 crop year (Statistics Canada, 2006). However, at present a major part of the barley produced is used for animal feed and brewing while little is used for direct human consumption.

While the antioxidant properties of fruits and vegetables have been investigated, relatively little attention has been focused on grains in general. A close scrutiny of scientific literature published over the last decade reveals that most of the work on grains has been directed toward wheat, buckwheat, and oat. Antioxidants are not evenly

distributed in the cereal grains. Salomonsson *et al.* (1980) indicated that *p*-coumaric acid was present in the lowest amount in the centre of the barley kernel and rapidly increased toward the outer layers, such as lignified husk. Some researchers have reported that phenolic acids are concentrated in the cell walls of the outer layers (Maillard and Berset, 1995), while others have indicated that phenolic acids are mainly present in the aleurone layer and endosperm (Goupy *et al.*, 1999). The highest content of ferulic acid occurred in the cell walls of the aleurone layer, which is rich in arabinoxylans (McNeil *et al.*, 1975). Maillard and Berset (1995) separated and identified trans-ferulic acid, trans-*p*-coumaric acid and cis-ferulic acid from barley and malt.

Based on the above summary, the following hypotheses were formulated for the present study on antioxidant potential of barley: (1) Six barley cultivars grown under similar agronomical conditions may possess similar antioxidant potential. It was further hypothesized that the phenolic compounds of barley function similarly in different antioxidant reactions; (2) Antioxidative compounds are evenly distributed in the kernel; and (3) Different phenolic fractions, namely free, soluble conjugate, and insoluble-bound possess the same antioxidant activity and contribute similarly toward total antioxidant activity.

In order to test the above hypotheses, several objectives were considered and these included: (1) Optimizing parameters for extraction of phenolic compounds from barley samples; (2) Evaluating phenolic extracts from whole kernel of six barley cultivars for their antioxidant, antiradical, and antiproliferative efficacies; (3) Determining how antioxidative compounds are distributed in the barley kernel, and (4) Determining the

contribution of free, soluble conjugate and insoluble-bound phenolic fractions towards total antioxidant activity.

Optimum conditions for solvent-extraction of antioxidative compounds from barley grains were determined by employing response surface methodology (RSM). Optimum extraction conditions (solvent composition, extraction temperature and time) were chosen based on total antioxidant capacity (TAC) of the extracts as determined by Trolox equivalent antioxidant capacity (TEAC) (Chapter 4). Antioxidative compounds of six barley cultivars were solvent-extracted by employing the optimum extraction conditions determined in Chapter 4. The extracts so obtained were assessed for their antioxidative, antiradical, and antiproliferative activities and results were reported in Chapter 5. In order to study the distribution of antioxidative compounds and their activity in barley kernel, two barley cultivars (Falcon and AC Metcalfe) were separately pearled into seven fractions and subsequently the antioxidative compounds were solvent-extracted using the optimum parameters determined in Chapter 4. The extracts from pearling fractions and pearled grain were evaluated for their antioxidant, antiradical and antiproliferative potentials and results are given in Chapter 6. The antioxidant potential of free, soluble conjugate, and insoluble-bound phenolic fractions and their contribution of each phenolic fraction towards the overall antioxidant potential were studied by separately hydrolyzing six barley samples. The resulting three phenolic fractions were separately evaluated for their antioxidant potential and the results so obtained are provided in Chapter 7.

This thesis is comprised of eight chapters in total with Chapter 1 presenting an introduction, Chapter 2 providing a review of literature, Chapter 3 outlining the

methodologies used Chapters 4 through 7 giving results of the studies conducted and Chapter 8 providing an overall summary and conclusions.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Cereals of the world

Cereals are the fruits of cultivated grasses, members of the monocotyledonous family, Poaceae. The principal cereal crops are wheat, rice, barley, oats, rye, maize, sorghum and millets. In addition, buckwheat, amaranth, wild rice, bulgur, kamut, quinoa, spelt, canary seeds and triticale (wheat-rye hybrid) are cultivated around the world in minor quantities. Cereals have been used as human food for thousands of years; their successful production and use have contributed to the development of modern civilization.

##### 2.1.1 World cereal consumption

Cereals occupy over 73% of harvested areas of the world and contribute over 60% of the food for the world's population by providing carbohydrate, protein, minerals, dietary fibre and vitamins required for human health (Charalampopoulos *et al.*, 2002). Grains provide approximately two-thirds of the calorie requirement and protein intake of the world, and even more in the developing countries (Penderson *et al.*, 1989). Wheat accounts for one-third of the total world grain production, while rice accounts for one fourth. Little barley is consumed in the western countries, but in Eastern countries, it continues to be a dietary staple. A major proportion of barley in Canada is used as animal feed while a small amount is used for brewing and flour production (Slavin *et al.*, 2000). Sorghum and millet, commonly grown for human consumption in arid and semi-arid areas of the world, are preferred to other cereals primarily due to their resistance to

disease and predators, as well as their tolerance of moisture stress and soil fertility. Oats are important because they are consumed whole and therefore contribute an important part of whole grain consumption in North America while whole rye is commonly consumed in Northern Europe (Slavin *et al.*, 2000).

### **2.1.2 Cereal production in North America**

North America produced 414,382 thousand metric tonnes of cereal in 2005 of which Canada contributed 50,363 thousand metric tonnes (EarthTrends, 2005). Grain products comprised the base of the USDA food guide pyramid, with 6-11 servings of grain products recommended daily (Slavin *et al.*, 2000). The new USDA food guide pyramid recommends 7-8 ounce equivalents of grains for males and 6 ounce equivalents for females (USDA, 2005). Dietary recommendations constantly stress the importance of grain choice, particularly whole grain products. It is generally accepted that a minimum of three grain servings per day should be whole grain. Cereal grains provide significant quantities of energy, proteins, and selected micronutrients to the animal and human diet. The chemical composition and bioavailability of nutrients varies with species and variety of grains and may be affected by processing (Zielinski and Kozłowska, 2000).

### **2.1.3 Composition of cereals**

For the majority of the world's human population, cereal-based foods constitute the main source of energy and other nutrients. In the poorest parts of the world, starchy foods, including cereals, supply 70% of total energy (Kent 1994). The mature grain of the common cereals consists of mainly carbohydrate (70-80%), protein (10-16%), fat

(1.4-4.6%), minerals and vitamins. The principal vitamins found in cereals include the vitamin B group; thiamine, nicotinic acid, riboflavin, pantothenic acid and pyridoxine, which are distributed non-uniformly throughout the grain. Moreover, cereal grains contain a myriad of biologically active substances including dietary fibre, sterols, phytoestrogens, tocopherols, and other phenolic compounds.

## **2.2 Barley**

Barley is among the most ancient cereal crops grown in the world. There is archeological evidence that barley cultivations existed 17,000 years ago in the Nile river valley, Egypt (Wendorf *et al.*, 1979) and 8000 BC in Iran (Bothmer and Jacobson, 1985). Barley is a major world crop, ranking among the top 10 crops and fourth among cereals (Nilan and Ulrich, 1993). Barley contributes significantly to the world's food supply as a human food, malt products and livestock feed.

### **2.2.1 Taxonomy of barley**

Barley is a grass belonging to the family Poaceae. The following are the full taxonomical details of barley.

Kingdom	: Plantae
Family	: Poaceae
Tribe	: Triticeae
Genus	: <i>Hordeum</i>
Species	: <i>vulgare</i>
Sub-species	: <i>vulgare</i>

The main taxonomic characteristic of *Hordeum* is its one-flowered spikelet (Bothmer and Jacobson, 1985). Three spikelets alternate on opposite sides at each node of the flat rachis of the spike (head) forming a triplet of spikelets at each node. When all three spikelets are fertile, the spike is described as six-rowed, when only the central spikelet is fertile; the spike is considered two-rowed (Nilan and Ullrich, 1993).

The cultivated barley form is considered to be belonging to the sub-species *vulgarae* while the wild forms are described as sub-species *spontaneum* of *H. vulgarae*. Sub-species *vulgarae* is both two-rowed and six-rowed with non-brittle rachis, whereas sub-species, *spontaneum* is two-rowed with brittle rachies (Nilan and Ullrich, 1993). Two-rowed barley is believed to have evolved from its immediate ancestor of two-rowed wild *H. vulgarae spontaneum*. The six-rowed forms are considered to be the result of mutation and hybridization (Nilan and Ullrich, 1993). Among 30 wild species, most are diploids ( $2n=14$ ), with about half being tetraploid ( $2n=28$ ) and hexaploid ( $2n=42$ ). **Table 2.1** lists important characteristics of some Canadian barley cultivars.

### **2.2.2 Barley cultivation**

Barley is a cool-season crop cultivated in the spring and summer at temperate latitudes as well as in the tropical regions. It is a moderately cold tolerant cereal crop and is considered to be one of the most drought, alkali, and salt tolerant crops (Poehlman, 1985) that grows best on well-drained, fertile loam soils under relatively cool temperatures with an annual rainfall of 500-1000 mm.

**Table 2.1 Characteristics of some Canadian barley cultivars**

Cultivar	Year registered	Row type	Predominant use	Hulled/hull-less
AC Metcalfe	1999	2R	Malting	Hulled
Falcon	1993	6R	Feed	Hull-less
Tyto	2002	6R	Feed	Hull-less
Peregrine	1999	6R	Feed	Hull-less
Phoenix	1992	2R	Feed	Hull-less
Tercel	1997	2R	Feed	Hull-less

The highest commercial yield is approximately 10MT/ha under optimum conditions with average yields varying between 1.7 and 5.0MT/ha under farming conditions (Nilan and Ulrich, 1993). The former USSR was consistently the world's leading barley producer with Canada ranking second in production. Canada produced 12.48 MMT of barley in the 2005 crop year. The United States, France, Spain, Germany, UK, Turkey, China and Denmark are the other important barley producing countries in the world.

### **2.2.3 Barley breeding**

Barley breeding programmes have improved productivity adaptations and quality over the last century. The major germ plasm collection in North America is the National Small Grains Collection of the USDA and Canada's Plant Gene Resources. Crossing various germ plasm sources and subsequent recombinations provide the basis for barley breeding and development of pure-line cultivars. Barley is a self-pollinated crop carrying perfect flowers, thus a commercially feasible hybridization is not feasible (Ramage, 1983).

### **2.2.4 Composition of barley**

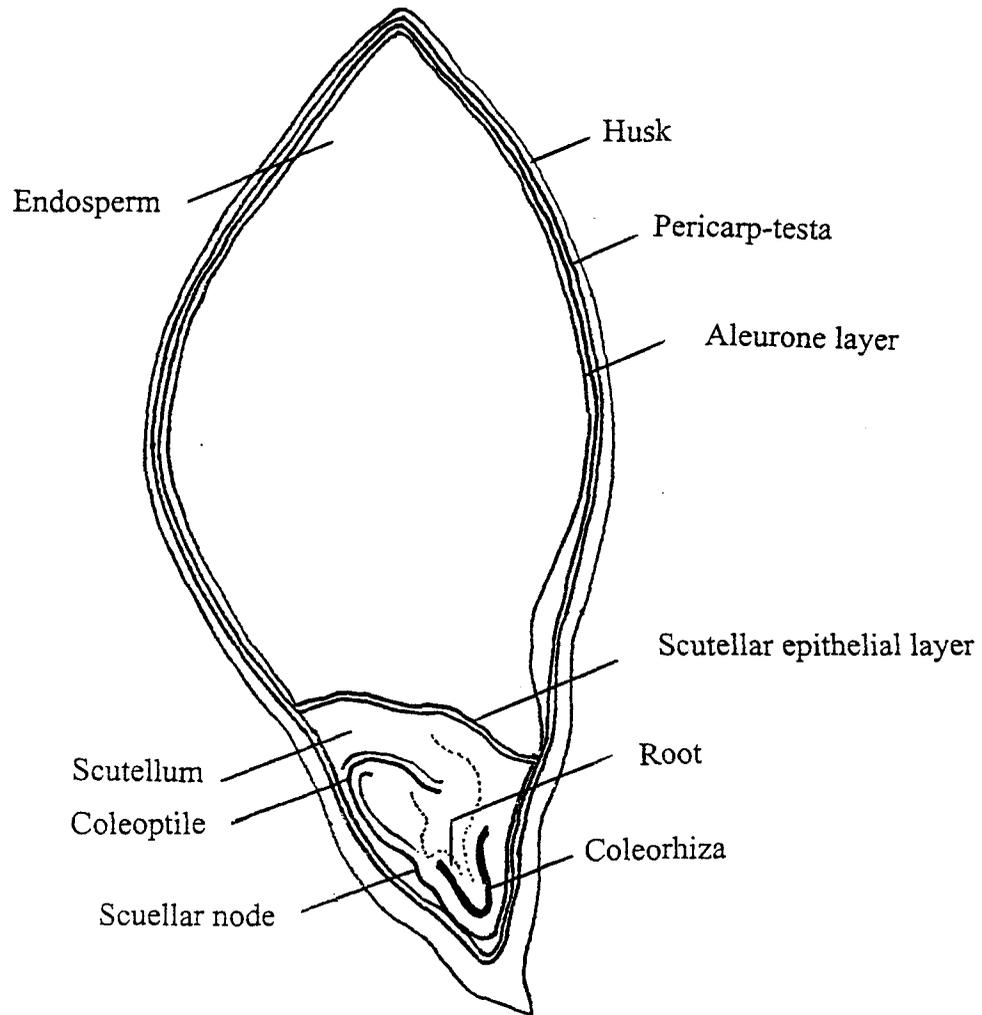
The mature grain of barley consists of about 80% carbohydrate. Starch is present exclusively in the endosperm of the mature grain. The cell walls consist of cellulosic microfibrills that reinforce a matrix comprised of arabinoxylans and (1→3), (1→4)- $\beta$ -glucan, commonly known as  $\beta$ -glucan. Arabinoxylan and  $\beta$ -glucan make up 95% of the cell walls in the mature grain (MacGregor and Fincher, 1993).  $\beta$ -glucan from barley endosperm consists of linear chains of  $\beta$ -glucosyl residues polymerized through both

(1→3) and (1→4) linkages. The husk and bran consist of cell wall remnants in which cellulose, silica and lignin contents are higher with heteroxylans (Fincher and Stone, 1986). Waxy barley has a higher content of  $\beta$ -glucan than non-waxy barley cultivars. Arabinoxylans are the building blocks of cereal cell walls and major components of cereal dietary fibre. They are composed of a main chain of unsubstituted and mono- and/or di-substituted (1→4)-linked  $\beta$ -D-xylopyranosyl residues with terminal  $\alpha$ -L-arabinofuranosyl residues. Variability in both the degree and distribution of substituents as well as molecular size determines the heterogeneity of arabinoxylan (Cyran *et al.*, 2004).

### **2.2.5 Structure of barley grain**

Barley, like rice and oat is harvested with the tightly adhering hull (husk) which consists of the lemma and palea. The caryopsis is composed of pericarp, seed coat, germ, and endosperm. Beneath the testa is the aleurone layer consisting of 2-3 cell layers (**Figure 2.1**). Usually the colour of aleurone layer is white, however, there are some cultivars bearing a blue colour aleurone. The endosperm cells which make up the bulk of the grain are packed with starch embedded in a protein matrix (Hoseney, 1994). Barley starch consists of a mixture of large lenticular granules of 15-25  $\mu\text{m}$  and small irregular shaped granules <10  $\mu\text{m}$  in length. During high temperature processing such as kilning, extrusion cooking, and pelleting, a part of the starch converts to resistant starch (McWilliams, 1972), which is considered beneficial in gastrointestinal health.

**Figure 2.1** Linear section of a barley grain illustrating the inner layers.  
*Adapted from Hosney (1994).*



### **2.2.6 Uses of barley**

Barley is used for malting, food, feed, and industrial applications. Barley malt has long been used to impart distinctive flavours and colours to a variety of foodstuff such as ice cream, biscuits, confectionary, malted drinks, soups, and cakes. The most extensive use of barley malt is in the brewing industry. Principal among the malt products are soluble extracts of coloured malts (Bamforth and Barclay, 1993).

### **2.3 Whole grains**

Whole grains became a part of the human diet with the advent of agriculture some 10,000 years ago (Spiller, 2002). Over the last 4000 years, a majority of the world's population has relied upon whole grains as a main source of their staple food. It is only within the last century that refined grain products entered the human diet with the introduction of the roller mill (Slavin, 2004). In different parts of the world, numerous terms are used to describe whole grains complicating the whole grain concept. In order to overcome this situation, the American Association of Cereal Chemists' (AACC) defined whole grain as 'the intact, ground, cracked or flaked caryopsis, whose principal anatomical components, the endosperm, germ, and bran, are present substantially in the same relative proportion as they are present in the natural caryopsis'. Whole grains contain fibre, vitamins, minerals, phenolic compounds, phytoestrogens, and other unmeasured constituents that are removed during the refining process (Slavin, 2003). The introduction of the roller mill in 1873 fuelled an increasing demand for refined grain products leading to a sharp drop in consumption of whole grains (Slavin, 2003). In the grain refining process, bran is either partially or completely removed, resulting in a

significant loss of minerals, vitamins, lignins, tocopherols, phytates, tannins, and other phenolic compounds, which are associated with improved health status.

Whole grains have been identified as a part of a healthful diet over the last 25 years by major governmental, scientific and non-profit organizations (USDA, 1995). Grain products were identified as an important component of the daily diet by dietary guidelines for Americans. However, for the first time, dietary guidelines for Americans have provided a quantitative recommendation for the intake of whole grains; > 3 ounce equivalents of whole grains or whole grain products per day (USDA, 2005). This recommendation is based on the link between consumption of whole grain and reduced risk of a number of chronic diseases including cardiovascular diseases (Jacobs *et al.*, 1998; Ekkila *et al.*, 2004), cancer (Kasum *et al.*, 2001; Larson *et al.*, 2005), type 2 diabetes (Meyer *et al.*, 2000; Monotonen *et al.*, 2003), higher body mass index (BMI) and higher insulin sensitivity (Steffen *et al.*, 2003). However, most North Americans consume far less than the recommended three servings of whole grains on a daily basis (Albeertson and Tobelmann, 1995).

Walker (1947), Burkitt (1952), Cleave (1956) and Trowell (1972) pioneered the concept that highly-refined foods significantly contribute to 'Western diseases' namely, diabetes, cardiovascular diseases (CVD), and obesity. Whole grain foods may protect against chronic diseases altering serum cholesterol profiles, exerting antioxidant and anti-thrombotic effects, and through their favourable effects on vascular reactivity and insulin sensitivity (Anderson and Hanna, 1999).

## 2.4 The role of whole grains in health

Whole grain is a rich source of both soluble and insoluble dietary fibre, resistant starch, and oligosaccharides. Trowell *et al.* (1976) defined dietary fibre as all polysaccharides and lignins in the diet that are not digested by the human digestive system. Most of the dietary fibre derives from the cell walls of fruits, vegetables, cereals, and oilseeds. The major components of dietary fibre are complex carbohydrates such as pectic polysaccharides, cellulose, hemicellulose, glycoproteins, arabinoxylans, and  $\beta$ -glucans, some of which are associated with polyphenolic components and protein while only a small percentage (5-10%) is non-carbohydrates in nature. Lignified tissues are the main source of dietary fibre in cereals and cereal products while paranchymatous tissues are the most important source in vegetables (Selvendran and Verne, 1990).

Dietary fibre can be divided into two major groups based on solubility; soluble dietary fibre and insoluble dietary fibre. Most of hemicellulose, cellulose and lignins are the main insoluble dietary fibre. From a physiological standpoint, these two classes appear to function differently in the digestive system.

Oats, rye and barley contain about one third of total fibre content as soluble fibre while wheat and rice contain very low amounts. Soluble fibre is associated with cholesterol lowering and improved glucose response while insoluble fibre is associated with improved laxation and speedy transit of stools (Marlett *et al.*, 2002) and bulky fecal matter. Oligosaccharide, inulin (C2-C20), and resistant starch act like soluble dietary fibre in the human gut (Huth *et al.*, 2000). Resistant starch and oligosaccharides reach the colon and are fermented by intestinal microflora to short chain fatty acids that lower serum cholesterol and decrease cancer risk (Slavin, 2003).

Ingestion of diets rich in dietary fibre may result in slower gastric emptying, delayed absorption in the small intestine, faster colonic transit, and bulky stools (Edwards, 1990). Many of these soluble polysaccharides are extensively fermented in the colon and have minimal effects on stool output (Edwards, 1990). In contrast, insoluble polysaccharides act as indigestible solids and have little effect on small intestine physiology. However, insoluble polysaccharides are more resistant to bacterial degradation and have a greater influence on stool output and colonic transit (Edwards, 1990).

#### **2.4.1 Whole grains and cardiovascular diseases (CVD)**

Epidemiological and clinical studies have clearly shown that oat tends to lower plasma total and LDL cholesterol. Barley and rice bran fibre may also have a similar effect, but wheat fibre does not (Truswell, 2002). In 1999, the US Food and Drug Administration (FDA) allowed a health claim for rolled oats, whole oat flour or oat bran as possible products for reducing the risk of coronary heart diseases (FDA, 1999). Five separate epidemiological studies, carried out in the USA and Europe involving over 100,000 human subjects, have shown a significant inverse association of cereal fibre of whole grains with coronary heart disease (CHD). The FDA permitted a health claim stating that 'diets rich in whole grain food and other plant foods low in total fat and saturated fat may reduce the risk of heart diseases and cancer' (FDA, 1999).

The effect of dietary fibre on human cholesterol profiles is dependent on the source of fibre. Out of 34 controlled human experiments carried out to investigate the cholesterol lowering effect of wheat fibre, in 27 experiments, plasma total cholesterol did

not go down (Truswell, 2002) – it even rose in two studies. In sharp contrast to wheat fibre, most researchers who examined the effect of oat fibre have found reduction of plasma total and LDL-cholesterol with no change in HDL or triacylglycerol (Truswell, 2002). The active components of dietary fibre in oat are  $\beta$ -glucan. Barley, which contains significant amounts of  $\beta$ -glucans, is reported to lower plasma total and LDL cholesterol by 6% compared to a control diet with wheat fibre (McIntosh *et al.*, 1991).

The most plausible mechanism of action of  $\beta$ -glucan is by its viscosity interfering with re-absorption of bile acids and hence producing a negative sterol balance. Three hypotheses have emerged regarding the cholesterol-lowering mechanism of dietary fibre: altering bile acid reabsorption, and modified lipid absorption; the effect of short chain fatty acids generated in the colon; and altering insulin or other hormones or tissue sensitivity to hormones (Anderson *et al.*, 1990a). Altering bile acid re-absorption, being one of the earliest hypotheses, postulates that dietary fibre increases fecal bile acid excretion by binding bile acids, forming gels, and interfering with micelle formation. Thus, the need for cholesterol for bile acids formation increases leading to low availability of cholesterol for lipoprotein synthesis (Story and Lord, 1987).

The high fibre diets are reported to lower insulin requirement in diabetic individuals and decreases serum insulin concentration of normal human subjects, increasing insulin sensitivity and decreasing serum glucagons (Anderson *et al.*, 1990b). All these effects lead to favourable changes in lipid metabolism. Additionally, whole-grains alter some of the risk factors contributing to a reduction in CVD; hypertriacylglycerolaemia, decreased HDL-cholesterol, hypertension, diabetes, obesity, insulin resistance, sub-optimal antioxidant status, hyperhomocysteinaemia, altered

vascular reactivity and inflammatory state (Anderson, 2003). It is evident that only a minor part of this protective effect can be explained by cholesterol lowering impact. The protective effect may be attributable to folate, vitamin E, and phytochemicals available in cereal grains.

#### **2.4.2 Whole grains and cancer**

Case control studies have substantiated that the consumption of whole grains has an inverse association with endometrial cancer, the fourth most common cancer among women in the USA and Canada (Kasum *et al.*, 2001). A great body of evidence indicates that cereal dietary fibre protects against colon cancer. Several mechanisms have been proposed to explain the effect and these include increased fecal bulk and decreased transit time that lower the potential of fecal mutagens to interact with intestinal epithelium (Andlauer and Fürst, 1999). Cereal fibre has been shown to lower fecal secondary bile acid concentration, which is presumed to be a cell proliferative agent. Another mechanism suggests that fibre protects against carcinogenesis through physical binding of mutagens and carcinogens (Andlauer and Fürst, 1999).

Lignans and isoflavones present in whole grains undergo enzymatic metabolic conversion in the digestive system, converting them to heterocyclic phenols that bear structures very similar to estrogen. They offer weak estrogenic and antiestrogenic effects depending on the concentration of circulating endogenous estrogens and estrogen receptors (Brezinski and Debi, 1999).

Certain hormone-related diseases such as breast cancer are associated with high levels of free circulating hormones. Whole grain consumption is associated with high excretion of sex hormones in the feces, leading to low levels of circulating hormones such as estrogen (plasma, estrone, estradiol) (Englyst and Kingman, 1990). Additionally, a high-fibre diet increases sex hormone binding globulins (SHBG), thus binding circulating estrogens and androgens, leaving low levels of hormone in the free form leading to a lower incidence of hormone-related diseases (Thompson, 1994).

### **2.4.3 Whole grains and diabetes**

Epidemiological studies have consistently indicated the inverse relationship of whole grain consumption with the risk of type 2 diabetes mellitus (Montonen *et al.*, 2003). Dietary fibre, vitamins B and E, and minerals may contribute to the protective effect in reducing type 2 diabetes through lowering the concentration of homocysteine (Boushey *et al.*, 1995). Additionally, whole grain consumption is inversely related to body mass index (BMI), waist circumference, and fasting insulin levels (Meyer *et al.*, 2000). Increased BMI is a leading factor in poor insulin resistance, a precursor for type 2-diabetes (Steffen *et al.*, 2003). Fibre may regulate body weight through its intrinsic effects and hormonal responses (Koh-Banerjee and Rimm, 2003). High fibre-diets promote satiety while providing low energy (Pereira and Ludwig, 2001). It has been shown that viscous soluble dietary fibre reduces the rate of absorption of simple carbohydrates through the gut and flattens the postprandial glycemia, which is a key factor in controlling diabetes (Landin *et al.*, 1992).

Until a decade ago, fibre was the major component in grains contributing to protective effects against diseases. The focus has now been shifted to whole grains as a rich source of other beneficial compounds besides fibre. There is increasing evidence indicating that phytochemicals such as phytates, lignans, tocopherols, and other polyphenolic compounds contribute to the disease preventing properties of whole-grains, partly due to their antioxidant properties (Kennedy and Knill, 2000). The antioxidant properties of cereals will be discussed in great detail in a later section.

## **2.5 Functional foods and plant bioactives**

The concept of functional foods includes food or food ingredients that exert a beneficial effect on health and/or reduce the risk of chronic diseases beyond basic nutritional functions. The interest in developing functional foods is thriving, driven largely by the market potential for foods that can improve the health and well being of consumers.

There are number of factors associated with dietary habits contributing to chronic diseases such as obesity, CVD and cancer. High-energy intake, smoked or salted products and consumption of saturated fats in large quantities are among the factors involved. On the other hand, some dietary habits are beneficial and contribute to mitigating some of these disease conditions. This fact has helped in the development of the health and functional food concept.

### **2.5.1 Cereals as a functional food**

Due to the multiple beneficial effects of cereals, they have a great potential as functional foods. Cereals can further be exploited in a number of ways leading to the

designing of novel food products that can target the functional food market. Some of the beneficial effects of cereals were discussed in the previous sections. Besides, non-digestible carbohydrates can be used to stimulate the growth of *Lactobacilli* and *Bifidobacteria* present in the colon as prebiotics (Charalambopoulos *et al.*, 2002). Water soluble fibre such as  $\beta$ -glucan, oligosaccharides, such as galacto- and fructo-oligosaccharides and resistant starch, have been suggested to fulfill the prebiotic concept (Charalambopoulos *et al.*, 2002). Cereals contain a wide array of bioactives including dietary fibre, minerals, phytoestrogens, lignans and other polyphenolic compounds. Among the bioactives present in cereals, much attention has been focused on antioxidative compounds.

## 2.6 Free radicals

Any species capable of independent existence that carries one or more unpaired electrons can be defined as a free radical. Superoxide radical ( $O_2^{\bullet}$ ), hydroxyl radical ( $^{\bullet}OH$ ), thiyl radical ( $RS^{\bullet}$ ), peroxy radical ( $ROO^{\bullet}$ ), alkoxy radical ( $RO^{\bullet}$ ), and nitrogen oxide radicals ( $NO^{\bullet}$ ,  $NOO^{\bullet}$ ) are some of the free radicals of biological importance. Free radicals are highly reactive and capable of generating new free radicals upon contact with non-radical species. Hence, the formation of reactive radicals *in vivo* is likely to set off free radical chain reactions (Halliwell *et al.*, 1995a). Radicals join their unpaired electrons in order to form covalent bonds.

Most organic and biological molecules contain even numbers of electrons that fill the available energy levels in pairs. The Pauli Exclusion Principle forbids any two electrons in a molecule to have exactly the same energy. Free radicals, on the other hand,

are molecules or part of molecules that carry an odd number of electrons in place of normal covalent chemical bonds consisting of pairs of valence electrons. Since the spin of an unpaired electron of a free radical is not mutually compensated by an orbital partner, the whole free radical molecule or molecule fragment will carry an uncancelled electron spin magnetic moment. Therefore, such molecules possess a non-quantized value of net electronic magnetic moment and hence are called paramagnetic species (Brog, 1993).

Although transitional metal ions are paramagnetic, strictly speaking, they are not considered free radicals as the unpaired electron in transitional metal ions is in the inner shell while the unpaired electron of free radicals exists in the outer shells (Brog, 1993). Since free radicals have unpaired electrons that are easily lost or paired up, they are highly reactive and therefore, short-lived. Although this holds true for most biomedically important free radicals, there are substantially unreactive free radicals as well i.e. ascorbyl, tocopheryl, and phenolic radicals. Steric protection of the paramagnetic centre by bulky groups is one way of imparting stability to free radicals (Brog, 1993).

### **2.6.1 Oxygen metabolism in organisms**

Each oxygen atom has one unpaired electron in its outer valence shell while molecular oxygen has two unpaired electrons. Thus, the oxygen atom is a free radical while molecular oxygen is a biradical. Tetravalent reduction of oxygen by the mitochondrial electron transport system (ETS) to produce water is considered to be a safe process, however, the reductive environment of the cellular milieu provides an opportunity for oxygen to undergo unscheduled reduction leading to the generation of a superoxide radicals,  $H_2O_2$  and hydroxyl radicals as common by-products (Davies, 1995).

The radical nature of oxygen permits some important reactions. In a non-enzymic, univalent, reduction pathway, oxygen can undergo four successive one-electron reactions (Davies, 1995).

Humans and other aerobic organisms are capable of tolerating oxygen as these organisms have evolved with ETS to utilize oxygen, and an antioxidant defence to protect against the toxic effects of oxygen (Aruoma, 1998). The fact that hydroxyl radicals and  $H_2O_2$  are generated as a result of regular metabolism was first reported approximately 55 years ago. During the early 1980s there was an explosion of research carried out in the area of free radical biology.

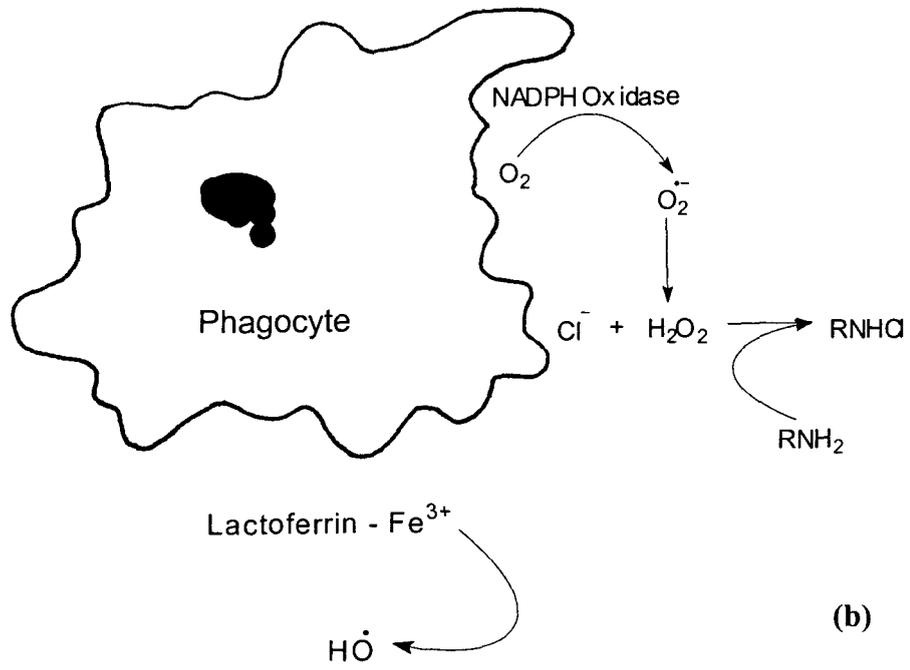
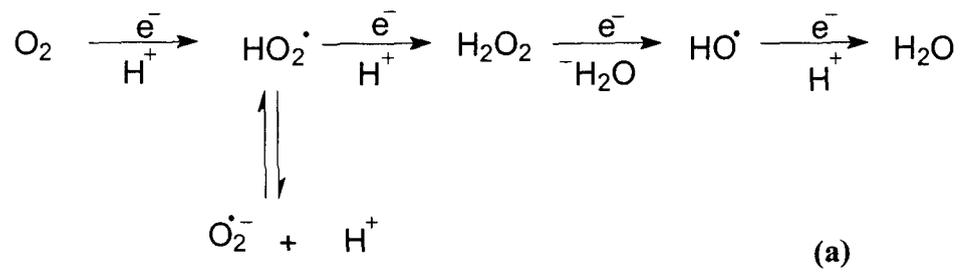
### **2.6.2 Reactive oxygen species (ROS)**

Recently, much interest has been focused on the oxygen-centred radicals such as superoxide and hydroxyl radicals. The term 'reactive oxygen species', widely used in the scientific literature encompasses oxygen-centred radicals and other related non-radical species such as singlet oxygen ( $^1O_2$ ), and hydrogen peroxide ( $H_2O_2$ ). These non-radical species constantly participate in free radical reactions mainly by generating new free radicals. ROS are constantly generated in the human body and have been implicated in the ethiology of several diseases, including cancer, atherosclerosis, malaria, rheumatoid arthritis (Aruoma, 1998), and a wide array of neurodegenerative diseases, including Alzheimers, Schizophrenia, Parkinson's, Huntington's Chorea, Tardine, and Dyskinesia (Armstrong, 1998).

ROS are basically generated in two ways: accidental generation, which encompasses leakage of electrons onto oxygen from the mitochondrial electron transport chain, microsomal cytochromes P450 and electron-donating enzymes (Halliwell and Gutteridge, 1989), and deliberate synthesis which includes production of ROS for useful biological purposes such as phagocytosis, intercellular signaling, and cell growth regulation (Mier *et al.*, 1990). **Figures 2.2 (a) and (b)** illustrate a schematic of production of superoxide radical and H<sub>2</sub>O<sub>2</sub> during ETS and phagocytosis, respectively. Superoxide radical and H<sub>2</sub>O<sub>2</sub> produced by phagocytes are utilized to kill microorganisms in the body while the superoxide radical may also serve as an intercellular signaling agent. In order to deal with these toxic ROS, all aerobic living organisms synthesize a series of antioxidant enzymes whose role is to intercept and inactivate ROS or garner from their surroundings a variety of water- and lipid-soluble antioxidants. Although very important, the antioxidative enzyme systems and antioxidants are not capable of completely preventing the damage caused by ROS left unintercepted by the above mechanisms. Therefore, a series of damage repair systems are in existence in aerobic organisms (Davies, 1995). All organisms are exposed to hydroxyl radical, which is generated by homolytic fission of O-H bonds in water. Hydroxyl radical is one of the most reactive ROS that reacts with any biological molecule with a second order rate constant of 10<sup>9</sup> to 10<sup>10</sup> M/s (Halliwell *et al.*, 1995a). The damage caused by hydroxyl radical is unavoidable and is dealt with by a repair process.

Reactive nitrogen species (RNS) also play an important role in the etiology of a number of disease conditions. Nitric oxide (NO<sup>•</sup>) radical is a widely considered endothelium-derived relaxing factor (EDRF) produced by vascular endothelium.

**Figure 2.2** Production of superoxide anion and hydroxyl radicals during electron transport system (ETS) **(a)**, and phagocytosis **(b)**.  
*Adapted from Grisham (1992) and Hiramatsu et al. (1997).*

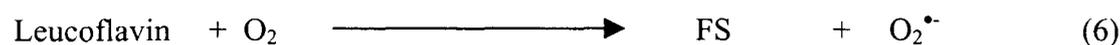
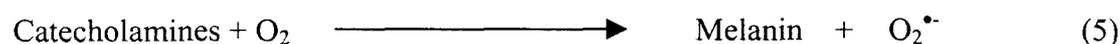
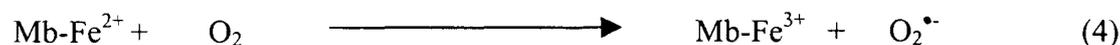
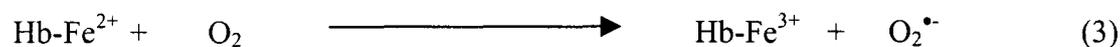
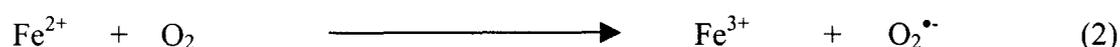
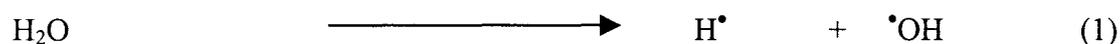


The role of the nitric oxide radical has been demonstrated in malaria, CVD, cancer and neurodegenerative diseases (Aruoma, 1998). The reaction between  $\text{NO}^\bullet$  and  $\text{O}_2^{\bullet-}$  leads to oxidative damage of DNA owing to the formation of  $\text{ONOO}^-$ , an extremely reactive species (Douki and Cadet, 1996).

### 2.6.3 Sources of reactive oxygen species

Biological systems encounter ROS generated from various types of endogenous as well as exogenous sources including external radiation, mitochondrial metabolism, phagocytosis, and by the action of xanthine oxidase as well as the microsomal mixed function oxidase (MFO) system (Grisham, 1992).

Exposure of an aqueous solution to radiation generates a number of radical species instantaneously following exposure (Schutte-Frohlinde and Von Sonntag, 1985). Excited water also produces  $\text{H}^\bullet$  and  $^\bullet\text{OH}$  as indicated in equation 1. Exposure to radiation for  $10^{-10} - 10^{-13}$  s is sufficient to cause free radical reaction, molecular alterations and formation of ROS (Kale, 2003). The following equations (1-6) illustrate the action of some nonenzymic endogenous sources of free radicals in biological systems:



*Hb – haemoglobin, Mb – myoglobin FS - Flavin semiquinone (Adapted from Grisham, 1992).*

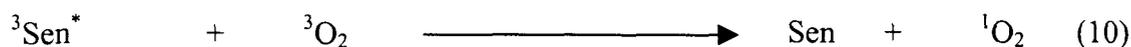
The MFO system generates  $O_2^{\bullet-}$  and  $H_2O_2$  during the metabolism of certain drugs and xenobiotics (Cadenas, 1985). It also activates triplet state oxygen to  $O_2^{\bullet-}$  under hyperoxic (elevated oxygen concentration) conditions. Interaction of ligands such as immune complexes with phagocytes through the action of nicotinamide adenine diphosphate (NADPH) oxidase leads to the generation of a large volume of  $O_2^{\bullet-}$ ,  $H_2O_2$  and  $\bullet OH$  (Grisham, 1992) (**Figure 2b**). Another source of ROS in biological systems is haemoprotein peroxidases in exocrine secretions such as milk, tears, and saliva, as well as in thyroid tissue and leukocytes. The enzyme catalyzes the oxidation of an electron donor leading to the generation of radicals (Grisham, 1992).

The xanthine oxidoreductase (XOR) system, which consists of xanthine dehydrogenase (XDH) and xanthine oxidase (XO), is another source of free radicals in biological systems. XOR is predominantly present in normal tissues as XDH and converts into free radical generating XO in the damaged tissues (Kale, 2003). XO uses molecular oxygen as an electron acceptor to produce  $O_2^{\bullet-}$  which may subsequently convert into a highly cytotoxic hydroxyl radical via Harber-weiss/Fenton reactions, or nitro peroxyxynitrite ( $ONOO^-$ ) through interaction with nitric oxide radicals.

Ingold *et al.* (1997) indicated that molecular oxygen reacts with the decomposition products of azo ( $X-N=N-Y$ ) compounds such as food colourants to generate superoxide radicals. Furthermore, photoactivation of haematoporphyrin is known to produce superoxide radicals. Light energy converts ground state tetrapyrroles (TPR) to their excited singlet ( $^1TPR^*$ ) state which subsequently becomes the excited triplet state ( $^3TPR^*$ ) upon releasing some of its energy.  $^3TPR^*$  reacts with molecular oxygen producing the superoxide radical (Buettner and Oberley, 1980; Haseloff and

Ebert, 1989) (equations 7 and 8). The superoxide radical can also be generated via the action of  $\gamma$ -radiation, a pulsed electric field, and microwave and ohmic heating of foods (Choe and Min, 2006).

$H_2O_2$  is formed by oxidases of xanthine, urate, and amino acids (Choe and Min, 2006). In the presence of sensitizers such as chlorophyll, methylene blue, eosin, curcumin, haemoporphyrin, and riboflavin molecules, oxygen can become singlet oxygen. The ground state sensitizer ( $^1Sen$ ) becomes excited ( $^1Sen^*$ ) upon exposure to light of a specific wavelength. The  $^1Sen^*$  returns to ground state via emitting light, fluorescence, or heat through internal conversion or intersystem crossing (Choe and Min, 2006). The intersystem crossing can lead to the production of excited triplet sensitizer ( $^3Sen^*$ ), which can abstract an electron or H atom from food constituents converting them to free radicals (equations 9 and 10). Alternatively,  $^3Sen^*$  can transfer energy to molecular oxygen to produce singlet oxygen (Choe and Min, 2006).



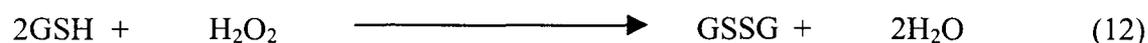
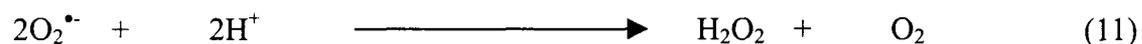
#### 2.6.4 Oxidative stress

While all aerobic organisms have to tolerate the background production of radicals, aerobic cells become exposed to a major risk if the production of radicals increases above a threshold level that may lead to toxicity (Roberfoid and Calderon,

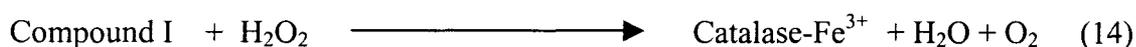
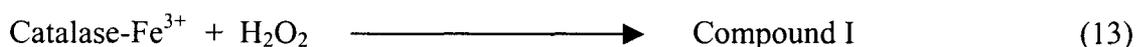
1995). Excessive ROS production leads to the so called ‘oxidative stress’ state. Some drugs, organic solvents such as CCl<sub>4</sub> (Brent and Rumack, 1993), pesticides such as paraquat (Cross *et al.*, 1993), and smoking (Halliwell and Gutteridge, 1989) also lead to oxidative stress due to production of large amounts of superoxide radical and H<sub>2</sub>O<sub>2</sub>. The intracellular generation of ROS can damage the cellular components such as enzymes, proteins, lipids, nucleic acids, and membranes, thus leading to organ dysfunction, tissue damage, cancer, degenerative disease and aging (Tarr and Samson, 1993).

### 2.6.5 Antioxidant defence system

The antioxidant enzyme system in living organisms protects the organism against unwanted ROS, mainly superoxide and H<sub>2</sub>O<sub>2</sub>. Superoxide dismutase (SOD), glutathione peroxidase (GSHPX), and glutathione reductase are the major antioxidant enzymes in organisms. In mammalian cells, there are two different isozymes of superoxide dismutase located in different subcellular compartments. Cytoplasm contains Cu-Zn SOD, which contains Cu and Zn while mitochondria have Mn containing SOD. Superoxide dismutase located in mitochondria removes superoxide radicals by accelerating their conversion to H<sub>2</sub>O<sub>2</sub> as indicated in equation 11. Some nutritional antioxidants such as α-tocopherol are associated with increasing the activity of SOD (Huang *et al.*, 1999).



H<sub>2</sub>O<sub>2</sub> generated by the reaction is converted to water by the action of catalases in peroxisomes and GSHPX (Halliwell *et al.*, 1995a). GSHPX removes H<sub>2</sub>O<sub>2</sub> by utilizing it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). GSH is responsible for the decomposition of peroxides using reduced glutathione (GSH) as the hydrogen donor. GSH is regenerated via the action of glutathione reductase, thus the ratio of GSH/GSSG is maintained at a high level to combat oxidative stress (Grisham, 1992). Haem containing catalase is present in almost all tissues especially in the liver and erythrocytes. Catalase catalyzes the decomposition of H<sub>2</sub>O<sub>2</sub> as summarized in equations 13 and 14,



where, compound I is an intermediate product that represents a haemoprotein associated free radical capable of oxidizing electron-donating substrates (Grisham, 1992). H<sub>2</sub>O<sub>2</sub> is converted to an extremely reactive hydroxyl radical through Fenton like reactions as follows.



In addition to the antioxidant defense enzymes, living organisms contain a variety of radical scavenging antioxidants, including  $\alpha$ -tocopherol, ascorbic acid, and uric acid (Halliwell *et al.*, 1995). It is accepted that enzymatic antioxidants are concentrated in the intracellular environment while non-enzymic antioxidants are found in higher

concentrations in extracellular fluids such as lymph and plasma (Grisham, 1992). Tissues such as liver, spleen and kidney contain relatively high levels of antioxidant enzymes whereas heart, brain and skeletal muscle contain small amounts (Grisham, 1992). Table 2.2 lists some nonenzyme antioxidants associated with extracellular fluids along with their properties.

## **2.6.6 Reactive oxygen species and human diseases**

### **2.6.6.1 Ischemia/reperfusion injury**

Ischemia, reduction of blood supply to tissues is a condition commonly caused by several conditions including atherosclerosis, thrombosis, and thrombo embolism (Knight, 1999). Production of large concentrations of free radicals has been postulated as one of the factors that lead to ischemic tissues (Knight, 1999). XDH catalyzes the conversion of hypoxanthine to xanthine, which in turn produces superoxide radical. Superoxide radical produced by xanthine reportedly causes ischemia (Knight, 1999). Once formed,  $O_2^{\bullet-}$  is converted to hydrogen peroxide and then inactivated by GSHPX or catalase (equations 13 and 14). However, in the presence of  $Fe^{2+}$ ,  $H_2O_2$  can be converted to hydroxyl radical. Xanthine oxidase plays a key role in releasing  $Fe^{2+}$  from ferritin and thereby increasing the risk of hydroxyl radical damage (Biemond *et al.*, 1986). Besides, there are several other possible sources of ROS in ischemia/reperfusion. Membrane-bound arachidonic acid released by various phospholipases results in free radical generation through fatty acids (Hammond *et al.*, 1985) which leads to ischemic injury (Knight, 1999). Additionally, nitric oxide radicals normally produced by endothelial cells, macrophages

**Table 2.2** Some nonenzymic antioxidants associated with extracellular fluids, their mechanisms of action, and solubility.

Antioxidant	Mechanism	Solubility
Ascorbic acid	FR and HOCl scavenger	Water soluble
Uric acid	FR scavenger + Fe chelator	Water soluble
Sulphydryl groups	FR + HOCl scavenger	Water soluble
Pyruvate	H <sub>2</sub> O <sub>2</sub>	Water soluble
$\alpha$ -Tocopherol	FR scavenger	Lipid soluble
Bilirubin	FR scavenger	Lipid soluble
Ubiquinol-10	FR scavenger	Lipid soluble
Transferrin	Fe chelator	Protein
Hemopexin	Binds free haeme	Protein
$\beta$ -Carotene	FR + singlet oxygen scavenger	Lipid soluble

*Adapted from Grisham 1992 and Halliwell and Gutteridge 1999; FR – free radical.*

via L-arginine oxidation is implicated in contributing to ischemia/reperfusion injury (Knight, 1999).

The central nervous system (CNS) contains the highest amount of polyunsaturated fatty acids (PUFA), thus it is highly susceptible to haemoprotein or metal-catalyzed oxidative reaction (Grisham, 1992). Post-haemorrhagic CNS dysfunction is believed to be caused by continuous generation of ROS (Sadzadeh and Eaton, 1988). The retina which contains nearly 50% PUFA is equally susceptible to oxidative damage caused by ROS. This situation is worsened by the fact that the retina maintains a very high rate of oxidative metabolism (Grisham, 1992) and a high concentration of ascorbate which assists in reducing  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  as shown in equation 17.



## 2.7 Lipid oxidation

Lipids, nucleic acids, enzymes and proteins, among others, are important target molecules for biological damage caused by ROS. In particular, PUFA in biological membranes are highly vulnerable to attack by ROS leading to lipid peroxidation through chain reactions. Peroxidation can be initiated by any chemical species that has sufficient energy to abstract a hydrogen atom from a methylene carbon in unsaturated fatty acids. Autoxidation of unsaturated fatty acids received much attention in the 20<sup>th</sup> century as it is associated with rancidity of foods and stability of lipids in biological systems. The classical lipid oxidation scheme explains that lipid oxidation occurs through chain

reactions. and proceeds through three steps; initiation, propagation and termination (Labuza, 1971).

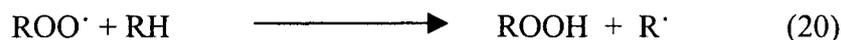
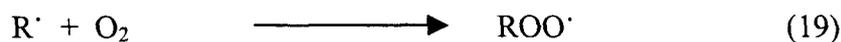
### 2.7.1 Lipid oxidation pathways

Autoxidation is a natural process that takes place between molecular oxygen and unsaturated lipids in the environment. PUFA have the potential for decomposition via autoxidation; these could be in the form of free fatty acids, triacylglycerols or phospholipids. An alternative pathway leading to lipid oxidation is photooxidation through excitation of lipids or oxygen in the presence of light and a sensitizer (Gordon, 2001). The main steps of lipid oxidation are as follows;

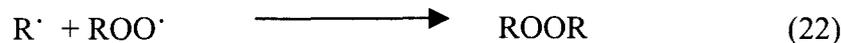
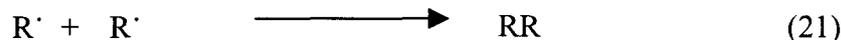
#### *Initiation*



#### *Propagation*



#### *Termination*



In the initiation step (equation 18), the free radical is formed from an unsaturated lipid molecule (RH) at an allylic methylene group, or a hydroperoxide (ROOH) by the action of an initiator. The activation energy of the reaction indicated in equation 18 ranges from 30-40 kCal/mol (Uri, 1961). This situation is thermodynamically unfavourable and therefore, the involvement of initiators is essential in order to overcome the energy barrier. Heaton and Uri (1961) postulated that the initial molecular oxygen reaction requires metal ion interaction. While it is well established that lipid oxidation occurs via chain reactions, the exact origin of the initial free radical is not known (St. Angelo, 1996). Although the source of the initial radical remains controversial, it is well established that the factors involved, including high temperature, the presence of singlet oxygen, photosensitizers, superoxide radicals, and radiation contribute to the activation of the initiation process (St. Angelo, 1996).

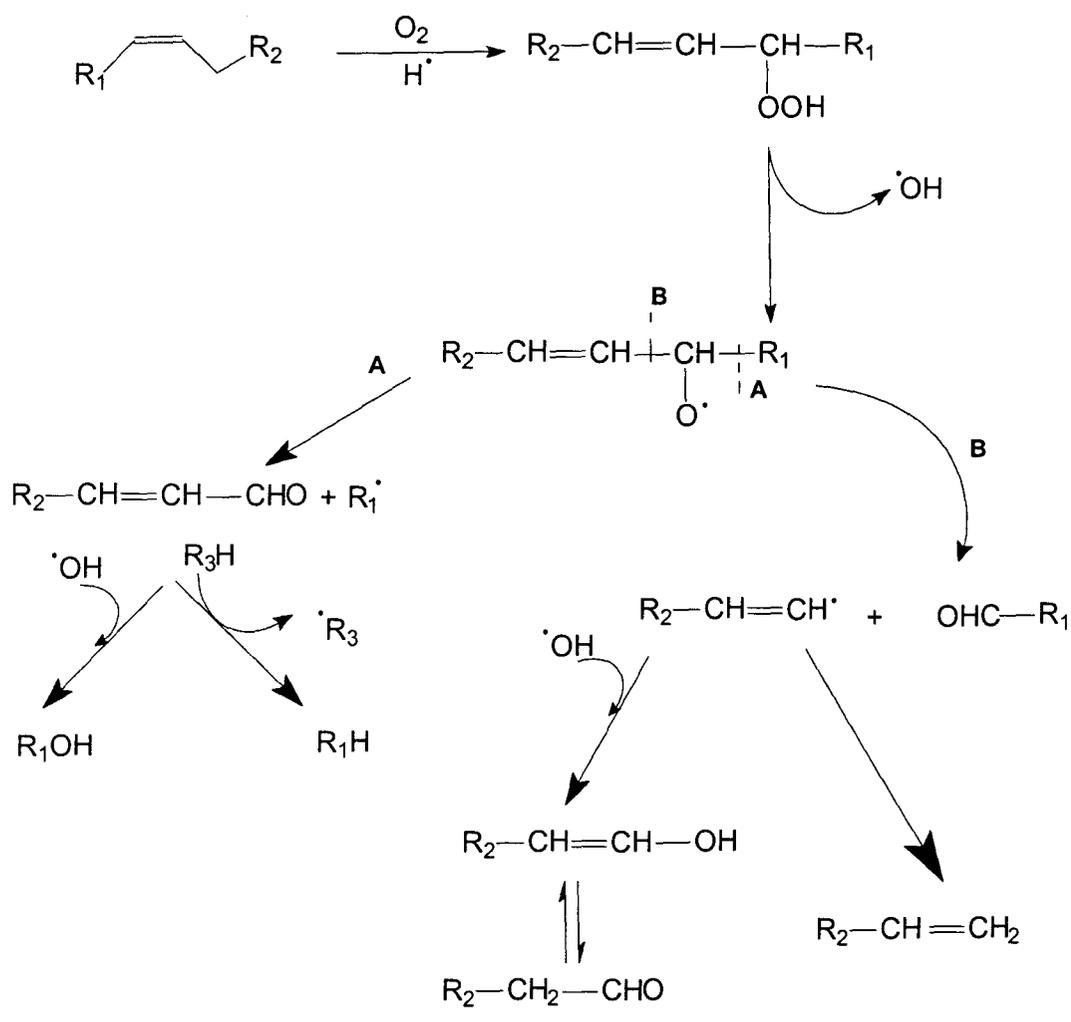
The reaction of a lipid with molecular oxygen, excited singlet oxygen state ( $^1\text{O}_2$ ), metal catalysts or by exposure to light can lead to the formation of lipid peroxy radicals (Shahidi and Wanasundara, 1992). In step 2 (equation 19), the free radical react with molecular oxygen to form peroxy radicals ( $\text{ROO}^\bullet$ ), which subsequently react with a fresh lipid molecule. After initiation, propagation reactions (equations 19 and 20) occur in which one lipid radical is converted into a different lipid radical. These reactions commonly involve abstraction of a hydrogen atom from a fresh lipid molecule or addition of oxygen to an alkyl radical. The enthalpy of this reaction is relatively low compared to that of the initiation reaction, so propagation reactions occur rapidly compared to initiation reactions. The oxygenation reaction is very rapid, having almost zero activation energy, therefore, the concentration of peroxy radical ( $\text{ROO}^\bullet$ ) is much higher than that of

alkyl radical ( $R^\bullet$ ) in foods in which oxygen is present (Shahidi and Wanasundara, 1992). Abstraction of hydrogen takes place preferentially at carbon atoms where the bond dissociation energy is low. Since the dissociation energy of the C-H bond is reduced by neighbouring alkene functionality, abstraction of hydrogen takes place most rapidly at the methylene group between two alkene groups *i.e.* diallylmethylene in a PUFA. The reaction is propagated by further abstraction of hydrogen atoms from other PUFA. During the formation of hydroperoxides, a shift in the position of the double bond could occur in order to take advantage of the resultant resonance stabilization. The radical formed initially from PUFA is delocalized across 5 carbon atoms of the 1,4-pentadiene moiety, and the reaction with oxygen occurs preferentially by addition at one of the end carbons of this structure (Gordon, 2001).

In the termination step (equations 21-23), two radicals react with one another to yield a product that does not sustain the propagation phase. Termination is also possible in the presence of antioxidants that possess free radical scavenging activity (St. Angelo, 1996). Although hydroperoxides produced (equation 20) are more stable than radical species, they are still weak oxidizing agents that themselves decompose to peroxy and alkoxy radicals, leading to secondary oxidation products, including aldehydes, ketones, alcohols, acids, and lactones that are no longer bound to the glycerol backbone (Benzie, 1996) (**Figure 2.3**). These secondary oxidation products are responsible for impaired taste, flavour, and texture of food and a number of deleterious reactions in biological systems (Kamal-Eldin *et al.*, 2003).

Once the first hydroperoxides are formed, in a medium containing PUFA and/or other oxidizable substrate, they increase the rate of initiation tremendously due to the

**Figure 2.3** Formation of secondary by products of lipid oxidation.  
*Adapted from Choe and Min (2006).*



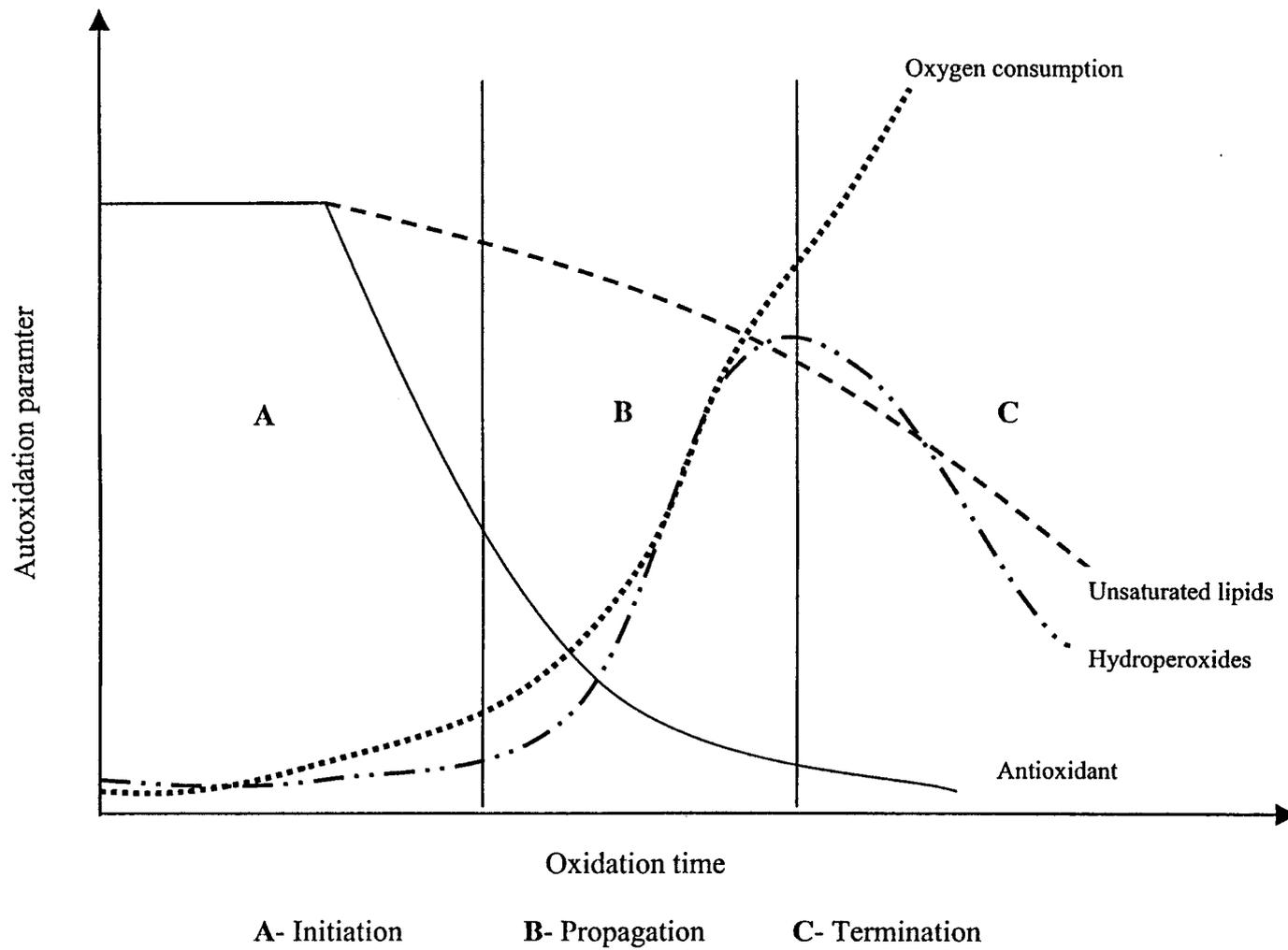
generation of re-initiating radicals by monomolecular and bimolecular hydroperoxide decomposition (Labuza, 1971). Hydroperoxide decomposition is believed to be responsible for the linear and exponential parts of the reaction (Figure 2.4). The lowest ROOH concentration limit that causes significant catalytic initiation is as low as  $10^{-6}$  M, corresponding to a peroxide value (PV) of 1-2 meq/kg (Knorre et al., 1957).

Abstraction of an H atom from a fresh lipid molecule (equation 18) is the slowest step in chain propagation (Kamal-Eldin, 2003). The susceptibility of fatty acids to H abstraction is dependent on the number of bis-allylic methylenes present in the fatty acid. In addition, the supermolecular orientation of ROOH also plays a significant role (Kamal-Eldin, 2003).

Hydroperoxides are fairly stable under favourable conditions such as low temperature, dilute concentrations and in the presence of antioxidants and the absence of prooxidants (Kamal-Eldin, 2003). However, they are easily broken down as explained earlier. Once ROOH concentrations reach a critical value, their decomposition starts and the rate of lipid oxidation continues to increase (**Figure 2.4**).

In the last stage of autoxidation,  $\text{ROO}^\bullet$  starts forming adducts. The combination of  $\text{ROO}^\bullet$  with other radicals yield ROOR (equation 23) which, supported by the Russel mechanism, is believed to be a termination step. However, Kamal-Eldin (2003) indicated that the major products of this reaction were  $\text{RO}^\bullet$ , which can undergo very fast rearrangement to form epoxyallyl radicals. Epoxyallyl radicals capture oxygen from epoxyperoxyl radicals (Gardner, 1991).

**Figure 2.4** Kinetic curves of autoxidation of polyunsaturated fatty acids (PUFA).  
*Adapted from Labuza (1971) and Kamal Eldin et al. (2003).*



### 2.7.2 Effect of lipid oxidation on lipid quality

ROS are mainly responsible for the initiation of oxidation reactions in foods. The ROO<sup>•</sup> reacts with lipids, proteins, sugars, and vitamins, producing undesirable changes in foods such as generation of volatile and non-volatile compounds, and deterioration of the nutritive value of foods by destroying essential fatty acids, and amino acids as well as vitamins, and the generation of potential mutagens and carcinogens (Choe and Min, 2006).

Hydroperoxides are non-volatile and odourless, but are relatively unstable and hence decompose either spontaneously or catalytically into a wide range of aroma compounds. The nature of the off-flavours developed depends on the fatty acid composition of the food (Gordon, 2001). Besides the development of rancid flavours, oxidative deterioration of lipid leads to bleaching of pigments such as carotenoids. Free radicals generated during the lipid oxidation process combine with vitamins, thus leading to a reduction in their nutritive value (Gordon, 2003).

Radical reactions of proteins, promoted by radicals generated during lipid oxidation, may lead to protein-protein or protein-lipid cross-linking, protein scission, and oxidation (Gardner, 1983). Free radical reactions involve hydrogen abstraction followed by  $\beta$ -scission of amino acid oxy radical addition (Genot *et al.*, 2003). Histidine, cysteine, methionine, lysine, tyrosine, and tryptophan are the most labile amino acids for free radical reaction (Gardner, 1979). Modification of proteins upon lipid oxidation leads to altered functionality loss of nutritive value, and destabilization of emulsions, among other deleterious effects.

### 2.7.3 Factors affecting lipid oxidation

The extent to which oxidation of fatty acids and their esters occurs in food depends on the chemical structure of the fatty acids involved and minor constituents present in the oxidizing system as well as environmental conditions where the food is being processed or stored. Oxygen pressure, temperature, irradiation, micro components such as free fatty acids, hydroperoxides, lipid hydroxyl compounds and transient-valency metal ions play a major role in lipid oxidation. Knowledge of the factors affecting lipid oxidation helps in adopting strategies to mitigate its occurrence.

#### 2.7.3.1 Role of transient-valency metal ions

All materials of biological origin contain small amounts of transitional metal ions such as those of Fe, Cu, Co, Mn, Zn, and Ni that cannot be completely eliminated during processing. Two mechanisms of oxidation promotion by metals have been proposed. Metals are believed to either interact with hydroperoxides or to react directly with lipid molecules producing lipid radicals. Transitional metal ions in their lower valence state ( $M^{n+}$ ) react very quickly with ROOH by acting as electron donors converting ROOH to  $RO^\bullet$  (equation 25). Oxidized metal ions are slowly reduced by the action of ROOH as indicated in equation 23. Also, metals can abstract H from fatty acids (equation 27).



It has been suggested that oxidative deterioration of the lipids in meat is caused by catalysis of haem compounds such as metmyoglobin (Kwoh, 1971). These metal-containing compounds catalyze hydroperoxide decomposition, thus producing free radicals, which initiate further chain reactions (Pokorny, 1987). The redox-active transition metals transfer single electrons during changes in oxidation states (Reische *et al.*, 2002). Even trace amounts of transition metal ions can promote electron transfer from lipids or hydroperoxides as the above reactions are cyclical, generating a lower valence state of metal ions (Reische *et al.*, 2002). However, it is not clear whether redox-active transition metals promote lipid peroxidation directly through the formation of metal-lipid complexes or by forming peroxy and oxy radicals (Reische *et al.*, 2002).

Ferrous ions in aerobic aqueous solutions can produce superoxide, hydrogen peroxide and hydroxyl radicals by Fenton reactions (Reactions 28 through 31). These reactions can be cycled either by superoxide radical (Harber-Weiss) or by reducing agents such as thiols or ascorbic acid (Redox-cycle). Ferrous ions can stimulate lipid peroxidation by generating hydroxyl radical from hydrogen peroxide but also by the breakdown of preformed lipid peroxides to form alkoxy radical (RO $\cdot$ ) (Reaction 31).



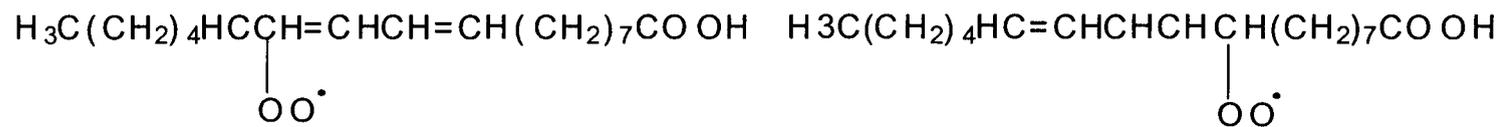
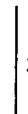
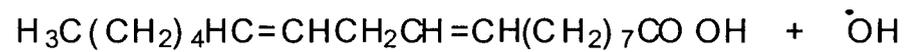
### 2.7.3.2 Role of oxygen species in lipid oxidation

PUFA are easily oxidized by ROS. Hydroperoxy radicals ( $\text{HOO}^\bullet$ ) can abstract hydrogen from lipid, thus producing a lipid radical  $\text{R}^\bullet$ . Linoleic, linolenic and arachidonic acids react with  $\text{HOO}^\bullet$  at a fast rate while oleic acid does not react.  $\text{HOO}^\bullet$  can also abstract hydrogen from lipid hydroperoxides producing peroxy radicals (**Figure 2.5**). Hydroperoxy radicals can also abstract H from lipid hydroperoxides and produce peroxy radicals. ROS with higher reduction potentials possess greater oxidizing capacity. Lipid peroxy radicals are good oxidizing agents with 1000 mv of reduction potential, which can abstract  $\text{H}^\bullet$  from fresh PUFA. Aromatic peroxy radicals are less reactive than the straight chain peroxy radicals due to delocalization of electrons in the aromatic ring (Choe and Min, 2006).

The hydroxyl radical with 2300 mv reduction potential is a strong electrophilic radical species that may be added onto double bonds of PUFA or it may abstract hydrogen from lipids (Lee *et al.*, 2004). The hydroxyl radical is mainly responsible for the initiation of lipid oxidation. The lipid radicals generated by the action of  $^\bullet\text{OH}$  react rapidly with triplet ground state oxygen producing  $\text{RO}^\bullet$  and  $\text{ROO}^\bullet$  as shown in equations 32 and 33.



**Figure 2.5** Involvement of hydroxyl radical in oxidation of linoleic acid.  
*Adapted from Choe and Min (2006).*



H<sub>2</sub>O<sub>2</sub> with a reduction potential of 320 mv (lower than the reduction potential of PUFA, 600 mv) is not directly involve in lipid oxidation. However, H<sub>2</sub>O<sub>2</sub> indirectly takes part in lipid oxidation by generating <sup>•</sup>OH (Choe and Min, 2006). Superoxide radical with 940 mv is not strong enough to abstract H<sup>•</sup> from unsaturated fatty acids (Bielski *et al.*, 1983).

#### **2.7.3.3 Role of water activity in lipid oxidation**

The role of water activity in lipid peroxidation has been examined extensively. Water activity has been manipulated as a means to control lipid oxidation in susceptible food products and to explain the relationship between lipid oxidation rates and moisture content. As water activity is decreased from 1, the rate of oxidation initially increases, reaching a maximum at a water activity of 0.6 – 0.8 and then decreases again as water activity range reaches a minimum of 0.3-0.4 and tends to increase afterwards (Kamal Eldin, 2000).

#### **2.7.3.4 Role of enzymes**

Formation of hydroperoxides by lipoxygenase (LOX) is an alternative pathway of lipid oxidation, which requires the presence of free PUFA. LOX occurs naturally in a variety of plants including soybeans, corn, potato, tomato, cucumber, oat and barley seeds. Four isozymes of LOX are found in soybeans (Gordon, 2001). LOX stereospecifically abstracts hydrogen from an active methylene group in the 1,4-pentadiene structure of PUFA. In plant tissues, various other enzymes have been found that cause the conversion of hydroperoxides to other products, some of which are flavour-

active compounds. Hydroperoxide lyase catalyzes formation of aldehydes and oxo acids; peroxygenase and epoxygenase catalyze the formation of epoxy and hydroxy fatty acids; hydroperoxide isomerase catalyzes the formation of epoxyhydroxy fatty acids (Gordon, 2001).

#### **2.7.4 Impact of lipid oxidation on human health**

Interest in the biochemistry of lipid peroxidation products, especially free radicals, has grown exponentially in the last two decades. There is an abundance of evidence that lipid peroxidation products are involved in the pathogenesis of certain human diseases and many tissue injuries. Lipid peroxidation leads to cell damage directly by attacking membrane structure and indirectly by releasing reactive products.

Esterbauer *et al.* (1982) have investigated the effect of free aldehydes resulting from breakdown of hydroperoxides in biological systems. Malonaldehyde (MA), a minor breakdown product of lipid hydroperoxides, originating from fatty acids with at least 3 double bonds, is reported to react with DNA through cross-linking with the amino groups of guanine bases of the DNA molecules (Crawford *et al.*, 1965). Hydroxyalkenals display cytotoxicity and also cytostatic effects in a wide range of cell types, including human diploid fibroblasts, umbilical vein endothelial cells, Chinese hamster ovary cells, hepatoma cells, freshly isolated rat hepatocytes, and erythrocyte tumour cells (Cheeseman, 1993).

### **2.7.5 Inhibiting lipid oxidation**

Optimum oxidative stability can be achieved by minimizing exposure of lipids and lipid-containing foods to air, light, high temperatures, and prooxidants during processing and storage. Deoxygenation, air-tight packaging, microencapsulation, hydrogenation of unsaturated oils, frozen storage, storage in dark containers, removal of metal ions, inactivation of enzymes, and addition of antioxidants are some of the methods widely practiced in the food industry in order to mitigate lipid oxidation of foods. Theoretically, the most elegant way of preventing lipid oxidation in fatty foods is to remove all the available oxygen from food during manufacturing and from the package (Yanishleiva-Maslarova, 2001).

## **2.8 Antioxidants**

Antioxidants can be defined as compounds capable of retarding or preventing the autoxidation process, when present at low concentrations compared to that of an oxidizable substrate (Halliwell and Gutteridge, 1999). Ingold (1968) classified all antioxidants into two groups, namely, primary chain-breaking antioxidants, which directly react with lipid radicals converting them to non-radical products, and secondary or preventive antioxidants.

### **2.8.1 Primary antioxidants**

Primary antioxidants can be defined broadly as the compounds, which can react with lipid radicals to convert them to more stable products. Antioxidants have the ability to donate a hydrogen atom to lipid radicals and neutralize them (equations 34 and 35).



The resulting antioxidant phenoxyl radical ( $\text{A}^\bullet$ ) does not initiate new free radicals and is not subject to rapid oxidation by a chain reaction. Antioxidant radicals may also participate in termination reactions of  $\text{ROO}^\bullet$ ,  $\text{RO}^\bullet$  and other antioxidant radicals, thus preventing propagation of chain reactions.

Reactions between antioxidant radicals and lipid molecules and oxygen (equations 39-41) are exothermic in nature and the activation energy increases with increasing A-H and R-H bond dissociation energy (Shahidi and Wanasudara, 1992). A molecule will be able to act as a primary antioxidant if it is able to donate a hydrogen atom to a lipid radical and if the radical derived from the antioxidant is much more stable than the lipid radical, or is converted to other stable products. Phenol itself is inactive as an antioxidant, but substitution of alkyl groups into the 2, 4 or 6 positions increases the electron density on the hydroxyl group by an inductive effect and this enhances the reaction with lipid radicals (Gordon, 1990).



The presence of bulky substituents in the 2 and 6 positions of the phenol ring also reduces the rate of reaction of the phenol with lipid radicals. The steric effect opposes the increased stabilization of the radical and both effects must be considered in assessing the overall activity of an antioxidant (Gordon, 1990). The introduction of a second hydroxyl or methoxy group at the *ortho*- or *para*- positions of the hydroxy group of a phenol increases its antioxidant activity.

Antioxidants are effective in extending the induction period only when added to unoxidized substrates. Antioxidants are virtually ineffective in retarding a deterioration, which has already begun. The effect of antioxidants depends on several factors including antioxidant structure, oxidation condition and the nature of the sample being oxidized (Gordon, 1990). The effectiveness of an antioxidant depends on the activation energy, rate constants, oxidation-reduction potential, and solubility properties (Nawar, 1996).

### **2.8.2 Secondary antioxidants**

The preventive antioxidants deactivate possible precursors of ROS by functioning as metal chelators, peroxide decomposers, singlet oxygen quenchers, and inhibitors of lipoxygenase and other related enzymes. Metal chelating agents have a significant effect on preventing lipid oxidation. Food lipids generally contain trace amounts of metal ions, which may arise from the presence of metal activated enzymes or their decomposition products (Gordon, 1990). Furthermore, metal ions may be included as contaminants from food processing and refining equipment, and storage vessels. Muscle food products contain a high concentration of iron in association with muscle myoglobin. Transition

metal ions such as Cu, Fe, and Mn reduce the length of the lag time and increase the rate of oxidation of lipids.

Metals act as prooxidants by electron transfer liberating radicals from fatty acids or hydroperoxides (equations 31). Chelation of metal ions by food components reduces the pro-oxidative effects of these ions and raises the energy of activation of the initiation reactions considerably (Gordon, 1999). Ethylenediaminetetraacetic acid, citric acid, polyphosphates, phytates, oxalates, and phosphoric, tartaric acid, malic acids, and phospholipids are some commonly used sequesterants in the food industry. EDTA forms thermodynamically stable complexes with all transition metal ions, thereby making the metal ions unavailable for chemical reactions. Amino acids and peptides also serve as metal chelators (Pokorny, 1987). The metal chelating characteristics of polyphenolic compounds such as flavonoids are also an important factor in their antioxidant activities. Carotenoids such as lycopene, zeaxanthin, lutein, and canthaxanthin quench singlet oxygen by either physical or chemical routes (Yanishlieva-Maslarova, 2001). The neutralization of singlet oxygen by carotenoids is predominantly through physical quenching that involves the transfer of excited energy from singlet oxygen to the carotenoids resulting in the formation of triplet ground state oxygen and triplet excited carotenoid (Stahl and Sies, 1993). Excited carotenoid dissipates absorbed energy through rotational and vibrational interactions converting back to ground state carotenoids (Stahl and Sies, 1993). The protonated phenolic is not a good ligand for metal chelation, but once deprotonated, it serves as a good chelator. Since, the presence of suitable cations, the proton is displaced at physiological pH levels (Hilder *et al.*, 2001).

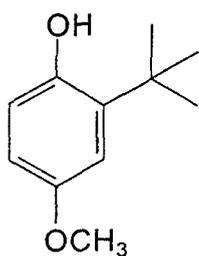
### 2.8.3 Synthetic antioxidants

Many compounds are active as antioxidants, but only a few are used in foods because of strict safety regulations. Most synthetic antioxidants are phenolic derivatives, usually substituted by more than one hydroxy or methoxy groups (Pokorny, 1999). Synthetic food antioxidants currently approved for use in foods are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), dodecyl gallate (DG) and tert-butylhydroquinone (TBHQ). The Food and Drug Administration (FDA) in the USA governs the application of antioxidants in foods and FDA regulations require that antioxidants and their carriers be declared in the ingredient label of the product. **Figure 2.6** illustrates the structure of the frequently used synthetic antioxidants.

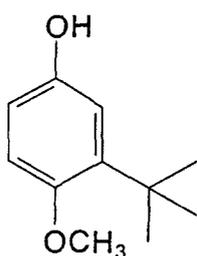
Approximately 40 countries reportedly permit the use of BHT. Food grade BHA, referred to as 2(3)-tert-butyl-4-hydroxyanisole, is generally a mixture of greater than 85% of 3-tert-butyl-4-hydroxyanisole (3-BHA) and 15% or less 2-tert-butyl-4-hydroxyanisole (2-BHA), while food-grade BHT which is 3,5-di-tert-butyl-4-hydroxytoluene is not less than 99% (w/w) pure (Williams *et al.*, 1999). Neither BHA nor its metabolites have shown adduct formation in genotoxicity studies (Williams *et al.*, 1999). BHA is metabolized to tert-butylhydroquinone (TBHQ) and tert-butylquinone (TBQ) in the liver. DNA damage has been reported with TBQ, but not with BHA or TBHQ (Morimoto *et al.*, 1991).

It has been found that BHA at high doses of above 3000 ppm induces forestomach squamous cell carcinoma in rodents, but not glandular cell or other types of neoplasms in the glandular stomach. Humans do not have a forestomach and therefore are less sensitive to exposures to BHA damage than rodents (Williams *et al.*, 1999).

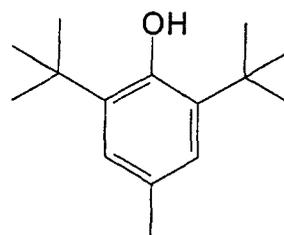
**Figure 2.6** Chemical structures of some of the synthetic antioxidants used for food applications.



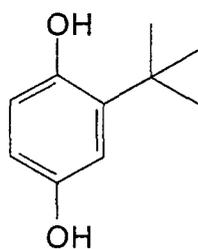
4-methoxy-2-*tert*-butylphenol (2-BHA)



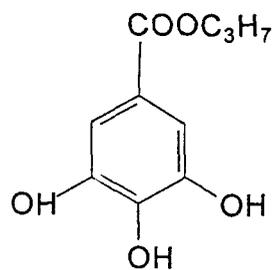
4-methoxy-3-*tert*-butylphenol (3-BHA)



2,6-di-*tert*-butyl-4-methylphenol (BHT)



*tert*-butylhydroquinone (TBHQ)



Propyl gallate (PG)

Moreover, these exposures to humans are well below those producing the epigenetic effects in rodents such as cell proliferation (Williams *et al.*, 1999).

#### **2.8.4 Natural antioxidants**

With mounting evidence on the protective effects of antioxidants *in vivo* against oxidative, stress-induced, degenerative and age-related disease conditions based on epidemiological, experimental, and clinical studies, the importance of antioxidants has received renewed attention. Antioxidative compounds naturally available in foods are well appreciated for both preserving foods and supplying essential antioxidants *in vivo* to combat oxidative stress related conditions.

Natural antioxidants include phytochemicals such as flavonoids, phytoestrogens, phenolic acids, carotenoids, tocopherols and other low-molecular-weight compounds. Natural antioxidants occurring in foods may be used as components of composite food formulations or may be extracted and subsequently added into foods *i.e.* extracts of green tea, sage, and rosemary can be added to a variety of foods, while tocopherols found in plant oils can be used in bulk oil to prevent oxidation. Vegetables, spices, herbs, fruits (especially berries), onions, teas, tomato, and oilseeds among others are rich in natural antioxidants, among others.

#### **2.8.5 Beneficial effects of phytochemicals**

Phytochemicals are biologically active, non-nutritive secondary metabolites present in plants. They may provide colour, flavour, and protective chemical agents that distract pests (Johnson, 2003). Phytochemicals fall into several major classes, including

phenolic compounds and carotenoids. Phenolic compounds include monophenolics, hydroxycinnamic acids, flavonoids, phytoestrogens, and tannins. Flavonoids are widely distributed in plants providing colour, taste, and protection against pests while phytoestrogens offer a weak oestrogen activity. Carotenoids have many members and these are distributed in different Cruciferae species, among others. Meanwhile, carotenoids are widely distributed in plants, with approximately 500 carotenoids having been identified.

Daily consumption of fruits and vegetables is reported to reduce the risk of coronary heart diseases (Joshi *et al.*, 2001), cancer mortality in general (Hertog and Bueno-de-Mesquita, 1996), lung, colon, breast, cervix, oesophagus, stomach, bladder, pancreas, and ovary cancer (Block *et al.*, 1992), and cardiovascular diseases (Gillman *et al.*, 1995). Furthermore, diets rich in fruits, vegetables, and grains have been shown to reduce oxidative damage to DNA, which is considered a crucial step in carcinogenesis (Djuric *et al.*, 1998). The disease prevention properties of fruits, vegetables and cereal grains are in part due to their polyphenolic content. Numerous studies have correlated the disease prevention properties and polyphenolic content (Nichenametla *et al.*, 2006). Polyphenols are common constituents of fruits, and vegetables as well as cereals and therefore are abundantly available in balanced human diets. Fruits like apple, cherry, grapes, and pear contain up to 200-300 mg of polyphenols per gram of fresh fruit (Scalbert *et al.*, 2005). Over the last decade, a large number of studies were carried out to explore the health benefits of plant phenolics and most of the studies concluded that their antioxidant activity significantly contributes toward their protective effect.

The total dietary intake of phenolic compounds is estimated to be about 1 g/day. It is 10 times higher than that of vitamin C and 100 times higher than vitamin E consumption (Scalbert and Williamson, 2000). Phenolic compounds, at low concentrations, act as antioxidants and protect plant foods against oxidative deterioration. They significantly contribute to human health through multiple biological effects, including antioxidant, antimutagenic, and anticarcinogenic, anti-inflammatory and antiatherogenic activities (Hollman and Katan, 1997; Parr and Bolwell, 2000; Shahidi and Naczk, 2004).

Antioxidant activities of phenolic compounds extracted from tea, fruits and vegetables, cereals, oilseeds, and herbs have been extensively studied using *in vitro* methods. The results of these studies indicated that phenolic compounds are powerful antioxidants. However, there is still controversy whether similar *in vitro* effects can be obtained *in vivo* due to lack of knowledge concerning the potential of phenolic compounds to stay for a sufficient time in an efficient chemical form in the human body (Karakaya, 2004).

## **2.9 Plant phenolics**

The term 'phenolic' embraces a wide range of plant substances which possess aromatic structures bearing one or more OH groups. Most are polyphenols having several hydroxy group substituents, one or more of which may be substituted with methyl or sugar groups (Harborne, 1999). Plant phenolics, a group of secondary metabolites that occur widely in plants are essential for growth and development as well as for pest and pathogen resistance.

### **2.9.1 Synthesis of plant phenolics**

Phenolics share a common biosynthetic origin with phenylalanine, one of the three amino acids formed from sedoheptulose via the Shikimate pathway (Harbourne, 1999). The enzyme phenylalanine lyase (PAL) catalyzes the release of ammonia from phenylalanine and leads to the formation of a carbon double bond, yielding *trans*-cinnamic acid. In certain other plants and grasses tyrosine is converted to 4-hydroxycinnamic acid (Shahidi and Naczki, 2004). *Trans*-cinnamic acids yield a number of phenolic acids such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid via subsequent hydroxylation and or methylation. These phenolic acids possess a phenol ring and C3 side chain and are thus collectively named phenylpropanoids (Shahidi and Naczki, 2004). Parallel to the phenylpropanoid series, hydroxylation and perhaps methylation of hydroxybenzoic acid leads to the formation of dihydroxybenzoic acid, syringic acid, and vanillic acid (Shahidi and Naczki, 2004). Customarily, the cinnamic acid family and benzoic acid derivatives are collectively termed 'phenolic acids'. Another important class of compounds, flavonoids, are formed in plants from phenylalanine tyrosine, and malonate (Harborne, 1986).

### **2.9.2 Structure of phenolic compounds**

#### **2.9.2.1 Flavonoids**

The main flavonoid classes of compounds are flavononol, flavonol, isoflavone, flavone, anthocyanins/ anthocyanidins and catechin. All flavonoids are characterized by the presence of three ring structures, designated A, B, and C. The above mentioned flavonoid classes differ in the level of oxidation and pattern of substitution in

the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (Pietta, 2000). Individual differences in the structure arise from variation in number and arrangement of the hydroxyl group, as well as from the nature and extent of alkylation and/or glycosylation of these groups (Karakaya 2004). The degree of hydroxylation is a determinant for the tendency to degrade in the colon and which degradation products are produced by colonic microflora (Rice-Evans *et al.*, 1996). Flavonoids that exist in foods are usually glycosylated with glucose, rhamnose, galactose, arabinose, xylose, glucouronic acids or other sugars (Karakaya, 2004). Usually the structures contain one sugar molecule; however, there may be more than one sugar molecule present in association. The glycosylation process greatly influences the chemical and biological properties of flavonoids (Scalbert and Williamson, 2000). The other flavonoid classes include biflavones, chalcones, aurones and coumarines. Hydrolysable tannins, condensed tannins, caffeates, lignins and lignans are all plant phenols that are classified separately. **Figure 2.7** illustrates the structure of different flavonoid classes.

### **2.9.2.2 Flavonols and flavones**

Flavonols and flavones bear the C ring structure with a double bond at 2-3 position. Flavonol has a hydroxyl group at the 3 position while flavone does not. Flavones and flavonols occur as aglycones in foods; approximately 200 flavonols and some 100 flavones have been identified in plant species (Shahidi and Naczki, 2004). Flavonols can be regarded as 3-hydroxyflavones and flavones as 3-deoxyflavonols. Within each class, individual flavonoids may vary in the number and distribution of

hydroxyl groups as well as in the degree of alkylation or glycosylation (Shahidi and Naczki, 2004). Flavonols are present mainly as 3-*O*-glycosides. Rutin and kaempferol 3-*(p*-coumaryl) glycoside found in fruits are the major flavonols.

### **2.9.2.3 Flavanones**

Some of the flavanones found in fruits include hesperidin, narirutin, hesperitin and visenin. Orange juice is considered to be one of the richest sources of flavanone, which contain 400-750 mg/L of flavanones (Karakaya, 2004).

### **2.9.2.4 Catechins and anthocyanins**

Catechins and anthocyanins are collectively known as flavans because of their lack of a carbonyl group in the C3 position. Catechin, epicatechin, epigallocatechin, and epicatechin gallate are some of the examples of catechins. Tea is a very rich source of catechins. Anthocyanins, the glycosidically bound anthocyanidins are the most important group of water-soluble pigments in plants. They are generally found in the form of glycosides and are responsible for the red, blue, and violet colours of fruits, flowers, and other foods such as blackberry, raspberry, cherry, currants, egg plants, red potato, and coloured beans (Mazza and Miniati, 1993). Most anthocyanins occur as monoglycosides and diglycosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Shahidi and Naczki, 2004).

### **2.9.2.5 Isoflavones**

Isoflavones are natural plant phenolics that resemble the structure of mammalian oestrogen and exhibit oestrogen activity. They possess a diphenylpropane structure in

which the B-ring is located at the C3- position. They are present in soybeans and soy products. Daidzein, formononetin, genistein, glycitein, daidzin, genistin, glycitin, dihydrodaizin, and dihydrogenistin are some of the isoflavones found in soybeans (Shahidi and Naczki, 2004; Hosmy and Rosazza, 2002). Daidzein, genistein, *O*-desmethylangolensin, and equol are some of the isoflavones commonly found in the Japanese diet (Karakaya, 2004). They are converted to the conjugated form after absorption and found in plasma after digestion. These isoflavones are present in the form of glycoside, malonylglucoside, acetylglucoside as well as in the free form (Karakaya, 2004; Shahidi and Naczki, 2004).

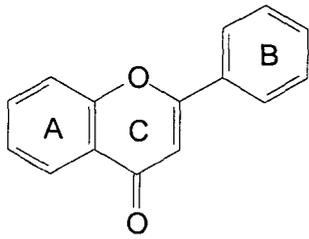
#### **2.9.2.6 Chalcones**

The most commonly available chalcones include phloretin, and its glucoside phloretin (phloretin 2-*O*-glucose), chalconaringenin, and arbutin. Phloretin and phloridzin are mainly found in apple, strawberry, bearberry, wheat, tea, coffee, red wine and broccoli (Robards *et al.*, 1999; Clifford, 2000).

#### **2.9.2.7 Phenolic acids**

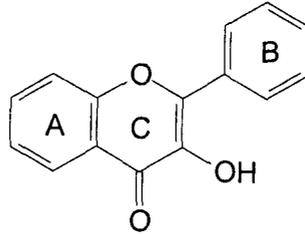
Phenolic acids are universally distributed in plants and are derivation of either hydroxycinnamic or hydroxybenzoic acids. Hydroxycinnamic acid derivatives include ferulic, sinapic, caffeic, and *p*-coumaric acids while the hydroxybenzoic acid group includes gallic, protocatechuic, vanillic, and syringic acids. Phenolic acids are widely distributed in substantial quantities in fruits, vegetables, and cereals.

**Figure 2.7** Chemical structures of different flavonoid classes as examples there of.



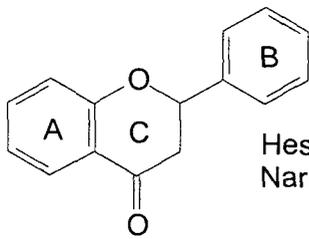
Luteolin  
Apigenin  
Rutin

Flavones



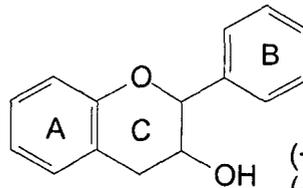
Quercetin  
Kaempferol

Flavonols



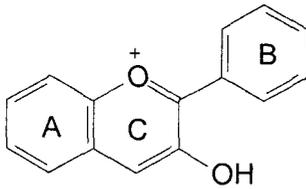
Hesperidin  
Naringin

Flavanones



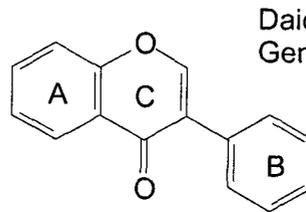
(+)-catechin  
(-)-epicatechin  
(+)-gallactocatechin  
(-)-epigallactocatechin

Flavanols  
(Catechins)



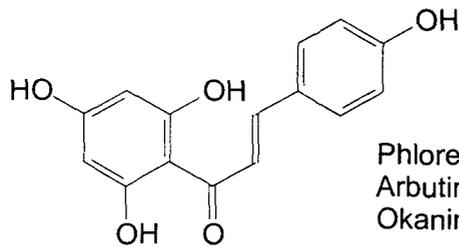
Peonidin  
Delphinidin  
Petunidin  
Cyanidin

Anthocaynins



Daidzein  
Genistein

Isoflavones



Phloretin  
Arbutin  
Okonin

Chalcones

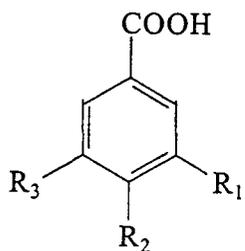
They occur in most plant tissues in a variety of conjugated forms such as ester, amide or glycoside, but seldom in the free form (Karakaya, 2004). The most abundant dietary hydroxycinnamates are ferulic and caffeic acids. Ferulic acid is covalently bound to plant cell walls and abundantly found in cereals.

Hydroxycinnamic acids occur most frequently as simple esters with carboxylic acids or glucose while hydroxybenzoic acids are mostly present in the form of glucosides (Nichenametla *et al.*, 2006). Caffeic acid is the major representative of hydroxycinnamic acid and occurs in foods as chlorogenic acid, which is an ester of caffeic acid and quinic acid. **Figure 2.8** illustrates structures of phenolic acids found in plant foods.

### **2.9.3 Structure-antioxidant activity relationship of phenolic compounds**

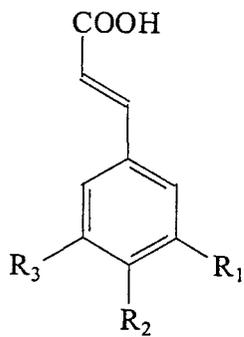
It has been found that plant phenolics, especially flavonoids, possess anti-tumour, anti-allergic, anti-platelet, anti-ischemic, and anti-inflammatory activities (Shi *et al.*, 2001). Polyphenolics are reported to be associated with low incidences of CVD, cancer and other degenerative diseases. Some of these beneficial effects stem from the antioxidant properties of polyphenolic compounds. Flavonoids can exert antioxidant activity by inhibiting enzymes including xanthine oxidase, lipoxygenase, and cyclooxygenase, by chelating metal ions, interacting with other antioxidative substances such as ascorbate, and by scavenging free radicals (Laughton *et al.*, 1991; Satoh and Sakagami, 1996)

**Figure 2.8** Chemical structures of phenolic acids found in plant foods.



### Hydroxybenzoic acids

Acid	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<i>p</i> -Hydroxybenzoic	H	OH	H
Protocatechuic	OH	OH	H
Vanillic	OCH <sub>3</sub>	OH	H
Gallic	OH	OH	OH
Syringic	OCH <sub>3</sub>	OH	OCH <sub>3</sub>



### Hydroxycinnamic acids

Acid	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<i>p</i> -Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH <sub>3</sub>	OH	H
Sinapic	OCH <sub>3</sub>	OH	OCH <sub>3</sub>

while additional OH groups at the 5' position enhances the antioxidant capacity (Rice-Evans *et al.*, 1996). Hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 are essential for efficient xanthine oxidase inhibiting activity (Cos *et al.*, 1998). For superoxide radical scavenging activity, the presence of OH groups at C-3' in ring B and C-3 is essential. Most flavonoids possess a significant reactivity with the hydroxyl radical. It is reported that reactivity of flavonoids towards the hydroxyl radical is generally at least four times higher than that towards the superoxide radical (Bors *et al.*, 1990).

Flavonoids such as catechins have been shown to possess efficient singlet oxygen scavenging ability (Tournaire *et al.*, 1993). Anthocyanidins are known to be potent scavengers of NO<sup>•</sup> (van Acker *et al.*, 1995) and NOO<sup>-</sup> (Haenen and Bast, 1999). Flavonoids are also good peroxy radical scavengers (Guo *et al.*, 1999) and therefore prevent LDL oxidation which is considered to be a key factor in atherogenesis.

#### **2.9.4 Bioavailability of phenolic compounds**

A number of factors affect the bioavailability of phenolic compounds. Partition coefficients seem to play an effective role in the absorption of phenolics that have no sugar or organic acid substitutes in their structure in the human digestive tract. Hydrophilic compounds are not absorbed in the upper GI tract (Scalbert and Williamson, 2000), whereas hydrophobic substances that have sugar or organic acids and ester linked substitutions are degraded by esterases in the colon (Scalbert and Williamson, 2000; Adam *et al.*, 2002). The number of sugar molecules also plays a key role in absorption. Phenolic compounds that carry glucose, galactose, or xylose are absorbed through the

small intestine by the action of cystolic  $\beta$ -glucosidase/lactase phlorizin hydrolase (Karakaya, 2004; Scalbert and Williamson, 2000). Acylated flavonoids, such as epicatechin, and epigallocatechin are absorbed without deconjugation or hydrolysis (Saclbert and Williamson, 2000). Phenolic compounds are metabolised by deconjugation and re-conjugation reactions. They are hydrolysed to their free aglycones, and subsequently are conjugated by methylation, sulphation, and glucuronidation. Food phenolics are found in plasma in the conjugated form, however, when higher doses are administered, they are found as the free form (Saclbert and Williamson, 2000). Large doses of phenolic compounds are metabolised by liver while small doses such as intake with food are metabolised directly by intestinal mucosa.

Plasma catechin levels are reported to significantly increase after the consumption of tea with the peak catechin levels being reached after 1 h (Hollman and Kattan, 1997). Catechins are mainly found in the conjugated form in the plasma. Most of absorption studies have revealed that anthocyanins differ from other flavonoids and are poorly absorbed. No anthocyanins have been detected in plasma at any time after feeding anthocyanin rich lyophilized blackberry powder in a study carried out using rats (Karakaya, 2004). Miyazawa *et al.* (1999) reported that the flavylum cation structure of anthocyanins could make them more suitable to bacterial hydrolysis. Hydrolysis of the glucose moiety in the small intestine is the main step in absorption. Chesson *et al.* (1999) reported that hydroxycinnamates are mainly absorbed from the foregut. It was further observed that 25% of phenolic acids ingested appeared in plasma within 2 h. Absorption of hydroxycinnamate occurs through a  $\text{Na}^+$ -dependent carrier-mediated transport process or passive diffusion (Chesson *et al.*, 1999). It was further found that ferulic acid is not

extracted in feces regardless of the intake in an experiment using Wistar rats. Forty five to 53% of ferulic acid was found in the conjugated form in peripheral tissues of rats. Absorbed ferulic acid is readily eliminated in the urine, and after 18 h no traces of ferulic acid were detected in the plasma.

## **2.9.5 Mechanism of action of phenolic compounds in disease prevention**

### **2.9.5.1 Chemoprevention**

There is a great body of evidence indicating that phenolics from fruits and vegetables are chemopreventive to many organ specific cancers. However, chemopreventive potential varies greatly with many factors. There are several mechanism of chemoprevention including the effect on cellular differentiation, growth, apoptosis, activation or deactivation of various enzymes, antioxidant action, antimutagenicity, antimetastasis activity, and direct interaction with carcinogens (Sailendra *et al.*, 2006). Most of the individual phenolic compounds have multiple overlapping effects that could be additive, synergistic or even antagonistic in nature (Sailendra *et al.*, 2006).

Most of the *in vitro* studies carried out examining the effect of phenolic acids on tumour cells suggest anticancer activity. Possible mechanisms of chemoprevention activity of phenolic acids include inhibition of carcinogen uptake and/or formation of carcinogens, deactivation or detoxification of carcinogens, preventing the carcinogen binding to DNA, and enhancing possible DNA repair (Sailendra *et al.*, 2006). Ferulic and chlorogenic acids are known to inhibit cytochrome p450 activity while caffeic acid has been shown to decrease oxidative damage to DNA in breast cancer cells (Sailendra *et al.*, 2006). Ferulic acid is reported to possess chemopreventive activity in many studies

(Huang *et al.*, 1988) while *p*-coumaric acid has been shown to possess antiproliferative activity (Hudson *et al.*, 2000). Caffeic acid exhibits both anticarcinogenic as well as carcinogenic activities. Furthermore, it has shown synergistic carcinogenic effects with BHA, sesamol, and catechol even at low doses (Hirose *et al.*, 1998).

#### **2.9.5.2 Cardiovascular diseases**

A number of animal studies have shown that polyphenols limit the development of atherogenesis. Polyphenols inhibit oxidation of LDL, which is a key factor in atherogenesis; however, evidence in humans remains controversial (Scalbert *et al.*, 2005). Soy isoflavones such as daidzen and genistein lower the incidence of CVD by a number of mechanisms including lowering blood cholesterol, improving the antioxidant status of the body, and myocardial activation of Akt (PKB protein kinase B) (Anderson *et al.*, 1995). Tea catechins have been shown to inhibit the invasion and proliferation of the smooth muscle cells in arterial walls (Lu *et al.*, 1998). Polyphenols have also been shown to modify lipid metabolism, however, the results are contradictory (Russ *et al.*, 2001). Furthermore, they are suggested to be antithrombic (Russ *et al.*, 2001) and known to improve endothelial differentiation, which is associated with atherogenesis (Scalbert *et al.*, 2005).

#### **2.9.5.3 Neurodegenerative diseases (NDD)**

NDD such as Alzheimer's and Parkinson's, caused by oxidative stress, are common in ageing populations. Animal studies have shown that low polyphenol

concentrations exhibit protective effects against the development of oxidative stress related disease conditions in rats (Scalbert *et al.*, 2005).

#### **2.9.5.4 Diabetes**

Plants and plant extracts have been used in traditional folk medicine for the treatment of diabetes in many cultures. Polyphenols may affect glycaemia through a number of mechanisms including the inhibition of glucose absorption in the gut or uptake by tissues (Scalbert *et al.*, 2005).

#### **2.9.6 Phenolic compounds in cereals**

Cereal grains contain unique phytochemicals that complement those of fruits and vegetables when consumed together. Various classes of phenolic compounds in cereals include derivatives of benzoic and cinnamic acids, anthocyanidins, flavonols, chalcones, flavones, flavonones, and amino phenolic compounds (Shahidi and Naczk, 2004). Ferulic acid that exists mainly in the seed coat is the major low-molecular-weight phenolic acid in many cereals. Senter *et al.* (1983) indicated that cereals contain 500 mg of phenolic acids per kg of their edible portion. Generally the phenolic compounds in cereal grains are concentrated in the outer layer (bran), while the endosperm contains less and the germ is, in general, rich in tocopherols and lipids. Cereals do not contain a high amount of flavonoids. In contrast, cereals are rich in lignans (Zielinski and Kozłowska, 2000), which are known to be potent anticarcinogens. **Table 2.3** lists some of the major phenolic compounds present in commonly available cereals.

**Table 2.3** Phenolic compounds in cereals

Cereal	Phenolic group	Phenolic compound	Quantity (mg/kg)	Reference
Barley	Phenolic acid	<i>p</i> -hydroxybenzoic acid	799-1180	Yu <i>et al.</i> (2001)
		vanillic acid	36-69	
		<i>p</i> -coumaric acid	1.68-35	
		ferulic acid	91-238	
		caffeic acid	5.84-12.66	
		chlorogenic acid	56-127	
		Total phenolics	110-1230	
Corn	Phenolic acid	β-tocotrienol		Zielinski <i>et al.</i> (1998)
		α-tocopehrol		Peterson (1993)
Millets	Phenolic esters Phenolic acids	<i>p</i> -hydroxybenzoic acid	1.3	Sosulki <i>et al.</i> (1982)
		vanillic acid	3.7	
		<i>p</i> -coumaric acid	19	
		ferulic acid	264	
		caffeic acid	4.5	
		chlorogenic acid	-	
		Stanol and sterols ferulates	31-70	
Sorghum	Phenolic acids	<i>p</i> -hydroxybenzoic acid	26-34	Hahn <i>et al.</i> (1983)
		vanillic acid	15.5-127	
		<i>p</i> -coumaric acid	86-232	
		ferulic acid	118-287	
		caffeic acid	23-50	
		chlorogenic acid	-	
		Anthocyanidins/ anthocyanins	cyanidin, pelargonidin peonidin, malvidin delphinidin, petunidin apigenidin, apigenidin-5- glucoside, luteolinidin, luteolinidin-5-glucoside	
Rye	Phenolic acids	<i>p</i> -hydroxybenzoic acid		Andreason <i>et al.</i> (2000)
		vanillic acid		
		<i>p</i> -cuomarcic acid	40-70	
		ferulic acid	900-1170	
		caffeic acid		
		Sinapic acid	70-140	
		Ferulic aciddehydrodimers	240-410	
Phenolic esters	Phenolic esters	Steryl ferulates		Seitz. (1989)
		Campestanlyl ferulates		
		Sitostanyl ferulates		
		Total phenolics	1390	

### 2.9.6.1 Barley

*Para*-hydroxybenzoic, vanillic, *o*-, *m*- and *p*-coumaric, syringic, ferulic and sinapic acids have been identified in barley grains, while ferulic and *p*-coumaric acids are quantitatively the most important phenolic acids (Nordvist *et al.*, 1984; Shahidi and Naczki, 2004). Ferulic and *p*-coumaric acids are known to be linked to lignin and arabinoxylan (Nordvist *et al.*, 1984). The highest content of total insoluble-bound phenolic acids are found in the husk, testa and aleurone layers of barley while the endosperm contains only trace amounts of insoluble phenolic acids. Apart from phenolic acids, the coloured barley grains contain anthocyanidins such as cyanidin, delphinidin, and pelargonidin (Briggs, 1978).

### 2.9.6.2 Other cereals

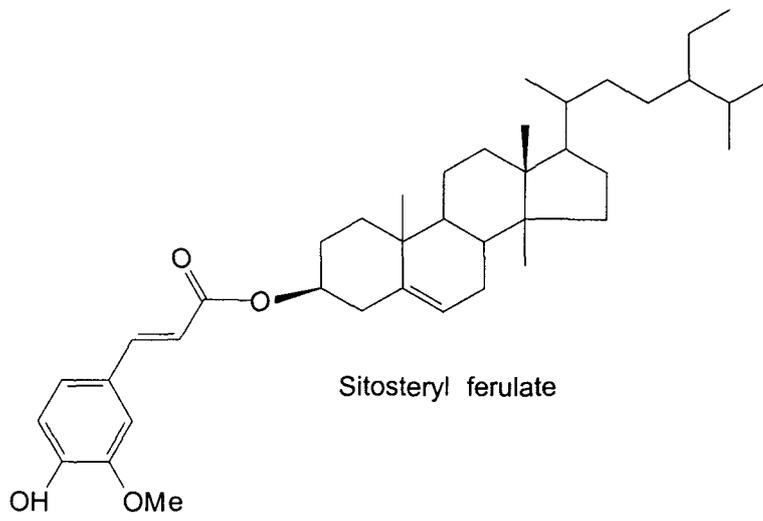
Rice, a staple food in many countries, contains a high amount of ferulic acid in the bound form and also, rice contains considerable amounts of sterol phenolic acid esters (Rogers *et al.*, 1993). Ferulic acid derivatives, including cycloartenyl, campesteryl, sitosteryl, stigmasteryl, sitostanyl, and campestanil ferulates have been identified in rice (Rogers *et al.*, 1993). Processing, including cooking and baking, may release bound phenolic acids leading to higher phenolic acid availability in the processed flour. During the traditional production of parboiled rice, a considerable amount of phenolics are leached into the soaking water, thus reducing the phenolic content (Shahidi and Naczki, 2004). A flavonoid, isovitexin, which provides chemical defence against post-harvest pests has been identified in rice hulls. **Figure 2.9** illustrates the structure of some of the predominant phenolic compounds reported in cereals.

Oat is used as a breakfast cereal, in snack foods, bread, cookies, gravies and soups. Oat contains a significant amount of  $\beta$ -glucan, a non-starch polysaccharide which is mainly responsible for cholesterol lowering effects. Sosulski *et al.* (1982) reported the presence of vanillic acid, sinapic acid, *p*-coumaric acid, caffeic acid, protocatechuic acid, siringic acid, and *p*-hydroxybenzoic acid in bound form in oat. Collins (1989) indicated that oat hulls contain at least 25 avenanthramides in.  $\alpha$ -Tocopherol has been identified as the main tocol in oat (Peterson and Qureshi, 1993).

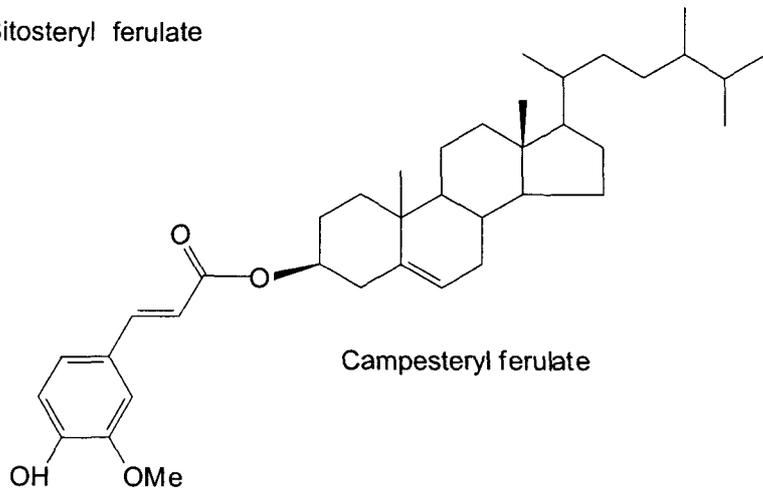
Bread made from whole rye flour (*Secale cereale* L.) is an important part of the diet in some North and East European countries. Unlike wheat, a considerable part of rye is consumed as whole meal products. Seven major phenolic acids are found in rye, with ferulic acid being most abundant. Phenolic acids are mainly linked to heteroxylans or lignans via esterification at the *O*-5 position and are mainly located in the bran fraction of the grain (Andreason *et al.*, 2000).

Polyphenolic compounds in sorghum include mainly, anthocyanidins, flavanols (condensed tannins), flavan-3-ol polymers and phenolic acids (Butler, 1989). The pigmented sorghum cultivars contain high levels of anthocyanins. Sorghum also contains a high amount of tannin, which is considered an antinutritive in food, mainly due to its protein binding capacity which reduces the bioavailability of proteins. Moreover, tannins are known to form insoluble complexes with iron rendering it unavailable for absorption in the gastrointestinal tract. In addition, sorghum contains protocatechuic, *p*-hydroxybenzoic, caffeic, *p*-coumaric, and ferulic acids in both free and bound forms. Many different types of phenolic acids are found in wheat with ferulic acid being most abundant.

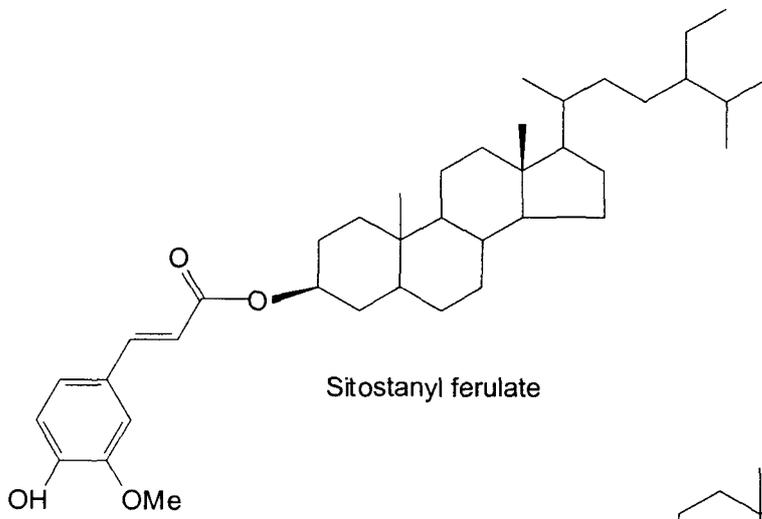
**Figure 2.9** Chemical structures of other phenolic compounds reported in cereals.



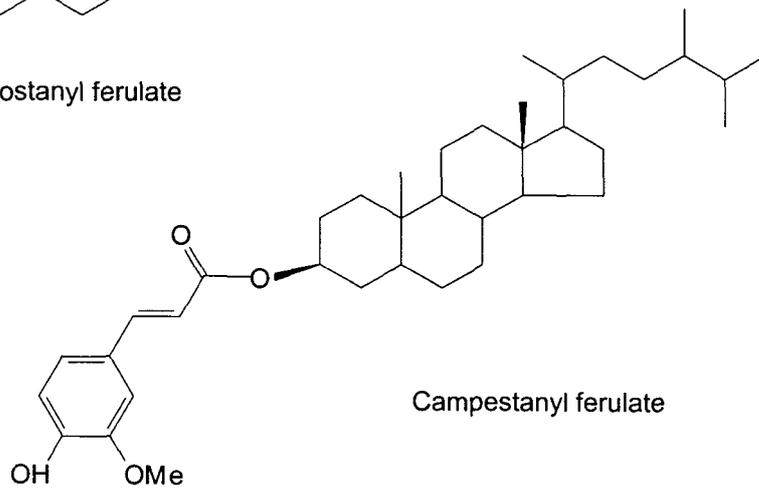
Sitosteryl ferulate



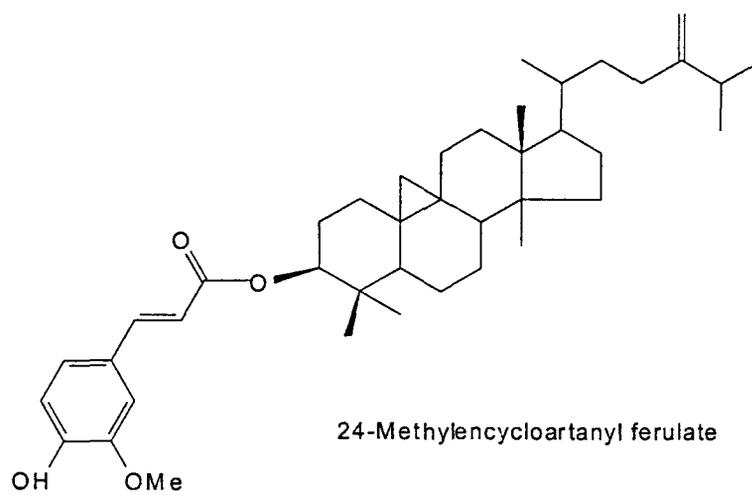
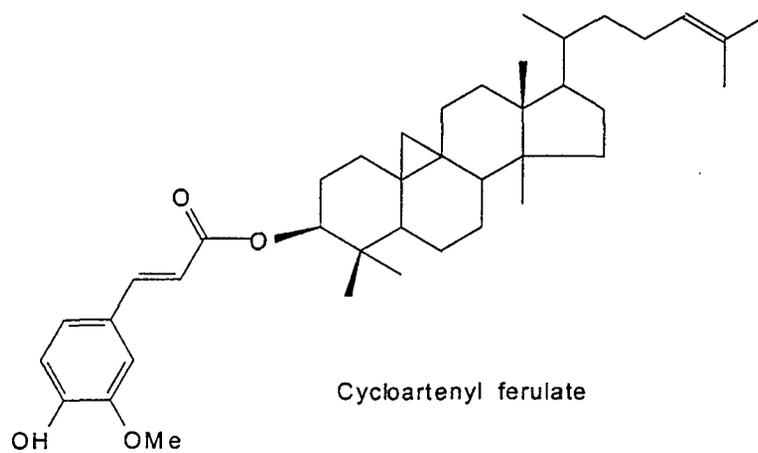
Campesteryl ferulate



Sitostanyl ferulate



Campestanol ferulate



Wheat has been reported to have the highest concentrations of alkylresorcinol (AR) (Ross *et al.*, 2003). AR are mainly found in the outer layers (bran fraction) of cereal grains and therefore, they are largely missing in refined cereal flour and conventional cereal products such as white bread and many breakfast cereals (Ross *et al.*, 2003). Both wheat germ and bran contain the same level of flavonoids. Tricin is the dominant flavone found in both cultivated and wild wheat cultivars and the content of flavonoids depends largely on the wheat class and variety.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Materials

Six barley cultivars namely, AC Metcalfe, Falcon, Phoenix, Tercel, Tyto and Peregrine were obtained from the Field Crop Development Center, Lacombe, Alberta, Canada in the crop year of 2002. The samples were stored at ambient temperature under low humidity conditions until the antioxidative compounds were extracted.

2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), Trizma base, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), nitroblue tetrazolium,  $\alpha$ -tocopherol, hydrogen peroxide, boric acid, ethidium bromide, sodium hydroxide, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2-thiobarbituric acid (2-TBA), 1,1,3,3-tetramethoxypropane, xanthine oxidase, hypoxanthine,  $\beta$ -carotene, linoleic acid, butylated hydroxytoluene (BHT), catechin, ferrous sulphate, Tween 40 (polyoxyethelene sorbitan monopalmitate), Folin Ciocalteu's phenol reagent, trichloroacetic acid (TCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), luminol, ascorbic acid, fluorescein, cobalt (II) fluoride tetrahydrate, 2,2' bipyridyl, Tris-HCl buffer, hydroxylamine.HCl, ferulic acid, picolinic acid, copper sulphate, ferric chloride, mono- and dibasic sodium and potassium phosphates, vanillin, ethylenediaminetetraacetic acid (EDTA), Trolox, human low density lipoprotein (hLDL), diethylenetriaminepentaacetic acid, acetonitrile, ferrous ammonium sulphate, and deoxyribonucleic acid (DNA) of PBR 322 (*E. coli* strain RRI) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Methanol, ethanol, acetone, dimethyl sulphoxide (DMSO), hexane, and chloroform (ACS grade or higher) were purchased from Fisher Scientific Company (Ottawa, ON).

Helium, hydrogen, nitrogen and compressed air were obtained from Air Liquide Ltd. (St. John's, NL). ATPlite 1 step luminescence kit was purchased from Perkin Elmer, Boston, MA. Caco-2 human cancer cells were purchased from the American Type Culture Collection (Rockville, MD). Cell culture media [McCoy's 5A medium modified with L-glutamine, antibiotic/antimycotic, and foetal bovine serum (FBS)], and 0.25% trypsin with 0.9 mM EDTA were purchased from Invitrogen (Carlsbad, CA). Disposable culture ware for cell culture assay was purchased from Corning glass works (Corning, New York, NY). Costar clear and black microplates (2650) were purchased from Fisher Scientific, Nepean, ON.

## **3.2 Methods**

### **3.2.1 Determination of proximate composition of barley**

#### **3.2.1.1 Moisture content**

Three grams of ground barley samples were weighed into pre-weighed aluminum dishes and placed in a preheated forced-air oven (Fisher Isotemp 300, Fair Lawn, NJ). Samples were maintained at  $105\pm 1^{\circ}\text{C}$  until a constant mass was obtained. The moisture content was then calculated as the percent ratio of the weight difference of the sample before and after drying to that of the original material (AOAC, 1990).

#### **3.2.1.2 Ash content**

Approximately 3-4 g of barley samples were accurately weighed into clean dry porcelain crucibles and charred over a Bunsen burner. Charred samples were then placed in a preheated muffle furnace (Blue M Electro Co., Blue Island, IL) and maintained at  $550\pm 1^{\circ}\text{C}$  until a gray ash was obtained. Crucibles were subsequently cooled in a

desiccator and weighed. Ash content was calculated as percent ratio of the mass of the ash obtained after ignition to that of the original material (AOAC, 1990).

#### **3.2.1.3 Crude protein content**

Approximately 0.3-0.4 g of powdered barley sample were weighed onto nitrogen-free paper and transferred into the digestion tube of a Büchi digester (Büchi 321, Büchi Laboratories, Flawil, Switzerland). The sample was digested with 20 mL of concentrated sulphuric acid, and two Kjeltab tablets (Profamo, Dorval, QC) for 45 min to obtain a clear solution. The digested samples were diluted with 50 mL of distilled water followed by the addition of 150 mL of a 25% (w/v) sodium hydroxide solution. The samples were steam-distilled (Büchi 321, Büchi Laboratories, Flawil, Switzerland) to release the nitrogen in the form of ammonia, which was trapped in 50 mL of a solution of 4% (w/v) boric acid containing an end point indicator (EM Science, Gibbstown, NL) in a receiving flask. Steam-distillation was continued for 6 min and the contents in the receiving flask were titrated against a 0.1 N standard solution of sulphuric acid to determine the content of nitrogen (AOAC, 1990). The crude protein content of the barley samples were calculated using a factor of 6.25.

#### **3.2.1.4 Total lipid content**

Total lipid content of the samples was determined using the procedure described by Bligh and Dyer (1959). Approximately 25 g of barley samples were accurately weighed and then extracted for 3 min period with a mixture of 25 mL of chloroform and 50 mL of methanol using a Polytron homogenizer (Brinkman Instruments, Rexdale, ON).

The samples were re-extracted using 25 mL of chloroform followed by homogenization. Approximately 25 mL of distilled water were added and the mixture was then filtered through a Buchner funnel under suction. The filtrate was allowed to separate overnight in a separatory funnel. 10 mL aliquots of the lipid extract in chloroform were transferred into a pre-weighed round bottom flask and the solvent was removed using a Büchi RE 111 rotorvapor (Büchi Laboratories, Fawil, Switzerland). The flask was then placed in a forced-air convection oven (Fisher Isotemp 300, Fairlawn, NJ) at 80°C for 1 h. After cooling in a desiccator, the round bottom flask containing the lipids was weighed and the total lipid content determined gravimetrically.

### **3.2.2 Preparation of barley samples**

Barley samples were manually hulled and ground to obtain a fine powder using a laboratory mill with a 60 mesh sieve (Tecator 3420, Tecator Inc., Boulder, CO). The barley meals so obtained were defatted by blending with hexanes (1:5 w/v, 5 min) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT) at ambient temperature. Defatted samples were vacuum-packaged in polythene pouches and stored at -20°C until used for analysis.

### **3.2.3 Solvent-extraction of phenolic compounds**

Six grams of each of the defatted barley samples were separately extracted with 100 mL of 80% (v/v) methanol under reflux conditions in a thermostated water bath at 60°C for 40 min. The resulting slurries were centrifuged for 5 min at 4000 x g (ICE Centra M; International Equipment Co., Needham Heights, MA) and supernatants were

collected. The residue was re-extracted with 80% methanol for another 30 min recentrifuged and the supernatants obtained were combined with those from the first extraction and the combined mixture was desolventized *in vacuo* at 40°C. The resulting concentrated solutions were lyophilized for 72 h at -49°C and  $25 \times 10^{-3}$  mbar (Freezone, Model 77530, Labconco Co., Kansas City, MO). Phenolic extracts so obtained were placed in air-tight screw-capped glass vials and stored in a freezer at -20°C until used for analysis.

### **3.2.4 Determination of total phenolic content (TPC)**

Extracts were dissolved in methanol to obtain a concentration of 3 mg/mL for whole kernel extract and pearling fractions 2-7 (F2-F7). As fraction 1 (F1) yielded an absorbance exceeding the appropriate range, it was diluted appropriately with methanol and used for the assay. TPC was determined according to a modified version of the procedure developed by Singleton and Rossi (1965). Folin Ciocalteu's reagent (1 mL) was added to centrifuge tubes containing 1 mL of methanolic extracts. Contents were mixed thoroughly and 8 mL of sodium carbonate (75 g/L) were added to each tube. To the mixture, 10 mL of distilled water were added and mixed thoroughly. Tubes were then allowed to stand for 2 h at ambient temperature and the contents were subsequently centrifuged for 5 min at 4000 x g (ICE Centra M5, International Equipment Co., Needham Heights, MA). Absorbance of the resultant supernatant was measured at 765 nm using appropriate blanks for background subtraction. Content of total phenolics in each barley extract was determined using a standard curve prepared for ferulic acid and expressed as mg of ferulic acid equivalents per gram of defatted material.

### 3.2.5 Total antioxidant capacity (TAC) by Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay is based on the scavenging of 2, 2'-azinobis-(3-ethylbenzothiazoline -6-sulphonate) radical anion (ABTS<sup>•-</sup>). A solution of ABTS<sup>•-</sup> was prepared in 100 mM phosphate buffered saline (pH 7.4, 0.15 M sodium chloride) (PBS) by mixing 2.5 mM 2,2'-azobis-(2-methylpropionamide) dihydrochloride (AAPH) with 2.0 mM ABTS<sup>2-</sup>. The solution was heated for 16 min at 60°C, protected from light by covering in tin foil, was used within 2 h as the absorbance of the radical itself depletes with time. Whole kernel extracts and pearling fractions were dissolved in PBS at a concentration of 3 mg/mL and diluted accordingly to have them fit in the range of values in the standard curve (drop of 0-0.3 of optical density). For measuring antioxidant capacity, 40 µL of the sample were mixed with 1.96 mL of ABTS<sup>•-</sup> solution. Absorbance of the above mixture was measured at 734 nm after 6 min as the extracts needed a minimum of 6 min in order to complete the reaction. The decrease in absorption at 734 nm after 6 min of addition of barley extract was used for calculating TEAC values. A standard curve was prepared by measuring the reduction in absorbance of ABTS<sup>•-</sup> solution at different concentrations of Trolox. Appropriate blank measurements (decrease in absorption at 734 nm due to solvent without any compound added) were carried out and the values recorded (van den Berg *et al.*, 1999). TEAC values were expressed as µmol Trolox equivalents per gram of defatted material.

### **3.2.6 DPPH radical scavenging assay**

The effect of extracts on the DPPH radical was monitored according to the method of Hatano *et al.* (1988). The extracts (100  $\mu$ L; 3 mg/mL) were added to a methanolic solution (1.9 mL) of DPPH radical (final concentration of DPPH radical was 5.7  $\mu$ M). The mixture was shaken vigorously, left standing at room temperature for 20 min and the absorbance was measured at 517 nm. DPPH radical scavenging capacity was expressed as  $\mu$ mol ferulic acid equivalents per gram of defatted material based on the depletion of absorbance after 20 min of the assay using a standard curve.

### **3.2.7 Determination of reducing power of barley extracts**

The reducing power of barley extracts was determined using the method explained by Oyaiza (1986). The assay medium contained 2.5 mL of extract (2 mg/mL) in 0.2 M phosphate buffer solution (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. After incubating at 50°C for 20 min, 2.5 mL of 10% TCA were added followed by centrifugation at 4000 x g for 5 min. 1 mL of supernatant was transferred into a tube containing 2.5 mL of deionized water and 0.5 mL of 0.1% FeCl<sub>3</sub>. The absorbance was measured at 700 nm and the results were expressed as Trolox and ascorbic acid using the appropriate standard curves.

### **3.2.8 Photo-induced chemiluminescent (PCL) detection of water- and lipid- soluble antioxidants**

Measurement of the photo-induced chemiluminescence was performed using the Photochem<sup>®</sup> apparatus (Analytik Jena, Konrad-Zuse-Strabe 1, Jena, Germany) according to the method developed Papov and Lewin (1999) and Madhujith *et al.* (2006). A 20  $\mu$ L

aliquot of the sample was mixed with 1480  $\mu\text{L}$  of diluent (water) and 1 mL of phosphate buffer (0.1 M, pH 10.5 containing 0.1 mM EDTA). The contents were mixed vigorously and 25  $\mu\text{L}$  of 1 mM luminol were added to the mixture. Immediately after the addition of luminol, the contents were mixed briefly and introduced to the sample intake port of the Photochem<sup>®</sup> apparatus. The luminescence generated by the reaction between the remaining radicals and the detection chemical was monitored and registered by the instrument for 80-160 s in water soluble (ACW) mode. Trolox (0.1 nM; 5-30  $\mu\text{L}$ ) was used as the standard. The lipid-soluble antioxidant content was measured as follows; an aliquot of 20  $\mu\text{L}$  of the sample was mixed with 2.3 mL of diluent (methanol) and 200  $\mu\text{L}$  of phosphate buffer (0.1 M, pH 10.5 containing 0.1 mM EDTA). The contents were mixed vigorously and 25  $\mu\text{L}$  of luminol (1 mM) were added to the mixture. Immediately after the addition of luminol, the contents were mixed briefly and introduced to the sample intake port of the Photochem<sup>®</sup> apparatus. The luminescence generated by the reaction between the remaining radicals and the detection chemical (luminol) was registered by the instrument for exactly 140 s in lipid soluble (ACL) mode. Alpha-tocopherol (0.275 nM; 5–30  $\mu\text{L}$ ) was used as a standard.

### **3.2.9 Determination of oxygen radical absorbance capacity (ORAC<sub>FL</sub>)**

The determination of ORAC<sub>FL</sub> was carried out using a Fluostar Optima plate reader (BMG Labtech, Durham, NC) equipped with an incubator and two injector pumps with fluorescein as the probe and AAPH as the radical generator. The reaction was carried out in 75 mM phosphate buffer (pH 7.4) medium at a final reaction mixture of 200  $\mu\text{L}$  in a 96-well Costar 2650 black plate. Fluorescein (120  $\mu\text{L}$ ; 64 nM, final concentration) was injected using the first injector pump into the wells containing the

extract (20  $\mu\text{L}$ ; 3  $\mu\text{g}/\mu\text{L}$  whole seed extract; 1  $\mu\text{g}/\mu\text{L}$  F1-F3; 3  $\mu\text{g}/\mu\text{L}$  F4-F7). The mixture was incubated for 20 min at 37°C in the built-in incubator and subsequently APPH solution (60  $\mu\text{L}$ ; 29 mM final concentration) equilibrated at 37°C was rapidly injected into the wells using the second pump. The plate was shaken for 4 s after each addition at 4 mm shaking width. To optimize the signal amplification in order to obtain maximum sensitivity, a gain adjustment was performed at the beginning by manually pipetting 200  $\mu\text{L}$  of fluorescein into a designated well. No more than 36 wells of the 96-well plate were used due to increased cycle time. Fluorescence was determined and recorded every minute for 60 min and the antioxidant activity of the extracts was calculated as Trolox equivalents using a standard curve prepared with 1-10  $\mu\text{M}$  (final concentration), control (phosphate buffer, fluorescein and AAPH) and positive control (phosphate buffer and fluorescein). Filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used (Prior *et al.*, 2003).

### **3.2.10 Hydroxyl radical averting capacity (HORAC<sub>FL</sub>)**

The determination of hydroxyl radical averting capacity was performed using fluorescein as the probe employing the method explained by Ou and co-workers (2002). A Fluostar Optima plate reader (explained in **Section 3.2.9**) was used to monitor the fluorescence. Fluorescein (180  $\mu\text{L}$ ; 96 nM, final concentration) dissolved in PBS (0.75 mM, pH 7.0) was pipetted into the wells containing 10  $\mu\text{L}$  of extract (2 mg/mL) or Trolox standard (0-10  $\mu\text{M}$ ; final concentration). A solution of hydrogen peroxide (10  $\mu\text{L}$ ; 1 M) dissolved in deionized water was injected into the mixture using the first injector pump and the mixture was incubated for 1min before the initial reading was taken. The second injector pump was set to inject a mixture of 10  $\mu\text{L}$  of cobalt fluoride (8 mM) and

picolinic acid (8 mM) dissolved in deionized water into the wells in order to initiate the reaction. Fluorescence was determined and recorded every minute for 40 min and the area under the decay curves (AUC) was calculated. Antioxidant activity of the extracts was expressed as ferulic acid equivalents. Control (buffer, fluorescein, H<sub>2</sub>O<sub>2</sub> and CoF<sub>2</sub>) and positive control (buffer and fluorescein) were used to compensate for the fluorogenic effect of reagents.

### **3.2.11 Determination of metal chelation activity**

Fe<sup>2+</sup> chelation activity of the barley extracts was measured by using a 2,2'-bipyridyl competition assay explained by Yu *et al.* (2002) and Yamaguchi *et al.* (2000). The reaction mixture contained 0.2 mL of 1 mM FeSO<sub>4</sub>, 1mL of Tris-HCl buffer (pH 7.4), 0.2 mL of extract (6 mg/mL whole seed extract; 2 mg/mL, pearling fractions) or EDTA standard, 0.4 mL of 10% hydroxylamine.HCl, 1mL of bipyridyl solution (0.1% in 0.2 M HCl), and 2.5 mL of ethanol. The reaction mixture was mixed well and the absorbance was measured at 522 nm. Metal chelation activity was expressed as EDTA equivalents using a standard curve prepared with 75-675 μM EDTA.

### **3.2.12 Superoxide radical scavenging assay**

Superoxide radicals were generated via an enzymatic reaction. The reaction mixture contained 1 mL of 3 mM hypoxanthine, 1 mL of 100 milli-International Units (mIU) of xanthine oxidase, 1 mL of 12 mM diethylenetriaminepentaacetic acid, 1 mL of 186 μM nitro blue tetrazolium and 1 mL of the extracts (final concentration of the extracts in the assay medium was 0.1 mg/mL). A standard curve was prepared using

Ferulic acid. All solutions were prepared in a 100 mM phosphate buffer (pH 7.4) solution. The absorbance values of the mixtures were read at 560 nm up to 60 min. Readings at 10 min were used to calculate superoxide radical scavenging capacities. The following equation was used to calculate superoxide radical scavenging capacity (Nishimiki *et al.*, 1972, Gaulejac *et al.*, 1999).

$$\text{Superoxide radical scavenging capacity, \%} = 100 - \left\{ \frac{\text{Abs}_{\text{additive}}}{\text{Abs}_{\text{control}}} \right\} \times 100$$

where,  $\text{Abs}_{\text{additive}}$  = absorbance of medium containing additive; and  $\text{Abs}_{\text{control}}$  = absorbance of the control medium.

### 3.2.13 Hydrogen peroxide scavenging assay

Extracts were dissolved in 3.4 mL of a 0.1 M phosphate buffer (pH 7.4) and mixed with 0.6 mL of 53 mM solution of hydrogen peroxide prepared in the same buffer solution. Immediately after mixing the zero time absorbance was read at 230 nm and subsequent readings were taken at 10 min intervals over 40 min. For each concentration, a separate blank sample devoid of hydrogen peroxide was used for background subtraction (Ruch *et al.*, 1989). A standard curve was prepared using ferulic acid. The hydrogen peroxide scavenging capacities were calculated using the following equation.

$$\text{Hydrogen peroxide scavenging capacity, \%} = 100 - \left\{ \frac{[\text{H}_2\text{O}_2]_{\text{additive}}}{[\text{H}_2\text{O}_2]_{\text{control}}} \right\} \times 100$$

$[\text{H}_2\text{O}_2]_{\text{additive}}$  = hydrogen peroxide concentration of medium containing the additive of concern; and  $[\text{H}_2\text{O}_2]_{\text{control}}$  = hydrogen peroxide concentration of the control medium.

### 3.2.14 Bulk stripped corn oil model system

The stability of stripped corn oil in the presence of barley extracts and standards (BHA and ferulic acid) under accelerated oxidative conditions was monitored over 7 days. Extracts (100 mg) were added into 30 mL screw-capped glass tubes containing 5 g of stripped corn oil. The mixture was thoroughly vortexed and placed under Schaal oven (Thelco, Model 2, Precision Scientifica Co., Chicago, IL) conditions at 60°C for 7 days. Samples for analysis of conjugated dienes (CD) and 2-thiobarbituric acid reactive substances (TBARS) analysis were drawn on days 0,1,3,5 and 7 as explained in **Sections 3.2.14.1 and 3.2.14.2.**

#### 3.2.14.1 Determination of conjugated dienes (CD)

A specified amount of oil (0.02–0.03 g) was weighed into a 25 mL volumetric flask, and made up to the mark with isooctane (2,2,4-trimethylpentane). The solution was thoroughly mixed and its absorbance read at 234 nm using a Hewlett Packard diode array spectrophotometer (Model 8452, Agilent, Mississauga, ON). Pure isooctane was used as the reference. Conjugated diene values were calculated using the following equation:

$$\text{CD} = \frac{\text{Absorbance of solution at 234 nm}}{C \times l}$$

where, C = concentration of the oil in g/100 mL; l = length of the cuvette in cm (IUPAC, 1987).

### **3.2.14.2 Determination of 2-thiobarbituric acid reactive substances (TBARS)**

The method explained by AOCS (1990) was employed to determine TBARS values. The oil (50-200 mg) was accurately weighed into a 25 mL volumetric flask and made up to volume with the n-butanol. 5 mL of this solution were transferred into a dry test tube to which freshly prepared 2-TBA reagent (200 mg 2-TBA in 100 mL 1-butanol) was added. Contents were thoroughly mixed and heated in a thermostated water bath at 95°C for 120 min. The samples were removed from the water bath and cooled under running tap water and the absorbance of the contents was read at 532 nm. A standard curve was prepared using 1, 1, 3, 3-tetramethoxypropane as the malonaldehyde (MA) precursor and the results were expressed as  $\mu\text{mol MA equivalents/g oil}$ .

### **3.2.15 $\beta$ -Carotene bleaching model system**

$\beta$ -Carotene-linoleate model system studies were carried out following the spectrophotometric method of Miller (1971) based on the ability of the extracts to decrease the oxidative bleaching of  $\beta$ -carotene in a  $\beta$ -carotene/linoleic acid emulsion. An 8 mg sample of crystalline  $\beta$ -carotene was dissolved in 10 mL of chloroform and 2 mL of the solution were pipetted into a 100 mL round bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40°C, 40 mg of linoleic acid, 400 mg of Tween 40 emulsifier and 100 mL of aerated distilled water were added to the flask with vigorous shaking. Aliquots of 4.8 mL of this emulsion were transferred into a series of tubes containing 200  $\mu\text{L}$  of 2 mg/mL barley extracts dissolved in methanol. Immediately after the addition of emulsion to the tubes, a zero time reading was measured at 470 nm using a Hewlett Packard diode array spectrophotometer absorbance

readings were recorded over a 2h period at 15 min intervals by keeping the stoppered samples in a thermostated water bath at 50°C. Blank samples devoid of  $\beta$ -carotene were prepared for background subtraction. A standard curve was prepared using ferulic acid and percentage  $\beta$ -carotene remaining after 2 h of incubation was calculated.

### 3.2.16 Accelerated oxidative stability test using Rancimat<sup>®</sup>

The effectiveness of barley extracts on delaying oxidation of stripped corn oil was measured under accelerated oxidative conditions using a Rancimat<sup>®</sup> apparatus (743 Rancimat<sup>®</sup>, Metrohm Ion Analysis Ltd., CH-9101, Herisau, Switzerland). The extracts (60 mg) were added into reaction vessels of the Rancimat<sup>®</sup> apparatus containing 3 g of stripped corn oil followed by sonicating for 10 min. A constant dry air stream (20 L/h), obtained by passing laboratory air through molecular sieve (0.3 nm) was blown through the samples in the reaction vessel which were maintained at 120°C throughout the experiment. The volatile oxidation products were collected in the measuring vessels containing 60 mL of deionized water. The conductivity of the aqueous solution was monitored continually and recorded. The inflection point (IP) was calculated by the software (743 Rancimat<sup>®</sup> PC software version 1.0, 2000, Metrohm Ion Analysis Ltd., CH-9101, Herisau, Switzerland) and recorded. A blank containing pure stripped corn oil devoid of extracts was used. Results were reported as protection factor (PF).

$$PF = \frac{IP_{\text{additive}}}{IP_{\text{control}}}$$

where,  $IP_{\text{additive}}$  = inflection point of oil mixture containing the additive;  
and  $IP_{\text{control}}$  = inflection point of pure oil.

### **3.2.17 Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity using electron paramagnetic resonance (EPR) spectrometry**

The DPPH radical scavenging assay described by Diaz and co-authors (2004) was adapted with slight modifications. Two millilitres of a 0.18 mM solution of DPPH in methanol were added to 400  $\mu$ L of various concentrations (whole barley sample; 0.67-3.33 mg/mL, final concentration and pearling fines; 0.17 mg/mL – 1.7 mg/mL, final concentration) of extracts dissolved in methanol. Contents were mixed well and after 1 min, the mixture was passed through the capillary tubing which guides the sample through the sample cavity of Bruker e-scan EPR spectrometer (Bruker E-scan, Bruker Biospin Co. Billerica, MA). The spectrum was recorded using the software (E-Scan analyzer, Bruker Biospin Co. Billerica, MA). The parameters were set as follows;  $5.02 \times 10^2$  receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000G sweep width, 3495.258 G centre field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. DPPH radical scavenging capacities of the extracts were calculated using the following equation:

DPPH radical scavenging capacity, % =  $100 - \left\{ \frac{\text{EPR signal intensity for the medium containing the additive}}{\text{EPR signal intensity for the control medium}} \right\} \times 100$

### **3.2.18 Determination of hydroxyl radical scavenging capacity using electron paramagnetic resonance (EPR) spectrometry**

The hydroxyl radical was generated via the  $\text{Fe}^{2+}$ -catalyzed Fenton reaction and spin-trapped with DMPO. The resultant DMPO-OH adduct was detected using a Bruker E-scan EPR. Barley extracts were dissolved in deionized water and diluted to obtain various concentrations (1.33 – 13.2 mg/mL, final concentrations). Extracts (100  $\mu$ L) were

mixed with 100  $\mu\text{L}$  of 10 mM  $\text{H}_2\text{O}_2$ , 200  $\mu\text{L}$  of 17.6 mM DMPO and 100  $\mu\text{L}$  of 1 mM. All the solutions were dissolved in deionized water except  $\text{FeSO}_4$  which was dissolved in deoxygenated distilled water in order to maintain reduced status until mixed with the other reagents. After 1 min the mixtures were introduced into the EPR spectrometer and the spectrum was recorded. Hydroxyl radical scavenging capacities of the extracts were calculated using the following equation;

Hydroxyl radical scavenging capacity, % =  $100 - \left\{ \frac{\text{EPR signal intensity for the medium containing the additive}}{\text{EPR signal intensity for the control medium}} \right\} \times 100$

### **3.2.19 Effect of barley extracts on preventing cupric ion-induced human low density lipoprotein (hLDL) peroxidation**

The method described by Andreasen *et al.* (2001) and Hu and Kitts (2000) was used to measure hLDL oxidation. Human LDL (in PBS, pH 7.4 with 0.01% EDTA) was dialyzed against 10 mM PBS (pH 7.4, 0.15 M NaCl) for 12 h under nitrogen at 4°C and EDTA-free hLDL was subsequently diluted to obtain a standard protein concentration of 0.2 mg/mL with PBS. The diluted hLDL solution (200  $\mu\text{L}$ ) was mixed with 1000  $\mu\text{L}$  of PBS and 10  $\mu\text{L}$  of extract (2 mg/mL) in a test tube. Oxidation of hLDL was initiated by adding 5.1 mM cupric sulphate solution resulting in a 4  $\mu\text{M}$  copper concentration in the reaction mixture. The mixture was incubated at 37°C for 100 min. The initial absorbance (t=0) was read at 232 nm immediately after mixing and every 5 min thereafter. The pattern of changing absorbance was plotted against time and percentage inhibition of CD formation was calculated as follows;

$$\% \text{ inhibition of formation of CD} = \left\{ \frac{\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{native}}} \right\} \times 100$$

where,  $\text{Abs}_{\text{oxidative}}$  = absorbance of hLDL mixture with  $\text{CuCl}_2$  only;  $\text{Abs}_{\text{sample}}$  = absorbance of LDL with extract/standard and  $\text{CuSO}_4$ ;  $\text{Abs}_{\text{native}}$  = absorbance of hLDL without  $\text{CuSO}_4$

### 3.2.20 Supercoiled strand DNA scission by peroxy and hydroxyl radicals

Plasmid supercoiled DNA (pBR 322) was dissolved in 10 mM PBS (pH 7.4, 0.15 mM sodium chloride). DNA (25 ng/ $\mu\text{L}$ ) was mixed with ferulic acid and barley extracts dissolved in the same PBS. Peroxyl radical was generated using AAPH (dissolved in PBS; pH 7.4, 0.15 mM sodium chloride) to attain a final concentration of 1 mM and mixed with the DNA and the extract mixture at a total volume of 12  $\mu\text{L}$ . The reactants were incubated at 37°C for 1 h in the dark (Hu *et al.*, 2000).

The hydroxyl radical generating system consisted of 100  $\mu\text{M}$  ferric chloride, 100  $\mu\text{M}$  ascorbic acid and 100  $\mu\text{M}$  hydrogen peroxide with the addition of 100  $\mu\text{M}$  EDTA for non site-specific hydroxyl radical generation (Halliwell *et al.*, 1987). The total volume was adjusted to 12  $\mu\text{L}$  with PBS and the reaction mixture was incubated at 37°C for 1 h in the dark. Upon completion of incubation, the loading dye (3  $\mu\text{L}$ ), consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose in distilled water, was added to the sample and loaded to a 0.7% (w/v) agarose gel prepared in Tris-acetic acid-EDTA buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5). Horizontal gel electrophoresis was performed at 32 v for 8 h. DNA strands were stained with 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide and visualized under ultraviolet light. Images were analyzed using AlphaEase™ stand

alone software (Alpha Innotech Co., San Leandro, CA). The protective effect of extracts and catechin was calculated based on the following equation.

$$\text{DNA retention, \%} = \left\{ \frac{\text{Intensity of supercoiled DNA in sample}}{\text{Intensity of supercoiled DNA in control}} \right\} \times 100$$

### 3.2.21 Inhibition of Caco-2 colon cancer cell proliferation

The Caco-2 colon cancer cell line assay was performed according to the method of Parry *et al.* (2006). The Caco-2 human colorectal adenocarcinoma cell line was propagated in T-150T flasks in McCoy's 5A media containing 10% fetal bovine serum albumin (FBS) and 1% antibiotic/antimycotic. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> (Qiao *et al.*, 1996 and Yoshida *et al.*, 2003). The media containing the cells were transferred into a 96-well microplate at a rate of 2,500 cells per well, and incubated overnight at 37°C. Extracts of whole barley and fractions (10µL) dissolved in 50% dimethyl sulfoxide (DMSO) were introduced into the wells containing 990 µL of cell medium to obtain final concentrations of 5mg/mL and 50 mg/mL. The control contained 990 µL of cell medium and 10 µL of 50% DMSO. The mixtures were incubated for four days at 37 °C and the live cells on each of the wells were counted daily using an ATPlite 1 step luminescence kit (Perkin Elmer, Boston, MA).

### 3.2.22 HPLC analysis of phenolic acids

Separation of phenolic acids (*i.e.* free and those liberated from soluble esters and soluble glycosides) from the extract was achieved according to the method of Amarowicz

and Weidner (2001). An aqueous suspension of extract (500 mg in 20 mL) was adjusted to pH 2 (6 M HCl), and free phenolics were extracted 5 times into 20 mL of diethyl ether using a separatory funnel. The extract was evaporated to dryness under vacuum at room temperature. The aqueous solution was neutralized to pH 7 with 2 M NaOH and then lyophilized. The residue was dissolved in 20 mL of 2 M NaOH and hydrolysed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 using 6M HCl, phenolic acids released from soluble esters were extracted from hydrolyzates 5 times into 30 mL of diethyl ether using a separatory funnel. The samples obtained in this way were injected onto an HPLC column and phenolic acids were analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of a LC-10AD pump, SCTL 10A system controller and SPD-M 10A photo-diode array detector. Phenolic acid separation was done using a prepacked LiChrospher 100 RP-18 column (4 × 250 mm, 5 µm; Merck, Darmstad, Germany). The mobile phase water-acetonitrile-acetic acid (88:10:2; v/v/v) was delivered at a rate of 1 mL/min and the detection was monitored at 320 and 260 nm. Phenolic acids were identified by DAD profiles using authentic standards based on the retention time (Amarowicz and Weidner, 2001).

### **3.2.23 Statistical analysis**

All experiments were carried out in triplicate and results were reported as mean ± standard deviation. The significance of differences among the values was determined at  $p < 0.05$  using analysis of variance (ANOVA) followed by the Holm-Sidak test using SigmaStat version 11 (Systat software Co., San Jose, CA).

## CHAPTER 4

### OPTIMIZATION OF EXTRACTION OF PHENOLIC COMPOUNDS

#### 4.1 Introduction

The yield and the activity of antioxidative compounds depend on the extraction method employed. In the literature, a number of methods, including cold and hot solvent extraction, supercritical extraction, and accelerated solvent extraction have been used to extract antioxidants from plant materials. Of these methods, hot solvent extraction has been widely used because of its simplicity and higher yields. The most important parameters that affect antioxidant activity in hot extraction include solvent composition, temperature, and extraction time.

A wide range of solvents and solvent combinations have been used by different researchers to extract antioxidative compounds from cereals and cereal products; including water (Martinez-Tome *et al.*, 2004), 80% chilled ethanol (Adom and Liu, 2002), acetone (4:1,v/v), ethanol (4:1, v/v), and methanol (4:1, v/v). Eighty percent methanol has been reported to yield the highest antioxidant activity with barley, rye, and wheat (Zielisnki and Kozłowska 2000). Methanol rendered the highest antioxidant activity in extracting antioxidative compounds from oat groats and hulls (Collins *et al.* 1991). Literature data indicate that the amount of components extracted by solvents increases with solvent polarity (Duh *et al.*, 1992; Przybylski *et al.*, 1998).

#### 4.2 Objectives

The objective of this work was to optimize the extraction conditions, namely composition of the aqueous extraction medium, extraction temperature, and time based

on total antioxidant capacity (TAC) as measured by the Trolox equivalent antioxidant capacity (TEAC).

### **4.3 Materials and methods**

#### **4.3.1 Materials**

The Falcon barley cultivar, obtained from Field Crop Development Centre, Lacombe, Alberta, Canada in the crop year 2000 was used as the representative barley cultivar for the optimization procedure.

#### **4.3.2 Methods**

##### **4.3.2.1 Preparation of Falcon barley samples**

The Falcon barley sample was prepared as explained in **Section 3.2.2**.

##### **4.3.2.2 Selection of appropriate extraction conditions**

Preliminary experiments were carried out to select an appropriate organic solvent using three different solvent systems, namely aqueous ethanol, methanol, and acetone, used in different proportions. The effect of solvent system on antioxidant activity of the extracts was tested by holding the other two variables (extraction temperature and time) constant. Phenolic compounds were prepared by extracting 6 g of ground defatted Falcon barley samples with 0-100% ethanol, methanol, and acetone (v/v, water/solvent) for 40 min in a thermostated water bath set at 80 °C under reflux. The resulting slurries were treated as described in **Section 3.2.3**. Total antioxidant activity of the crude phenolic extracts obtained was determined as detailed in **Section 3.2.5** and based on TEAC value,

the best organic solvent and its best three compositions (lower, middle, and upper levels) were selected.

In the next step, the best range of temperature for extraction of phenolic compounds from Falcon barley was determined. Phenolic compounds were extracted using the middle solvent composition selected above in a thermostated water bath at various temperatures ranging from 30 to 80°C (one temperature treatment at a time) for 40 min. Crude phenolic extracts were obtained as explained by centrifuging and subsequently desolventing as explained above and in **Section 3.2.3**. Total antioxidant capacity of the crude phenolic extracts obtained was determined as detailed in **Section 3.2.5** and based on TEAC value, the best three values (lower, middle, and upper levels) for temperature was selected.

The objective of the final step of the preliminary experiments was to determine the best extraction time. Phenolic compounds were extracted using the best solvent system (from step 1), the middle temperature value (from step 2), for various times (20 – 70 min). The resulting crude phenolic extracts were assessed for TAC and based on the TEAC values, the best three values (lower, middle, and upper levels).

#### **4.3.2.3 Use of response surface methodology (RSM) to establish the optimum extraction parameters**

A three-factor, three-level face centered cube design with 17 different design points was adapted for this purpose (Mason *et al.*, 1989; Snedcore and Cochran, 1980) (**Table 4.1**). Three independent factors studied were composition of organic solvent, extraction temperature, and extraction time. Response (Y) was the antioxidative index [Trolox equivalent antioxidant capacity (TEAC) values] calculated for the extracts. The

extractions were carried out according to the **Table 4.1** and the TEAC value of each extraction was determined. The following generalized second order polynomial model was used in the RSM.

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i < j=1}^3 \beta_{ij} X_i X_j$$

where,  $\beta_0$ ,  $\beta_{ii}$  and  $\beta_{ij}$  are regression coefficients for intercept, linear, quadratic and interaction terms, respectively.  $X_i$  and  $X_j$  are independent variables.

Antioxidant index expressed as (TEAC) values were analyzed using a general linear model (GLM) and response surface regression coefficients were substituted in the quadratic polynomial equation. Response surface and contour plots were obtained using the fitted model.

#### **4.3.2.4 Verification of the model used**

Verification experiments were carried out using combinations of variables at different levels within the experimental range in order to determine the adequacy of the model fitted. The extraction parameters established by RSM were used to extract the phenolic components from barley extracts.

### **4.4 Results and discussion**

#### **4.4.1 Selection of three levels of factors (extraction parameters) for design points**

##### **4.4.1.1 Selection of factor levels for solvent composition ( $X_1$ )**

The most important parameters affecting the efficacy of extraction of antioxidative phenolic compounds are solvent system and its composition ( $X_1$ ), temperature ( $X_2$ ) and time of extraction ( $X_3$ ). The main objective of the preliminary experiments was to select three optimal levels (lower, middle, and upper) for each of the

parameters mentioned above. These three levels of independent variables were selected based on TAC as determined by TEAC. **Figures 4.1 (a), (b), and (c)** illustrate the effect of the composition of aqueous methanol, ethanol, and acetone, respectively, on TEAC value.

TEAC value increased with increasing organic solvent content for all solvents, reached a maximum and then started to decrease. Maximum antioxidant activity was obtained when the proportion of organic solvent in the aqueous extraction medium was 80% for methanol and ethanol and 70% for acetone. Among the solvents used, methanol yielded the highest antioxidant activity as measured by TEAC. Thus, aqueous methanol concentrations of 70, 80 and 90% were selected as the lower, middle and upper design points, respectively.

#### **4.4.1.2 Selection of factor levels for temperature ( $X_2$ )**

In the second step, the effect of temperature on TAC was investigated. The effect of extraction temperature on antioxidant activity also followed a polynomial function with a correlation coefficient of  $r^2 = 0.93$  [**Figure 4.1(d)**]. Extraction at low temperature yielded lower antioxidant activity as measured by TEAC value, which gradually increased up to about 60°C and then exhibited a downward trend. Therefore, 40, 60 and 80°C were selected as the lower, middle and upper points, respectively. Extraction of antioxidative compounds was not efficient at low temperatures while high temperatures might lead to destruction of antioxidative components, thus leading to low TEAC values.

#### 4.4.1.3 Selection of factor levels for time ( $X_3$ )

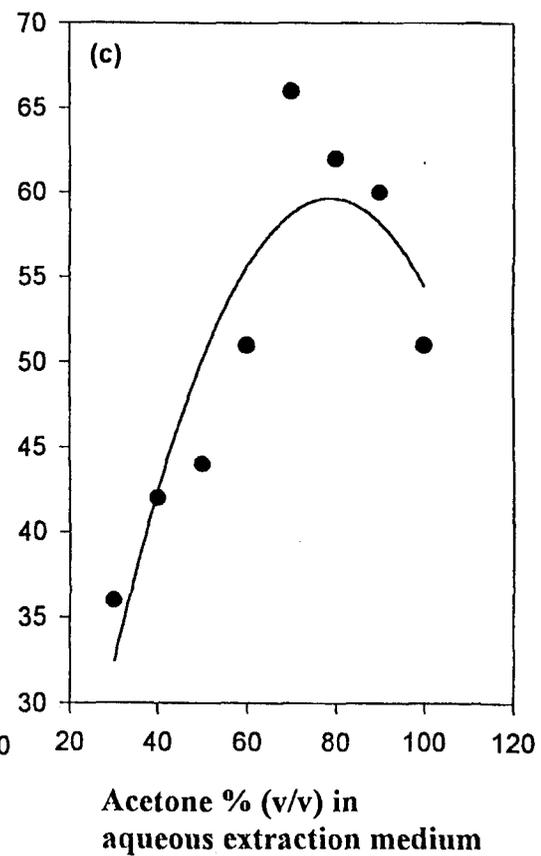
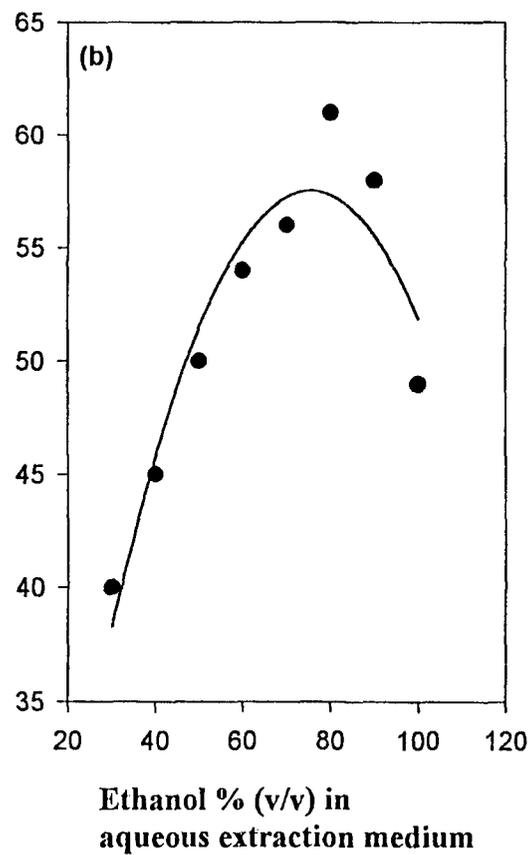
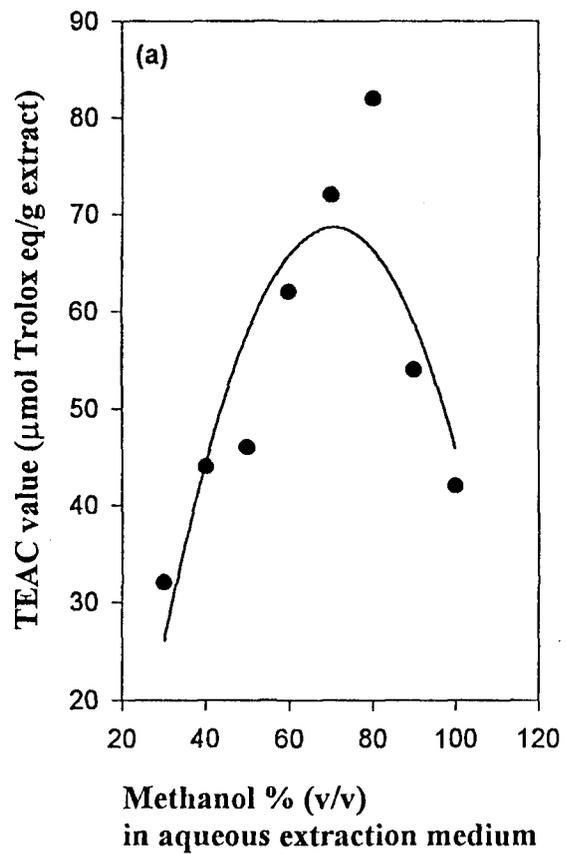
The third factor investigated was the time length of the extraction (20-70 min) and a similar trend was observed. The regression analysis showed that the effect of time on total antioxidant capacity followed a second order polynomial pattern with a regression coefficient of  $r^2 = 0.94$  [Figure 4.1(e)]. With the increase of extraction time, antioxidant capacity gradually increased reaching a plateau around 40 min and then started decreasing. Thus, times of 20, 40 and 60 min were selected as the lower, middle, and upper levels for RSM.

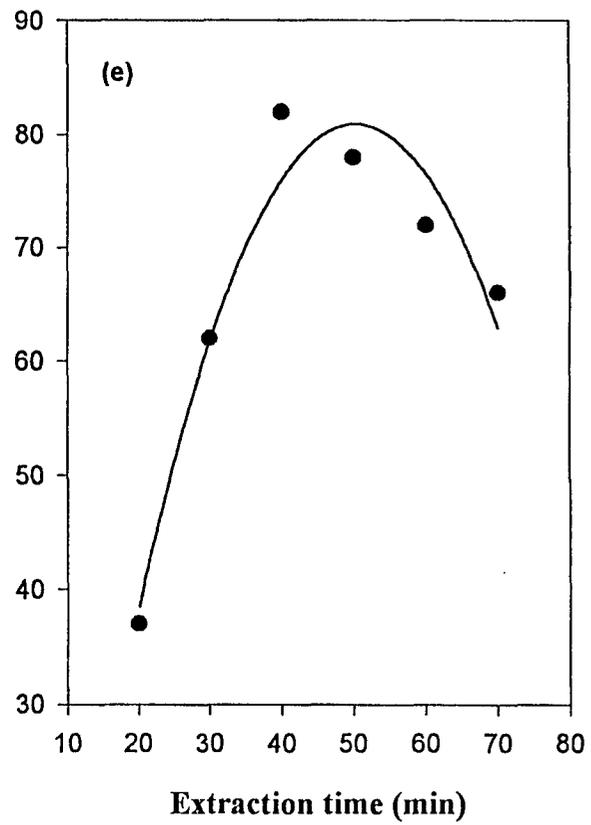
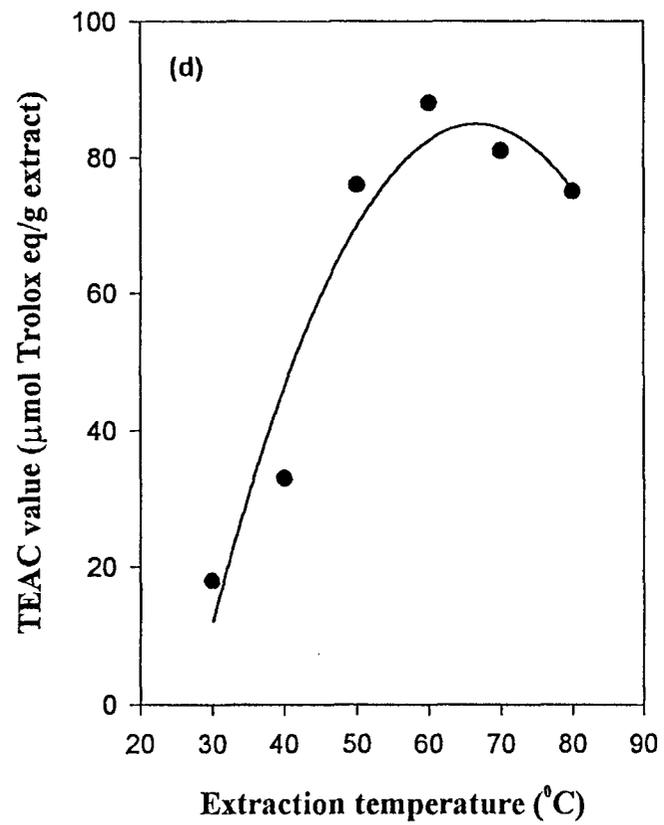
Times less than 30 min did not yield high antioxidant activity due to insufficient time available for the extraction process while prolonged extraction time might lead to structural changes in antioxidative compounds.

#### 4.4.2 Fitting data to experiment model and data analysis

The three independent variables, solvent composition ( $X_1$ ), temperature ( $X_2$ ), and time ( $X_3$ ) were assigned a code (Table 4.2). The centre point of each independent variable level was given a code of zero while the lowest and highest levels of interest of each variable were coded minus and plus one, respectively.

**Figure 4.1** Effect of varying organic solvents methanol (**a**), ethanol (**b**), and acetone (**c**), extraction temperature (**d**), and time (**e**) on the total antioxidant capacity (TAC) as measured by Trolox equivalent antioxidant capacity (TEAC). Each data point represents the average of two determinations.





While a number of different designs are available for fitting a response surface to experimental data (Mason *et al.*, 1989), a special form of central composite  $2^3$  factorial designs was selected for fitting data in this study. The design known as face-centred cube design has gained popularity due to a lesser number of determinations being required as opposed to factor factorial experiments. Having 3 levels instead of 5 levels in most classical methods, the face-centred cube design has the advantage of using a fairly small number of design points. Three replicates were taken at the design centre (0,0,0) so that the total number of points remain at 17 ( $n = 8 + 6 + 3 = 17$ ). **Table 4.1** lists the response values (Y) obtained against design points. Seventeen different combinations of factors and the response obtained (TEAC value) were analyzed using the face-centred cube design model. Regression coefficients of intercepts, linear, quadratic and interaction terms of the experimental model were calculated and their levels of significance were determined using the t-tests.

Multiple regression coefficients, obtained by employing a least-squares procedure to predict the quadratic polynomial model for the antioxidant capacity, are summarized in **Table 4.3**. Examination of these parameters with the t-tests indicated that the linear and quadratic terms of solvent percentage were highly significant ( $p=0.0001$ ).

As with most of the RSM problems, in this case, the form of relationship between the response (Y) and independent variables ( $x_1$ ,  $x_2$ , and  $x_3$ ) is unknown. Thus, the first step is to find a suitable approximation for the function (f). As explained previously, since antioxidant activity followed a quadratic function of solvent composition, temperature, and time, so a quantitative polynomial model was selected for RSM (**Section 4.4.1**).

**Table 4.1** Face-centred cube design and observed responses<sup>1</sup>

Design point	Independent variable <sup>2</sup>			Response (Y) <sup>6</sup>
	X <sub>1</sub> <sup>3</sup>	X <sub>2</sub> <sup>4</sup>	X <sub>3</sub> <sup>5</sup>	
1	(-1) 70	(-1) 40	(-1) 20	63.1
2	(-1) 70	(-1) 40	(+1) 60	64.3
3	(-1) 70	(+1) 80	(-1) 20	65.4
4	(-1) 70	(+1) 80	(+1) 60	66.2
5	(+1) 90	(-1) 40	(-1) 20	68.6
6	(+1) 90	(-1) 40	(+1) 60	67.2
7	(+1) 90	(+1) 80	(-1) 20	66.9
8	(+1) 90	(+1) 80	(+1) 60	65.9
9	(-1) 70	(0) 60	(0) 40	74.9
10	(+1) 90	(0) 60	(0) 40	75.8
11	(0) 80	(-1) 40	(0) 40	87.6
12	(0) 80	(+1) 80	(0) 40	90.6
13	(0) 80	(0) 60	(-1) 20	92.7
14	(0) 80	(0) 60	(+1) 60	96.7
15	(0) 80	(0) 60	(0) 40	99.2
16	(0) 80	(0) 60	(0) 40	102
17	(0) 80	(0) 60	(0) 40	101

<sup>1</sup>Non-randomized<sup>2</sup>See Table 1 for actual values<sup>3</sup>X<sub>1</sub>: Solvent (methanol) content (% v/v) in the extraction media<sup>4</sup>X<sub>2</sub>: Extraction temperature (°C)<sup>5</sup>X<sub>3</sub>: Extraction time (min)

Y : TEAC value (μmol Trolox equivalents / gram extract)

<sup>6</sup>Mean of duplicate determinations except for points 15-17 where the trials were run in triplicate.

**Table 4.2** Variable ( $X_1$ ,  $X_2$  and  $X_3$ ) levels used for response surface methodology

Variable	Symbol	Coded variable levels		
		(-1)	(0)	(+1)
Solvent (methanol, % v/v)	$X_1$	70	80	90
Extraction temperature ( $^{\circ}\text{C}$ )	$X_2$	40	60	80
Extraction time (min)	$X_3$	20	40	60

It is assumed that the independent variables  $X_1, X_2, \dots, X_k$  are continuous and controllable by the experimenter with negligible error while the response (Y) is assumed to be a random variable (Montgomery, 1976). In this particular case, the variables were assumed to be continuous and controllable while the response (TEAC value) was assumed to be a random variable. The observed response,  $y$  can be expressed as a function of the independent variable

$$Y = f(X_1, X_2, X_3) + \varepsilon$$

where,  $f$  is function and  $\varepsilon$  is random error.

**Table 4.3** shows the results of the analysis of variance and multivariate regression employed in the determination of regression coefficients. Multiple regression coefficients of intercept, linear, quadratic, and interaction terms were obtained by employing the least square technique and determining the significance against the  $t$  test. Examination of the parameters revealed that the linear and quadratic effects of the organic solvent content in the extraction medium ( $x_1$ ) was highly significant ( $p < 0.0001$ ). All interaction terms were insignificant ( $p > 0.05$ ) while the polynomial model fitted to experimental data was highly significant ( $p < 0.05$ ). The coefficient of determination ( $R^2$ ) was 0.98 indicating that the model explained most of the observed variation while the coefficient of variation (CV) of 2.90% indicated that the model was reproducible. The high correlation coefficient obtained indicated that the polynomial function adequately explained the variation. Furthermore, error analysis showed that the lack of fit was not significant ( $p > 0.05$ ). The fitted model was as follows:

$$Y = 98.906 - 2.7693 w_1^2 - 8.3585 w_2^2 - 22.1418 w_3^2$$

where, Y is response (TEAC) and  $w_1$ ,  $w_2$  and  $w_3$  are the axes of response surfaces.

The response surface analysis revealed that the methanol content ( $X_1$ ) in the extraction medium had the greatest effect on the antioxidant activity of extract followed by a linear effect of temperature ( $X_2$ ) and time ( $X_3$ ).

#### **4.4.3 Analysis of response surface**

RSM is an optimization technique that determines optimum process conditions by combining special experimental designs with modeling by first or second order polynomial equations in a stepwise experimental procedure.

RSM is capable of testing a number of variables at a time, thus reducing the number of required determinations. It is a collection of mathematical and statistical techniques useful for analyzing problems where several independent variables influence a dependent variable or response, and the goal is to optimize the response (Montgomery, 1976).

##### **4.4.3.1 Determination of stationary points using canonical analysis**

Canonical analysis is a mathematical approximation that can be used to determine the stationary point and whether it represents the maximum, minimum, or saddle point (Mason *et al.*, 1989). Canonical analysis was performed to examine the nature of the stationary point of response surfaces.

**Table 4.3.** Estimated regression coefficients of the quadratic polynomial model for extraction of antioxidative components from barley

Parameter <sup>1</sup>	Estimated coefficient	Standard error
Intercept		
$\beta_0$	-1461.254****	149.227
Linear		
$\beta_1$	36.2222****	3.8645
$\beta_2$	2.6576	0.9196
$\beta_3$	1.1681	0.7637
Quadratic		
$\beta_{11}$	-0.2256****	0.0239
$\beta_{22}$	-0.0283	0.0059
$\beta_{33}$	-0.0068	0.0059
Interaction		
$\beta_{12}$	-0.0077	0.0069
$\beta_{13}$	-0.0091	0.0069
$\beta_{123}$	--	--
$R^2$	0.9895	
F value	73.41	
P value	<0.0001	
CV,%	2.9034	

<sup>1</sup>Parameters refer to the general linear model

where  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$ ,  $\beta_{ij}$ , and  $\beta_{ijk}$  are regression coefficients for intercept, linear, quadratic and interaction terms, respectively.

$R^2$  = regression coefficient

CV% = coefficient of variation

\*\*\*\* Significant at 0.0001 level

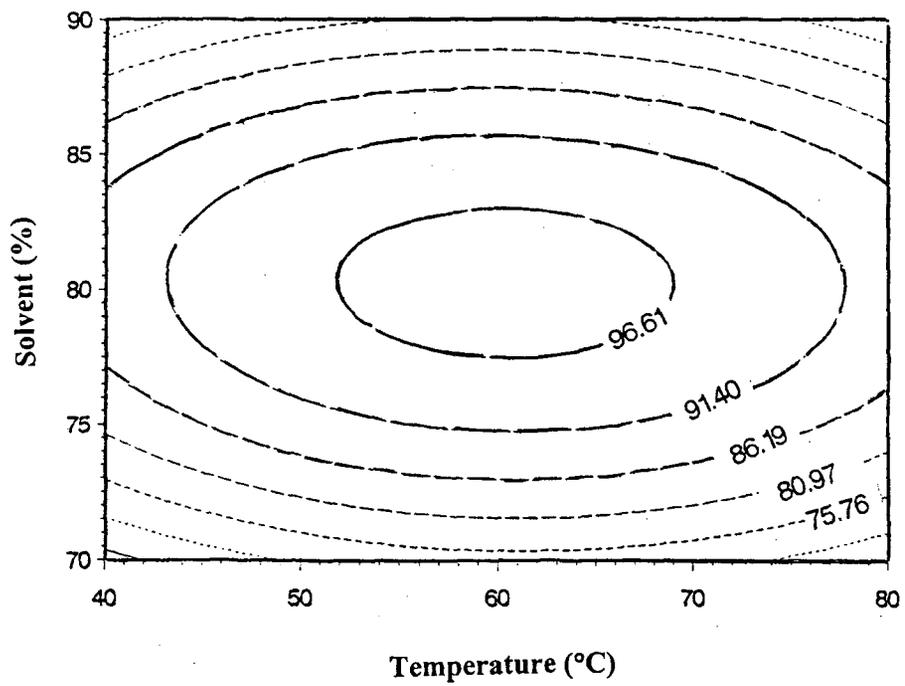
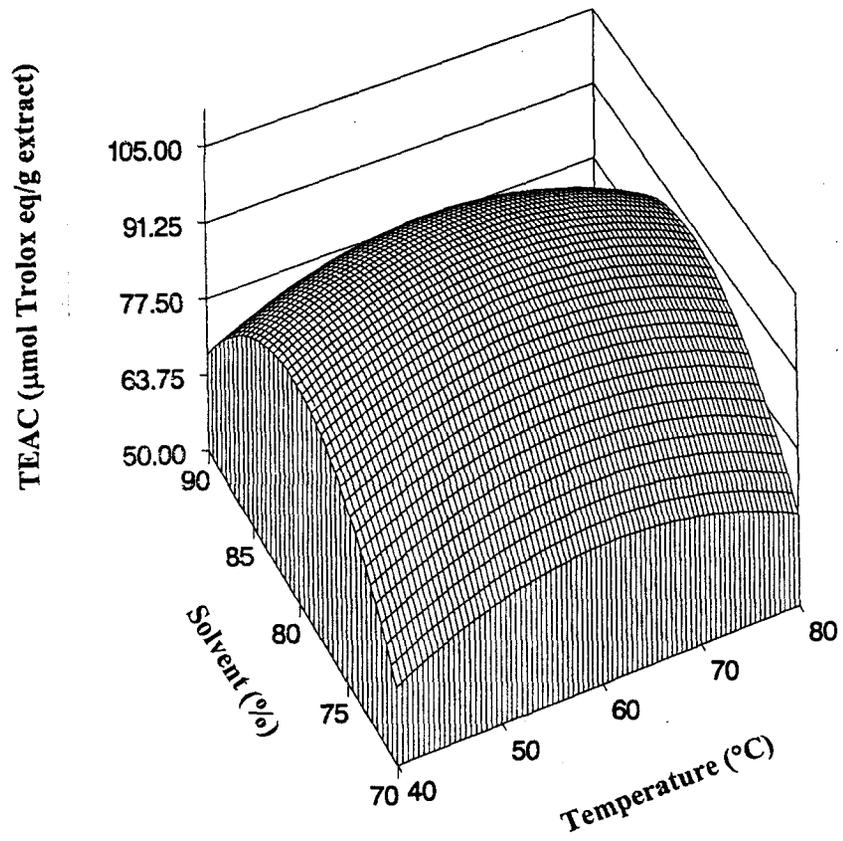
Contour plots were generated using data obtained from canonical analysis. The contours represent constant response as a function of  $x_1$  and  $x_2$ . These contour plots show the location of a maximum response within the experimental region.

All eigen values were negative indicating a maximum or minimum stationary point. Based on the response surface mesh plots, the stationary point was a maximum. Canonical analysis revealed that the critical values were  $X_1 = 80.24 \%$ ,  $X_2 = 60.47^\circ\text{C}$  and  $X_3 = 38.36 \text{ min}$ . The response surfaces were generated as a function of two factors with the third being held at a fixed level. The response surfaces were analyzed to determine the combination of factors that produce the maximum response (**Figure 4.2a** and **4.3a**). Alternatively, these results can be presented as contour plots (**Figure 4.2 b** and **4.3b**) by drawing the  $X_1$ , and  $X_2$  axes in the plane of the paper and visualizing the  $E(Y)$  axis perpendicular to the plane of paper.

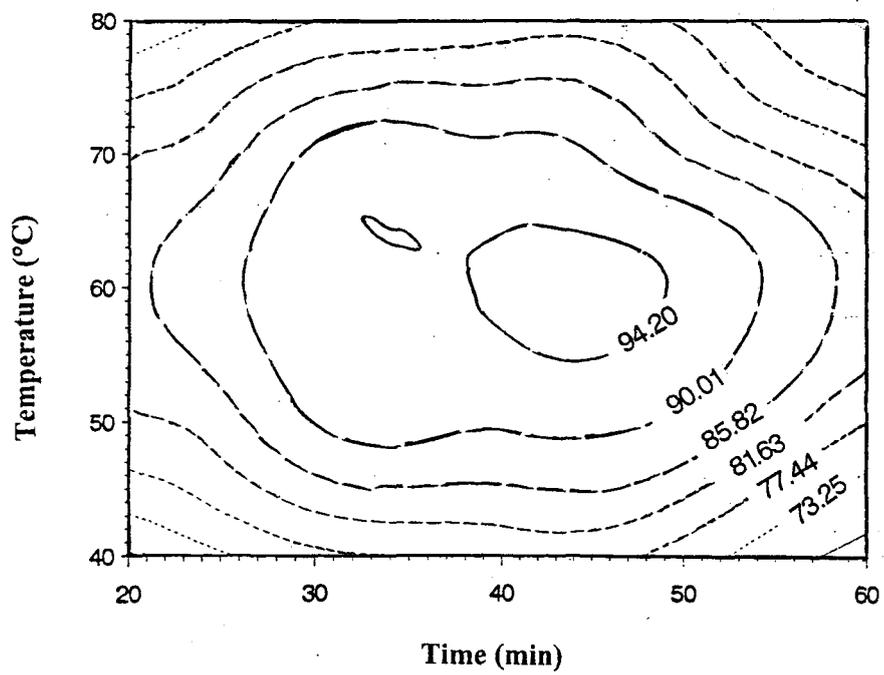
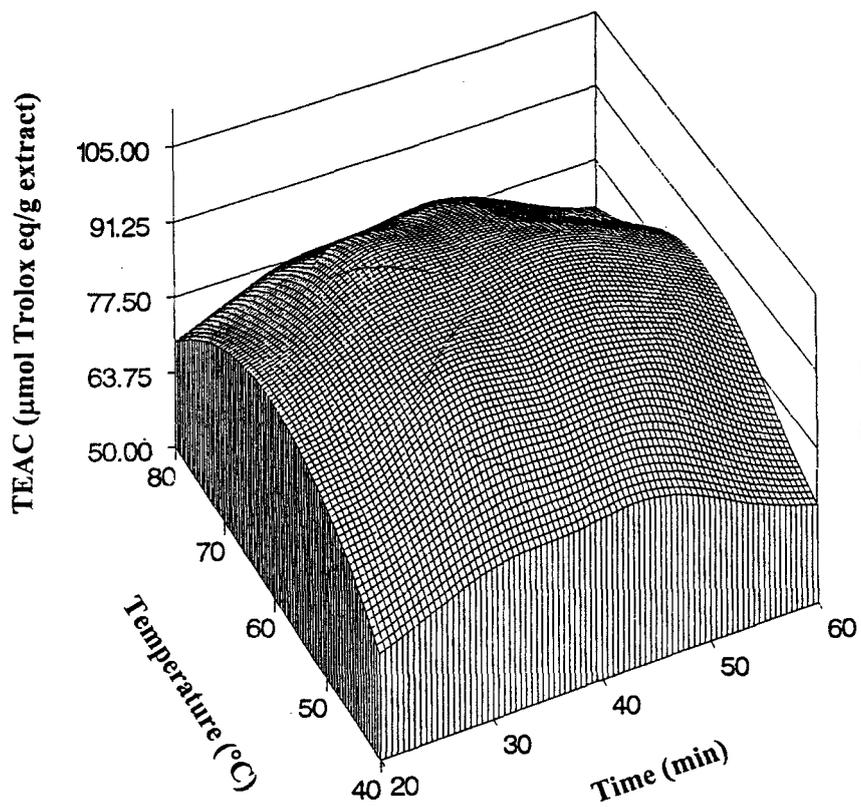
#### **4.4.4 Verification of the model**

Extractions carried out using the parameters predicted by the model indicated that the observed responses were in agreement with predicted values. Optimum response on a response surface can be mathematically investigated. If a maximum or minimum response value exists it corresponds to the predictor variable values that are a solution of the set of equations obtained by setting each of partial derivatives of the estimated response factors with respect to the predictor variables equal to zero (Mason *et al.*, 1989). The predictor variable values that satisfy the set of equations are called a stationary point.

**Figure 4.2** Response surface **(a)** and contour plot **(b)** illustrating the relationship among extraction parameters.



**Figure 4.3** Response surface (a) and contour plot (b) illustrating the relationship among extraction parameters.



#### **4.5 Conclusions**

The optimum conditions for extraction of phenolic compounds from whole barley were 80.24% aqueous methanol (v/v), 60.47°C, and 38.36 min.

## CHAPTER 5

### ANTIOXIDANT ACTIVITY OF WHOLE BARLEY EXTRACTS

#### 5.1 Introduction

Barley is used around the world for production of pasta and other baked products, brewing, and malted products. Over the last few years, it has gained increasing attention due to its positive dietary role. A number of bioactives such as  $\beta$ -glucan, tocopherols and phenolic compounds have been identified in barley. Phenolic antioxidative compounds such as benzoic and cinnamic acid derivatives, proanthocyanidins, quinones, flavanols, and flavones (Hernay *et al.*, 2001; Bonoli *et al.*, 2004). A great body of evidence has been accumulated indicating that plant polyphenols are an important class of defense antioxidants. Grains contain unique phytochemicals that complement those in fruits and vegetables when consumed together. A wide array of phenolic compounds are present in grains and these include derivatives of benzoic and cinnamic acids, anthocyanidins, quinones, flavanols, chalcones, flavones, flavanones, and aminophenolic compounds, among others (Shahidi and Naczk, 2004).

Estimated barley production in Canada was 10.287 million metric tons in 2006 (Statistics Canada, 2006). The major portion of Canadian barley production is used for animal feed production while the rest is used for bakery products, baby food, malting and production of nutraceuticals (e.g.  $\beta$ -glucan). Over the past decade an increasing interest in barley for human consumption has been observed, mainly due to health-related bioactives. Health benefits of barley are mainly attributable to its dietary fibre content, however, there is increasing evidence that antioxidative properties rendered by phenolic compounds present in barley significantly contribute toward the overall benefits.

## **5.2 Objectives**

The objectives of this phase of the research were to determine antioxidative, antiradical and antiproliferative efficacies of six different barley cultivars, namely AC Metcalfe, Falcon, Phoenix, Tercel, Tyto, and Peregrine that are grown in the Canadian prairies.

## **5.3 Materials and methods**

### **5.3.1 Materials**

Six barley cultivars, Falcon, AC Metcalfe, Tyto, Tercel, and Peregrine were obtained from the Field Crop Development Centre, Lacombe, Alberta, Canada, during the 2002 crop year. The samples were stored in the dark at ambient temperature until analyzed.

### **5.3.2 Methods**

#### **5.3.2.1 Preparation of samples**

Barley samples were prepared and analysed as explained in **Section 3.2.2** and analysed as described earlier.

## **5.4 Results and discussion**

### **5.4.1 Proximate composition of barley**

Moisture, ash, crude protein, and total lipids of Falcon, AC Metcalfe, Tyto, Tercel, Phoenix, and Peregrine cultivars of barley are listed in **Table 5.1**

#### 5.4.2 Yield of extracts

Optimum extraction parameters determined by RSM (**Section 4.3**) were used for extraction of antioxidative compounds from six barley cultivars, namely Falcon, AC Metcalfe, Tyto, Tercel, Phoenix, and Peregrine. The percentage yield obtained under optimum extraction conditions established by RSM varied between 4.6 and 5.7% based on defatted weight (**Table 5.2**). These values lie within the range reported by Zielinski and Kozłowska (2000). Eighty percent methanol was used for all the extractions, which yielded the highest antioxidant activity as measured by TEAC. Xing and White (1997) reported that methanol was one of the best solvents for extracting phenolics and other polar materials from cereals.

#### 5.4.3 Total phenolic content (TPC)

Folin-Ciocalteu method was used to quantify TPC of the whole barley extracts in this study. TPC ranged from 13.58 to 22.93 mg ferulic acid equivalents per gram dry weight. The corresponding values on the basis of defatted weight ranged from 0.81 to 1.38 mg ferulic acid equivalents per gram.

The six barley cultivars in order of total phenolic content were Peregrine > AC Metcalfe > Falcon > Tyto > Phoenix > Tercel (**Table 5.2**). Zielinski and Kozłowska (2000) reported that barley cultivars Mobek and Gregor contained 26.9 and 24.3 mg catechin equivalents of total phenolics per gram of lyophilizate extracted using 80% methanol. Barley cultivars tested in this study contained more total phenolics than wheat, rye and oat as reported by Zielinski and Kozłowska (2000).

**Table 5.1** Proximate composition of barley cultivars

Barley cultivar	Proximate composition (% w/w)			
	Moisture	Protein	Lipid	Ash
Falcon	14.21 ± 1.22 <sup>ab</sup>	8.72 ± 0.07 <sup>a</sup>	1.98 ± 0.06 <sup>b</sup>	1.45 ± 0.00 <sup>b</sup>
AC Metcalfe	15.30 ± 1.31 <sup>c</sup>	8.82 ± 0.09 <sup>a</sup>	1.63 ± 0.01 <sup>a</sup>	1.33 ± 0.02 <sup>a</sup>
Tyto	13.98 ± 0.97 <sup>a</sup>	9.34 ± 0.34 <sup>b</sup>	1.49 ± 0.05 <sup>a</sup>	1.62 ± 0.02 <sup>c</sup>
Tercel	14.63 ± 1.52 <sup>b</sup>	9.02 ± 0.65 <sup>b</sup>	2.02 ± 0.01 <sup>b</sup>	1.45 ± 0.06 <sup>b</sup>
Phoenix	13.77 ± 0.28 <sup>a</sup>	9.67 ± 0.03 <sup>b</sup>	2.34 ± 0.07 <sup>c</sup>	1.30 ± 0.03 <sup>a</sup>
Peregrine	14.32 ± 1.62 <sup>ab</sup>	8.32 ± 0.01 <sup>a</sup>	1.88 ± 0.03 <sup>a</sup>	1.56 ± 0.01 <sup>c</sup>

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

The Folin-Ciocalteu method, used traditionally for quantifying TPC in antioxidative extracts, was used for measuring, the TPC of the whole barley extracts. This method, initially intended for the analysis of proteins, was later developed as a total phenolic assay by Singleton and Rossi (1965). The Folin-Ciocalteu assay actually measures the reducing power of the sample (Huang *et al.*, 2005) and involves two components in the reaction mixture, antioxidant and oxidant (also serves as the probe), which follows the following electron transfer reaction.



The probe is an oxidant that accepts an electron from the antioxidant, causing colour change of the probe (Huang *et al.*, 2005). Folin-Ciocalteu reagent consists of a heteropoly phosphotungstic-molybdic complex that is oxidized by reducing agents present in the extract, mainly phenolic groups. Thus, this method is not specific to phenolic compounds as the complex can also be reduced by non-phenolic compounds such as ascorbic acid and Cu(I).

The testing system is a mixture of tungstic and molybdate in a highly basic medium, which promotes abstraction of the phenolic proton leading to the formation of phenolate ion, which is capable of reducing the reagent. This results in the formation of a blue colour complex, which has an absorption maximum at 750 nm (Roginski and Lissi, 2005). The blue complex formed is independent of the structure of the phenolic compound (Huang *et al.*, 2005).

**Table 5.2** Yield, total phenolic content (TPC) and total antioxidant capacity (TAC) of whole barley extracts

Barley cultivar	Extract yield (%, w/w)	TPC <sup>1</sup>	TPC <sup>2</sup>	TAC <sup>3</sup>
Falcon	5.1 ± 0.3 <sup>ab</sup>	16.31 ± 0.22 <sup>d</sup>	0.83 ± 0.01 <sup>b</sup>	5.27 ± 0.03 <sup>b</sup>
AC Metcalfe	5.7 ± 0.9 <sup>b</sup>	19.99 ± 0.15 <sup>c</sup>	1.14 ± 0.02 <sup>c</sup>	5.33 ± 0.20 <sup>c</sup>
Tyto	4.7 ± 0.7 <sup>a</sup>	15.53 ± 0.23 <sup>c</sup>	0.73 ± 0.02 <sup>a</sup>	4.92 ± 0.04 <sup>a</sup>
Tercel	5.2 ± 0.6 <sup>ab</sup>	13.58 ± 0.09 <sup>a</sup>	0.71 ± 0.01 <sup>a</sup>	3.74 ± 0.05 <sup>a</sup>
Phoenix	4.6 ± 0.6 <sup>a</sup>	14.87 ± 0.10 <sup>b</sup>	0.68 ± 0.01 <sup>a</sup>	4.23 ± 0.24 <sup>a</sup>
Peregrine	5.2 ± 0.9 <sup>ab</sup>	22.93 ± 0.07 <sup>f</sup>	1.19 ± 0.03 <sup>c</sup>	6.82 ± 0.26 <sup>d</sup>

<sup>1</sup>TPC was expressed as mg ferulic acid/g extract.

<sup>2</sup>TPC was expressed as mg ferulic acid/g defatted material.

<sup>3</sup>TAC was measured by Trolox equivalent antioxidant capacity (TEAC) and expressed as µmol/g defatted material.

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

A strong relationship existed between the TPC and TAC of whole barley extracts as determined by TEAC (**Figure 5.1a**). TAC was strongly correlated with TPC ( $r^2 = 0.87$ ). On the other hand, efficacy of inhibiting DNA scission, DPPH and hydroxyl radical scavenging capacities did not correlate well with TPC, indicating that factors other than TPC may play a role in antioxidant activity of 80% methanolic extracts (**Figures 5.2 and 5.3**). The narrow range of total phenolic content might also have attributed to the poor correlation. Moreover, all phenolics do not render the same level of antioxidant efficacy, since phenolic compounds may have antagonistic or synergistic effects with themselves or with other constituents of the extracts (Rice-Evans *et al.*, 1996). Other than phenolic compounds, protein in barley that was extracted in aqueous methanol may contribute to antioxidant activity and Iwama *et al.* (1997) reported that cereal proteins exert a strong antioxidant activity.

Barley phenolics include phenolic acids, flavan-3-ols including catechin, and epicatechin, while the most abundant dimers are prodelfphinidin B<sub>3</sub> and procyanidin B<sub>3</sub> (McMurrough, 1996; Goupy *et al.*, 1999). The major trimers were procyanidin C<sub>2</sub> and proanthocyanidin T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>. Monomeric, dimeric and trimeric flavan-3-ols accounted for 58-68% of total phenolic content (McMurrough *et al.*, 1983).

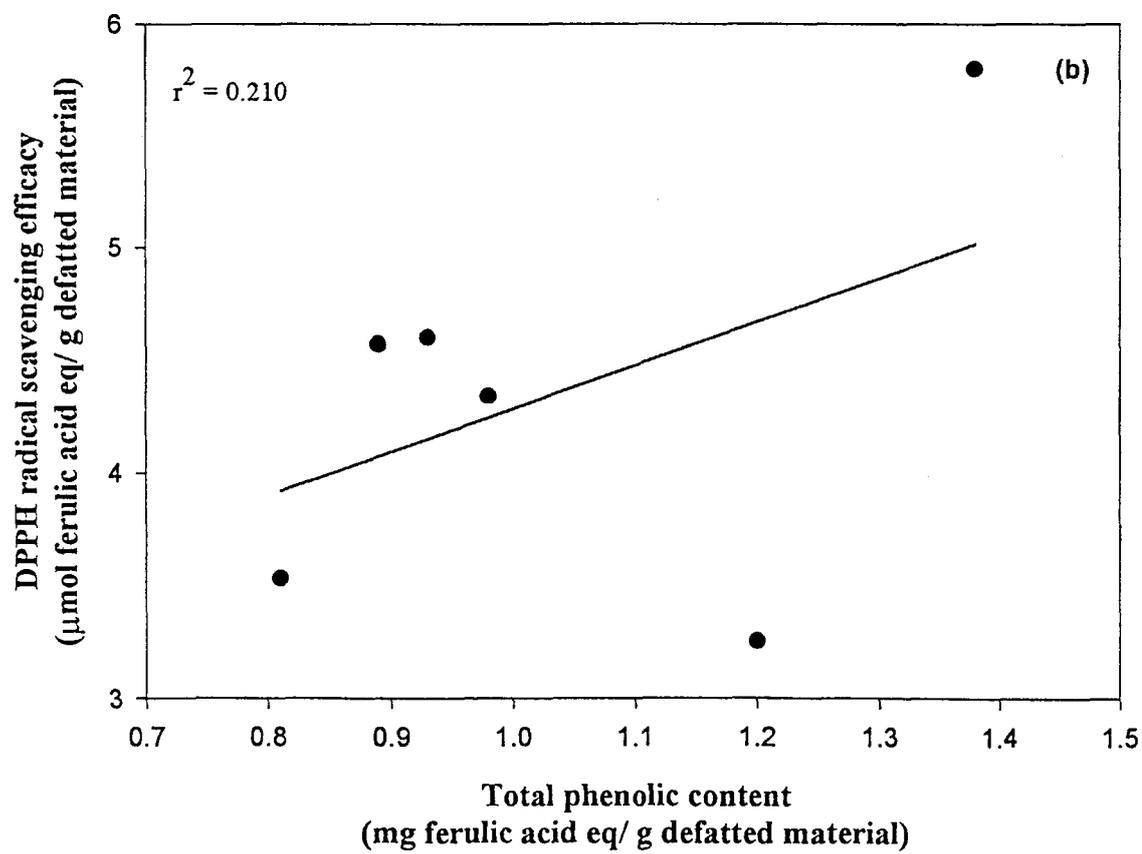
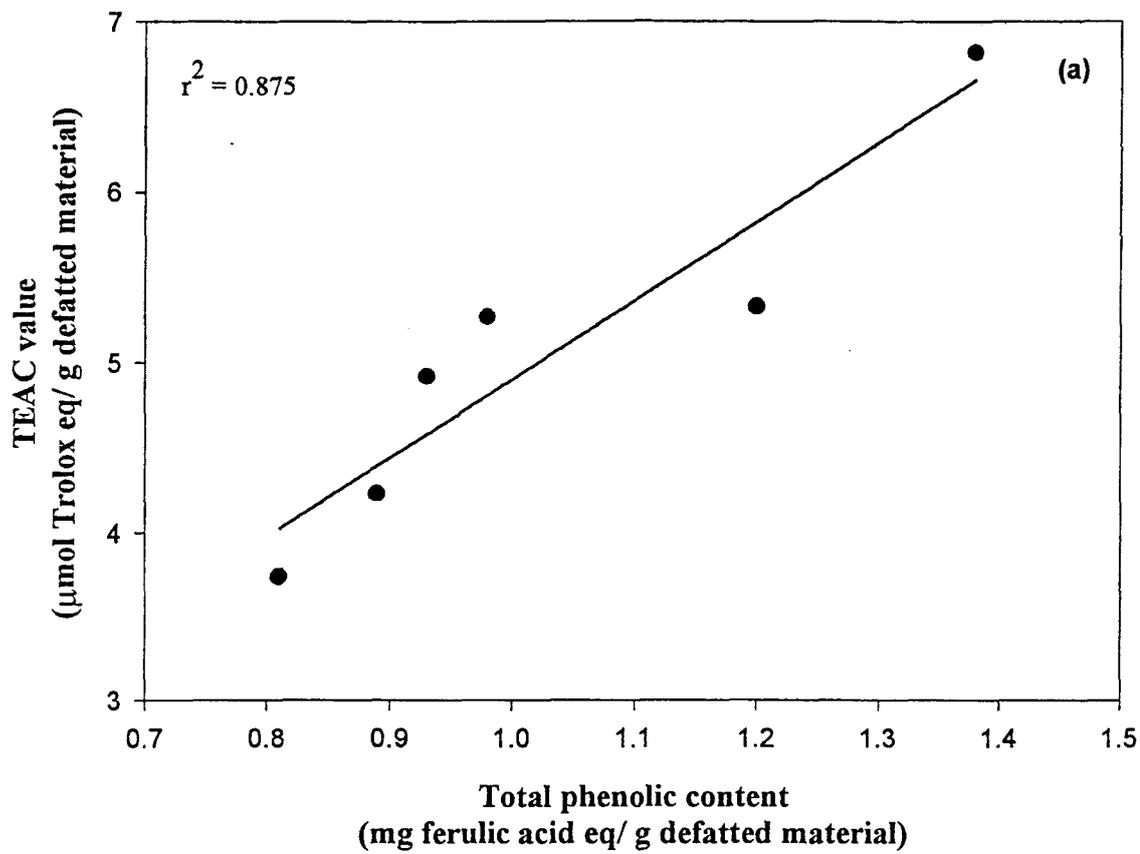
It was reported that ferulic acid is the major phenolic acid present in barley among other phenolic acids, namely vanillic acid, *p*-coumaric acid and caffeic acid (Du and Gebicki, 2004). Hydroxycinnamic and hydroxybenzoic acids are the primary phenolic antioxidants acting as free radical acceptors (Goupy *et al.*, 1999).

#### 5.4.4 Total antioxidant capacity (TAC) measured by Trolox equivalent antioxidant capacity (TEAC)

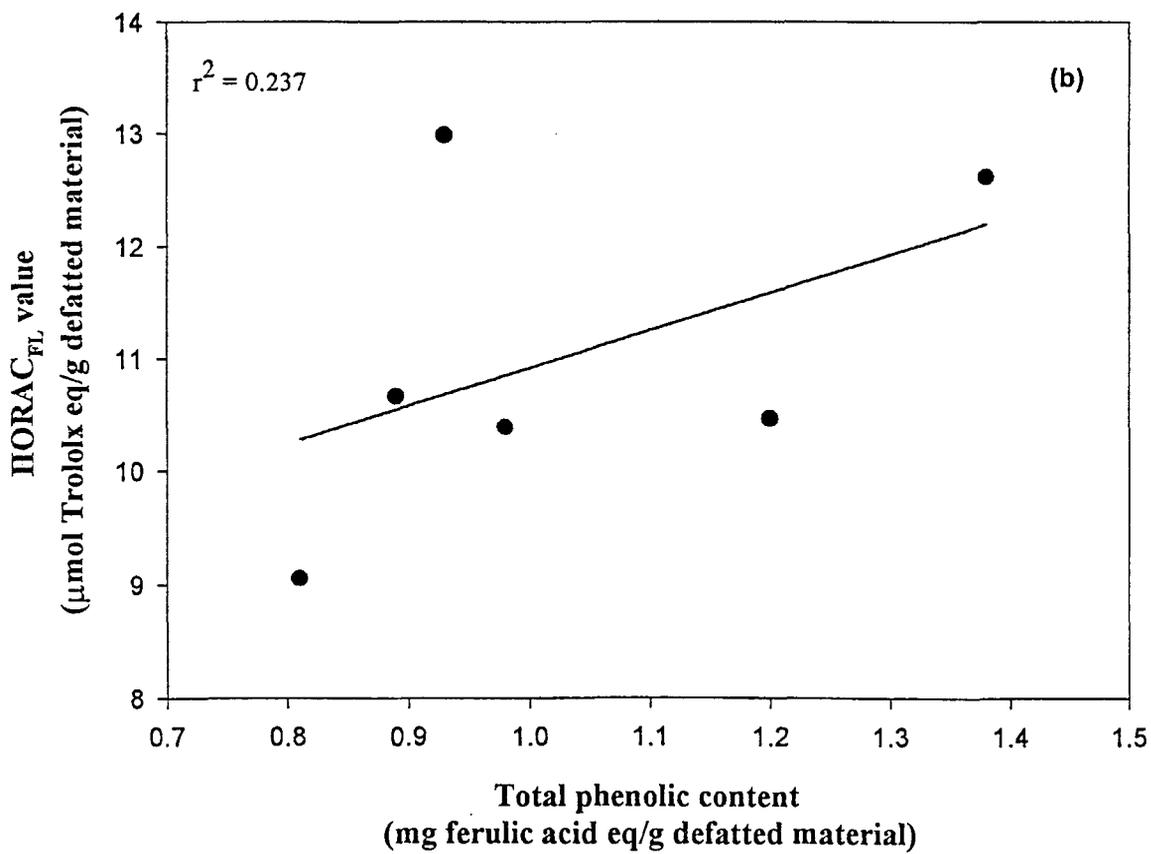
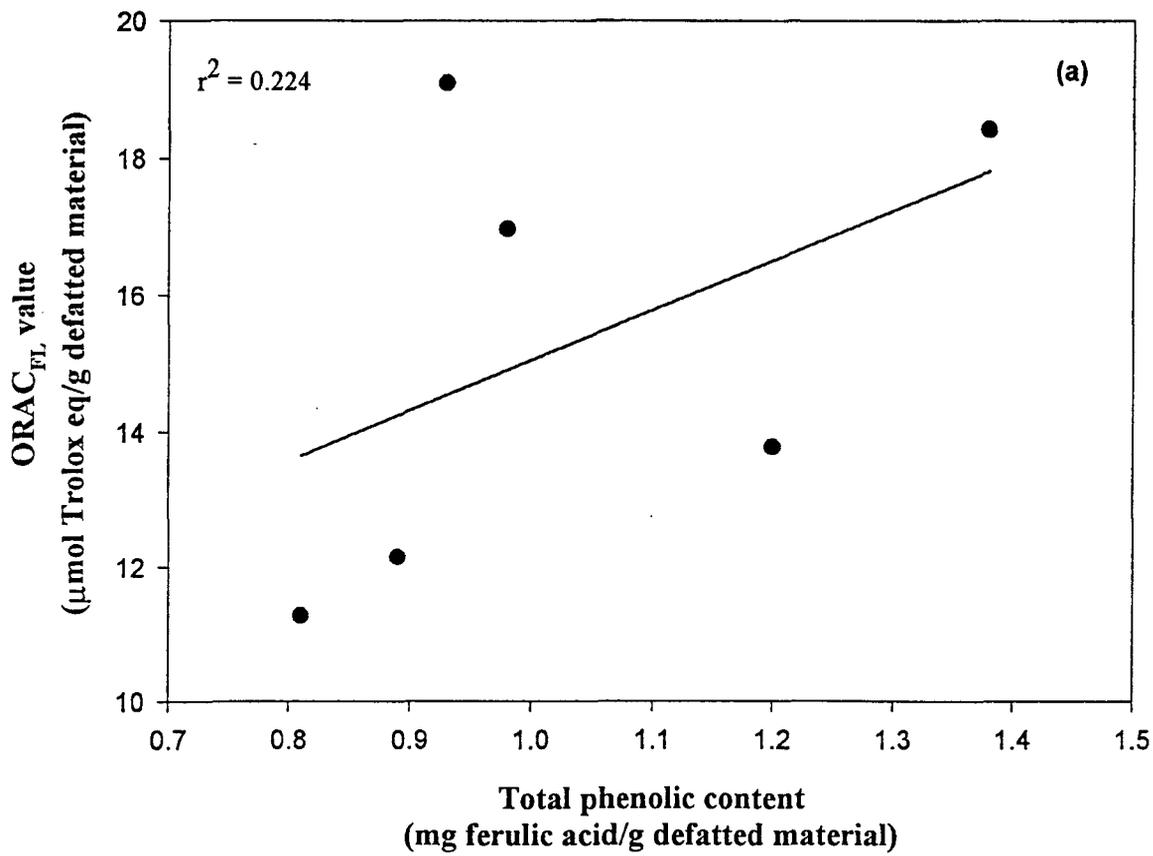
TEAC value for barley cultivars ranged from 3.74 to 6.82  $\mu\text{mol/g}$  defatted material. The order of TEAC values of the six cultivars was Peregrine > AC Metcalfe > Falcon > Tyto = Phoenix > Tercel (**Table 5.2**). TEAC values were well correlated ( $r^2 = 0.87$ ) with total phenolic contents of the cultivars (**Figure 5.1a**). The TEAC value of a compound represents the concentration of Trolox (a water soluble vitamin E analogue without the side chain moiety) that has the same antioxidant capacity as the compound or a mixture of compounds of interest (van den Berg *et al.*, 1999).

ABTS<sup>2-</sup> is oxidized by an oxidizing agent, leading to the formation of ABTS<sup>•-</sup>, which is intensely coloured. The antioxidative capacity of test compounds is assessed by measuring the ability of test compounds to decrease the colour reacting directly with the ABTS radical. ABTS<sup>•-</sup> can be generated chemically by oxidizing ABTS<sup>2-</sup> using ferrylmyoglobin, magnesiumoxide, 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH), potassium persulphate or through enzymatic reactions. Metmyoglobin, haemoglobin, and horseradish are some of the enzymes that can be used for generating ABTS<sup>•-</sup> (Prior *et al.*, 2005). The original TEAC assay developed by Miller *et al.* (1993) employs metmyoglobin and hydrogen peroxide to generate ferrylmyoglobin, which then reacts with ABTS<sup>2-</sup> to obtain ABTS<sup>•-</sup>, a procedure, which involves post-generation of the radical in the assay medium. However, the use of oxidizing agents in the assay medium allows the antioxidant compounds to directly react with the oxidizing agents, thus leading to erroneous estimations.

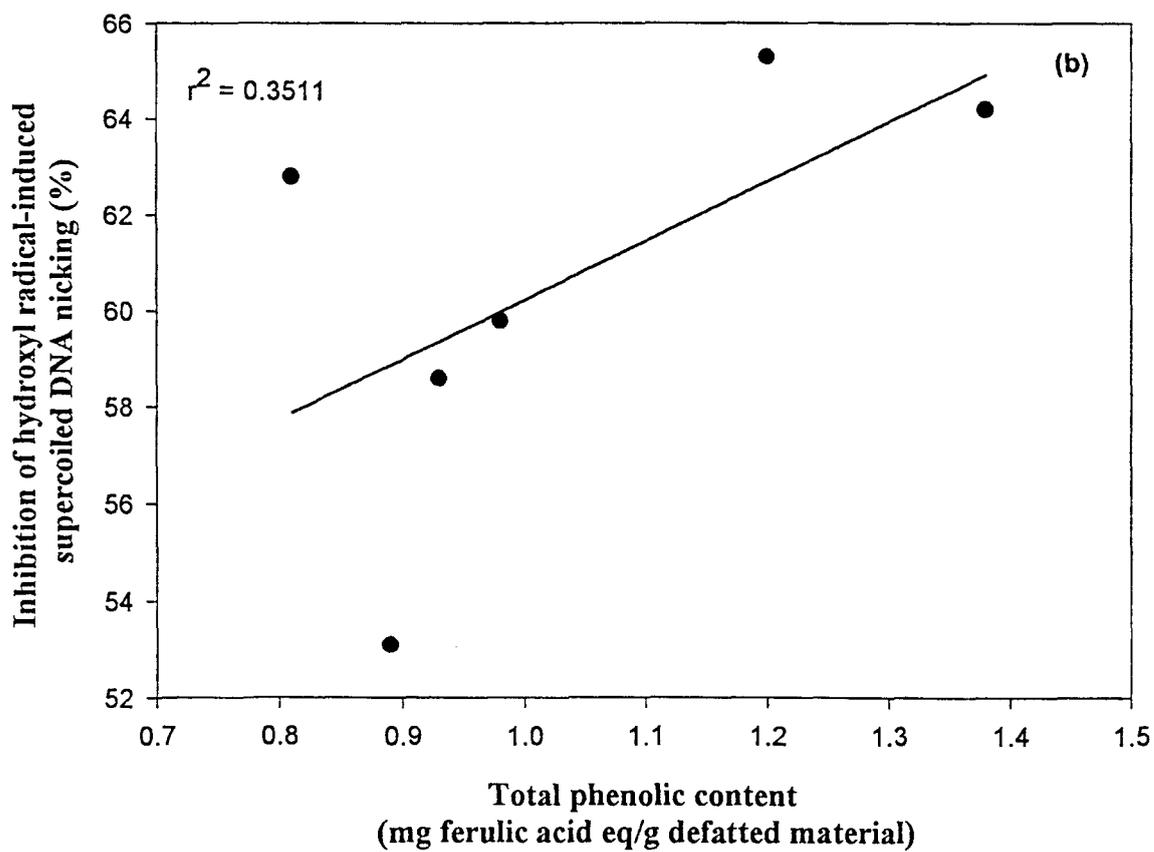
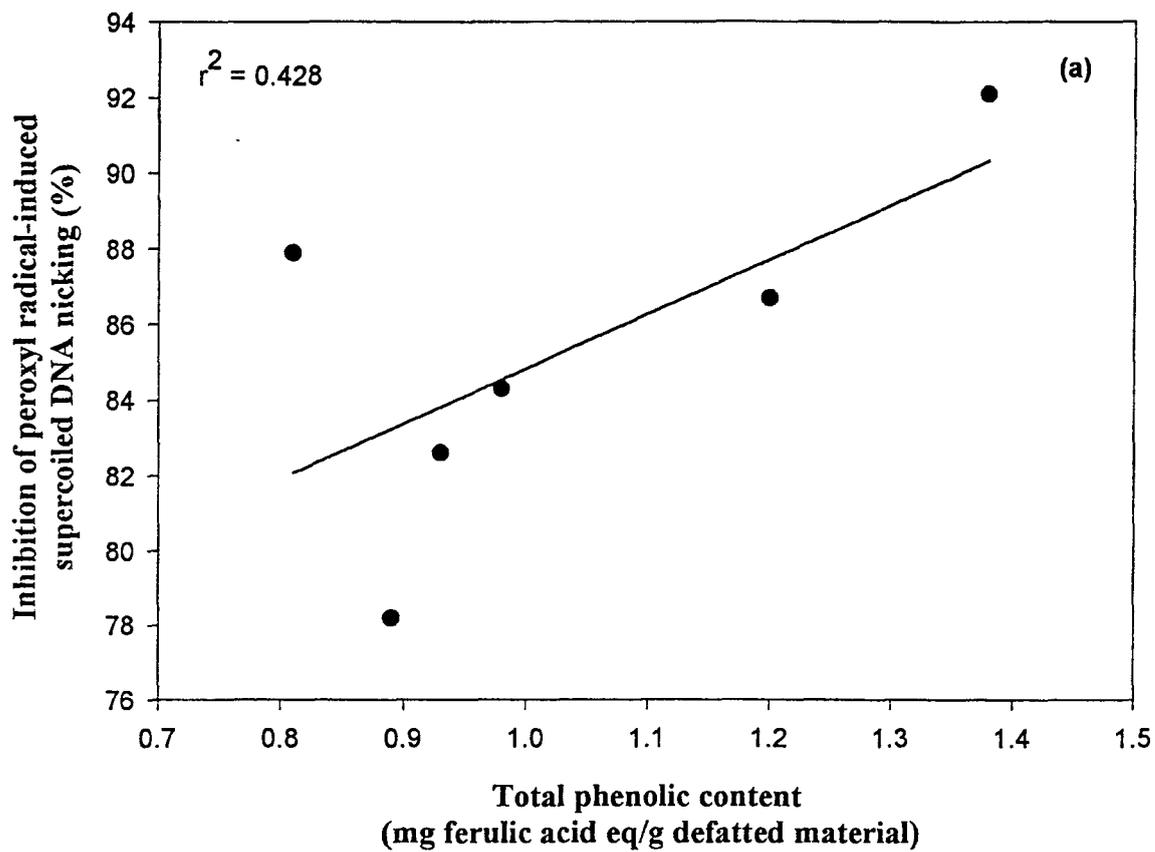
**Figure 5.1** Relationships between total phenolic content (TPC) of whole barley extracts and Trolox equivalent antioxidant capacity (TEAC) **(a)**, and DPPH radical scavenging efficacy **(b)** at 0.05 level of significance.



**Figure 5.2** Relationships between total phenolic content (TPC) of whole barley extracts and oxygen radical absorbance capacity (ORAC<sub>FL</sub>) (**a**), and hydroxyl radical absorbance capacity (HORAC<sub>FL</sub>) (**b**) at 0.05 level of significance.



**Figure 5.3** Relationships between total phenolic content (TPC) of whole barley extracts and percentage inhibition of AAPH induced supercoiled DNA **(a)**, and percentage inhibition of hydroxyl radical-induced supercoiled DNA **(b)** at 0.05 level of significance.



The assay used in this study utilizes pre-generated  $\text{ABTS}^{\bullet-}$  by reacting  $\text{ABTS}^{2-}$  with AAPH. The generation of the radical before the antioxidative compound is added prevents interference of compounds which affect the radical formation, hence improving the accuracy of the test (van den Berg *et al.*, 1999). The  $\text{ABTS}^{\bullet-}$  produced as a result of the reaction between AAPH and  $\text{ABTS}^{2-}$ , reacts instantly with Trolox and the reaction is completed within 1 min, however, the extracts may take up to 6 min to complete the reaction indicating a biphasic pattern. Therefore, a 6-min period was used before reading the final absorbance values in this study.

The absorption maxima of the ABTS radical are 415, 645, 734, and 815 nm, however, 415 and 734 nm are adopted widely by researchers.  $\text{ABTS}^{\bullet-}$  reacts rapidly with antioxidants and is soluble in both aqueous and organic solvents making the TEAC test suitable for measuring the activity of both hydrophobic as well as hydrophilic antioxidants. The TEAC assay also lends itself to automation and therefore, can be adopted for use with microplate readers. Due to these advantages, the TEAC assay is widely used to assess the antioxidative capacity of compounds from various sources.

Thermodynamically, phenolic compounds, which possess redox potential lower than that of  $\text{ABTS}^{\bullet-}$  (0.86V), can reduce  $\text{ABTS}^{\bullet-}$ . However, some compounds may take longer periods to complete the reaction and therefore, measuring the TEAC within 4-6 min may lead to underestimations (Prior *et al.*, 2005). Thus, the TEAC test is more useful in ranking the test compounds rather than quantifying the antioxidative capacity (van den Berg *et al.*, 1999).

#### 5.4.5 DPPH radical scavenging capacity

In the DPPH assay, antioxidants reduce DPPH<sup>•</sup>, one of the few stable organic nitrogen radicals which has an absorption maximum at 515 nm. One of the advantages of using DPPH<sup>•</sup> is that it does not require preparation of the radical before the assay. The assay is based on the measurement of the reducing ability of antioxidants toward DPPH<sup>•</sup>, which can be monitored by measuring the decrease in absorbance spectrophotometrically or by EPR signal (Prior *et al.*, 2005).

The reduction of the DPPH radical, in the presence of extracts and standards was monitored spectrophotometrically at 515 nm after 20 min of mixing. DPPH radical scavenging capacity of the whole barley extracted ranged from 3.25 to 5.80  $\mu\text{mol}$  ferulic acid equivalents per gram of defatted material. The order of DPPH radical scavenging capacity was Peregrine > Tyto = Phoenix > Falcon > Tercel > AC Metcalfe.

#### 5.4.6 Reducing power

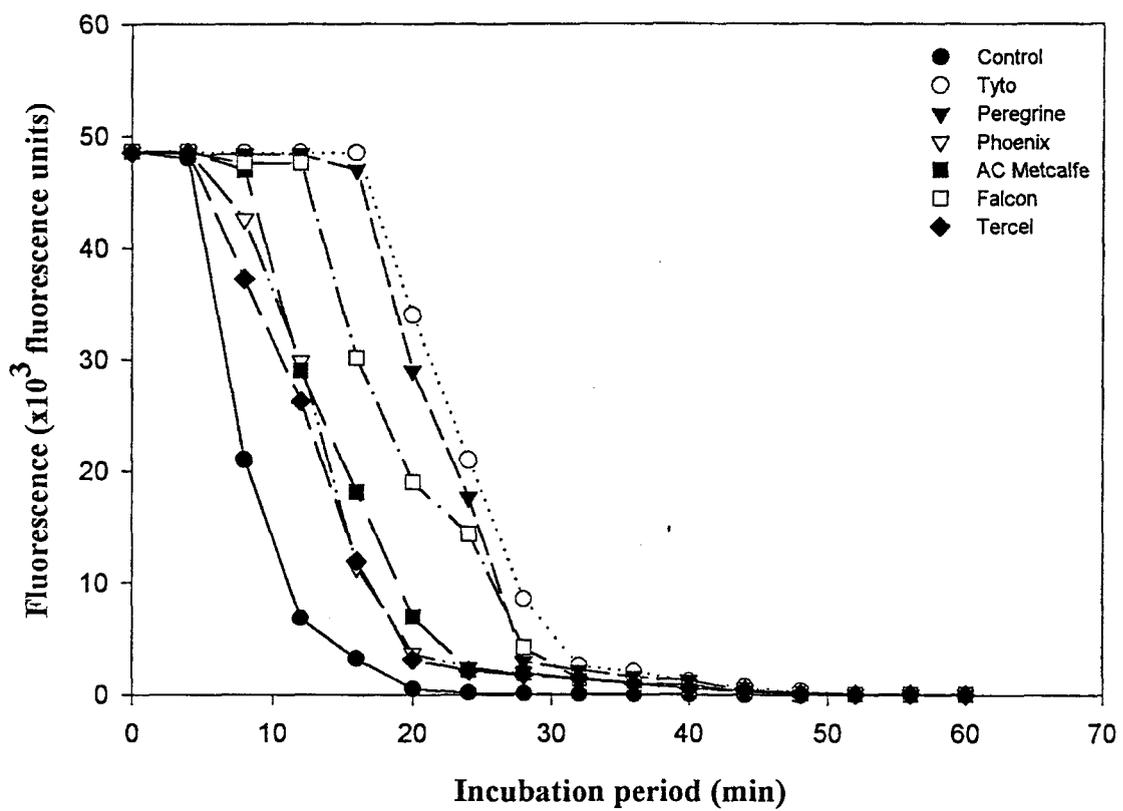
The reducing power of an antioxidative extract serves as a significant reflection of the antioxidative activity. In this assay the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of the extracts (Zou *et al.*, 2004). Ascorbic acid and Trolox were used as standards in this study and the results were expressed as ascorbic acid and Trolox equivalents. Reducing power values obtained as Trolox and ascorbic acid were well correlated with each other. The order of reducing power of the whole barley extracts was Peregrine > AC Metcalfe > Tyto > Falcon > Phoenix > Tercel (**Figure 5.4**). Reducing power of whole barley extracts varied from 3.92 to 8.01 and 3.52 to 7.2  $\mu\text{mol}$  ascorbic acid and Trolox equivalents/g of defatted material, respectively.

#### 5.4.7 Antioxidant activity as measured by photochemiluminescence (PCL)

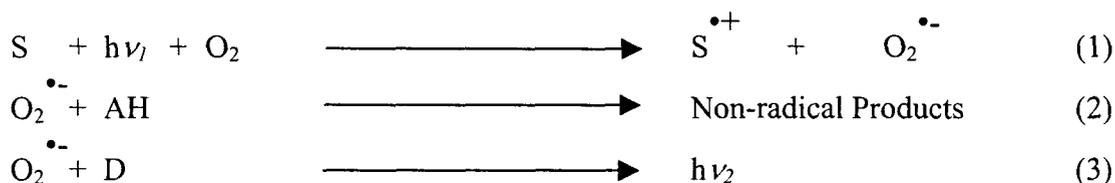
The antioxidant activity of whole barley extracts was assayed using the Photochem<sup>®</sup> developed by Analytik Jena AG (Konrad-Zuse-Strabe 1, Jena, (Germany) based on the principle described by Popov and Lewin (1999a). The activity of the water-soluble and lipid-soluble compounds were determined separately and expressed as Trolox and  $\alpha$ -tocopherol equivalents, respectively. Antioxidant activity ranged from 11.22 to 18.66  $\mu\text{mol}$  Trolox equivalents and 9.09 to 16.88  $\mu\text{mol}$   $\alpha$ -tocopherol equivalents per gram of defatted material, respectively. The content of the water soluble fraction was higher than that of the lipid soluble fraction with all barley extracts except Tyto (**Figure 5.5**). TEAC and water-soluble antioxidant activity were strongly correlated ( $r^2 = 0.856$ ) while TEAC and lipid-soluble antioxidant activity showed an even stronger correlation of 0.913. Water-soluble antioxidant activity was correlated well with TPC ( $r^2 = 0.81$ ), however, it did not show a strong correlation with DPPH ( $r^2 = 0.23$ ).

This assay is based on the reaction of radical oxidants with marker compounds to produce excited state species that emit chemically induced light (chemiluminescence). Any compound that interferes with the initiation of radicals inhibits the light production. Chemiluminescence is characterized by very low emission intensity, ten to a few thousands counts per second in comparison to millions of counts for fluorescence. Therefore, chemiluminescence detection requires special equipment that places the sample close to the detector which detects light at a single photon level. Chemiluminescence methods are very sensitive in the detection of low level reactions.

**Figure 5.4** Reducing power of whole barley extracts expressed as ascorbic acid and Trolox equivalents.



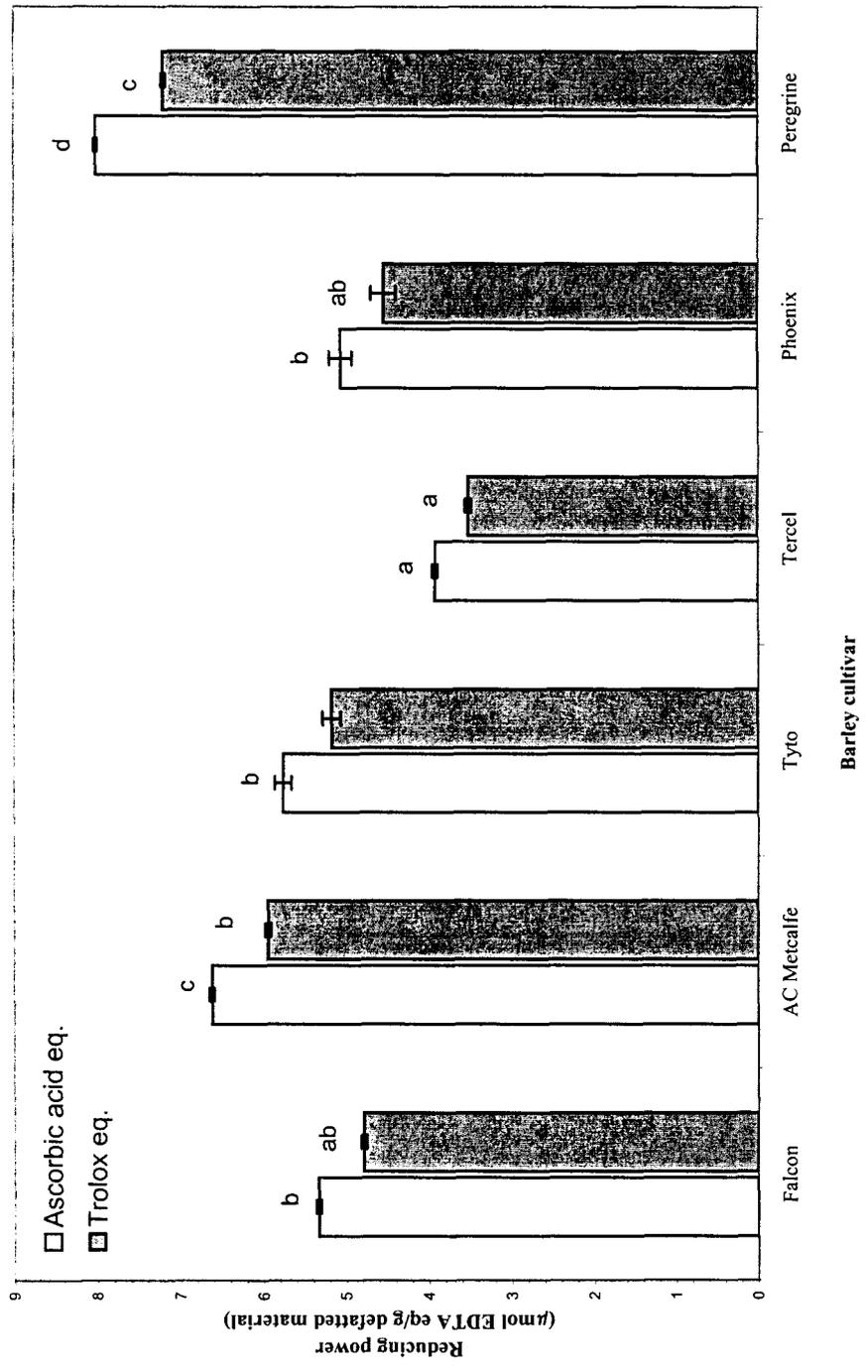
Measurement of the antioxidant activity of a substance can be carried out in a system containing a free radical generator and a detector which indicates changes of the signal being measured in the presence of an antioxidant (Popov and Lewin, 1999a). The Photochem<sup>®</sup> photochemiluminometer generates free radicals photochemically by UV radiation of a photosensitizer solution. During irradiation, the assay mixture is continuously pumped through the measuring cell of the chemiluminometer which registers the light being produced by the photochemically generated free radicals in reactions illustrated below.



where, S = photosensitizer; AH = antioxidant and D = free radical detecting compound (Popov and Lewin, 1999).

UV irradiation ( $h\nu_1$ ) of the assay mixture containing the photosensitizer (luminol) generates superoxide radicals. These radicals are scavenged, to different extents, by the antioxidant compounds present in the extract/standard. After a certain period of time the remaining radicals are quantified by reacting with luminol, which generates UV light ( $h\nu_2$ ). Antioxidants in the sample were quantified by comparing their inhibitory effect on luminescence generation against that of a standard.

**Figure 5.5** Bar chart illustrating the antioxidant activity of water- and lipid-soluble components in whole barley extracts as measured by photochemiluminescence (PCL).  
*Water- and lipid-soluble antioxidant activities are expressed as  $\mu\text{mol}$  Trolox and  $\alpha$ -tocopherol equivalents per gram defatted material, respectively.*



In the strictest sense, Photochem<sup>®</sup> measures the antiradical capacity of a test compound. The hydrophilic antioxidant activity is assayed by means of the lag phase (L) in seconds while the lipophilic antioxidant activity is assayed by the degree of PCL inhibition (I), according to the following equation.

$$I = 1 - S/S_0$$

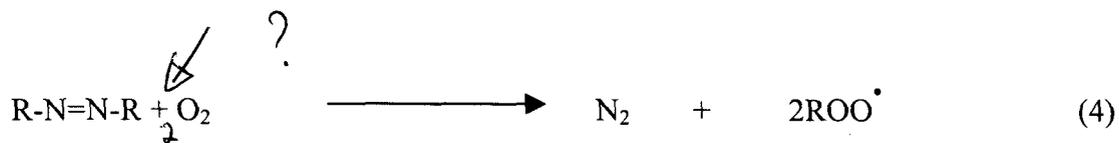
where,  $S_0$  = the integral under the blank curve and S is the integral under the sample.

#### 5.4.8 Oxygen radical absorbance capacity (ORAC<sub>FL</sub>)

The ORAC<sub>FL</sub> method is becoming popular for assessing antioxidant capacity in biological and food samples. The ORAC<sub>FL</sub> value is based on the inhibition of the peroxy radical-induced oxidation initiated by thermal decomposition of azo compounds such as 2,2'-azinobis [3-ethylbenzthiazoline-6-sulphonic acid] (AAPH). This is the only assay that combines both inhibition time and degree of inhibition into a single quantity (Prior *et al.*, 2005), that is  $\mu\text{mol Trolox equivalents/g}$  defatted material. The ORAC<sub>FL</sub> value of whole barley extracts ranged from 11.28 to 19.10  $\mu\text{mol Trolox equivalents per gram}$  of defatted material. The order of ORAC was as follows. Tyto = Peregrine = Falcon > AC Metcalfe = Phoenix = Tercel (**Table 5.3**).

The ORAC<sub>FL</sub> values and antioxidant activity as measured by photochemiluminescence were strongly correlated with each other (ORAC<sub>FL</sub> and water-soluble antioxidant activity:  $r^2 = 0.98$ , ORAC<sub>FL</sub> and lipid-soluble counterparts;  $r^2 = 0.95$  for Falcon cultivar). **Figure 5.6** illustrates the time course of the reaction of fluorescein with AAPH (decay curves) for whole barley extracts. The peroxy radical generated by AAPH reacts with the fluorescent probe, fluorescein (3',6'-dihydroxy

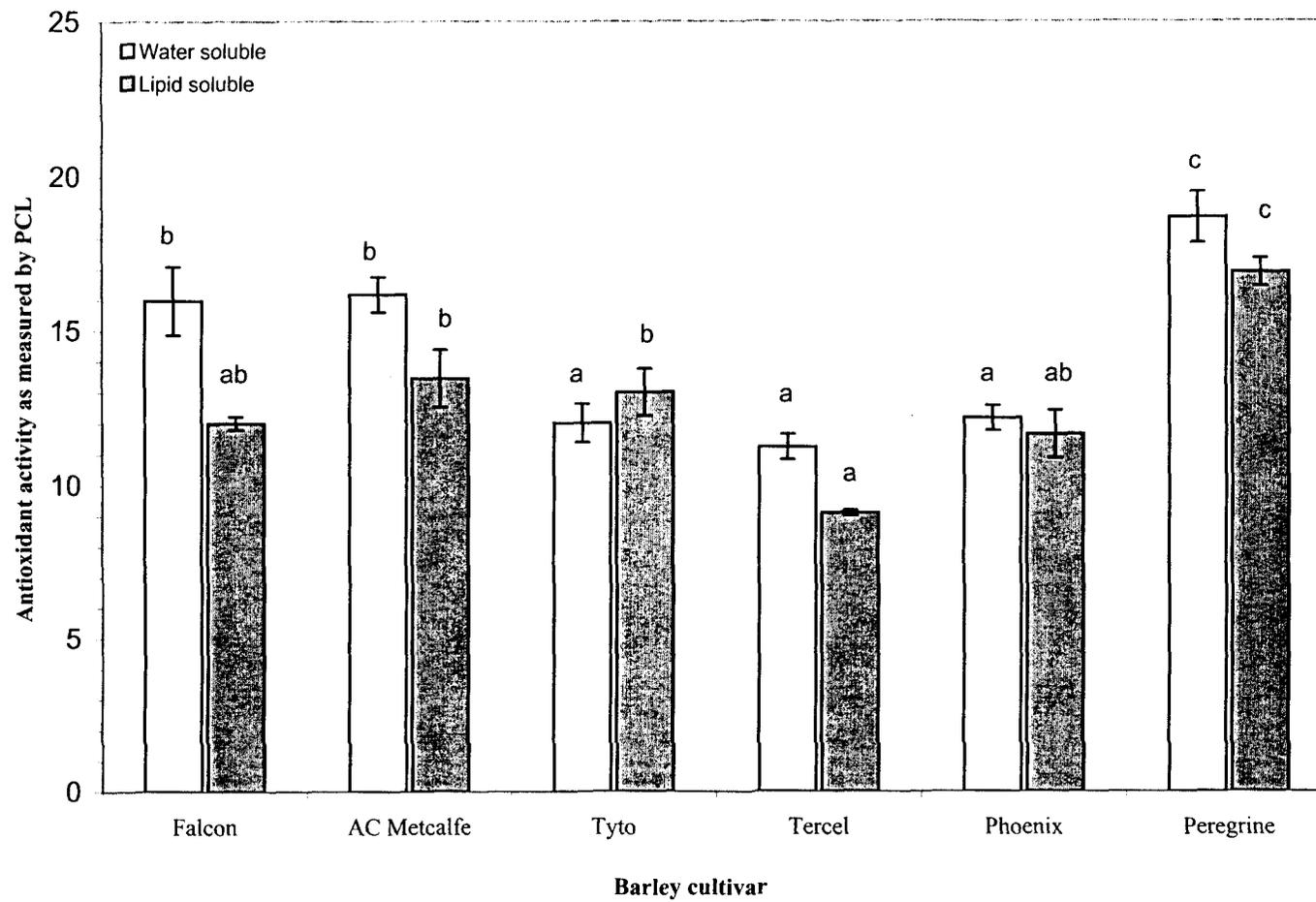
spiro[isobenzofuran-1[SH] 9'[9H]-xanthan-3-one), to form a non-fluorescent product, and the decrease in fluorescence can be quantified (Prior *et al.*, 2005).



Protective effect of antioxidant is calculated from the net integrated area under the fluorescence decay curve (AUC) as illustrated in **Figure 5.6** and reported as Trolox equivalents.

The ORAC<sub>FL</sub> assay is among the standard assays accepted for measuring the antioxidative activity of botanicals, herbs and nutraceuticals. It has been widely applied to the assessment of free radical scavenging capacity of human plasma, proteins, DNA, pure antioxidants and plant/food extracts (Davalos *et al.*, 2004). The assay, as performed in this study, is limited to the measurement of the hydrophilic chain breaking antioxidant capacity against peroxy radical and ignores lipophilic antioxidants. Moreover, the assay disregards some other physiologically important radicals such as  $\cdot OH$ ,  $\cdot OOH$ ,  $ONOO$ ,  $O_2^\cdot$ . It can be adapted to quantify the activity of lipophilic antioxidants as well by changing the solvent system, however, the hydrophilic component of ORAC<sub>FL</sub> constitutes more than 90% of the total ORAC<sub>FL</sub> for most foods (Wu *et al.*, 2004) and therefore, the ORAC<sub>FL</sub> measured in this study is sufficient to assess the extracts.

**Figure 5.6** Change of relative fluorescence with incubation time (fluorescence decay curves) in the presence of whole barley extracts in ORAC<sub>FL</sub> assay.



**Table 5.3** ORAC<sub>FL</sub> value and ORAC<sub>FL</sub>/TPC ratio of whole barley extracts.

Barley cultivar	ORAC <sub>FL</sub> Value	ORAC <sub>FL</sub> /TPC
Falcon	16.97 ± 1.8 <sup>c</sup>	20.45 ± 2.1 <sup>c</sup>
AC Metcalfe	13.77 ± 1.1 <sup>b</sup>	12.08 ± 0.97 <sup>a</sup>
Tyto	19.10 ± 1.0 <sup>d</sup>	26.16 ± 1.32 <sup>d</sup>
Tercel	11.28 ± 0.8 <sup>a</sup>	15.89 ± 0.98 <sup>b</sup>
Phoenix	12.15 ± 0.4 <sup>ab</sup>	17.87 ± 0.52 <sup>b</sup>
Peregrine	18.43 ± 1.5 <sup>cd</sup>	15.49 ± 1.34 <sup>b</sup>

<sup>1</sup> HORAC<sub>FL</sub> value is expressed as µmol Trolox equivalents/g defatted material.

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

The use of FL as the probe is more effective and overcomes problems such as lot to lot variance and the poor photostability of  $\beta$ -phycoerythrins (PEs) which were used in the original assay as the fluorescent probe. The other main advantage of using fluorescein is its inactivity towards the antioxidative compounds tested. ORAC<sub>FL</sub> and TPC of whole barley extracts did not correlate well ( $r^2 = 0.224$ ). Kaur and Kapoor (2002) also reported a linear relationship between ORAC<sub>FL</sub> and TPC; however, Wu *et al.* (2004) reported that this linearity is not correlated across all foods.

Wu *et al.* (2004) ranked foods into different categories based on the ORAC<sub>FL</sub>/TPC ratio. This ratio ranged from 1.7 in green pepper to 156.4 in garlic powder while most of the foods fell in the range from 5 to 15. The ORAC<sub>FL</sub>/TPC ratio for whole barley extracts ranged from 12.08 to 26.16. The order of ORAC<sub>FL</sub>/TPC ratio was as follows. Tyto > Falcon > Phoenix > Tercel = Peregrine > AC Metcalfe (**Table 5.3**). Furthermore, the common foods were grouped into four classes (0-499, 500-999, 1000-1999, and 2000-14000) based on ORAC<sub>FL</sub> per serving.

#### **5.4.9 Hydroxyl radical absorbance capacity (HORAC<sub>FL</sub>)**

Hydroxyl radical absorbance capacity (HORAC<sub>FL</sub>) of whole barley extracts ranged from 9.06 to 12.99  $\mu\text{mol}$  Trolox equivalents/g defatted material. The order of HORAC<sub>FL</sub> of the whole barley extracts was Tyto = Peregrine > AC Metcalfe = Phoenix = Falcon = Tercel (**Table 5.4**). The order of HORAC<sub>FL</sub> values were more or less similar to that of ORAC<sub>FL</sub> described in **Section 5.4.8**. HORAC<sub>FL</sub>/TPC ratio indicates the hydroxyl radical scavenging efficacy of phenolic compounds.

**Table 5.4** HORAC<sub>FL</sub> value and HORAC<sub>FL</sub>/TPC ratio of whole barley extracts.

Barley cultivar	HORAC <sub>FL</sub> Value <sup>1</sup>	HORAC <sub>FL</sub> /TPC
Falcon	10.39 ± 0.24 <sup>ab</sup>	12.52 ± 0.31 <sup>b</sup>
AC Metcalfe	10.47 ± 0.53 <sup>b</sup>	9.19 ± 0.50 <sup>a</sup>
Tyto	12.99 ± 0.83 <sup>c</sup>	13.68 ± 0.95 <sup>b</sup>
Tercel	9.06 ± 0.49 <sup>a</sup>	14.17 ± 0.62 <sup>bc</sup>
Phoenix	10.67 ± 0.43 <sup>b</sup>	15.70 ± 0.78 <sup>c</sup>
Peregrine	12.62 ± 0.01 <sup>c</sup>	8.93 ± 0.01 <sup>a</sup>

<sup>1</sup> HORAC<sub>FL</sub> value is expressed as µmol Trolox equivalents/g defatted material.

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

Metal ion-reduced hydroxyl radical generation can conveniently be monitored by the decay of fluorescein. Ou *et al.* (2002) tested the Fe(II), Co(II), and Cu(II) ions and found that Co(II) is the most effective and was the ion used in this study. Cu(I) salts are not soluble while Fe(II) salts are too air sensitive and do not decay fluorescein. Although Co(I) salts are not soluble in water, their complex with picolinic acid is rapidly soluble in water (Ou *et al.*, 2002).

The hydroxyl radical is a biologically important species that can cause severe damage to biomolecules. Quantitative measurement of the hydroxyl radical has been a challenging task due to the lack of a controllable hydroxyl radical source (Ou *et al.*, 2002). In the presence of low concentrations of Fe(II), H<sub>2</sub>O<sub>2</sub> is converted to hydroxyl radical through Fenton reactions. Many other transition metal ions such as Cu(II), Ti(II), Cr(II), and Co(II) also react with H<sub>2</sub>O<sub>2</sub> in a similar manner as Fe(II) generating hydroxyl radical. It is reported that reactions proceed through an inner sphere electron transfer, in which H<sub>2</sub>O<sub>2</sub> forms a complex with transitional metal ions (Goldstein *et al.*, 1993). Generation of hydroxyl radical both *in vitro* and *in vivo* involves the presence of oxidizable metal ions and H<sub>2</sub>O<sub>2</sub>. Thus, the presence of a transitional metal ion-H<sub>2</sub>O<sub>2</sub> mixture is fatal to living cells.

Ou *et al.* (2002) evaluated a number of fruits using HORAC<sub>FL</sub> assay. The HORAC<sub>FL</sub> value of selected fruits was as follows: Apple, 15  $\mu$ mol gallic acid equivalents (GAE)/g fruit; grapes 40  $\mu$ mol GAE/g fruit; blueberry, 95  $\mu$ mol GAE/g fruit; and elderberry, 333  $\mu$ mol GAE/g fruit. The HORAC<sub>FL</sub> value of green tea varied from 476 to 624  $\mu$ mol GAE/g, while HORAC<sub>FL</sub> values of whole barley extracts tested were lower

than any fruit tested by Ou *et al.* (2004). This is understandable as the phenolic content of barley extracts was comparatively less compared to that of most of these fruits.

Ou *et al.* (2002) evaluated a number of authentic phenolic compounds for HORAC<sub>FL</sub> and ORAC<sub>FL</sub> and revealed that HORAC<sub>FL</sub> is consistently lower than ORAC<sub>FL</sub> across the phenolic compounds tested as was true for most of the foods tested. A similar trend was observed for whole barley extracts as well. Ou *et al.* (2002) observed that the phenolic compounds that have metal chelation potential show higher values while the compounds with poor metal chelation activity show negligible HORAC<sub>FL</sub> values. Vitamin C and Trolox, well known antioxidants showed no hydroxyl radical prevention capacity under the experimental conditions although they have shown some activity towards ORAC<sub>FL</sub>. It is suggested that vitamin C and Trolox act as prooxidants by initiating a catalytic cycle of hydroxyl radical generation (Ou *et al.*, 2004). Catechins have shown higher HORAC<sub>FL</sub> values compared to phenolic acids. HORAC<sub>FL</sub> values of phenolic acids fell within a narrow range of 1.0 – 1.51 GAE. Glucose and rutinose groups on phenolic compounds contributed positively towards HORAC<sub>FL</sub> (Ou *et al.*, 2004).

Phenolics act as metal chelators by coordination to Co(II) thereby blocking the reaction sites for H<sub>2</sub>O<sub>2</sub>. This coordination leads to a reduced concentration of Co(II) and effectively reduces generation of hydroxyl radicals. HORAC values do not correlate with the number of hydroxyl groups or the number of chelating sites and is mainly decided by the stability of the Co(II)-phenol complex formed (Ou *et al.*, 2004).

The HORAC<sub>FL</sub> value mainly reflects the metal chelation potential while ORAC<sub>FL</sub> primarily indicates the peroxy radical absorption capacity. Therefore, theoretically, no

correlation exists between these two parameters.  $ORAC_{FL}$  and  $HORAC_{FL}$  values obtained in this study showed a poor correlation ( $r^2 = 0.23$ ). Genestein, quercetin, and kaempferol with very high  $ORAC_{FL}$  values have shown only modest  $HORAC_{FL}$  values while EGCG and rutin have shown high values for both (Ou *et al.*, 2004).

Direct scavenging of hydroxyl radical by dietary antioxidants in biological systems is extremely unlikely as the cellular concentration of dietary antioxidant is negligible compared to other biological molecules. However, it is possible to prevent the formation of hydroxyl radicals by deactivating free metal ions using antioxidants. Therefore, measuring the capacity of antioxidant compounds to prevent hydroxyl radical generation is more meaningful than measuring direct hydroxyl radical scavenging capacity. The net area under the curve is sensitive to the concentration of antioxidants.

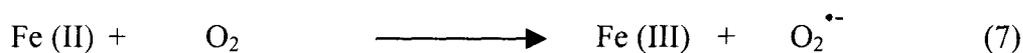
#### **5.4.10 Metal chelation activity**

Metal chelation activity of whole barley extracts as measured using the 2,2'-bipyridyl competition assay. 2,2'-Bipyridyl can quantitatively form a red colour complex with Fe(II). In the presence of metal chelating agents, the complex formation is disrupted as they compete with bipyridyl, hence leading to lower colour intensity. Metal chelation activity of whole barley extracts as measured by the 2,2'-bipyridyl competition assay ranged from 1.10 to 2.10  $\mu\text{mol}$  EDTA equivalents/g defatted material. The order of metal chelation activity was Peregrine = Falcon > AC Metcalfe = Phoenix > Tercel = Tyto (Figure 5.7).

Metal chelation is a key aspect of phenolic antioxidants that helps in mitigating the generation of radicals in biological systems. Metal chelation is an example of

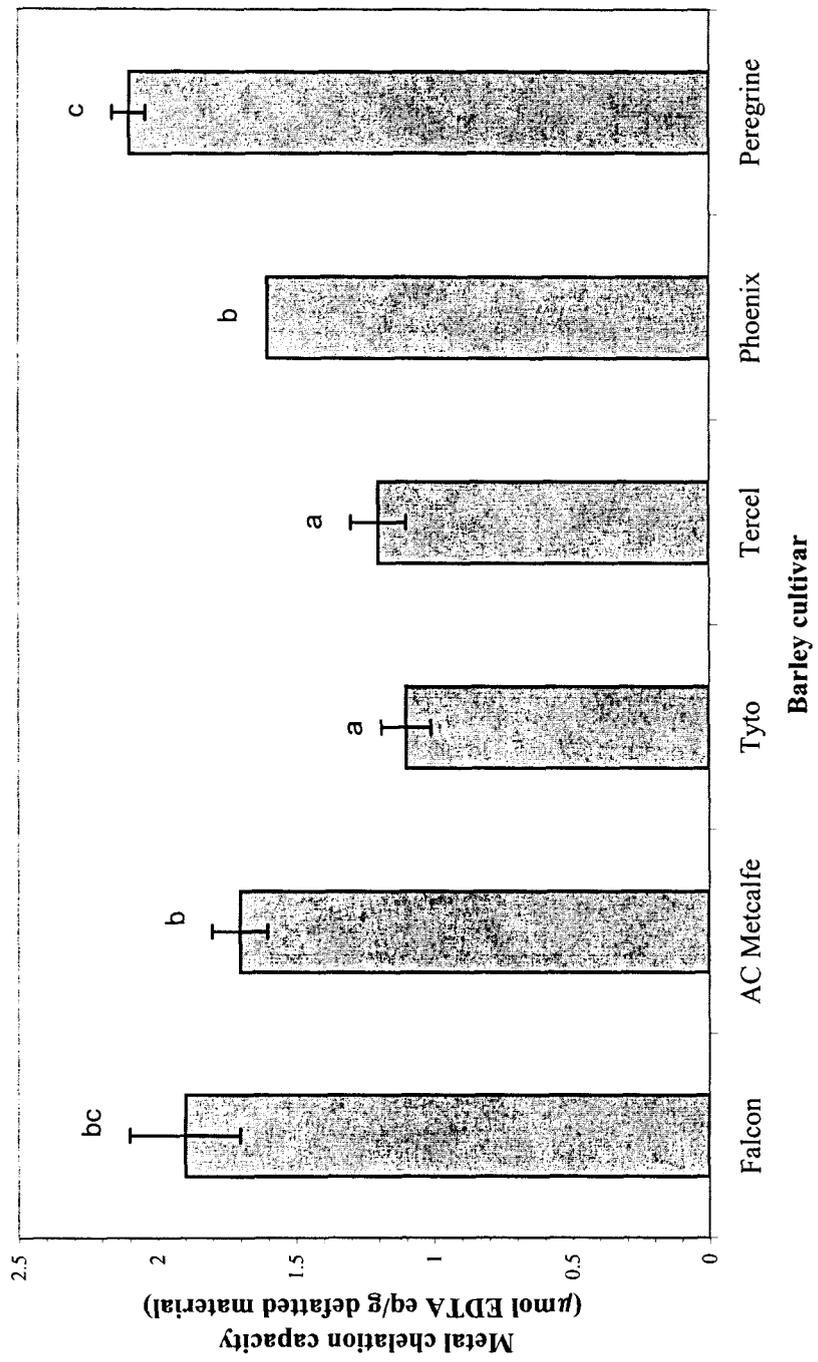
secondary antioxidant mechanism by which antioxidants can influence the oxidation process. Transition metal ions such as iron and copper participate in the generation of ROS which are associated with many pathological conditions.

The following reactions (equations 7-9) indicate the contribution of metal ions in generating radicals.



The protonated phenolic group is not a good ligand for metal cation binding, however, once deprotonated, an oxygen centre is generated that possesses a high charge density. Although the  $P_{ka}$  value of most phenols is in the range of 9-10, in the presence of suitable metal cations, the proton is displaced at pH values of 5-8. Therefore, phenolic compounds are able to chelate metal ions under physiological conditions (Hilder *et al.*, 2001). Binding of iron to flavonoid antioxidants can suppress the accessibility of iron to oxygen molecules. Preferential ligand binding to Fe(II) changes the redox potential for converting Fe(II) to Fe(III) and thereby inhibit generation of ROS (Khokhar and Apenten, 2003).

**Figure 5.7** Metal chelation effect of whole barley extracts as determined by 2,2'-bipyridyl competition assay.



#### 5.4.11 Superoxide radical scavenging capacity

The superoxide radical is one of the ROS that causes damage to biomolecules and hence it is important to assess the antioxidative extracts for their potential in inhibiting this radical. Superoxide radical scavenging capacity of the whole barley extracts ranged from 62.23 to 73.53%. The superoxide radical scavenging potential of five extracts tested was not statistically significant ( $p > 0.05$ ). The order of the whole barley extracts was Ferulic acid > Peregrine = Falcon = AC Metcalfe = Tercel = Phoenix > Tyto (**Table 5.5**). As expected, the scavenging capacity of ferulic acid, the reference antioxidant, was the highest (95.94%). Various methods are available for producing superoxide radical in the laboratory. There are generally two superoxide anion sources used; enzymatic and non-enzymatic sources. In the present study, superoxide radical was enzymatically produced using hypoxanthine/xanthine oxidase (X/XO) system. The generation of superoxide radical was characterized by the development of a blue colour in the assay medium due to the reduced nitro blue tetrazolium (Nishikimi *et al.*, 1972) and this was used to monitor its production.

The observed antioxidant effect may be due to a combination of the effects on superoxide radical and XO. The effect of antioxidant extracts on XO can be evaluated using metabolite conversion of xanthine to uric acid (Valentao *et al.*, 2001). However, this was not performed in this study. Kaempferol has exhibited high XO inhibition activity while most phenolic acids exhibit both XO and radical inhibitory activities. Sinapic acid has been identified as the most active superoxide radical scavenger (Valentao *et al.*, 2001), while flavonoids and phenolic acids are reported to be good free radical scavengers.

**Table 5.5** Percentage hydrogen peroxide and superoxide radical scavenging capacity of whole barley extracts

Sample identity	Superoxide radical scavenging capacity <sup>1</sup>	H <sub>2</sub> O <sub>2</sub> Scavenging capacity
Falcon	73.53 ± 3.90 <sup>b</sup>	40.07 ± 2.38 <sup>a</sup>
AC Metcalfe	73.47 ± 5.90 <sup>b</sup>	64.81 ± 2.15 <sup>c</sup>
Tyto	62.23 ± 6.70 <sup>a</sup>	40.07 ± 0.24 <sup>a</sup>
Tercel	73.53 ± 3.20 <sup>b</sup>	50.84 ± 3.33 <sup>b</sup>
Phoenix	72.36 ± 9.80 <sup>b</sup>	39.98 ± 3.45 <sup>a</sup>
Peregrine	72.72 ± 3.50 <sup>b</sup>	78.87 ± 1.79 <sup>d</sup>
Ferulic acid	95.94 ± 6.80 <sup>c</sup>	76.94 ± 3.89 <sup>d</sup>

<sup>1</sup>Scavenging capacity is calculated based on the absorbance values at 10min of the assay. Results are means of three determinations ± standard deviation. Values in each column having the same superscript are not significantly different (p>0.05).

Rice-Evans *et al.* (1996) reported that dihydroxybenzoic acids such as ferulic and caffeic acids effectively quench free radicals including superoxide radical.

In enzymatic X/XO systems, the scavenging effect may come directly from the radical-quenching effect and/or the enzyme-inhibiting effect (Shi and Noriko, 2001). The structure-activity relationship of flavonoids as inhibitors of xanthine oxidase and as scavengers of the superoxide radical was recently reported (Cos *et al.*, 1998). The presence of hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 are essential for a high inhibiting activity on xanthine oxidase. On the other hand, for a high superoxide scavenging activity, the presence of a hydroxyl group at C-3' in the B ring and at C-3 is essential (Shi and Noriko, 2001). Based on the effect on xanthine oxidase and superoxide radical, flavonoids are classified into six groups; superoxide radical scavengers without inhibiting activity on xanthine oxidase, xanthine oxidase inhibitors without any additional superoxide scavenging, xanthine oxidase inhibitors with an additional superoxide scavenging activity, xanthine oxidase inhibitors with an additional prooxidant activity, and flavonoids with marginal effect on xanthine oxidase but with prooxidant effect on the production of superoxide radical.

#### **5.4.12 Hydrogen peroxide scavenging capacity**

Hydrogen peroxide is inert at low concentrations such as physiological concentrations and therefore, the direct effect of H<sub>2</sub>O<sub>2</sub> is minimal. However, it is believed to be involved in the generation of extremely potent hydroxyl radical through Fenton reactions. Therefore, it is worth evaluating antioxidative extracts for their potential in scavenging H<sub>2</sub>O<sub>2</sub>.

Whole kernel extracts scavenged 39.98 to 78.87% of H<sub>2</sub>O<sub>2</sub> present at the beginning of the assay while ferulic acid, the reference antioxidant used, scavenged 76.94% of H<sub>2</sub>O<sub>2</sub>. The highest amount of H<sub>2</sub>O<sub>2</sub> was scavenged by Peregrine extract while, extracts of Tyto and Phoenix scavenged the least. The order of activity of the extracts was Peregrine > AC Metcalfe > Tercel > Falcon = Phoenix = Tyto (**Table 5.5**). Hydrogen peroxide scavenging capacity showed a strong correlation ( $r^2 = 0.82$ ) with TPC. Hydrogen peroxide is a weak oxidizing agent that can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. However, the ability of hydrogen peroxide to produce other ROS such as hydroxyl radical cannot be ignored. Hydrogen peroxide is found to be toxic to cells at 10–100  $\mu$ M levels and can cross biological membranes rapidly to form hydroxyl radicals (Halliwell and Gutteridge, 1999). Furthermore, DNA is an important target to be damaged when hydrogen peroxide is added to mammalian cells (Halliwell and Gutteridge, 1989). Any biological system generating superoxide radical can produce hydrogen peroxide by dismutation reactions, unless all of the superoxide radicals are intercepted by other molecules such as cytochrome C. Hydrogen peroxide formation has been frequently derived from mitochondria and microsomes. There are also several enzymes that produce hydrogen peroxide without intermediacy of free superoxide radical which include glycollate oxidase, D-amino acid oxidase and urate oxidase (Halliwell and Gutteridge, 1989).

Martinez-Tome *et al.* (2004) evaluated different oat and wheat bran for H<sub>2</sub>O<sub>2</sub> scavenging activity and the highest activity was shown by wheat bran (84% inhibition). Except for gentisic and protocatecheuic acids all the other phenolic acids, and standards tested did not yield good inhibition.

#### 5.4.13 Bulk corn oil model system

Use of different model systems to assess antioxidative capacity of test compounds is common. In the bulk corn oil model system, a known weight of antioxidative extract is mixed with commercially available corn oil, stripped of its endogenous antioxidants. The oxidation of the oil is accelerated by employing elevated temperatures and the progression of autoxidation is monitored by measuring conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) in the treated oil. CD measures the formation of primary oxidation products, whereas TBARS measures secondary oxidation products. The data are used to assess the efficacy of different extracts in preventing oxidative deterioration of stripped corn oil.

CD and TBARS values of the oil samples collected on days 0,1,3,5 and 7 are listed in **Tables A-5.1** and **A-5.2**. CD values of treated oils increased by a factor of about 2.4 – 3.8 at the end of the 7-day period while the control showed approximately 7.8 fold increase. TBARS values of treated oils increased by about 2.3 -3.6 fold at the end of the 7-day storage period whereas the control showed about a 5.6 fold increase. Both CD and TBARS followed the same pattern. **Figure 5.8** depict the percentage inhibition of the formation of CD and TBARS of stripped corn oil at 100 mg/5 g oil concentration at the end of the 7<sup>th</sup> day of storage. At this concentration, the formation of CD and TBARS was reduced by 10 to 31% and 27 to 44%, respectively. The order of effectiveness was Tyto > Peregrine > Falcon > Phoenix > AC Metcalfe > Tercel. The results show that barley extracts are effective in controlling autoxidation of stripped corn oil in the bulk corn oil model system. The difference in phenolic content and chemical composition of the barley cultivars might be attributable to the differential antioxidant activity exhibited.

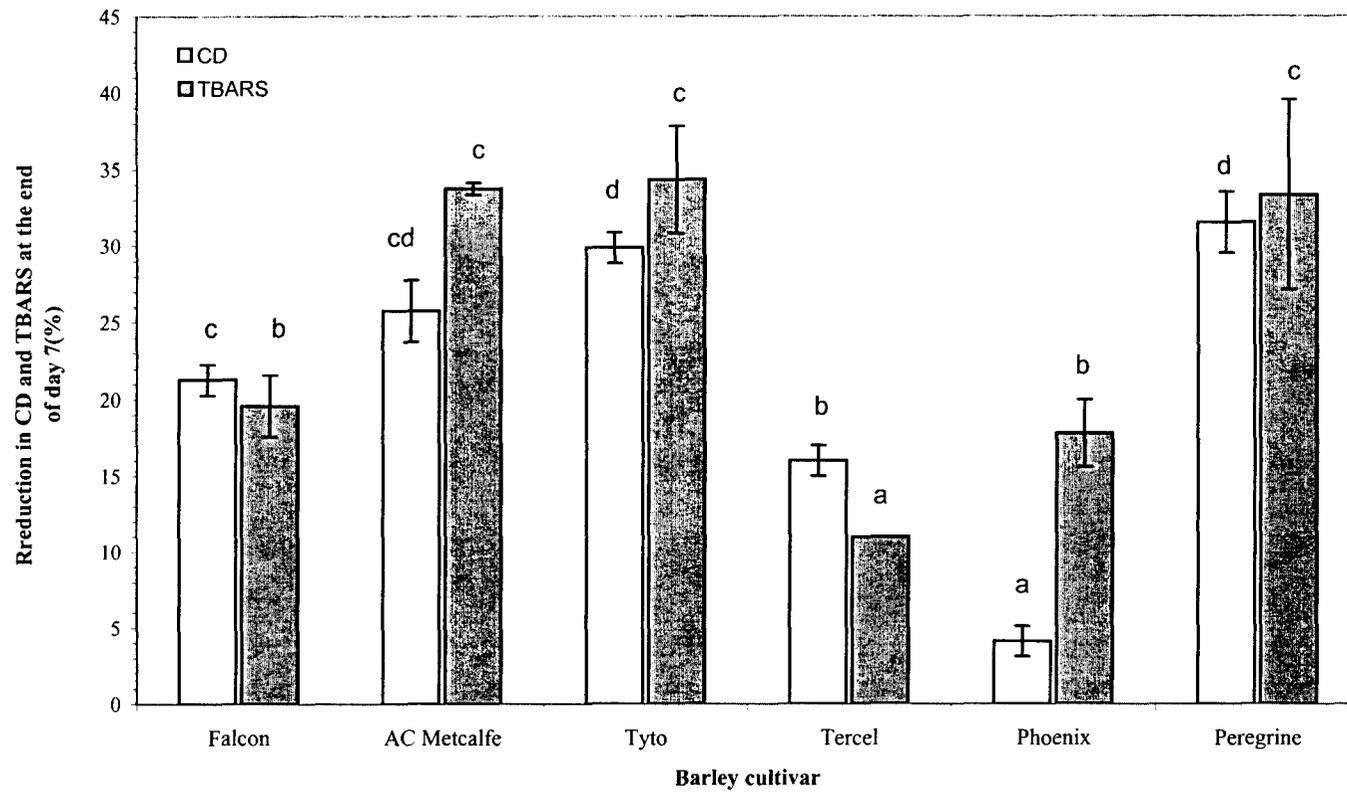
Similar storage studies have been used by Onyeneho and Hettiarachchy (1991) who found that navy bean hull extracts were effective against oxidation of soybean and sunflower oils stored at ambient temperature for 12 months or at 37°C for 9 months. The extracts were found to be more effective than the synergistic effects of BHA and BHT and rosemary antioxidants in inhibiting peroxide value (PV) in soybean or sunflower oil.

#### 5.4.14 $\beta$ -Carotene model system

$\beta$ -Carotene has a natural orange colour which degrades upon oxidation. The linoleic acid radical formed upon abstraction of a hydrogen atom may attack the highly conjugated  $\beta$ -carotene, thus leading to the loss of its natural orange colour. The presence of phenolic antioxidants prevents the loss of colour by neutralizing the linoleic acid free radical. The greater the potential of the antioxidant, the lesser the depletion of colour. Thus, this system can be employed to evaluate the efficacy of unknown antioxidative compounds (Wettasinghe and Shahidi, 1999).

In the  $\beta$ -carotene-linoleate model system the amount of  $\beta$ -carotene remaining in the assay medium after 120 min was used as an indicator of efficacy of antioxidants. **Figure 5.9** depicts the change of  $\beta$ -carotene concentration over a 120 min period. The initial content of  $\beta$ -carotene was 76  $\mu\text{g}$ . **Table 5.6** lists the amount of  $\beta$ -carotene in  $\mu\text{g}$  left after 120 min of assay and protection capacity percentage with different antioxidative extracts at 2 mg/mL concentration after 120 min of the assay. Protection capacity offered by whole barley extracts varied from 63.15 to 75.58% while only 7.96% of  $\beta$ -carotene was left at the end of the assay in the control devoid of any extracts. Ferulic acid, the reference antioxidant used, provided 92.18% protection at the end of 120 min.

**Figure 5.8** The effect of whole barley extracts on the formation of conjugated dienes (CD), and 2-thiobarbutiric acid reactive substances (TBARS) at 60°C on day 7 in a corn oil model system.

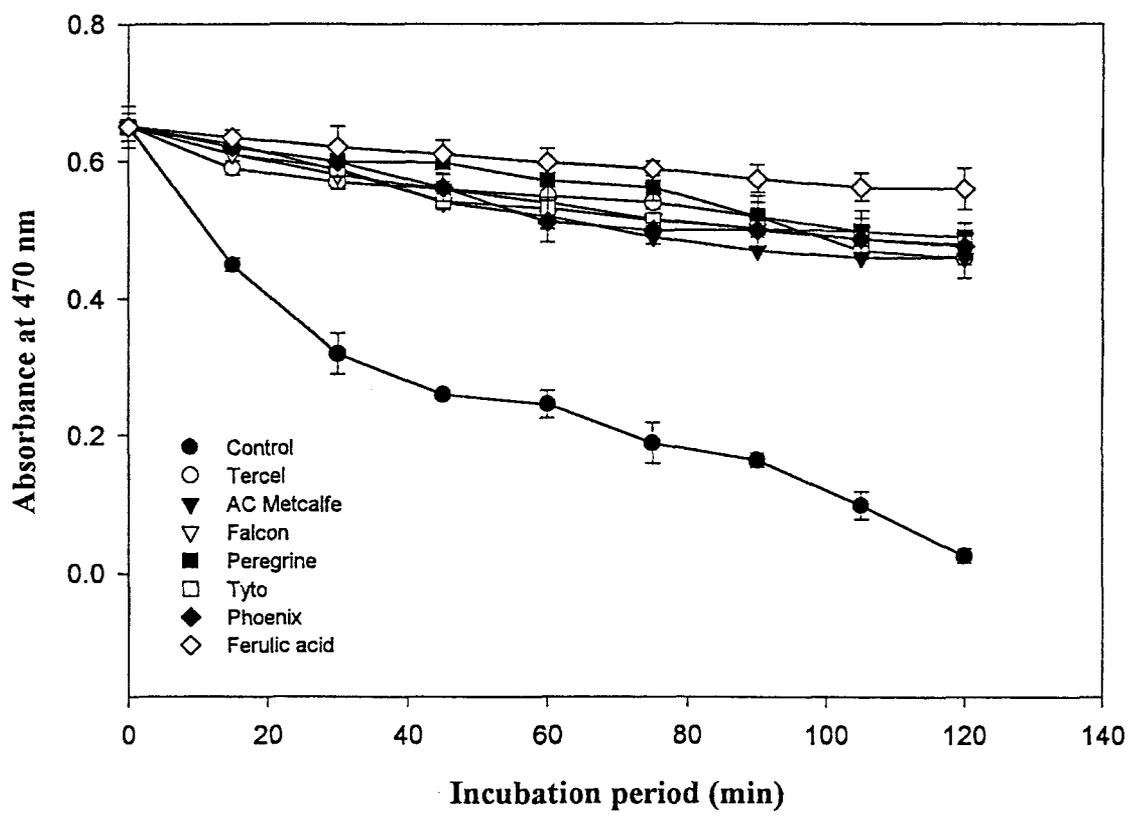


The linoleic acid free radical attacks the highly conjugated  $\beta$ -carotene molecule, thus reducing the  $\beta$ -carotene content. Therefore, as shown in **Figure 5.9**, the amount of  $\beta$ -carotene present is decreased at a fast rate. On the other hand, the presence of extracts changes the pattern of  $\beta$ -carotene loss. In the presence of additives, the loss of  $\beta$ -carotene occurred according to a second order polynomial function. As expected, ferulic acid, the reference antioxidative compound, exerted the strongest antioxidative effect. The  $\beta$ -carotene-linoleate system is comparable to an oil-in-water emulsion. Therefore, it can be speculated that hydrophobic antioxidants can be more effective in oil-in-water type emulsion. In the assay, the hydrophobic fraction of antioxidants might have performed effectively in protecting  $\beta$ -carotene. It is evident that the protective effect of the extracts is attributed to their phenolic content although structural effects are also very important.

#### **5.4.15 Evaluation of antioxidant activity using the Rancimat<sup>®</sup> method**

Whole kernel extracts were evaluated for their potential in inhibiting accelerated autoxidation of commercial stripped corn oil (stripped by passing through columns of adsorbants). The experiment was carried out using the Rancimat<sup>®</sup> apparatus and the protection factor was calculated for each extract. In the Rancimat<sup>®</sup> apparatus, dry air is bubbled at a constant speed through oil samples containing extracts. The oil samples are brought to an elevated temperature (120<sup>0</sup>C) and maintained at that temperature over the course of the experiment. Due to these accelerated conditions (high temperature and bubbling of air) lipid oxidation starts in the oil samples. As a result of autoxidation, different volatile compounds are generated, which are channelled to a separate container filled with a constant volume of deionized water.

**Figure 5.9** The effect of whole barley extracts on bleaching of  $\beta$ -carotene over 120 min in a  $\beta$ -carotene-linoleate model system.



**Table 5.6** Inhibition of bleaching of  $\beta$ -carotene by whole barley extracts.

Sample identity	Amount of $\beta$ -carotene retained after 120 min ( $\mu\text{g}$ )	Protection capacity (%) <sup>1</sup>
Falcon	54.23 $\pm$ 2.57 <sup>bc</sup>	72.31 $\pm$ 2.90 <sup>cd</sup>
AC Metcalfe	53.07 $\pm$ 3.61 <sup>c</sup>	70.77 $\pm$ 3.90 <sup>cd</sup>
Tyto	48.75 $\pm$ 2.09 <sup>b</sup>	63.15 $\pm$ 2.23 <sup>b</sup>
Tercel	49.58 $\pm$ 1.78 <sup>bc</sup>	65.23 $\pm$ 1.98 <sup>bc</sup>
Phoenix	53.21 $\pm$ 4.02 <sup>bc</sup>	70.95 $\pm$ 4.41 <sup>bcd</sup>
Peregrine	56.68 $\pm$ 2.39 <sup>c</sup>	75.58 $\pm$ 2.50 <sup>c</sup>
Ferulic acid	69.92 $\pm$ 1.56 <sup>d</sup>	92.18 $\pm$ 1.80 <sup>e</sup>
Control	5.86 $\pm$ 1.03 <sup>a</sup>	7.69 $\pm$ 1.18 <sup>a</sup>

<sup>1</sup>Protection capacity is calculated based on the absorbance values at 120min of the assay.

Results are means of three determinations  $\pm$  standard deviation.

Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

The volatile compounds dissolved raises the conductivity of deionized water and this change of conductivity is sensed by a probe and recorded. The progression of oxidation of the sample is monitored by obtaining conductivity data and recording using appropriate software.

Oxidation of unsaturated lipids proceeds very slowly during the initial stages, but it increases abruptly at a particular point, which is called the inflection point (IP). The time elapsed duration from the beginning to the IP is the induction period. This inflection point can easily be identified by plotting conductivity data obtained through the probe. Oils resistant to autoxidation take a long time to reach the IP while those vulnerable to autoxidation take a short time. Rancimat<sup>®</sup> results are conventionally expressed as protection factor (PF), which is defined as the ratio of the IP of the oil sample containing the test material and the IP of pure oil devoid of any additive. **Figure A-5.3** illustrates the change of conductivity in the solutions contained in the of collection vessels in the Rancimat<sup>®</sup> apparatus during the accelerated oxidative study.

Corn oil used in this experiment is stripped of any natural antioxidant present in it. The intrinsic resistance, if any, is eliminated from PF by taking the IP of pure oil into consideration as mentioned above. The protection factor for whole barley extracts varied between 1.31 and 1.59 while the PF for ferulic acid, the reference antioxidant used was 0.97 (**Figure 5.10**).

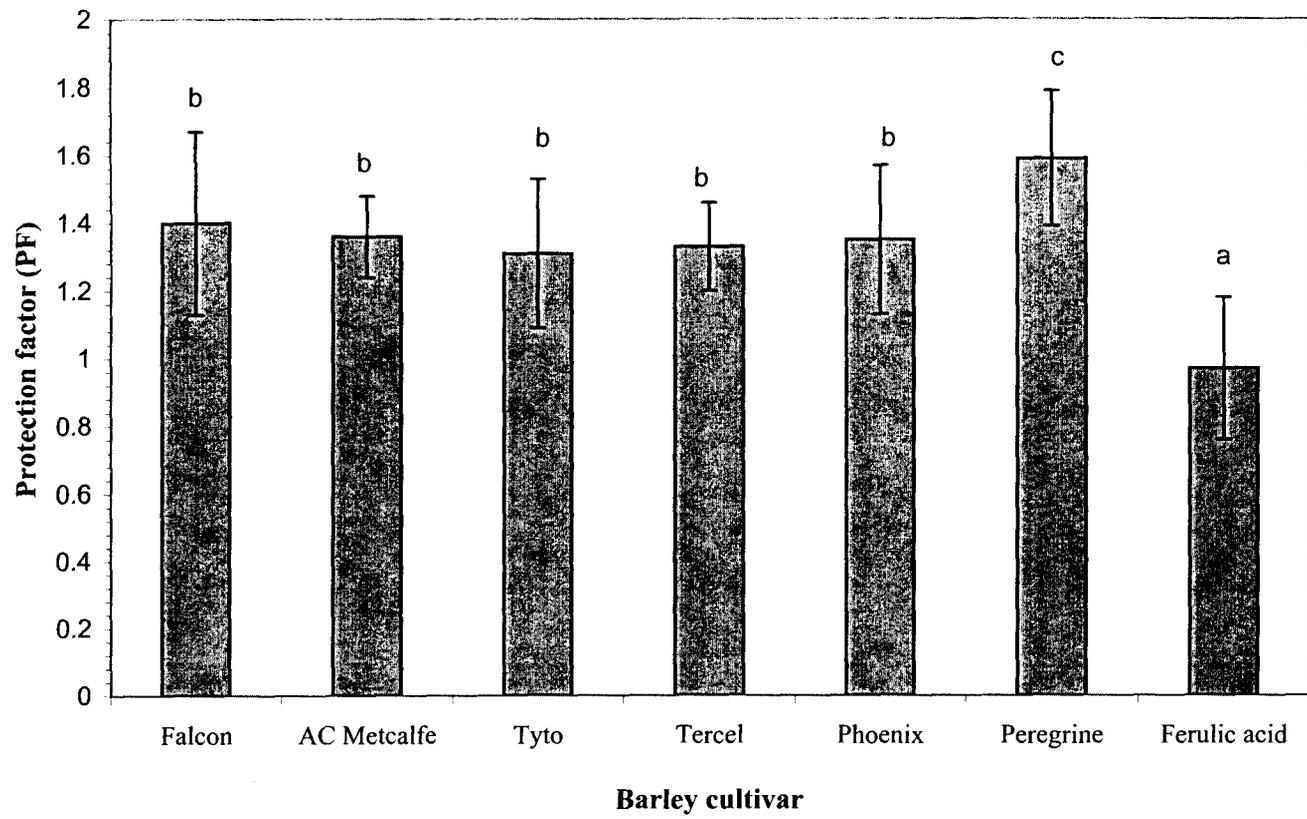
Ley *et al.* (2002) assessed different antioxidative compounds using the Rancimat<sup>®</sup> method. The PF for  $\alpha$ -tocopherol, BHT, and ascorbic acid were 5.1, 4.6, and 1.2, respectively. 3, 4-dihydroxymandelic acid (DHMA), which occurs in mammalian heart and other tissues showed the highest PF of 9.1 among the antioxidants tested. The PF of

whole barley extracts fell into the low region and was comparable to that of ascorbic acid. The Rancimat<sup>®</sup> test is used to determine the shelf life of fats and oils. As different substances can potentially accelerate or inhibit the formation of formic acid, it is possible to evaluate the protective effect offered by an ingredient/additive. Martinez-Tome *et al.* (2004) evaluated the effect of oat and wheat bran on butter using the Rancimat<sup>®</sup> method. The PF for wheat and oat bran ranged from 1.23 to 1.91 and 1.47 to 2.11, respectively while most of the phenolic acids tested yielded PF values of less than 1. All of the bran types produced higher PF values than the standard antioxidants used in the study. Among the authentic antioxidants tested, Trolox and propyl gallate yielded very high PFs of 7.17 and 6.48, respectively, followed by BHA (2.40) and BHT (1.40).

#### **5.4.16 DPPH radical scavenging activity by electron paramagnetic resonance (EPR) spectrometry**

The DPPH test is the oldest indirect method for measuring antioxidant activity. The test is based on the capability of suitable DPPH radical to react with H-donors. DPPH is more selective than ABTS<sup>••</sup> in the reactions with H-donors (Roginski and Lissi, 2005). In contrast to ABTS<sup>••</sup>, DPPH<sup>•</sup> does not react with flavonoids containing no hydroxyl groups in the B-ring (Yokozawa *et al.*, 1998) and with aromatic acids containing only one OH group (van Gadov *et al.*, 1997). The DPPH radical has been widely used in model systems to investigate the scavenging activities of antioxidative compounds.

**Figure 5.10** The effect of whole barley extracts on prevention of autoxidation of stripped corn oil as measured by Rancimat<sup>®</sup>.



DPPH<sup>•</sup>, a stable nitrogen containing free radical, is widely used in evaluating free radical scavenging properties and to assess the efficiency of the antioxidant potential of whole barley extracts using EPR. Antioxidative capacity of the extracts was expressed as the IC<sub>50</sub> value, which is defined as the amount of extract (mg/mL) required to lower the initial DPPH radical concentration by 50% and this was extrapolated from the dose-dependent curves. All barley extracts tested exhibited a strong antioxidant activity against DPPH<sup>•</sup> in a concentration-dependent manner.

The DPPH test can be carried out in two ways: dynamic, where the rate of DPPH decay is measured after addition of antioxidant, and static, where the amount of DPPH scavenged by the antioxidant is measured. While the first version characterizes the reactivity, the second determines the stoichiometry of the reaction of DPPH with H-donor for the antioxidant compound (Roginski and Lissi, 2005). Antioxidant activity of the test compound is expressed as T<sub>IC50</sub> (time needed to reach 50% of original DPPH concentration) with dynamic version while it is customary to express the antioxidant activity as IC<sub>50</sub> (concentration of test compound needed to reduce the original concentration of DPPH by 50%) with the static version. In this study the static version was used to evaluate the whole barley extracts. Sanchez-Moreno and co-workers (2002) classified the kinetic behavior of antioxidant activity of test compounds into three categories based on IC<sub>50</sub> value as rapid (< 5 min), intermediate (5-30 min) and slow (>30 min).

The IC<sub>50</sub> values obtained for six barley cultivars ranged from 1.51 to 3.20 mg/mL with smaller IC<sub>50</sub> values corresponding to greater radical scavenging activity. The IC<sub>50</sub> value for Tercel could not be extrapolated as it did not yield 50% reduction even at the

highest concentration used. The order of DPPH radical scavenging activity of the extracts was as follows: Peregrine > AC Metcalfe > Falcon > Tyto > Phoenix > Tercel (Table 5.7). Figure A-5.4 depicts the EPR spectra obtained for whole kernel extracts at 2.67 mg/mL concentration while Figure A-5.5 depicts the EPR spectra obtained for whole kernel Falcon and AC Metcalfe extracts at various concentrations. Figures A-5.6 and A-5.7 illustrate the dose dependent curves of DPPH<sup>•</sup> for whole kernel extracts. The reduction of DPPH<sup>•</sup> in the presence of additives was monitored by measuring the intensity of the EPR signal. Hydrogen donated by antioxidants can neutralize DPPH<sup>•</sup> into a non-radical (DPPH)H, as follows (Blois, 1958).

**Table 5.7** IC<sub>50</sub> values of barley extracts for DPPH<sup>•</sup> and hydroxyl radicals as measured by EPR spectrometry.<sup>1</sup>

Extract	DPPH radical	Hydroxyl radical
Falcon	2.12 ± 0.21 <sup>b,c</sup>	2.20 ± 0.04 <sup>a</sup>
AC Metcalfe	1.65 ± 0.17 <sup>a,b</sup>	2.81 ± 0.12 <sup>b</sup>
Tyto	2.60 ± 0.02 <sup>c</sup>	2.40 ± 0.06 <sup>a</sup>
Tercel	n.d.	9.65 ± 0.04 <sup>e</sup>
Phoenix	3.20 ± 0.09 <sup>d</sup>	5.31 ± 0.13 <sup>d</sup>
Peregrine	1.51 ± 0.02 <sup>a</sup>	3.14 ± 0.11 <sup>c</sup>

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

<sup>1</sup>IC<sub>50</sub> values are expressed as mg extract/mL in the final assay medium.

n.d.; not determined as the highest concentration used did not yield 50% reduction of radical.



The scavenging effect of various antioxidants on the DPPH radical was strongly concentration dependent (Lai *et al.*, 2001). In general, the scavenging effect on the DPPH radical increases with increasing antioxidant concentration to a certain extent and then levels off with further increase (Lai *et al.*, 2001). The scavenging percentage on the DPPH radical was found to be 68.6% for crude Hsian-tsoa plant extract at 1.25 mg/mL and 71.7% for  $\alpha$ -tocopherol and BHT at a dose level of 0.31 mg/mL (Lai *et al.*, 2001).

Although the DPPH assay is technically simple, some disadvantages are associated with the test. DPPH radical is long-lived nitrogen radical, which does not bear any similarity to physiological ROS. Many antioxidants react quickly with physiological ROS such as  $\text{ROO}^\bullet$ , but react slowly with  $\text{DPPH}^\bullet$  or may even be inert towards  $\text{DPPH}^\bullet$  (Huang *et al.*, 2005). It is also reported that the reaction between DPPH and some phenolic compounds such as eugenol are reversible (Bondet *et al.*, 1997), thus this may lead to underestimation of the results.

#### **5.4.17 Hydroxyl radical scavenging capacity by electron paramagnetic resonance.**

Hydroxyl radical is an extremely reactive, short-lived species that can hydroxylate DNA, protein and other biomolecules (Du and Gebicki, 2004). Thus, it is important to assess the hydroxyl radical scavenging activity of antioxidative extracts. Hydroxyl radicals generated were spin-trapped with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) due to the very short life of the radical. DMPO-adduct, a relatively stable free radical can easily be detected with EPR. The intensity of the characteristic 1:2:2:1 quartet with a

hyperfine coupling constant of 14.9G (Yen and Chen, 1995) diminished with the addition of the extract.

Since most of the antioxidants possess some metal chelation activity, this could either be due to scavenging of the hydroxyl radical by the extract, chelation of Fe(II) by the extract or, most likely, due to the combination of both actions. It is impossible to distinguish which is responsible for the activity. The IC<sub>50</sub> value is defined as the amount of extract (mg/mL) required to lower the initial hydroxyl radical concentration by 50% and was extrapolated from dose response curves. IC<sub>50</sub> values for hydroxyl radical ranged from 2.20 to 9.65 mg/mL with lower IC<sub>50</sub> values corresponding to higher antioxidant potential. Falcon, with an IC<sub>50</sub> value of 2.2 mg/mL, was the most active hydroxyl radical scavenger. The hierarchy of extracts based on their hydroxyl radical scavenging activity was Falcon > Tyto > AC Metcalfe > Peregrine > Phoenix > Tercel (**Table 5.7**). This pattern is different to the pattern observed for DPPH radical scavenging activity and is possibly due to the existing differences in kinetics and scavenging power of the two radicals. **Figure A-5.8** depicts the EPR spectra obtained for whole kernel extracts at 6.67 mg/mL while **Figure A-5.9** depicts the EPR spectra obtained for Falcon and AC Metcalfe whole kernel extracts at various concentrations. **Figures A-5.10 and A-5.11** depict the concentration dependence of antioxidant activity.

Hydroxyl radical is generated through Fenton reactions in the presence of Fe (II) and H<sub>2</sub>O<sub>2</sub>. Known antioxidants such as ascorbic acid may act as prooxidants by reducing Fe (III) to Fe (II) leading to catalytic generation of <sup>•</sup>OH. Zhu *et al.* (2000) reported that <sup>•</sup>OH can be generated through organic Fenton reaction using tetrachlorohydroquinone (TCHQ)/H<sub>2</sub>O<sub>2</sub> mixture. If this mixture can be used, the effect of Fe (II) can be eliminated

from the assay. However, the applicability of this mixture for the assay is yet to be determined.

#### **5.4.18 Inhibition of Cu (II)-induced human LDL cholesterol oxidation**

Oxidation of LDL *in vivo* may contribute to the pathology of atherosclerosis (Steinberg *et al.*, 1989; Steinbrecher, 1987). Thus, this has led to increased interest in investigating the role of natural antioxidants in preventing oxidation of LDL and membrane lipids. Antioxidants are known to offer protective effects in controlling oxidative modification of LDL cholesterol, thus reducing the chances of developing atheroma in the arteries. One approach to study the effect of test antioxidant against LDL cholesterol oxidation is by subjecting LDL to oxidation in the presence of a known concentration of the test compound and monitoring the progression of oxidation. LDL is susceptible to oxidation, which can be initiated by different agents such as AAPH, Cu(II), and hypochlorite. In this study, human LDL cholesterol samples (0.2 mg/mL) were mixed with Cu(II) in the presence of whole barley extracts (2 mg/mL) and the development of CD was measured at timed intervals over 100 min. Conjugated diene is often used as an indicator of the level of peroxidation of LDL in antioxidant studies. The inhibition of the LDL oxidation of the extracts was expressed as percentage inhibition based on the CD value after 100 min of incubation and the results are illustrated in **Figure 5.11**. The whole kernel extracts exhibited 19.64 to 33.93% inhibition, with Peregrine offering the highest inhibition followed by AC Metcalfe extract. The order of effectiveness was as follows. Peregrine > AC Metcalfe > Tyto = Tercel = Falcon. Ferulic acid exhibited the highest inhibition of 53.57% at 70  $\mu\text{g/mL}$  concentration (**Table 5.8**).

None of the samples showed any indication of oxidation during the first 10 min of incubation, however, all samples showed a rise in CD after 10 min of incubation. During 10 – 40 min of incubation, all extracts except Peregrine indicated higher level of oxidation as indicated by CD compared to the blank containing native LDL and Cu(II) devoid of any additive. During this period, whole barley extracts demonstrated prooxidant activity. However, after 40 min, the development of CD in the blank was far greater than in the samples containing whole barley extracts. This type of inconsistent behaviour was also observed by Andreasen *et al.* (2001) for authentic phenolic acids. Ferulic acid, ferulic acid dihydromers, sinapic acid, and, *p*-coumaric acid were tested against LDL oxidation at various concentrations (10-60  $\mu$ M) over 100 min. Andreasen *et al.* (2001) determined the potency of phenolic extracts made from rye flour, bran, and whole grain in the prevention of LDL oxidation and determined that they behaved in a similar manner. Furthermore, it was found that extracts from bran inhibited LDL oxidation in a dose-dependent manner while the extracts from whole grain showed a very weak activity. The inhibition offered by whole rye extracts (33%) was comparable to that of Peregrine (33.9%) and AC Metcalfe (26.8%). All phenolic acids, except sinapic acid and *p*-coumaric acid, at some concentrations indicated a prooxidant activity compared to the native LDL (blank) sample at the beginning of the incubation however, subsequently showed antioxidant activity as the incubation continued.

Caffeic acid and gallic acid were the most active phenolic acids in inhibiting CD development and completely blocked the formation of CD at all concentrations tested (5-60  $\mu$ M). In contrast, ferulic acid did not inhibit LDL oxidation by prolonging lag time.

The development of CD in the samples containing Peregrine extract and ferulic acid exhibited a similar pattern throughout the incubation period (Andreasen *et al.*, 2001).

Native LDL is heterogeneous, containing a large number of components sensitive to oxidation. Therefore, oxidation of LDL leads to an infinite number of products with varying degrees of oxidation of cholesterol, phospholipids, cholesteryl esters, triacylglycerols, and proteins (Chisom and Steinberg, 2000).

**Figure 5.11** Development of conjugated dienes (CD) in LDL cholesterol in the presence of whole barley extracts over 100 min of incubation.



**Table 5. 8** Percentage inhibition of Cu(II)-induced human LDL oxidation by whole barley extracts.

Sample identity	Inhibition (%) <sup>1</sup>
Falcon	26.79 ± 3.2 <sup>abc</sup>
AC Metcalfe	19.64 ± 2.7 <sup>a</sup>
Tyto	21.43 ± 1.2 <sup>ab</sup>
Tercel	21.11 ± 0.98 <sup>ab</sup>
Phoenix	28.57 ± 4.2 <sup>bc</sup>
Peregrine	33.93 ± 2.8 <sup>bc</sup>
Ferulic acid	53.57 ± 3.7 <sup>d</sup>

<sup>1</sup>Inhibition percentage is calculated based on the conjugated diene values at 100min of incubation.

Results are means of three determinations ± standard deviation.

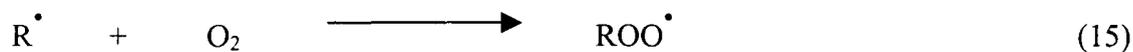
Values in each column having the same superscript are not significantly different (p>0.05).

The effect of the phenolic extracts *in vivo* is still uncertain as there is a gap in knowledge about the mechanism of absorption and the bioavailability. It is also controversial if these phenolic acids are present in plasma as glucouronic acid and/or sulphate conjugates (Andreasen *et al.*, 2001). The conjugated forms of hydroxycinnmates may act differently towards the inhibition of LDL oxidation as exhibited by the extracts *in vitro*.

Lipid peroxidation in LDL by Cu (II) is initiated by endogenous or contaminated peroxy radicals as follows.



The generated  $\text{ROO}^\bullet$  and  $\text{RO}^\bullet$  are believed to initiate oxidation of parent PUFA through hydrogen atom abstraction and oxygenation as follows (Burkit, 2001).



Reduction of Cu(II) to unstable Cu(I) aggravates the oxidation process. It is accepted that  $\alpha$ -tocopherol, ascorbic acid and thiols (especially cystein, and glutathione) can bring about this reduction, thus, leading to enhanced oxidation. Similarly, other minor constituents present in the extracts may also affect the oxidation. Therefore it can be

deduced that the chemical compositional differences among the barley cultivars might be responsible for the differential behaviour of the extracts in preventing LDL oxidation.

Some researchers use lag time (time taken to show a sharp rise in CD) to assess the antioxidant efficacy of the extracts against LDL oxidation. However, the use of lag time in this study seems to be inappropriate as all the extracts showed very similar lag times. All the curves corresponding to whole extracts, except Peregrine, indicated a steep upward slope at the beginning indicating a prooxidant activity, however, the total CD developed was low in the samples containing barley extracts.

The predominant phenolic compounds present in methanolic barley extracts are phenolic acids. Therefore, the protective effect observed against LDL oxidation can mainly be attributable to the effect of phenolic acids. Andreasen *et al.* (2001) reported the order of effectiveness of phenolic acids against LDL oxidation as caffeic acid > sinapic acid > ferulic acid > *p*-coumaric acid.

The protective effect can be in part attributable to the Cu(II) chelation potential of the phenolic acids. Recent studies have shown that Cu (II)-mediated oxidation of the LDL can exhibit different kinetics depending on Cu (II) concentration. Propagation can proceed when antioxidants are depleted, whether Cu(II) is present in high or low concentrations (Ziouzenkova *et al.*, 1998). At high molar ratios of at least 10 Cu(II) ions per one LDL molecule, oxidation continues to propagate after all the available antioxidants are consumed (Ziouzenkova *et al.*, 1998). The increase in CD during the propagation phase was reported to be mainly due to the formation of cholesteryl linoleate hydroperoxides and substantial amounts of cholesteryl linoleate hydroxides (Esterbauer *et al.*, 1991).

#### **5.4.19 Supercoiled DNA scission study**

Free radicals found in biological systems are reactive towards biomolecules in their immediate vicinity including DNA and can trigger chain reactions leading to damages. More importantly, the radical reactions promote modifications to DNA which may lead to mutagenesis and subsequently initiate carcinogenesis. Oxidants produced as by-products of mitochondrial electron transport and products from lipid peroxidation that escape the numerous antioxidant defense systems can cause damage to cellular macromolecules including DNA (Ames and Shigenaga, 1993). Oxidative damage of DNA results in a wide range of scission products, which include strand breaks and sister chromatid exchange, DNA-DNA and DNA-protein cross-links, as well as base modifications (Ames and Shigenaga, 1993). DNA damage is often measured as single strand-breaks, double strand-breaks or chromosomal aberrations (Breimer, 1990). There is compelling evidence that antioxidants prevent the DNA damage by neutralizing free radicals. One approach to studying the protective effect offered by the test antioxidative extract against DNA damage is to expose double stranded DNA to a known concentration of radicals in the presence of a known quantity of extract. Free radicals are capable of breaking open the double stranded DNA and nicking. After incubating for a sufficient period of time, the protective effect is evaluated by visualizing the DNA bands i.e. double strands and nicked DNA. In the present study, the whole barley extracts were evaluated for their capacity to inhibit peroxy and hydroxyl radical-induced scission of DNA supercoiled (Form 1) strands.

#### 5.4.19.1 Inhibition of supercoiled plasmid DNA scission induced by peroxy radical

Peroxy radical generated through AAPH led to breakage of supercoiled plasmid DNA. All barley extracts exhibited strong protection against peroxy radical-induced DNA breakage in a concentration-dependent manner. The level of protection against breakage was presented as percentage inhibition by comparing the amount of DNA remaining at the end of incubation with the amount of DNA present in the native DNA sample (**Table 5.9**). Extracts were tested at different concentrations of 1.33, 2.62, 4.00 and 6.67 mg/mL and they exhibited DNA protective effect in a dose-dependent manner (**Figures A-5.12 and 5.13**). The protective effects, in general, increased rapidly up to 4 mg/mL and then started to level off. Therefore, 4 mg/mL concentration was used in the calculation of protective effect. Radicals cleave supercoiled plasmid DNA (form I) to nicked circular DNA (form II) or, at higher concentrations of radicals, to linear DNA (form III). The presence of peroxy radical resulted in a dramatic scission of supercoiled DNA which was clearly seen in the wells, where the reaction mixture did not contain any antioxidant. The radical concentration used in the present study was not sufficient enough to destroy the nicked circular DNA, which is more difficult to destroy than form I DNA. This is clearly seen in lane 2 of **Figures 5.12a and b**, which depict the effect of peroxy radical on supercoiled DNA incubated without any extracts. As the concentration of antioxidative extract was increased, the protective effect against nicking of supercoiled DNA was also increased. S and N represent supercoiled and nicked DNA bands, respectively. The high intensity S band with a low intensity N band in lane 1 indicates a high concentration of supercoiled DNA and low concentrations of nicked DNA in the native DNA sample. Lane 2 represents the native supercoiled DNA sample without any

additives. The presence of a high intensity N band and the disappearance of the S band in lane 2 indicate that supercoiled DNA was completely nicked. Wells 3 through 6 contained supercoiled DNA, along with the same concentration of radical together with increasing concentrations of whole kernel extracts (1.33-6.67 mg/mL). The intensity of the S band gradually increased from lane 3 through 6 reflecting a higher level of retention of supercoiled DNA due to the protection offered by increasing concentrations of barley extracts. On the other hand, the intensity of the N band gradually decreased from lane 3 through 6 depicting less nicked DNA concentrations with an increasing level of protection offered by the extracts. In order to compare the DNA protection efficiency among the barley extracts, 4 mg/mL concentration was chosen as the reference concentration. The percentage inhibition offered by the extracts ranged from 78.2 to 92.1% at an extract concentration of 4 mg/mL. The ferulic acid standard yielded 99.2% protection at 60 µg/mL concentration which is equivalent to the extracts in terms of total phenolic content. Peregrine exhibited the highest inhibition against DNA scission while Phoenix exhibited the lowest inhibition at 78.2%. It is important to note that no pro-oxidant activity was observed at any level for all the extracts and all extracts showed a similar pattern of concentration dependence in protecting supercoiled plasmid DNA. The affinity of reactive oxygen species to initiate DNA damage has been well characterized *in vitro*; however, the effect of peroxy radical on breakage of DNA has only recently been established (Hu and Kitts, 2001). Peroxyl radicals are more stable than other oxygen radicals (Ross and Bielski, 1990) and have the ability to diffuse relatively far from the site of their generation before they react with a target molecule (Morrero and Marnett,

1993). It is well documented that the frequently associated oxidative stress occurring in biological systems is attributable to peroxy radicals.

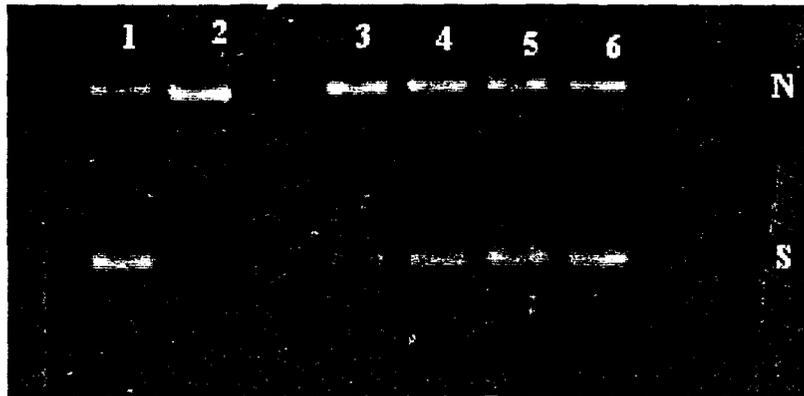
In the absence of any antioxidant, it may be expected that the peroxy radical abstracts a hydrogen atom from the nearby DNA to generate new radicals, which in turn evokes a free radical chain reaction resulting in the destruction of the DNA molecule. However, in the presence of antioxidants, this chain reaction is terminated by abstracting a hydrogen atom from the antioxidant molecule (Hu and Kitts, 2000).

#### **5.4.19.2 Hydroxyl radical-induced supercoiled plasmid DNA scission**

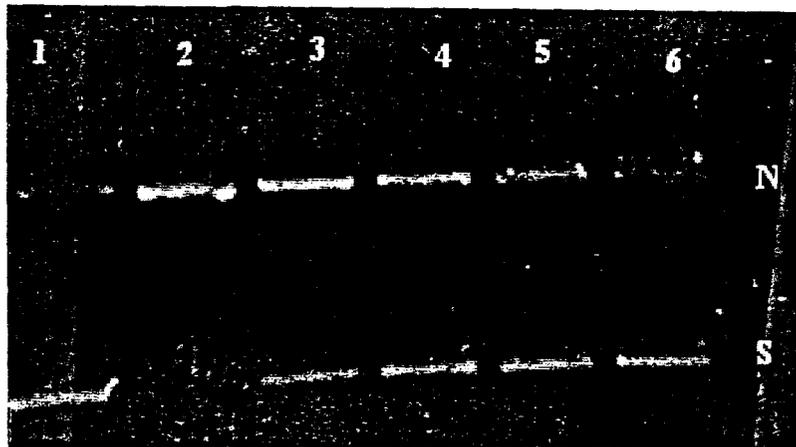
Whole barley extracts were effective in suppressing hydroxyl radical-induced DNA damage in a non-site specific protocol in a concentration-dependent manner (**Figures A-5.14-A-5.15**).

**Figure 5.12** Representative gel pictographs illustrating the effect of whole AC Metcalfe **(a)** and Falcon **(b)** extracts at different concentrations against peroxy radical-induced nicking of supercoiled DNA double strands. *Lanes 1 through 6 contained DNA. Lanes 2-6 contained 1 mM AAPH; In addition, lanes 3 through 6 contained 1.33, 2.67, 4.00 and 6.67 mg/mL of whole kernel extracts, respectively.*

(a)



(b)



**Table 5.9** Effect of barley extracts at 4 mg/mL on the retention of supercoiled strand of PBR322 DNA under peroxy and hydroxyl radical-induced scission.

Sample identity	DNA protection (%)	
	Peroxy radical	Hydroxyl radical
Falcon	84.3 ± 2.3 <sup>a</sup>	59.8 ± 2.7 <sup>a,b</sup>
AC Metcalfe	86.7 ± 1.7 <sup>a</sup>	65.3 ± 3.1 <sup>b,c,d</sup>
Tyto	82.6 ± 4.6 <sup>a</sup>	58.6 ± 1.2 <sup>a,d</sup>
Tercel	87.9 ± 5.2 <sup>a,b</sup>	62.8 ± 3.7 <sup>b,c</sup>
Phoenix	78.2 ± 2.9 <sup>a</sup>	53.1 ± 3.3 <sup>a</sup>
Peregrine	92.1 ± 3.2 <sup>b,c</sup>	64.2 ± 4.2 <sup>c,b</sup>
Ferulic acid <sup>1</sup>	99.2 ± 4.7 <sup>c</sup>	78.2 ± 2.6 <sup>c</sup>

<sup>1</sup> 70 µg/mL.

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

Extracts were tested at concentrations of 1.33, 2.67, 4.00 and 6.67 mg/mL. The protective effects, in general, increased rapidly up to 4 mg/mL and then started leveling off. Therefore, for the calculation of protective effect, 4 mg/mL concentration was considered. At this concentration, the whole barley extracts exhibited 53.1 to 65.3% inhibition. It was clearly demonstrated that the level of protection is substantially lower than against peroxy radical. The results of the experiment are listed in **Table 5.9**.

The reactions of hydroxyl radical occur mainly through addition to the double bond of pyrimidine bases and abstraction of hydrogen from the sugar moiety resulting in chain scission of DNA. These effects can cause cell mutagenesis and carcinogenesis (Namiki, 1990). The results of the experiment extracts toward protecting plasmid DNA strand scission provide further evidence of the antioxidant efficacy exhibited by barley.

On the other hand, it is possible to prevent the formation of hydroxyl radical by deactivating free metal ions such as Fe(II) through chelation. Non site-specific protocol distinguishes Fe(II) chelation and hydroxyl radical scavenging capabilities of test extracts by eliminating the contribution of the extracts toward the Fe(II) chelation effect. Therefore, this protocol identifies the true hydroxyl radical scavenging capacity of the extracts. This study demonstrated that barley contained substantial amounts of phenolic antioxidants that effectively scavenge free radicals. They were very effective against peroxy, DPPH and hydroxyl radicals. The Peregrine, Falcon and AC Metcalfe cultivars were, in general, more effective than the rest of the cultivars, perhaps due to their higher content of phenolics. The genetic differences are mainly attributable to the different antioxidant capacities among the cultivars examined.

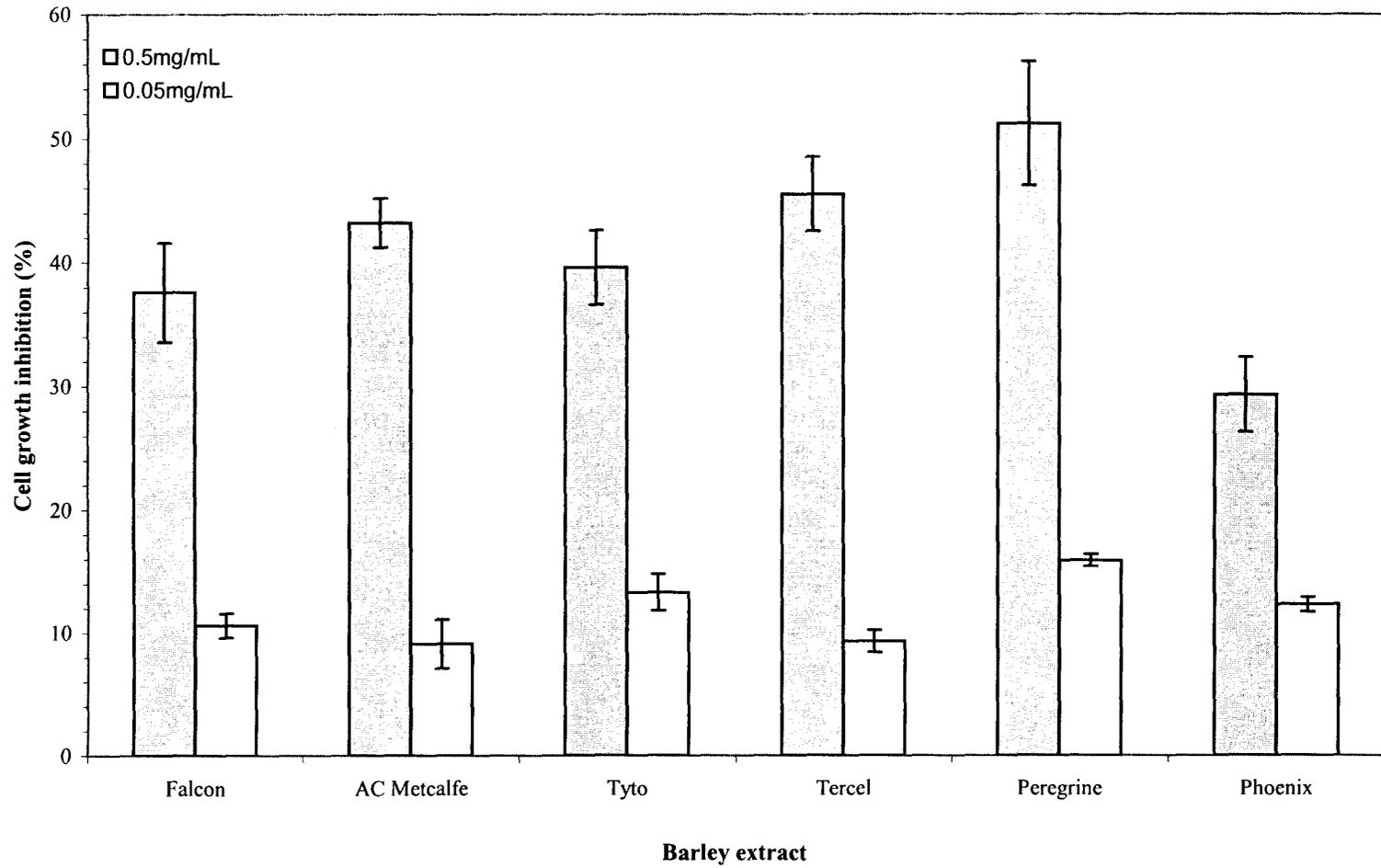
It is important to note that no prooxidant effects were observed in the range of concentrations used in this study. The concept of a site-specific effect of the hydroxyl radical was described by Gutteridge (1984). In the absence of EDTA, iron ions bind to deoxyribose molecules and bring about a site-specific reaction in the molecule. However, in the presence of EDTA, the iron ion is removed from the binding site to form an EDTA-metal complex and produce a hydroxyl radical that can be removed by hydroxyl radical scavengers. Most hydroxyl radical scavengers show poor inhibitory effects for site-specific hydroxyl reactions. In a separate study, Aruoma (1998) reported that hydroxytyrosol present in extra virgin olive oil strongly inhibited LDL oxidation, but exhibited extensive cleavage of DNA in a hydroxyl radical-induced-DNA model system. When tested in human lymphocytes, using the Comet assay, ascorbic acid was found to induce a marked dose-dependent increase in DNA breakage (Anderson and Phillips, 1999). Furthermore, high plasma ascorbic acid concentrations increased the percentage of cell aberrations in *in vivo* studies (Anderson and Phillips, 1999).

#### **5.4.20 Effect of whole barley extracts on prevention of proliferation of Caco-2 colon cancer cell line.**

Colorectal cancer accounts for the second highest cause of death due to cancer in North America and the American Cancer Society estimated that 56,300 deaths were caused by colorectal cancer in 2005 (Jemal *et al.*, 2005). Thus, it is important to investigate the effect of antioxidative extracts on colorectal carcinoma cells. The Caco-2 cell line is widely used to evaluate different extracts and additives *in vitro*. Whole kernel extracts were evaluated for their potential antiproliferative efficacy at 0.5 and 0.05mg/mL levels using Caco-2 human cancer cells. In this study, the antiproliferative activity was

measured and reported as percent inhibition. None of the barley extracts showed significant antiproliferative activity by the end of day 1. The inhibition effect ranged from 5.7 to 10.4% and 1.1 to 3.3% at 0.5 and 0.05 mg/mL concentrations, respectively after 24 h of incubation. However, the effect of barley extracts on cell proliferation became evident over the incubation period and was clearly seen at the end of day 4. The inhibition effect of whole barley extracts varied from 29.3 to 51.2% at the 0.5 mg/mL concentration and from 12.3 to 15.9 at of 0.05 mg/mL (**Figure 5.13**). Peregrine extracts rendered the highest inhibition effect at both concentrations while the Phoenix extract showed the lowest activity at 0.5 mg/mL. Peregrine extracts contained higher total phenolic content and consistently showed higher antioxidant activity with most of the other assays. Inhibition of cell proliferation was well correlated with total antioxidant capacity as measured by TEAC ( $r^2 = 0.835$ ) and showed fair correlation with TPC ( $r^2 = 0.784$ ). However, there are many additional factors, such as bioavailability that may affect antiproliferative action *in vivo*. The higher concentration (0.5 mg/mL) used was quite effective in inhibiting cell proliferation, however, the lower concentration (0.05 mg/mL) was not effective even at the end of the day 4 in controlling the growth of Caco-2 cells. The discrepancy in inhibition of cell proliferation may be attributable to the different chemical composition of the barley cultivars.

**Figure 5.13** Effect of whole barley extracts on inhibition of growth of Caco-2 adenocarcinoma cells at the end of day 4 of incubation in an *ex vivo* study.



Parry *et al.* (2006) tested a number of fruit seed flours against Caco-2 cell proliferation and observed that the effectiveness of the extracts increased over four days and attained the highest value at the end of day 4. This trend is very similar to what was observed in this study.

## 5.5 Conclusions

Of the six barley cultivars tested, Peregrine yielded the highest total phenolic content followed by AC Metcalfe and Falcon. TPC of Tercel and Phoenix were significantly lower compared to that of previously mentioned cultivars. TAC as measured by TEAC followed a similar trend to TPC. Total phenolic content and TEAC showed a strong correlation, however, TPC did not show a good correlation with DPPH radical scavenging capacity measured spectrophotometrically. Peregrine and AC Metcalfe extracts exhibited the highest reducing power while Tercel exhibited the lowest. Tyto showed the highest  $ORAC_{FL}$  as well as  $ORAC_{FL}/TPC$  ratio. Although Peregrine exhibited the highest antioxidant activity, in general, the  $ORAC_{FL}/TPC$  ratio of Peregrine was moderate among the cultivars tested. The order of activity of  $HORAC_{FL}$  was different from that of  $ORAC_{FL}$  suggesting that the mechanism of hydroxyl radical and peroxy radical scavenging were different. Metal chelation activity of barley cultivars, in general was poor compared to many other food stuffs, however, it remains on par with the metal chelation ability of other cereals such as wheat. Falcon, Peregrine, and AC Metcalfe exhibited the highest metal chelation among the extracts tested.

In  $\beta$ -carotene and corn oil model systems all barley cultivars tested exhibited very high antioxidant activity compared to that of the control. The tested barley cultivars also

exhibited substantial protection against copper-induced LDL oxidation and radical-induced DNA nicking.

## CHAPTER 6

### ANTIOXIDANT PROPERTIES OF PEARLING FINES OF BARLEY

#### 6.1 Introduction

Cereals, including barley, are generally milled before being used for food in order to make them more palatable. Milling generally involves the removal of certain cereal components such as hull, and bran (pericarp, seed coat, nucellar, epiderm, and aleurone and sub aleurone layers). In addition to these seed components the germ, which is rich in oil, is also removed during milling operations. It is the general practice to clean and condition cereal grains prior to milling. Magnetic separators, gravity tables and disk separators, among others, are used for cleaning grains. Conditioning or tempering cereal grains is performed following cleaning in order to toughen the bran, thus making it resistant to breakage during subsequent milling operations. Another objective of conditioning is to soften the endosperm (Hoseney, 1994) thus, making it easily breakable during milling operations. Tempering is achieved by adding water to dry grains and allowing the grains to rest for a sufficient period of time before milling.

The pearling is an important process used in food barley preparation. Pearling refers to gradual removal of grain tissues through abrasive action starting from the outer tissues. In the pearling process, the outer layers, mainly bran that contains pericarp, testa, aleurone and sub-aleurone layers and germs are sequentially removed. These layers, removed from the barley grain, are called pearling fines. Pearling leads to a number of pearling fines and a bright white coloured kernel called pearled grain that has many food applications (Yeung and Vasanthan, 2001). A major proportion of pearling fines is utilized for animal feed production while little is used for soups and as rice extenders.

Historically barley has been used to extend rice when rice is in short supply or is expensive. Novel uses of barley pearling fines, which are rich in  $\beta$ -glucan, and include the production of functional pasta and nutraceuticals (Yeung and Vasanthan, 2001).

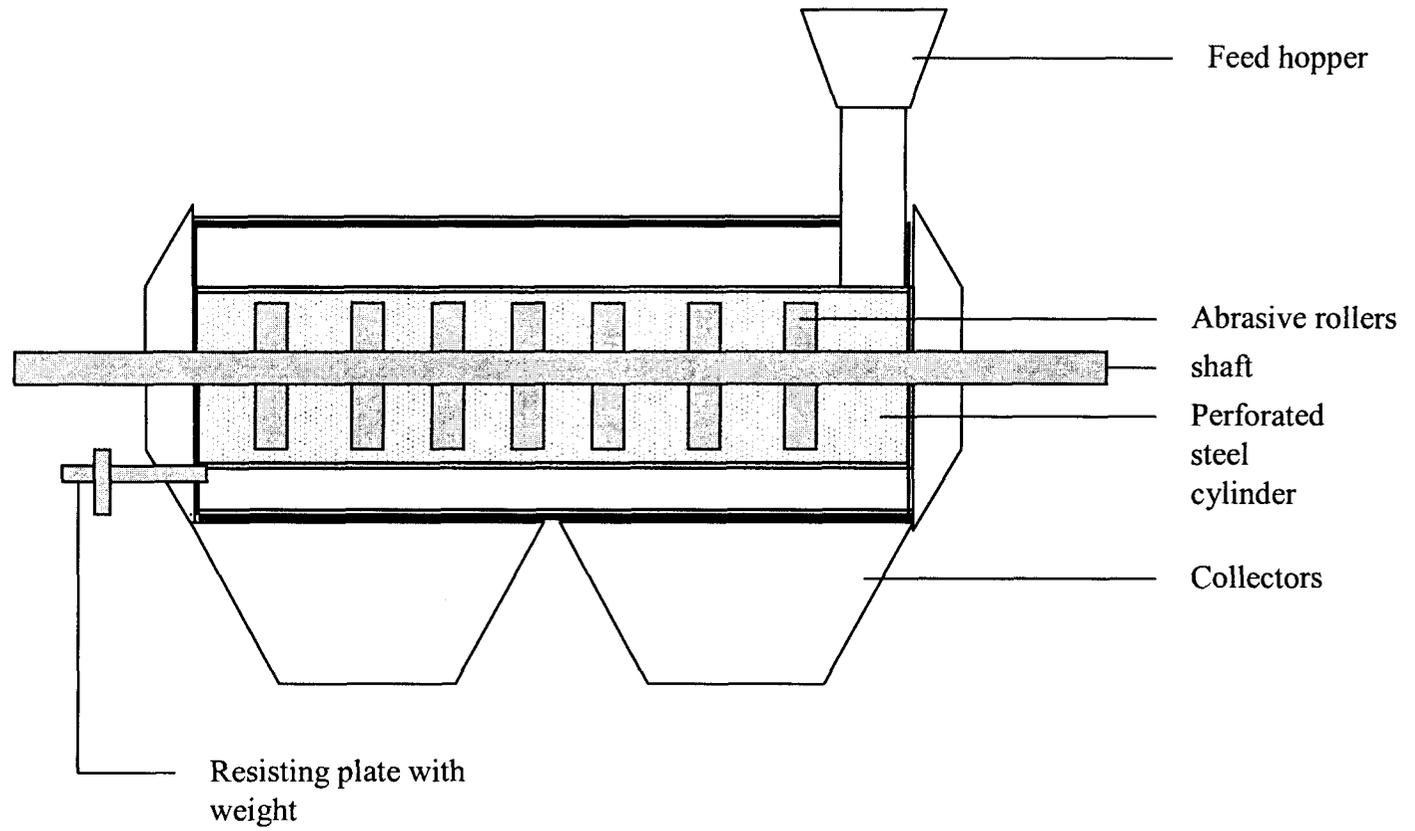
Unlike wheat, barley does not have a long tradition of being fractionated by milling. Interest in fractionation or pearling stems from current demand for barley fractions with a unique composition and functionality. As the distribution of various barley components is not uniform throughout the kernel, grain fractionation may prove to be an efficient way to obtain products enriched in specific functional components of bioactives. (Izydorczyk *et al.*, 2003).

The pearling process is carried out in a special milling device known as pearler, which consists of 6-8 abrasive carborandum or emery-coated disks that rotate at a controllable speed (450 rpm) enclosed in a perforated chamber (Leonard and Martin, 1963). In a typical pearler, the grains enter through a flow-regulating valve and are then conveyed by a screw to the pearling chamber, where the rotating disks cause the grains to rub against each other, abrading off the bran. Most of the bran is removed by abrading the grain against other grains while a small amount is also removed by rubbing against steel screen (Hoseney, 1994). At the discharge end of the pearling chamber is a resisting plate held by a weight. The amount of weight determines the back pressure applied to the contents in the pearling chamber, therefore, the degree of pearling can be controlled by varying the weight. Pearling is usually carried out as a batch process rather than a continuous process. **Figure 6.1** illustrates a schematic of a barley pearler.

The term 'degree of pearling' is commonly used in the milling industry to characterize the percent removal of outer layers, however, the use of the term is not universal. According to North American usage, degree of pearling refers to the percentage weight of the outer layers removed. For example, 25% pearled refers to 25% of pearling fines and 75% pearled grain. In Japan, the degree of pearling refers to the amount of pearled grain left intact. Therefore, 75% pearled refers to 75% of pearled grain and 25% pearling fines (Yeung and Vasanthan, 2001).

Phenolic antioxidants are not evenly distributed in cereal grains. Salomonsson *et al.* (1980) indicated that *p*-coumaric acid was present in the lowest amount in the centre of the barley kernel and rapidly increased toward the outer layers, such as lignified husk. Some researchers have reported that phenolic acids are concentrated in the cell walls of outer layers (Maillard and Berset, 1995), while others indicated that phenolic acids were mainly present in the aleurone layer and endosperm (Goupy *et al.*, 1999). The content of ferulic acid was highest in the cell walls of the aleurone layer, which is rich in arabinoxylans (McNeil *et al.*, 1975). Pearling of barley has also been identified as an important processing step in production of animal feed for swine. In cereal grains, the presence of deoxynivalenol, a trichothecene mycotoxin produced by *Fusarium* species presents a challenge to the production of animal feed especially for swine since the presence of this mycotoxin in feed causes anorectic effects in swine and other animals (Vesonder *et al.*, 1973). Pearling has been suggested as an effective means of reducing the deoxynivalenol level in barley (House *et al.*, 2003).

**Figure 6.1** Schematic diagram illustrating a barley pearler.  
*(Adapted from Hosney, 1994)*



## **6.2 Objectives**

The objectives of this phase of the research were to determine antioxidative, antiradical, and antiproliferative efficacies of pearling fines (fractions) of two barley varieties, namely Falcon (a hullless cultivar) and AC Metcalfe (a hulled cultivar) and thereby to understand the distribution of antioxidative activity within the barley grain. AC Metcalfe was the only hulled cultivar used in the study. Falcon was selected as the representative hullless cultivar as it yielded an average antioxidant activity.

## **6.3 Materials and methods**

### **6.3.1 Materials**

Two barley cultivars, the hull-less variety, Falcon, and hulled variety, AC Metcalfe, were obtained from the Field Crop Development Centre, Lacombe, Alberta, Canada, during the 2002 crop year. The samples were stored at room temperature under low humidity conditions until the antioxidative compounds were extracted.

### **6.3.2 Preparation of barley samples**

The two barley samples were tempered to 14% moisture over a 65-h period and dehulled prior to further processing. The dehulled barley samples were pearled with a Satake type TM pearler (Model TM05, Satake Co., Tokyo, Japan) and the pearling by-products (pearling fines) were collected at ~5-10% weight intervals by successively abrading the kernels up to 50% of their original weight. Pearled grains were collected separately. **Figure 6.2** illustrates a schematic diagram of the pearling action. The successive abrasion of the grains resulted in seven fractions designated as fractions 1-7

(F1 to F7) throughout the following text with F1 and F7 representing the outermost and innermost fractions, respectively.

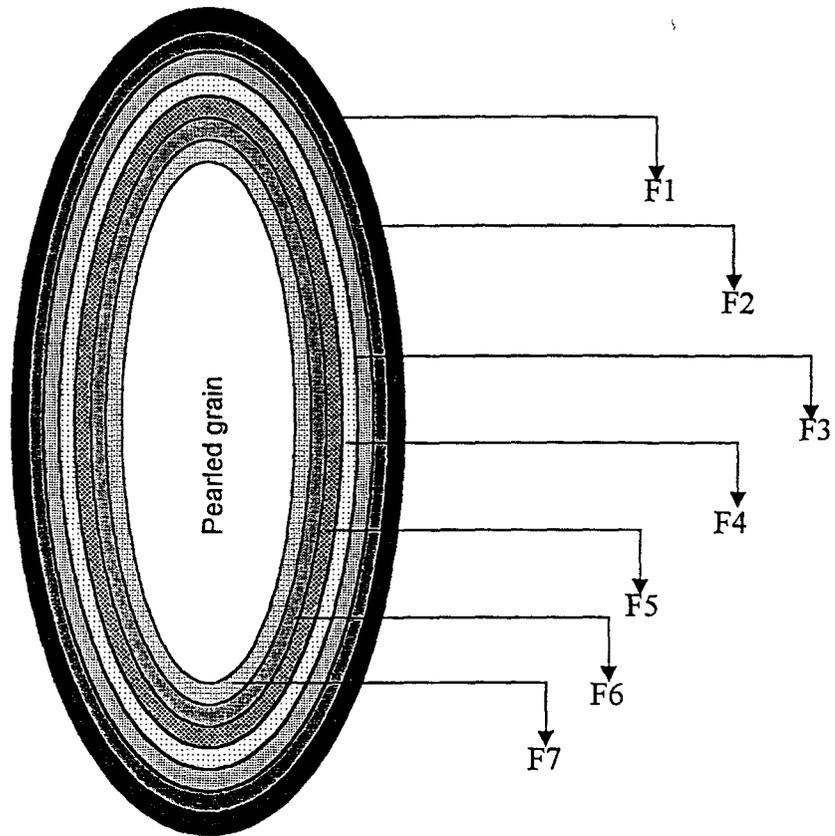
The barley fractions so obtained were prepared as explained in **Section 3.2.2**. Phenolic extracts were prepared from Falcon and AC Metcalfe fractions according to the method explained in Section 3.2.3 using 80% methanol at 60°C and over a 40 min period. Total phenolic content of the extracts so obtained was determined according to the method explained in **Section 3.2.4**. Antioxidative capacity of the extracts was determined according to the methods explained in **Sections 3.2.5 – 3.2.11**, and **3.2.17 – 3.2.20**. Antiproliferative activity of the extracts was determined as explained in **Section 3.2.21**. Content of phenolic acids was determined as explained in **Section 3.2.22**.

## **6.4 Results and discussion**

### **6.4.1 Pearling and extraction yields**

**Table 6.1** summarizes the yield data of pearling fines obtained on a weight basis. Pearling was performed in such way that yields gradually decreased in each successive layer. Pearling yield ranged from 4.6 to 9.7% for Falcon fractions and 4.6 – 9.4% for AC Metcalfe fractions. **Table 6.1** lists the percentage weight of each fraction of the Falcon and AC Metcalfe cultivars.

**Figure 6.2** Schematic diagram illustrating the sequential removal of outer layers of Barley grain. *F1-F7* represent *pearling fractions*. *Thickness of layers shown in diagram does not exactly represent the true thickness of layers.*



Nearly 50% of the grain was obtained as fractions while the rest of the grain (pearled grain) was not subjected to further pearling.

The extraction yields obtained varied from 3.5% to 13.9% for Falcon and 3.3% to 14.0% for AC Metcalfe. The extraction parameters established in **Chapter 4** were used to extract phenolic compounds from barley fractions. Optimization experiments established that extraction with 80% methanol at 60°C for 30 min yields the maximum antioxidant activity as reflected in Trolox equivalent antioxidant capacity (TEAC) values. When moving from fraction 1 (F1) to fraction 7 (F7), the recovery of antioxidative compounds declined gradually in both cultivars (**Table 6.1**). The decline in the yield can be explained by the composition of fractions. Outer fractions, especially F1 and F2, contain pericarp and aleurone layers that are rich in phenolic compounds leading to a higher recovery of phenolic compounds in the outer fractions. The inner fractions (F3-F7) contain increasing amounts of endosperm, which is a poor source of phenolic compounds, thus leading to low phenolic yields.

#### **6.4.2 Total phenolic content of extracts**

Total phenolic content (TPC) of Falcon barley fractions, reported as ferulic acid equivalents (**Table 6.2**), ranged from 0.36 to 6.26 mg per gram of defatted material, while that of AC Metcalfe ranged from 0.17 to 4.16 mg per gram. TPC of Falcon and AC Metcalfe pearled grain was 0.59 and 0.23 mg ferulic acid per gram of defatted material, respectively. A general trend of decreasing TPC was observed when moving from the exterior to the centre of the barley grain in both cultivars. The average TPC of Falcon was significantly higher ( $p < 0.05$ ) than, that of AC Metcalfe. The outermost layer

(F1) of Falcon, which is basically the bran, contained the highest TPC of 6.26 mg ferulic acid equivalents per gram defatted material. Fraction 2 also showed a significantly higher TPC compared to the rest of the inner fractions. TPC of F1 and F2 of Falcon was 6.26 and 2.35 mg ferulic acid equivalents per gram defatted material, respectively, which represents a 62% drop. TPC of AC Metcalfe F1 and F2 were 4.16 and 2.42, respectively, while the drop of TPC from F1 to F2 was 41.83%. This indicates that phenolic compounds are mainly concentrated in the outermost layer (approximately 10% of the grain on a weight basis) in both cultivars. Therefore, removal of the bran causes a significant loss of phenolic compounds during milling operations. Total phenolic content of Falcon F3 showed an 81% decrease from F1 while the corresponding AC Metcalfe fractions showed a 67% decrease. TPC of F7 of the Falcon cultivar showed a 91% decrease compared to its F1 while the corresponding value for AC Metcalfe cultivar was 96%. Total phenolic contents of Falcon F1 and F2 were 7.5 and 2.8 times higher than that of whole Falcon extract.

#### **6.4.3 Total antioxidant capacity (TAC) measured by Trolox equivalent antioxidant capacity (TEAC)**

The TEAC value of a compound represents the concentration of Trolox (a water-soluble vitamin E analog without the side-chain moiety) that has the same antioxidant capacity as the compound or a mixture of compounds of interest (van den Berg *et al.*, 1999).

**Table 6.1** Pearling and extraction yields obtained from two barley cultivars

Cultivar/ pearling fraction	% (w/w) Kernel weight range	% (w/w) Yield of pearling	% (w/w) Yield of extraction
Falcon			
F1	0 – 9.7	9.7	13.9 ± 0.6 <sup>a</sup>
F2	9.8 – 18.8	9.1	9.8 ± 0.7 <sup>b</sup>
F3	18.9 – 26.7	7.9	7.0 ± 0.7 <sup>c</sup>
F4	26.8 – 33.3	6.6	8.4 ± 1.2 <sup>d</sup>
F5	33.4 – 39.8	6.5	6.3 ± 1.3 <sup>e</sup>
F6	39.9 – 45.3	5.5	3.1 ± 0.5 <sup>f</sup>
F7	45.4 – 49.9	4.6	3.5 ± 0.8 <sup>d</sup>
AC Metcalfe			
F1	0 – 9.4	9.4	14.0 ± 1.2 <sup>a</sup>
F2	9.5 – 17.7	8.3	10.9 ± 0.9 <sup>b</sup>
F3	17.8 – 25.5	7.8	9.6 ± 0.8 <sup>c</sup>
F4	25.6 – 32.4	6.9	7.4 ± 0.6 <sup>c</sup>
F5	32.5 – 38.0	5.6	6.8 ± 0.5 <sup>c</sup>
F6	38.1 – 43.1	5.1	4.6 ± 0.6 <sup>d</sup>
F7	43.2 – 47.7	4.6	3.3 ± 0.9 <sup>e</sup>

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

**Table 6.2** Total phenolic content (TPC) and total antioxidant capacity (TAC) of barley fractions.

Fraction	TPC <sup>1</sup>		TEAC <sup>2</sup>	
	Falcon	AC Metcalfe	Falcon	AC Metcalfe
F1	6.26 ± 0.11 <sup>f</sup>	4.16 ± 0.08 <sup>c</sup>	59.71 ± 3.11 <sup>g</sup>	56.09 ± 3.20 <sup>h</sup>
F2	2.35 ± 0.00 <sup>e</sup>	2.42 ± 0.08 <sup>d</sup>	17.72 ± 1.30 <sup>f</sup>	25.15 ± 0.01 <sup>g</sup>
F3	1.18 ± 0.02 <sup>d</sup>	1.38 ± 0.04 <sup>c</sup>	10.99 ± 0.30 <sup>e</sup>	11.99 ± 0.42 <sup>f</sup>
F4	0.58 ± 0.01 <sup>b</sup>	1.26 ± 0.23 <sup>c</sup>	3.83 ± 0.21 <sup>d</sup>	7.85 ± 0.34 <sup>e</sup>
F5	0.99 ± 0.03 <sup>c</sup>	1.24 ± 0.00 <sup>c</sup>	2.72 ± 0.21 <sup>c</sup>	6.31 ± 0.30 <sup>d</sup>
F6	0.36 ± 0.01 <sup>a</sup>	0.48 ± 0.00 <sup>b</sup>	1.19 ± 0.30 <sup>h</sup>	5.2 ± 0.21 <sup>c</sup>
F7	0.51 ± 0.00 <sup>b</sup>	0.17 ± 0.00 <sup>a</sup>	0.45 ± 0.00 <sup>a</sup>	0.69 ± 0.02 <sup>b</sup>
Pearled grain	0.59 ± 0.01 <sup>b</sup>	0.23 ± 0.00 <sup>a</sup>	0.58 ± 0.01 <sup>a</sup>	0.52 ± 0.01 <sup>a</sup>

<sup>1</sup>TPC is expressed as mg ferulic acid equivalents/g defatted material.

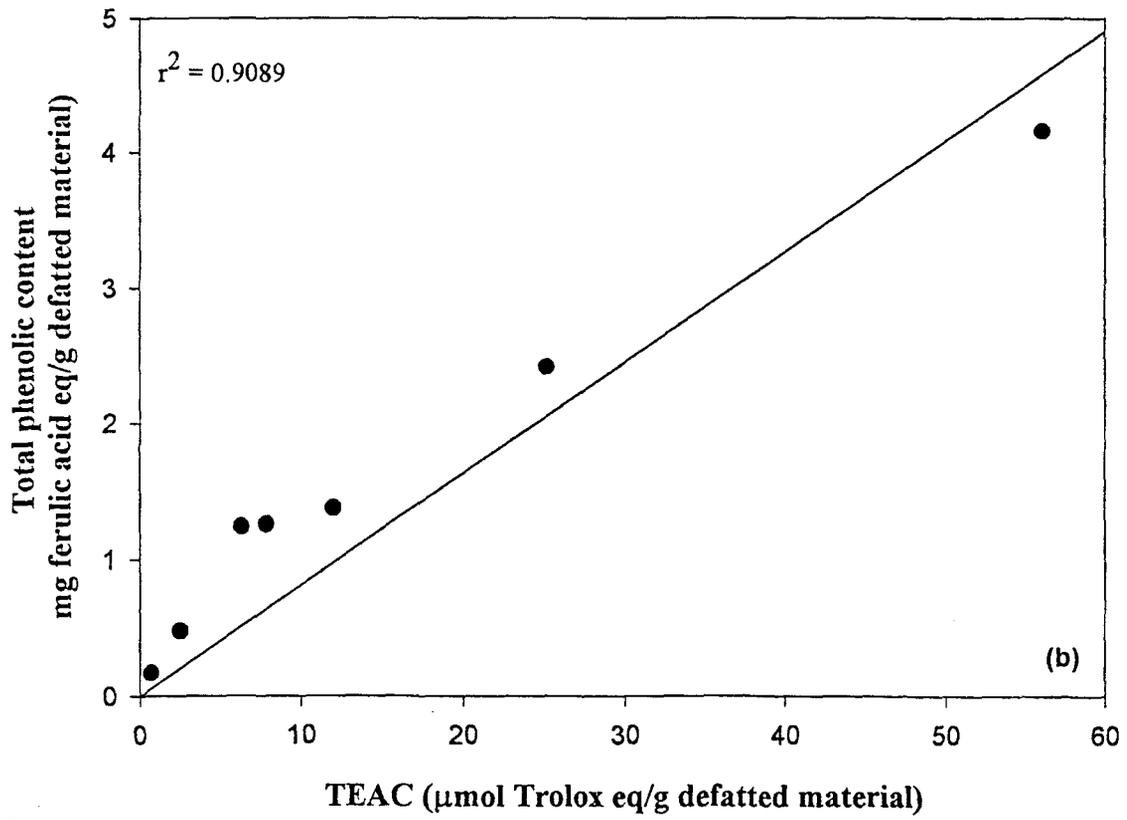
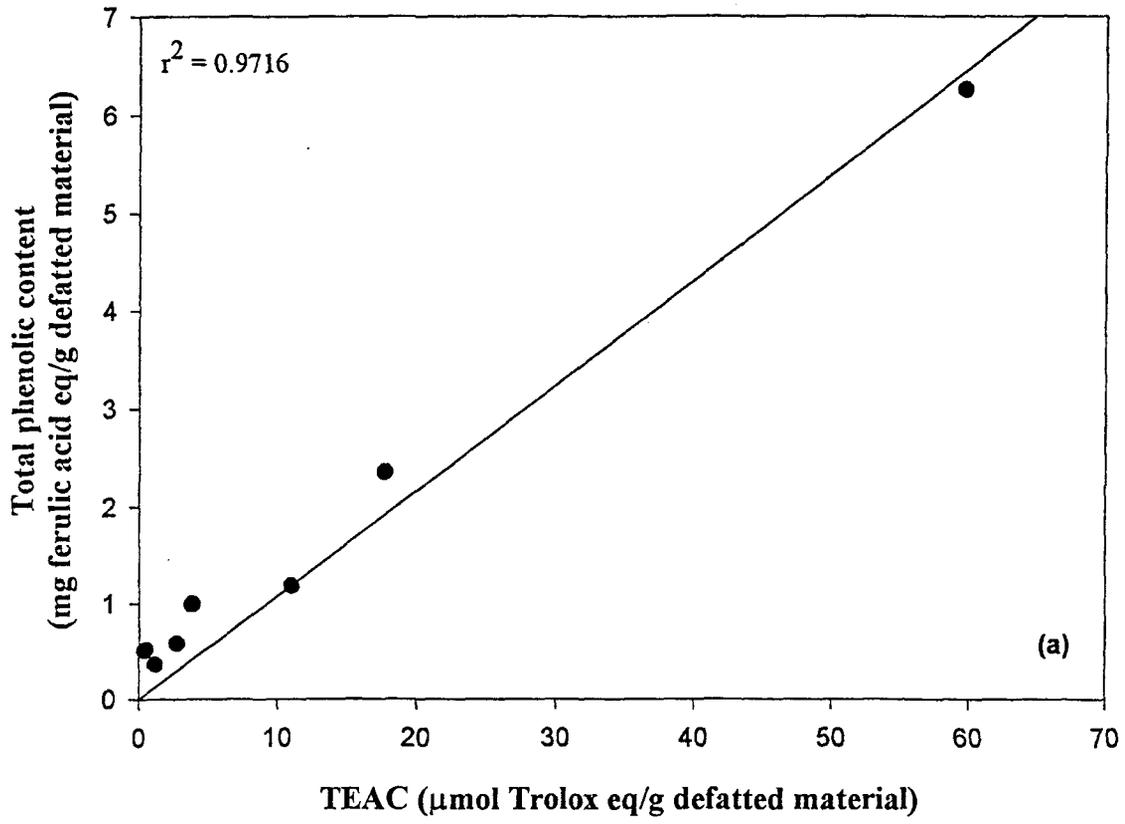
<sup>2</sup>TEAC values are expressed as μmol/g defatted material.

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

The  $\text{ABTS}^{\cdot-}$  produced as a result of the reaction between AAPH and  $\text{ABTS}^{2-}$ , reacts instantly with Trolox and the reaction is completed within 1 min, however, using extracts, the reaction may take up to 6 min to complete indicating a biphasic pattern. Therefore, a 6-min period was observed before reading the final absorbance values. TEAC for Falcon and AC Metcalfe ranged from 0.45 to 59.71 and 0.69 to 56.09  $\mu\text{mol}$  Trolox equivalents per gram defatted material, respectively, based on scavenging during 6 min. TEAC for Falcon and AC Metcalfe pearled grain was 0.58 and 0.52  $\mu\text{mol}$  Trolox equivalents per gram defatted material, respectively (**Table 6.2**). As expected, TEAC values gradually decreased from the outer fractions to inner fractions in both cultivars keeping in line with TPC. The high TEAC of F1 in both cultivars might be attributable to its highest phenolic content. Both barley cultivars exhibited a strong correlation between TPC and TEAC (Falcon,  $r^2 = 0.97$  and AC Metcalfe,  $r^2 = 0.91$ ). **Figures 6.3a** and **b** depict the correlation between TEAC and TPC for the two cultivars. TEAC value of fraction 1 of Falcon was approximately 131 times higher than that of fraction 7 while the corresponding value for AC Metcalfe was 81. Removal of F1 in Falcon caused a 61% reduction in TEAC value while the removal of both F1 and F2 caused an 80% reduction in TEAC value. The corresponding values for AC Metcalfe stood at 50 and 72%.

**Figure 6.3** Relationship between Trolox equivalent antioxidant capacity (TEAC) and total phenolic content (TPC) for Falcon (a) and AC Metcalfe (b) fractions at 0.05 level of significance.



#### 6.4.4 DPPH Radical scavenging activity

The reduction of the DPPH radical in the presence of extracts and standards was monitored spectrophotometrically at 515 nm after 20 min of mixing. The DPPH radical scavenging capacity of Falcon fractions varied from 1.18 to 69.3  $\mu\text{mol}$  ferulic acid equivalents per gram defatted material while the corresponding values ranged from 0.8 to 46.59 in AC Metcalfe (**Table 6.3**). The DPPH radical scavenging capacity gradually diminished from F1 to F7, which is attributable to the decrease in TPC. Falcon F1 and F2 were 32 and 16 times more efficient than F7, respectively, while the corresponding AC Metcalfe fractions were 59 and 19 times more efficient than its F7. The average DPPH radical scavenging capacity of Falcon was superior to that of AC Metcalfe. DPPH radical scavenging capacity displayed a strong correlation with TEAC in both cultivars (Falcon,  $r^2 = 0.93$  and AC Metcalfe,  $r^2 = 0.98$ ). **Figure 6.4** depicts the relationship between the TEAC and DPPH radical scavenging efficacy for the two cultivars.

#### 6.4.5 Reducing power

The reducing power of Falcon and AC Metcalfe fractions varied from 1.89 to 26.34 and 2.12 to 27.62, respectively, as  $\mu\text{mol}$  ascorbic acid equivalents per gram defatted material. The reducing power of Falcon F1 was approximately 14 times higher than its F7 while that of AC Metcalfe was 13 times higher based on ascorbic acid equivalents. Reducing power of the barley fractions were also expressed as Trolox equivalents. Although ascorbic acid is physiologically more relevant than Trolox, both of these standards are commonly used in expressing reducing power. The corresponding values were 1.54-24.22 and 1.86-24.19 as  $\mu\text{mol}$  Trolox equivalents per gram defatted material (**Table 6.4**).

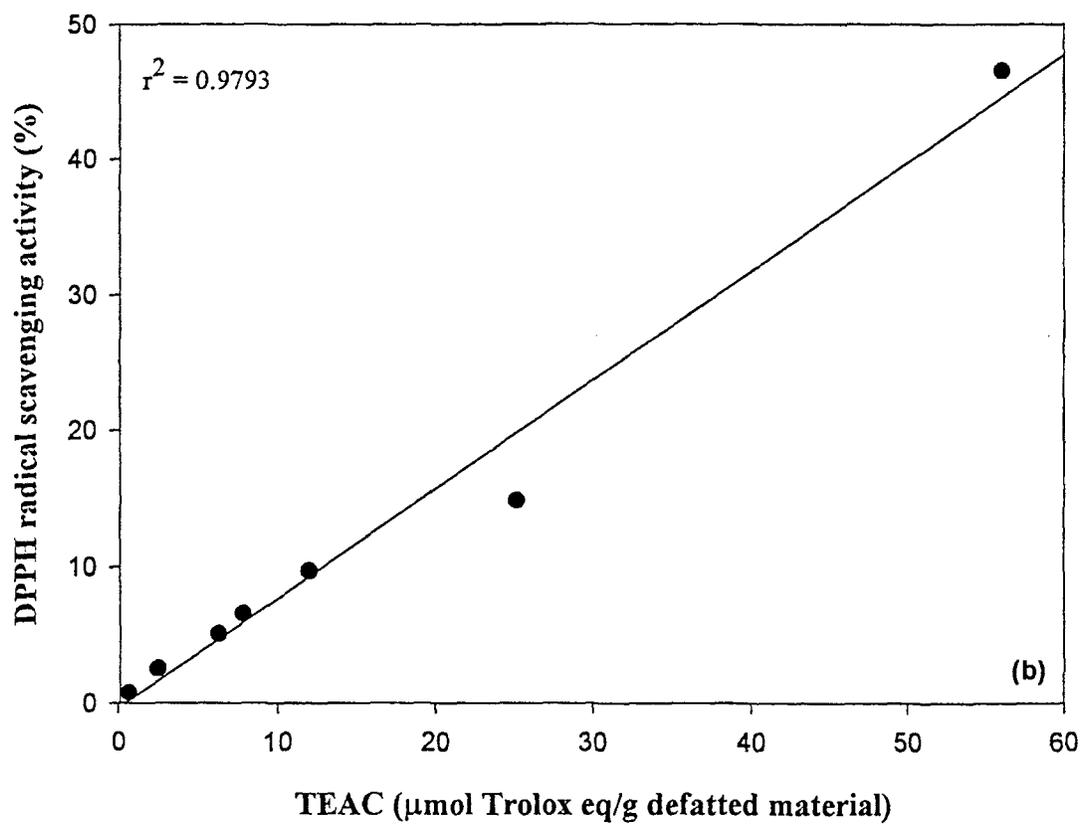
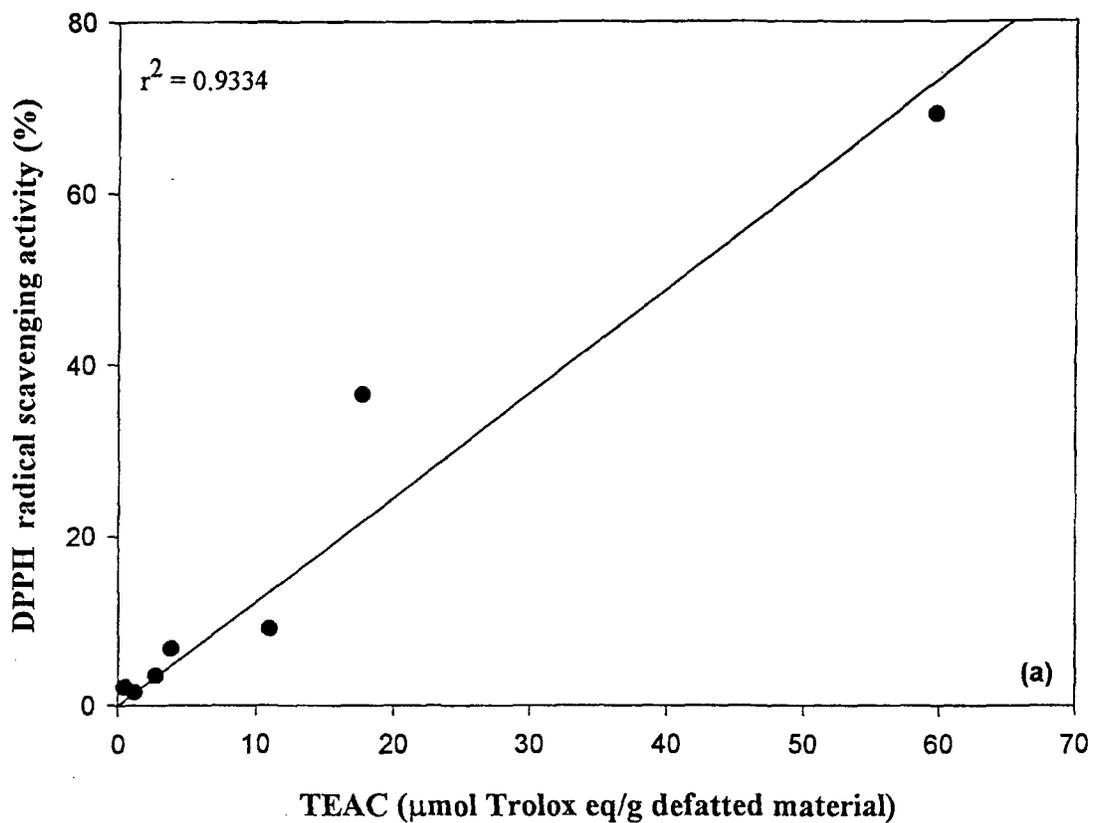
**Table 6.3** Percentage DPPH radical scavenging capacity of barley fractions.

Fraction	Falcon	AC Metcalfe
F1	69.30 ± 0.1 <sup>g</sup>	46.59 ± 0.00 <sup>g</sup>
F2	36.53 ± 1.33 <sup>f</sup>	14.84 ± 0.19 <sup>f</sup>
F3	9.11 ± 0.05 <sup>e</sup>	9.68 ± 0.74 <sup>e</sup>
F4	6.72 ± 0.48 <sup>d</sup>	6.59 ± 0.35 <sup>d</sup>
F5	3.55 ± 0.49 <sup>c</sup>	5.10 ± 0.55 <sup>c</sup>
F6	1.57 ± 0.41 <sup>b</sup>	2.58 ± 0.43 <sup>b</sup>
F7	1.77 ± 0.22 <sup>b</sup>	2.98 ± 0.01 <sup>b</sup>
Pearled grain	1.18 ± 0.11 <sup>a</sup>	0.80 ± 0.00 <sup>a</sup>

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

**Figure 6.4** Relationship between Trolox equivalent antioxidant capacity (TEAC) and DPPH radical scavenging capacity for Falcon (a) and AC Metcalfe (b) fractions at 0.05 level of significance.



**Table 6.4** Reducing power of barley fractions

Fraction	Falcon		AC Metcalfe	
	Ascorbic acid equivalents ( $\mu\text{mol/g}$ defatted material)	Trolox equivalents ( $\mu\text{mol/g}$ defatted material)	Ascorbic acid equivalents ( $\mu\text{mol/g}$ defatted material)	Trolox equivalents ( $\mu\text{mol/g}$ defatted material)
F1	$26.34 \pm 2.2^c$	$24.22 \pm 1.92^d$	$27.62 \pm 0.48^f$	$24.19 \pm 0.42^f$
F2	$17.85 \pm 1.9^d$	$15.6 \pm 1.51^d$	$11.09 \pm 0.65^e$	$9.48 \pm 0.57^c$
F3	$9.50 \pm 0.03^c$	$8.22 \pm 0.02^c$	$7.74 \pm 0.01^d$	$6.72 \pm 0.01^d$
F4	$7.62 \pm 0.05^{bc}$	$6.67 \pm 0.04^c$	$6.22 \pm 0.01^c$	$5.41 \pm 0.01^c$
F5	$6.43 \pm 0.67^b$	$5.63 \pm 0.59^{bc}$	$5.32 \pm 0.11^b$	$4.65 \pm 0.09^b$
F6	$3.22 \pm 0.01^a$	$2.82 \pm 0.01^{ab}$	$2.62 \pm 0.03^a$	$2.29 \pm 0.02^a$
F7	$1.89 \pm 0.03^a$	$1.54 \pm 0.02^a$	$2.12 \pm 0.04^a$	$1.86 \pm 0.03^a$
Pearled grain	$2.33 \pm 0.01^a$	$2.14 \pm 0.00^{ab}$	$2.31 \pm 0.03^a$	$2.02 \pm 0.02^a$

Results are means of three determinations  $\pm$  standard deviation.

Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

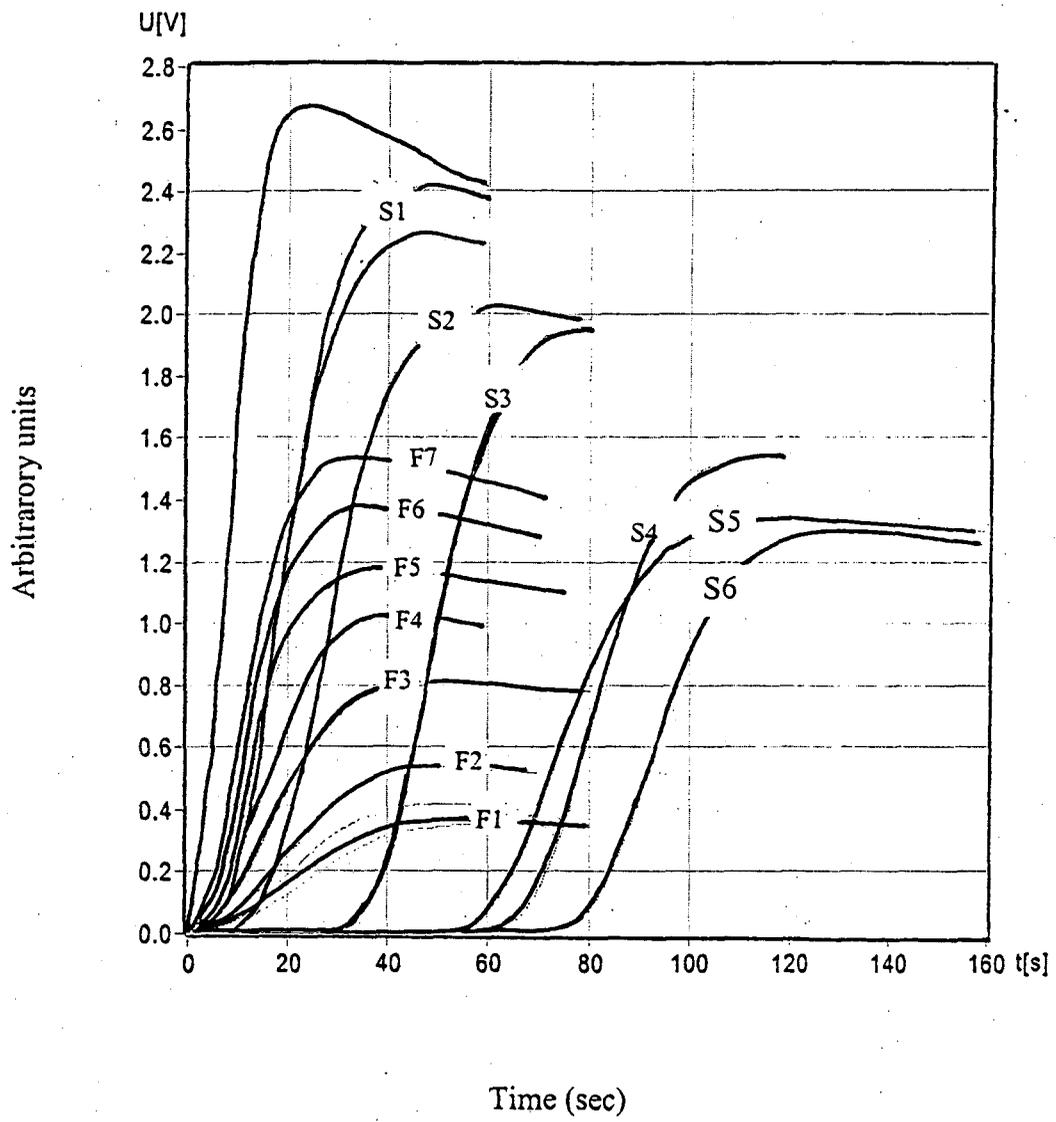
The reducing power of both barley fractions gradually decreased from F1 through F7 keeping in line with TPC and TEAC. Reducing power of AC Metcalfe decreased by 2.45 times from F1 to F2 while the reducing power of F7 showed a 13 fold decrease as compared to F1. Similarly, reducing power of Falcon decreased by 1.45 times from F1 to F2 while the reducing power of F7 showed a 13.9 fold decrease.

#### **6.4.6 Photo-induced chemiluminescent (PCL) detection of antiradical activity**

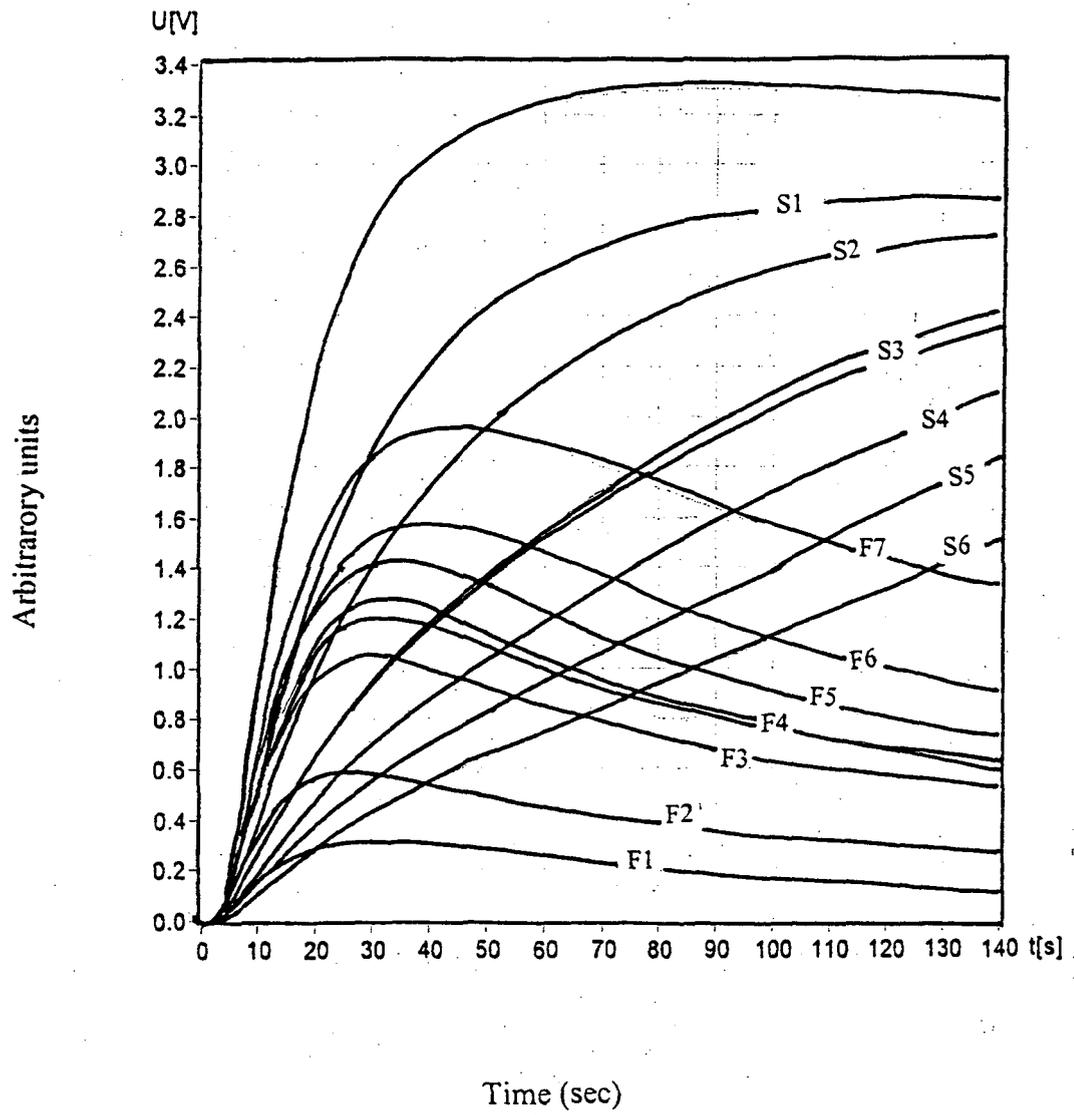
Measurement of antioxidant activity of barley fractions was carried out using a Photochem<sup>®</sup> (Analytik Jena, Konrad-Zuse-Strabe 1, Jena, Germany) that generates free radicals. The remaining free radicals, after reacting with the extracts, were quantified using a detection chemical (luminol). The measurement of antioxidant activity was carried out in two different systems, namely water- and lipid-soluble systems. The standards used for water- and lipid-soluble antioxidant modes were Trolox and  $\alpha$ -tocopherol, respectively, and the antioxidative capacities were expressed as equivalent units of the corresponding standards. **Figure 6.5** illustrates the on-line plot of the change of luminescence for water-soluble antioxidative components from the Falcon cultivar while **Figure 6.6** illustrates the change of luminescence of lipid-soluble counterparts.

Antioxidant activity of water- and lipid-soluble fractions from two barley varieties is depicted in **Table 6.5**. The water-soluble antioxidant amounts of Falcon fractions ranged from 1.07 to 179 while the pearled grain contained 0.82  $\mu\text{mol}$  Trolox equivalent per gram defatted material, while corresponding values for AC Metcalfe fractions and pearled grain were 4.92 to 80.5 and 1.86  $\mu\text{mol}$ .

**Figure 6.5** Change of luminescence of detector chemical (luminol) in the presence of water-soluble antioxidative components from Falcon pearling fractions (F1-F7) and Trolox standards (S1-S6).



**Figure 6.6** Change of luminescence of detector chemical (luminol) in the presence of lipid soluble antioxidative components from Falcon pearling fractions (F1-F7) and  $\alpha$ -tocopherol standards (S1-S6).



**Table 6.5** Inhibition of photo-induced chemiluminescence by water- and lipid-soluble components of antioxidants from barley fractions.

Fraction	Water-soluble <sup>1</sup>		Lipid-soluble <sup>2</sup>	
	Falcon	AC Metcalfe	Falcon	AC Metcalfe
F1	179.12 ± 1.6 <sup>a</sup>	80.50 ± 5.9 <sup>a</sup>	93.5 ± 0.5 <sup>a</sup>	65.8 ± 5.7 <sup>a</sup>
F2	55.48 ± 0.3 <sup>b</sup>	49.05 ± 1.5 <sup>b</sup>	59.5 ± 1.2 <sup>b</sup>	44.4 ± 6.6 <sup>b</sup>
F3	29.82 ± 0.3 <sup>c</sup>	39.7 ± 0.1 <sup>c</sup>	34.0 ± 2.3 <sup>c</sup>	31.6 ± 0.1 <sup>c</sup>
F4	13.87 ± 0.0 <sup>d</sup>	30.50 ± 0.2 <sup>d</sup>	14.9 ± 0.8 <sup>d</sup>	4.3 ± 0.1 <sup>d</sup>
F5	1.42 ± 0.0 <sup>e</sup>	26.48 ± 0.8 <sup>d</sup>	22.2 ± 0.0 <sup>e</sup>	2.7 ± 0.1 <sup>d</sup>
F6	0.91 ± 0.0 <sup>e</sup>	17.56 ± 0.1 <sup>e</sup>	1.1 ± 0.0 <sup>f</sup>	1.7 ± 0.1 <sup>d</sup>
F7	1.07 ± 0.1 <sup>e</sup>	4.92 ± 0.0 <sup>f</sup>	1.3 ± 0.0 <sup>f</sup>	0.5 ± 0.0 <sup>d</sup>
Pearled grain	0.82 ± 0.0 <sup>c</sup>	1.86 ± 0.0 <sup>g</sup>	1.2 ± 0.2 <sup>f</sup>	0.62 ± 0.1 <sup>d</sup>

<sup>1</sup> expressed as μmol Trolox equivalents per gram defatted material.

<sup>2</sup> expressed as μmol α-tocopherol equivalents per gram defatted material. Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

The lipid-soluble counterparts ranged from 1.1 to 93.5 and 0.5 to 65.8  $\mu\text{mol}$   $\alpha$ -tocopherol equivalents per gram defatted material for Falcon and AC Metcalfe fractions, respectively. The corresponding values for pearled grains of Falcon and AC Metcalfe were 1.2 and 0.62, respectively. The water-soluble antioxidant activity was significantly higher with F1 and F2 of both cultivars, while this pattern was not consistent with the rest of the fractions. The contents of water-soluble and lipid-soluble antioxidants were reasonably well correlated ( $r^2 = 0.79$ ) with each other while TEAC and water-soluble antioxidant activity were strongly correlated ( $r^2 = 0.99$ ) in the Falcon cultivar. However, the lipid-soluble antioxidant content was not well correlated with TEAC in both cultivars. The standards (S1-S6) were selected to yield an antioxidant activity over a wide range so that the activity of extracts certainly falls within the range exhibited by the standards (Figures 6.5 and 6.6).

#### **6.4.7 Oxygen radical absorbance capacity (ORAC<sub>FL</sub>)**

The ORAC<sub>FL</sub> value of Falcon and AC Metcalfe cultivars ranged from 8.8 to 188.1 and 4.2 to 123.5  $\mu\text{mol}$  Trolox equivalents per gram defatted material, respectively. ORAC<sub>FL</sub> values for Falcon and AC Metcalfe pearled grains were 10.22 and 6.37  $\mu\text{mol}$  Trolox equivalents per gram defatted material, respectively (Table 6.6). Fractions F1 and F2 of the Falcon cultivar exhibited a significantly ( $p < 0.05$ ) higher antioxidant activity compared to that of AC Metcalfe, however, the rest of the fractions did not show any consistent pattern. The ORAC<sub>FL</sub> value of F7 of the Falcon cultivar was 21 fold lower than that of F1 while the corresponding value for AC Metcalfe was 29. The overall average ORAC<sub>FL</sub> value of Falcon stood significantly ( $p < 0.05$ ) higher than that of AC Metcalfe

(Section 5.4.8).  $ORAC_{FL}$  values and antioxidant activity as measured by photochemiluminescence were strongly correlated with each other ( $ORAC_{FL}$  and water soluble antioxidant activity:  $r^2 = 0.98$ ,  $ORAC_{FL}$  and lipid soluble counterparts;  $r^2 = 0.95$  for the Falcon cultivar). Moreover,  $ORAC_{FL}$  values were strongly correlated ( $r^2 = 0.92$ ) with TEAC values in Falcon cultivars, however, a poor correlation existed for AC Metcalfe ( $r^2 = 0.57$ ). Figure 6.7 illustrates the time course of the reaction of fluorescein with AAPH (decay curves) for Falcon fractions. Removal of the first layer (F1) of Falcon and AC Metcalfe barley resulted in a loss of 45 and 31%  $ORAC_{FL}$  activity, respectively. Further removal of F2 and F3 of the Falcon cultivar caused a loss of 67 and 79%, respectively. The corresponding values for the AC Metcalfe cultivar stand at 50 and 65%.

#### 6.4.8 Hydroxyl radical scavenging capacity ( $HORAC_{FL}$ )

The hydroxyl radical is a biologically important radical that can cause severe damage to biomolecules. Therefore, it is of paramount importance to evaluate the hydroxyl radical scavenging capacity of antioxidative extracts. The hydroxyl radical scavenging capacity of Falcon and AC Metcalfe barley extracts ranged from 5.68 to 151.34 and 3.26 to 126.32  $\mu\text{mol Trolox equivalents per gram defatted material}$ , respectively. The corresponding values for pearled grains were 5.22 and 4.02. Fraction 1 of both showed the highest  $HORAC_{FL}$  values of 151.34 and 126.32  $\mu\text{mol Trolox/ g defatted material}$ , respectively. The  $HORAC_{FL}$  value of F1 and F2 of Falcon was significantly different ( $p < 0.05$ ) from that of AC Metcalfe. However, as in the case of  $ORAC_{FL}$ , the trend was not consistent with F3 through F7 (**Table 6.6**). Higher  $HORAC_{FL}$  values displayed by the outer fractions, mainly F1 and F2, can be attributed to the

presence of the components of bran in those fractions, which contain high concentrations of phenolic compounds. HORAC<sub>FL</sub> of F1 of Falcon and AC Metcalfe cultivars was approximately 15 and 12 times higher than those of respective whole grain extracts. The effect of dilution the outer layers with the endosperm contents, which do not contain substantial amounts of antioxidative constituents, yields poor HORAC<sub>FL</sub> values in whole grain extracts of both cultivars. Removal of the first layer (F1) of Falcon and AC Metcalfe barley resulted in a HORAC<sub>FL</sub> reduction of 48%. Further removal of F2 and F3 of the Falcon cultivar caused a reduction of 68 and 81% of HORAC<sub>FL</sub> activity, respectively. The corresponding values for the AC Metcalfe cultivar stood at 67 and 83%.

#### **6.4.9 Metal chelation activity**

Metal chelation activity is an important aspect of antioxidative compounds. Metal ions, especially transition metal ions, are involved in the production of punitive hydroxyl radical in biological systems which leads to their alteration and destruction. Thus, it is very important to quantify the metal chelation activity of antioxidative extracts. Metal chelation activity of Falcon and AC Metcalfe extracts ranged from 3.50 to 92.14 and 1.34 to 89.20  $\mu\text{mol}$  EDTA equivalents per gram defatted material, respectively. Metal chelation activity of F1 of Falcon extract was approximately 67 fold higher than that of F7 while the metal chelation capacity of F1 of AC Metcalfe was approximately 26 fold higher than that of F7. Metal chelation capacity gradually decreased from F1 to F7 in extracts of both Falcon and AC Metcalfe cultivars. The metal chelation activity of pearled grain was negligible and therefore not reported (**Figure 6.8**).

**Table 6.6** Oxygen radical absorbance capacity (ORAC<sub>FL</sub>) and hydroxyl radical absorbance capacity (HORAC<sub>FL</sub>) values of barley fractions.

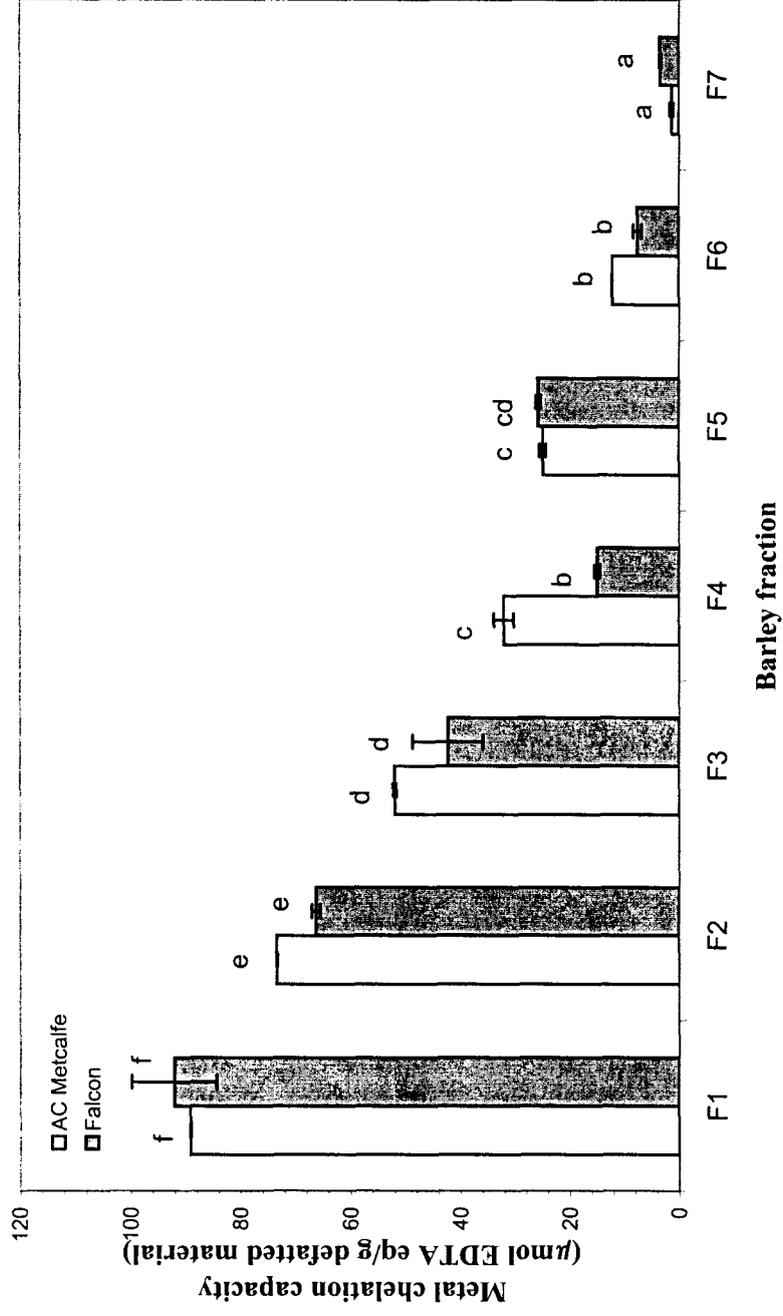
Fraction	ORAC <sub>FL</sub> <sup>1</sup>		HORAC <sub>FL</sub> <sup>2</sup>	
	Falcon	AC Metcalfe	Falcon	AC Metcalfe
F1	188.08 ± 8.7 <sup>a</sup>	123.46 ± 5.9 <sup>a</sup>	151.34 ± 2.5 <sup>f</sup>	126.32 ± 3.48 <sup>f</sup>
F2	89.17 ± 1.8 <sup>b</sup>	72.02 ± 10.7 <sup>b</sup>	62.80 ± 3.3 <sup>e</sup>	51.69 ± 1.93 <sup>e</sup>
F3	49.13 ± 2.1 <sup>c</sup>	60.77 ± 8.9 <sup>b,c</sup>	41.07 ± 1.5 <sup>d</sup>	40.23 ± 2.21 <sup>d</sup>
F4	35.14 ± 5.4 <sup>d</sup>	55.54 ± 0.9 <sup>b,c</sup>	32.21 ± 2.2 <sup>c</sup>	22.68 ± 1.76 <sup>c</sup>
F5	17.65 ± 0.0 <sup>e</sup>	45.68 ± 5.6 <sup>c</sup>	11.35 ± 0.98 <sup>b</sup>	10.99 ± 0.98 <sup>b</sup>
F6	14.18 ± 1.7 <sup>e</sup>	23.31 ± 2.1 <sup>d</sup>	6.10 ± 21.1 <sup>ab</sup>	5.23 ± 0.23 <sup>ab</sup>
F7	8.77 ± 0.9 <sup>e</sup>	4.23 ± 0.2 <sup>e</sup>	5.68 ± 0.56 <sup>a</sup>	3.26 ± 0.14 <sup>a</sup>
Pearled grain	10.22 ± 0.3	6.37 ± 0.1	5.22 ± 0.21 <sup>a</sup>	4.02 ± 0.34 <sup>a</sup>

<sup>1,2</sup>ORAC<sub>FL</sub> and HORAC<sub>FL</sub> values are expressed as  $\mu\text{mol}$  Trolox equivalents per gram defatted material. Results are means of three determinations  $\pm$  standard deviation. Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

**Figure 6.7** Time course of the reaction of fluorescein with peroxy radicals (decay curves) for Falcon fractions obtained in the ORAC<sub>FL</sub> assay.



**Figure 6.8** Metal chelation activity of Falcon and AC Metcalfe extracts as determined by 2,2' bipyridyl competition assay.

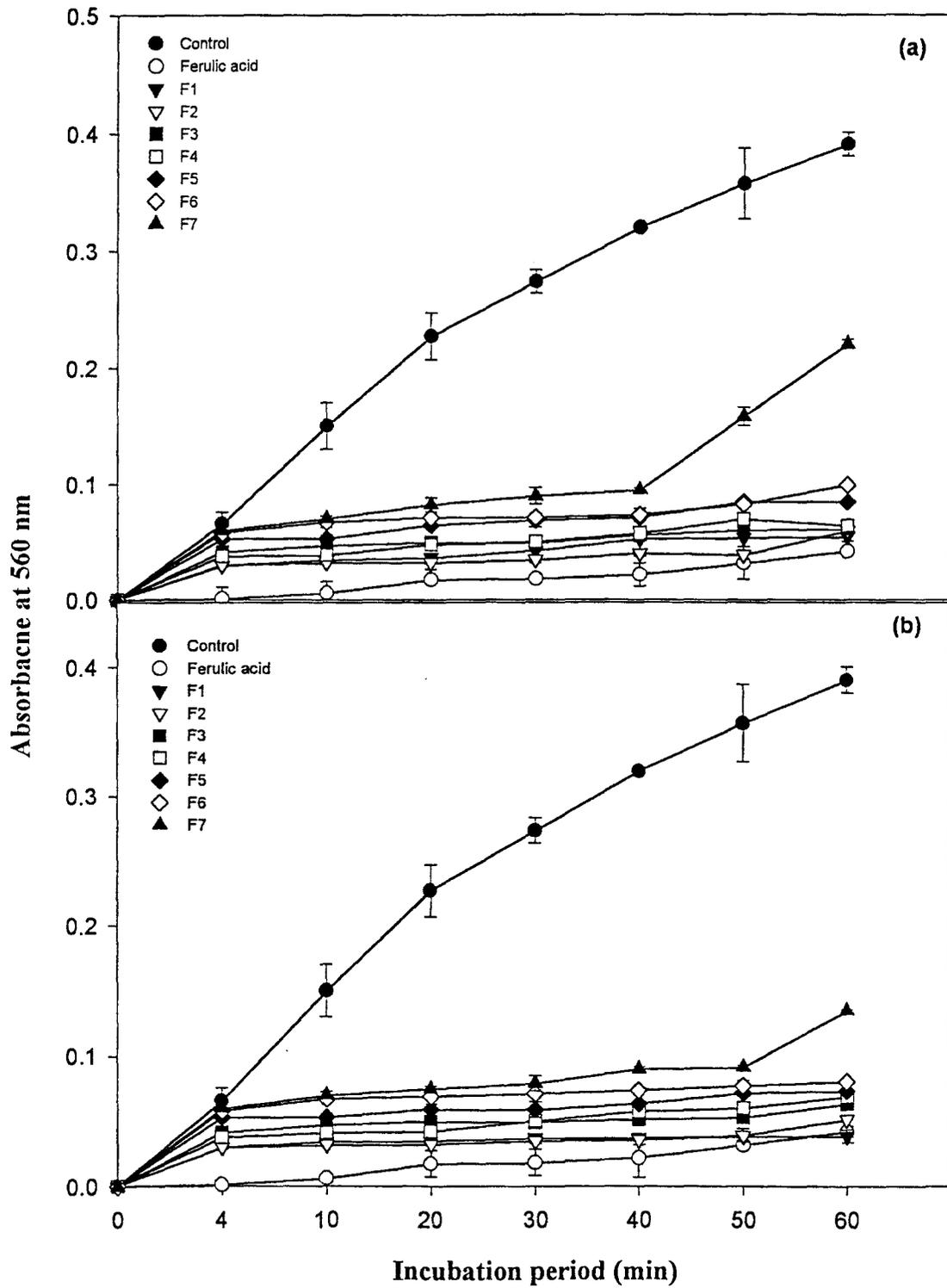


Higher metal chelation capacity exhibited by the outermost fractions, especially F1 and F2, of both cultivars may be attributable to the higher phenolic content of the outer layers. Inner layers, F6 and F7 exhibited significantly lower ( $p < 0.05$ ) metal chelation activity as compared to the outer layers owing to the poor phenolic content in the endosperm.

#### **6.4.10 Superoxide radical scavenging capacity**

In the control, the colour developed continuously and levelled off after 1 h. **Figures 6.9 a and b** illustrate the development of blue colour in the presence of 0.2 mg/mL of Falcon and AC Metcalfe fractions, respectively. The superoxide radical scavenging capacity of Falcon and AC Metcalfe ranged from 82 to 94% and 76 to 87%, respectively while the activity of the pearled grains was 84 and 81%, respectively (**Table 6.7**). It is interesting to note that even the inner layers of both barley cultivars exhibited strong superoxide radical scavenging activity at the concentration tested. In Falcon, the superoxide radical scavenging capacity of F1 was 1.4 times higher than that of its F7 while F1 of AC Metcalfe was 1.5 times higher than that of F7. In both cultivars, F1 exhibited the highest activity, which gradually decreased toward F7. In general, all the extracts were quite capable of scavenging enzymatically-generated superoxide radical. Barley extracts showed a very high efficacy in scavenging superoxide radical that any other radical. This may be due to the fact that compared to the other radical species, superoxide radical is easily scavenged by most of the antioxidative extracts.

**Figure 6.9** The effect of Falcon **(a)** and AC Metcalfe **(b)** barley fractions, respectively, on scavenging enzymatically generated superoxide anion radical.



**Table 6.7** Percentage superoxide radical scavenging capacity of barley fractions<sup>1</sup>.

Sample identity	Falcon	AC Metcalfe
F1	94.3 ± 2.68 <sup>b</sup>	87.8 ± 5.21 <sup>b</sup>
F2	91.6 ± 1.78 <sup>b</sup>	83.1 ± 5.80 <sup>b</sup>
F3	90.8 ± 2.14 <sup>b</sup>	82.4 ± 3.32 <sup>ab</sup>
F4	88.8 ± 4.56 <sup>ab</sup>	80.6 ± 7.60 <sup>a</sup>
F5	88.0 ± 3.10 <sup>ab</sup>	79.0 ± 6.40 <sup>a</sup>
F6	82.0 ± 6.03 <sup>a</sup>	78.4 ± 3.85 <sup>a</sup>
F7	81.2 ± 2.79 <sup>a</sup>	76.2 ± 3.25 <sup>a</sup>
Pearled grain	84.2 ± 3.61 <sup>a</sup>	80.9 ± 2.27 <sup>a</sup>
Ferulic acid		95.9 ± 3.44 <sup>c</sup>

<sup>1</sup>Scavenging capacity is calculated based on the absorbance values at 10 min of the assay. Results are means of three determinations ± standard deviation. Values in each column having the same superscript are not significantly different (p>0.05).

#### **6.4.11 DPPH radical scavenging capacity by electron paramagnetic resonance (EPR)**

The barley fractions were tested at four different concentrations (0.33, 1, 1.34, and 1.7 mg/mL). All the fractions from Falcon and AC Metcalfe barley cultivars that were tested, with the exception of F6 and F7, exhibited strong antioxidant activity against DPPH<sup>•</sup> in a concentration-dependent manner. F1 and F2 of Falcon exhibited strong radical scavenging activity against the DPPH radical while the activity of corresponding AC Metcalfe fractions was significantly lower ( $p < 0.05$ ). Radical scavenging capacity gradually decreased from F1 through F6 in both cultivars. It was not possible to determine the  $IC_{50}$  value for F6 and F7 of both Falcon and AC Metcalfe cultivars as the highest concentration of F6 and F7 extracts used in the assay failed to yield 50% scavenging of the initial DPPH radical concentration. As expected, the activity of pearled grain was also low and therefore it was not possible to determine the  $IC_{50}$  value.  $IC_{50}$  values of whole extracts of Falcon and AC Metcalfe cultivars were 2.12 and 1.65, respectively. **Table 6.8** lists  $IC_{50}$  values for DPPH radical for Falcon and AC Metcalfe fractions.

#### **6.4.12 Hydroxyl radical scavenging capacity by electron paramagnetic resonance (EPR)**

Fractions 6 and 7, and pearled grains of both cultivars did not yield 50% scavenging of the initial DPPH level at the concentrations used in the study. As expected, the  $IC_{50}$  value gradually decreased from F1 to F5. F1 and F2 of both cultivars exhibited the highest activity as was the case with the DPPH radical scavenging assay.

**Table 6.8** IC<sub>50</sub> value of Falcon and AC Metcalfe barley fractions for DPPH and hydroxyl radicals<sup>1</sup>.

Fraction	DPPH radical		Hydroxyl radical	
	Falcon	AC Metcalfe	Falcon	AC Metcalfe
F1	0.27 ± 0.02 <sup>a</sup>	0.51 ± 0.04 <sup>a</sup>	0.51 ± 0.06 <sup>a</sup>	0.68 ± 0.01 <sup>a</sup>
F2	0.74 ± 0.01 <sup>b</sup>	1.31 ± 0.06 <sup>b</sup>	1.51 ± 0.03 <sup>b</sup>	2.11 ± 0.04 <sup>b</sup>
F3	1.75 ± 0.00 <sup>c</sup>	2.01 ± 0.15 <sup>c</sup>	1.75 ± 0.01 <sup>c</sup>	2.68 ± 0.02 <sup>c</sup>
F4	3.50 ± 0.04 <sup>d</sup>	3.25 ± 0.01 <sup>d</sup>	2.21 ± 0.07 <sup>d</sup>	3.13 ± 0.01 <sup>d</sup>
F5	3.71 ± 0.07 <sup>e</sup>	3.51 ± 0.03 <sup>e</sup>	3.30 ± 0.02 <sup>e</sup>	3.75 ± 0.06 <sup>e</sup>
F6	n.d.	n.d.	n.d.	n.d.
F7	n.d.	n.d.	n.d.	n.d.
Pearled grain	n.d.	n.d.	n.d.	n.d.

<sup>1</sup>IC<sub>50</sub> values are expressed as mg/mL.

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

n.d.; not determined.

Fraction 1 of Falcon was 6.47 times more active in scavenging the DPPH radical than F7 while F1 of AC Metcalfe was 5.51 times more active than F7. In general, the IC<sub>50</sub> for the hydroxyl radical was significantly ( $p < 0.05$ ) higher than for the DPPH radical in both cultivars. A similar trend was observed with the whole extracts as well. **Table 6.8** lists IC<sub>50</sub> values for the hydroxyl radical.

#### **6.4.13 Inhibition of Cu (II)-induced human low density cholesterol oxidation.**

The contribution of oxidation of LDL cholesterol towards atherogenesis that leads to cardiovascular diseases is well established. Oxidized LDL may be endocytosed in an uncontrolled manner by macrophages, resulting in the generation of cholesterol-laden foam cells, which characterize atherosclerotic lesions (Berliner and Heinecke, 1996). Furthermore, oxidized LDL is believed to be chemotactic to leukocytes and can induce smooth muscle cell proliferation with many other atherogenic effects (Rebecca and Leake, 2000).

In this study, human LDL cholesterol samples (0.032 mg/mL; final concentration) were mixed with Cu(II) in the presence of Falcon and AC Metcalfe barley extracts (0.06 mg/mL; final concentration) and the development of CD was measured at timed intervals over 100 min. Conjugated diene is often used as an indicator of oxidation in LDL studies. The inhibition of LDL oxidation of the extracts was expressed as percentage inhibition based on CD values at the end of the 100 min incubation period by comparing with the level of oxidation in the control. Falcon fractions exhibited percentage inhibition 10.44 – 63.4% inhibition while AC Metcalfe fractions exhibited 8.6 – 56.52% inhibition against Cu (II) induced LDL cholesterol oxidation. **Figure 6.10** illustrates the progression of CD

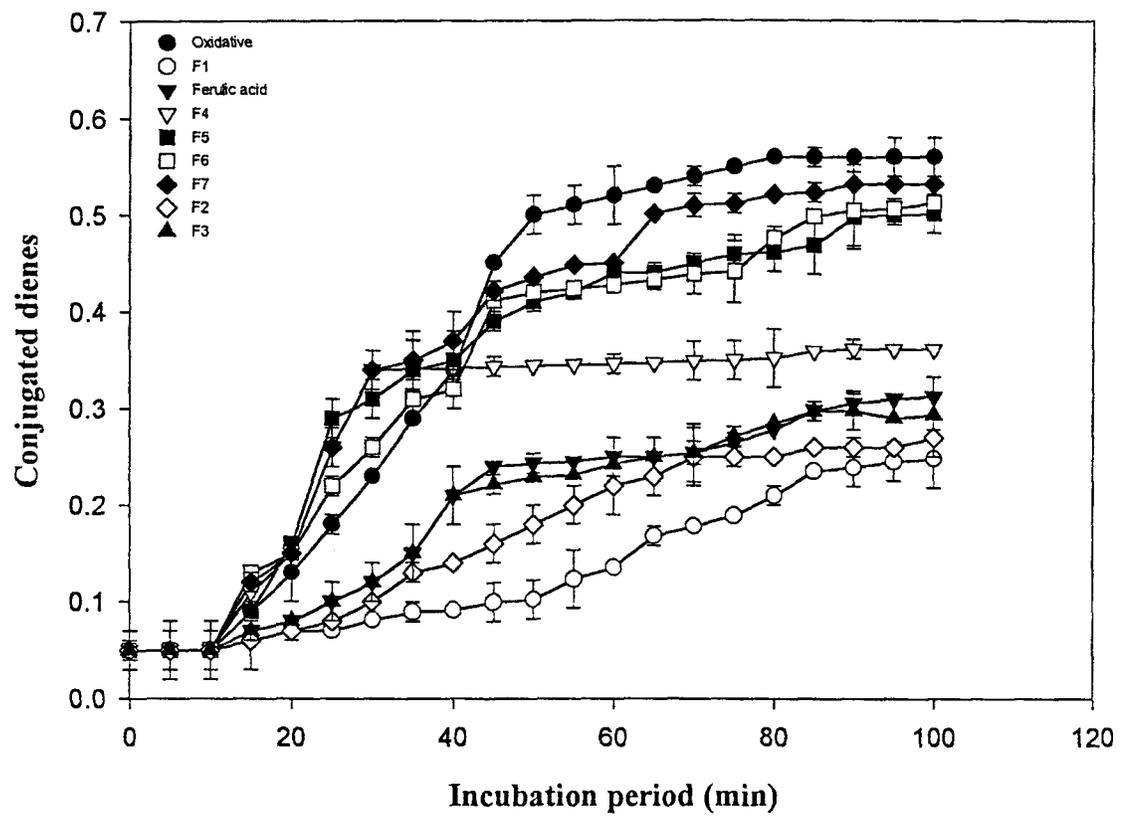
formation in the LDL cholesterol sample in the presence of Falcon barley fractions, and ferulic acid over 100 min of incubation. LDL oxidation prevention capacity decreased gradually from F1 to F7 with both Falcon and AC Metcalfe cultivars. The trend of LDL oxidation prevention is quite similar to that of metal chelation and total phenolic content. The effect of ferulic acid on LDL oxidation was superior to that of inner fractions, however, Falcon F1-F3 and AC Metcalfe F1 and F2 were more potent (**Figure 6.11**).

#### **6.4.14 Supercoiled DNA scission study**

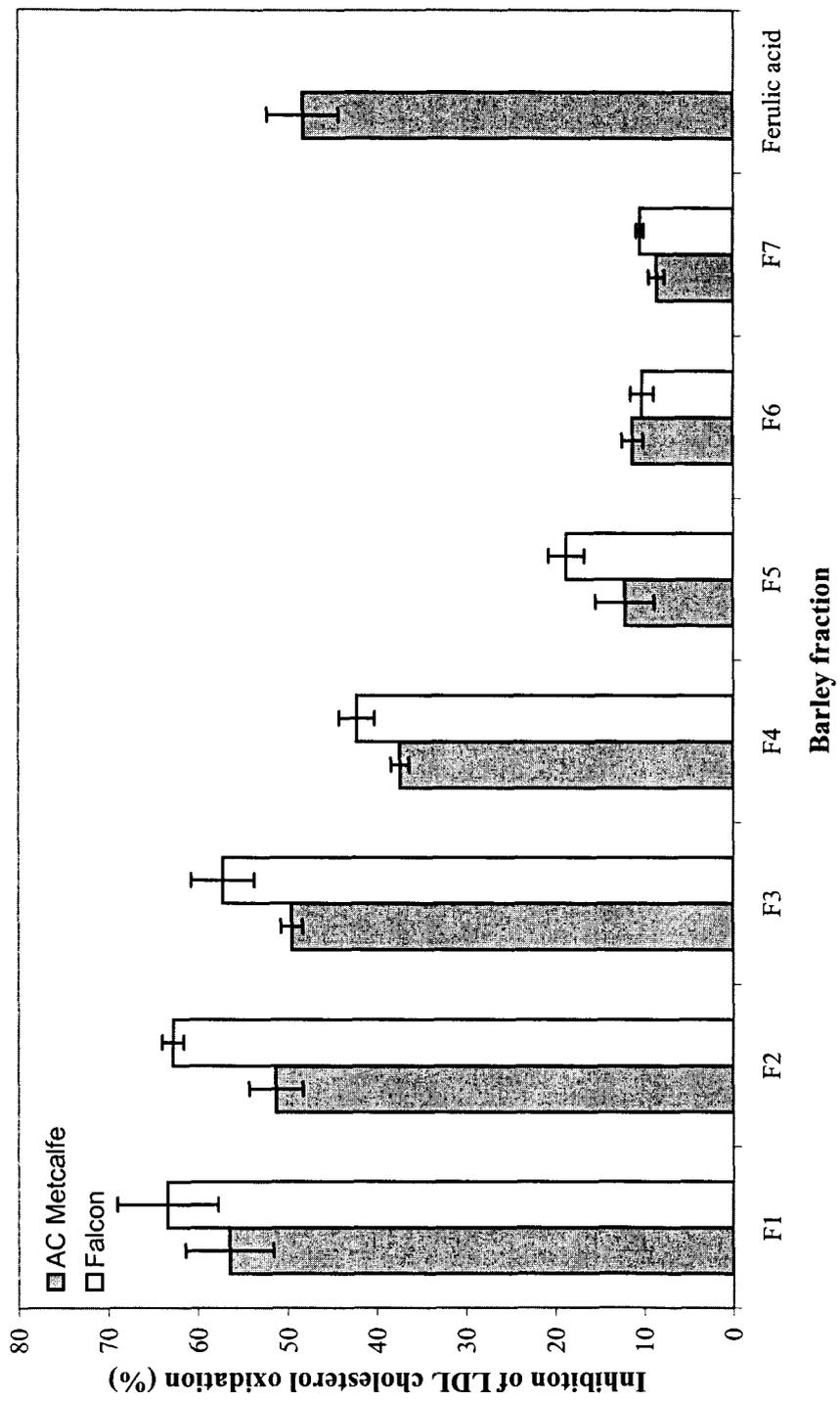
##### **6.4.14.1 Inhibition of supercoiled plasmid DNA scission induced by peroxy radical**

Peroxy radical generated by AAPH leads to breakage of supercoiled plasmid DNA. Both barley extracts exhibited strong protection against peroxy radical-induced DNA breakage in a concentration-dependent manner. The level of protection against breakage was presented as percentage inhibition by comparing the amount of DNA remaining at the end of the incubation period with the amount of DNA present in the native DNA sample (**Table 6.9**). Extracts were tested at different concentrations of 1.33, 2.67, 4.00 and 6.67 mg/mL. The protective effects of F1-F3 increased rapidly up to 2.67 mg/mL and then started to level off. However, the protective effects of F4-F7 continued to increase up to 4.00 mg/mL and then started to level off. This large variation in the protective effect was attributable to the wide variation of total phenolic content and antioxidant activity among the fractions.

**Figure 6.10** Effect of antioxidative extracts from Falcon fractions in inhibition of development of conjugated dienes (CD) in Cu(II)-induced LDL cholesterol oxidation over 100 min of incubation.



**Figure 6.11** Effect of antioxidatve extracts from Falcon and AC Metcalfe fractions in inhibition of Cu(II)-induced LDL cholesterol oxidation



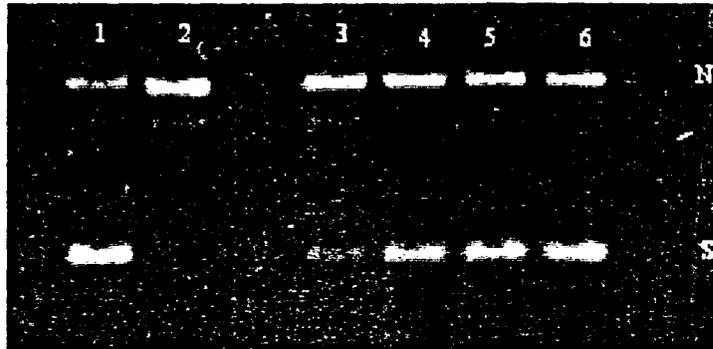
In order to express results on a common scale, a concentration of 2.67 mg/mL was used in the calculation of the protective effect for all extracts although the activity showed an increasing trend after 2.67 mg/mL in F4-F7. At this concentration, the Falcon fractions exhibited 42.08 – 89.22% inhibition while the corresponding values for AC Metcalfe were 47.65 – 82.57%. Pearled grains of Falcon and AC Metcalfe exhibited 40.88 and 44.5% inhibition, respectively. Radicals cleave supercoiled plasmid DNA (form I) to nicked circular DNA (form II) or at higher concentrations to linear DNA (form III). The presence of peroxy radical resulted in a dramatic scission of supercoiled DNA. This was clearly seen in the wells, where the reaction mixture did not contain any antioxidant (**Figure 6.12 Lane 2**). The radical concentration used in the present study was not sufficient enough to destroy the nicked circular DNA, which is more difficult to destroy as compared to form I DNA. This is clearly seen in lane 2 of **Figure 6.12**, which depicts the effect of peroxy radical on supercoiled DNA incubated without any extracts. As the concentration of antioxidative extract was increased, the protective effect against nicking of supercoiled DNA was also increased.

S and N represent supercoiled and nicked DNA bands, respectively. Lane 1 represents the native supercoiled DNA sample without any additives. The high intensity S band with a low intensity N band indicates a high concentration of supercoiled DNA and low concentrations of nicked DNA in the native DNA sample, respectively. Lane 2 shows the presence of supercoiled DNA, radical and PBS. The presence of a high intensity N band and the disappearance of the S band in lane 2 indicates that supercoiled DNA was completely nicked.

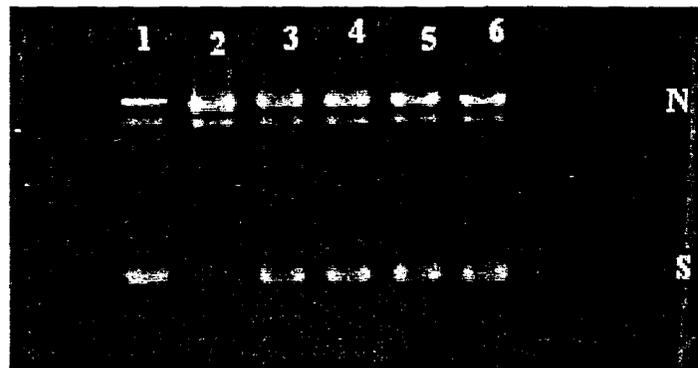
**Figure 6.12** Representative gel pictographs illustrating the effect of F1 fraction from Falcon **(a)** and AC Metcalfe **(b)**, respectively, at different concentrations against peroxy radical-induced nicking of supercoiled DNA double strands.

*Lanes 1 through 6 contained DNA. Lanes 2-6 contained 1mM AAPH; In addition, lanes 3 through 6 contained 1.33, 2.67, 4.00 and 6.67 mg/mL of extracts, respectively*

(a)



(b)



Wells 3 through 6 contained supercoiled DNA, along with the same concentration of radical together with increasing concentrations of barley extracts (1.33-6.67 mg/mL). The intensity of S band gradually increased from lanes 3 through 6 reflecting a higher level of retention of supercoiled DNA due to the protection offered by increasing concentrations of barley extracts. On the other hand, the intensity of the N band gradually decreased from lane 3 through 6 depicting less nicked DNA concentrations with an increasing level of protection offered by the extracts. In general, Falcon extracts exhibited a higher protective effect than AC Metcalfe extracts. However, this trend was not consistent among all fractions.

#### **6.4.14.2 Inhibition of supercoiled plasmid DNA scission induced by hydroxyl radical**

Falcon and AC Metcalfe barley fraction extracts were effective in suppressing hydroxyl radical-induced DNA damage in a non-site specific protocol in a concentration-dependent manner. Extracts were tested at concentrations of 1.33, 2.62, 4.00 and 6.67 mg/mL. The protective effects, in general, increased rapidly up to 2.67 mg/mL, and then started to level off. Therefore, for the calculation of protective effect, a concentration of 2.67 mg/mL was considered. Falcon fraction extracts exhibited 18.24 to 52.34% inhibition while AC Metcalfe exhibited 20.42 to 46.99%. Falcon and AC Metcalfe pearled grain fraction exhibited 21 and 20.2% inhibition, respectively. It was clearly seen that the level of protection against hydroxyl radical is substantially lower than that against peroxy radical. The level of protection exerted by whole barley extracts at 2.67 mg/mL listed in **Table 6.9**.

The reactions of hydroxyl radical occur mainly through addition to the double bond of pyrimidine bases and abstraction of hydrogen from the sugar moiety resulting in chain scission of DNA. These effects can cause cell mutagenesis and carcinogenesis (Namiki, 1990). The effects of barley extracts toward protecting plasmid DNA strand scission provide further evidence of antioxidant efficacy exhibited by barley.

#### **6.4.15 Effect of barley extracts on prevention of proliferation of Caco-2 colon cancer cell line**

Falcon and AC Metcalfe barley fractions were evaluated for their potential antiproliferative efficacy at 0.5 mg/mL and 0.05 mg/mL levels against Caco-2 human cancer cells. The antiproliferative activity of the barley extracts was measured and reported as percent control cells. Falcon F1 showed 75 and 29% inhibition at 0.5 mg/mL and 0.05 mg/mL concentrations, respectively. The corresponding values for AC Metcalfe were 57 and 17%. The effectiveness gradually increased over the four days of incubation and achieved the highest level of antiproliferative activity, which could clearly be observed at the end of day 4 of incubation. All fractions of Falcon and AC Metcalfe rendered significant antiproliferative effect against Caco 2 colon cancer cells in a concentration dependant manner.

**Table 6.9** Effects of barley fractions at 2.67 mg/mL on the retention of supercoiled strand of PBR322 DNA under peroxy and hydroxyl radical-induced scission<sup>1</sup>.

Sample identity	Peroxy radical		Hydroxyl radical	
	Falcon	AC Metcalfe	Falcon	AC Metcalfe
F1	89.22 ± 2.12 <sup>a</sup>	82.57 ± 2.26 <sup>d</sup>	52.34 ± 3.34 <sup>d</sup>	46.99 ± 1.33 <sup>e</sup>
F2	82.64 ± 3.21 <sup>e</sup>	78.89 ± 7.67 <sup>d</sup>	49.25 ± 1.23 <sup>cd</sup>	45.23 ± 0.33 <sup>d</sup>
F3	78.23 ± 1.63 <sup>e</sup>	67.45 ± 2.34 <sup>c</sup>	44.23 ± 4.42 <sup>bc</sup>	40.77 ± 2.28 <sup>d</sup>
F4	70.12 ± 0.98 <sup>d</sup>	61.02 ± 3.34 <sup>bc</sup>	36.88 ± 2.34 <sup>b</sup>	30.38 ± 1.23 <sup>c</sup>
F5	64.29 ± 2.41 <sup>d</sup>	56.98 ± 1.23 <sup>b</sup>	24.53 ± 4.3 <sup>a</sup>	28.35 ± 3.45 <sup>bc</sup>
F6	42.08 ± 5.34 <sup>a</sup>	52.34 ± 0.77 <sup>ab</sup>	18.24 ± 2.26 <sup>a</sup>	25.33 ± 1.22 <sup>b</sup>
F7	51.78 ± 3.36 <sup>c</sup>	47.65 ± 2.23 <sup>a</sup>	19.98 ± 1.54 <sup>a</sup>	20.42 ± 2.53 <sup>a</sup>
Pearled grain	40.88 ± 2.33 <sup>a</sup>	44.49 ± 1.22 <sup>a</sup>	20.91 ± 3.56 <sup>a</sup>	20.22 ± 1.74 <sup>a</sup>
Ferulic acid	94.65 ± 4.59 <sup>c</sup>		77.34 ± 1.23 <sup>e</sup>	

<sup>1</sup>Effect of barley extracts on retention of DNA scission is expressed as a percentage.

Results are means of three determinations ± standard deviation.

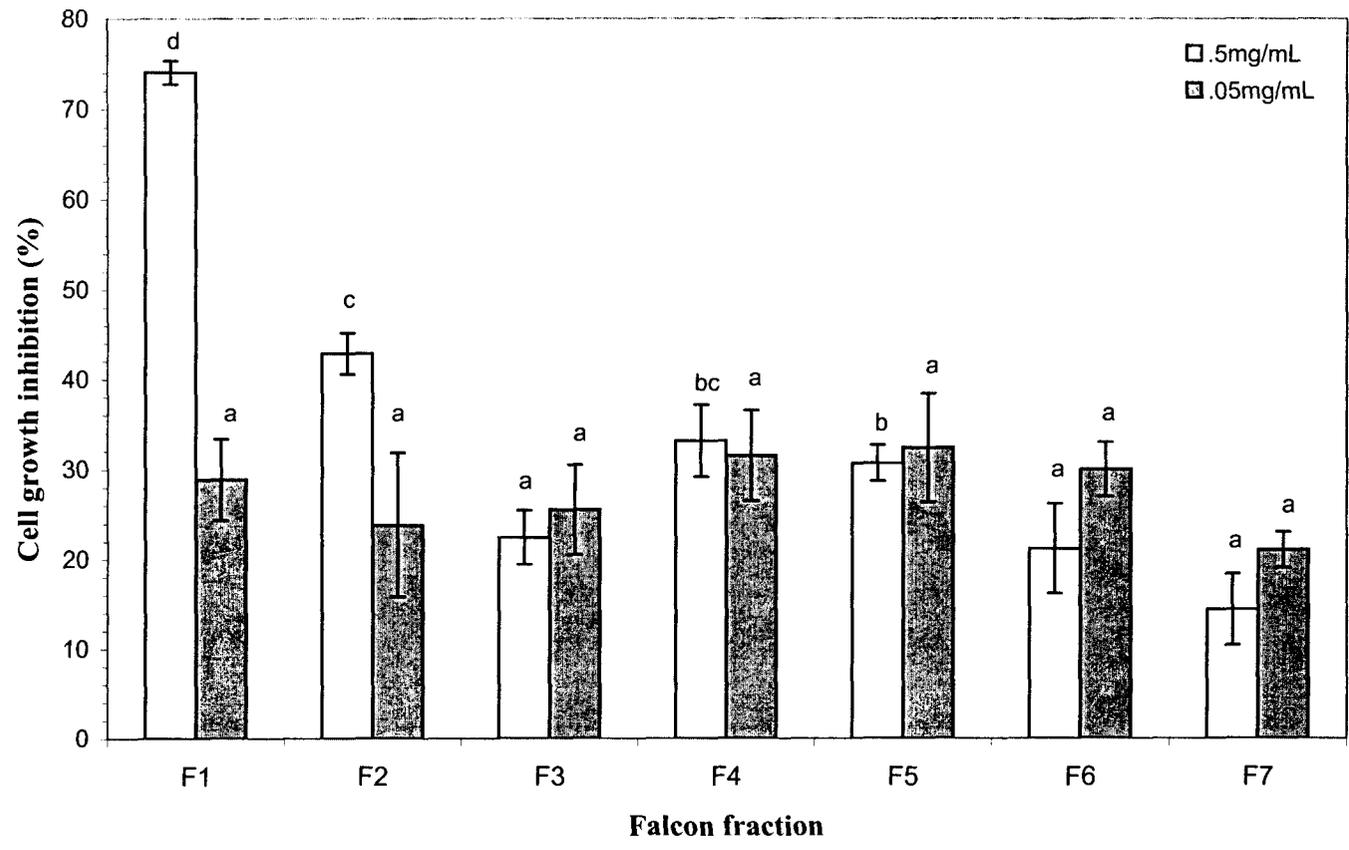
Values in each column having the same superscript are not significantly different (p>0.05).

Falcon fractions offered significantly higher antiproliferative protection compared to that of AC Metcalfe. **Figure 6.13** illustrates the percentage cell inhibition observed at the end of day 4 in the presence of 0.5 mg/mL of Falcon fractions, while **Figure 6.14** illustrates the percentage cell inhibition in the presence of AC Metcalfe fractions at 0.5 and 0.05 mg/mL concentrations. In order to have a better understanding, additional research is required to evaluate the extracts using other cancer cell lines as well as normal cells. The discrepancy in inhibition of cell proliferation may be attributable to the different chemical composition of barley cultivars.

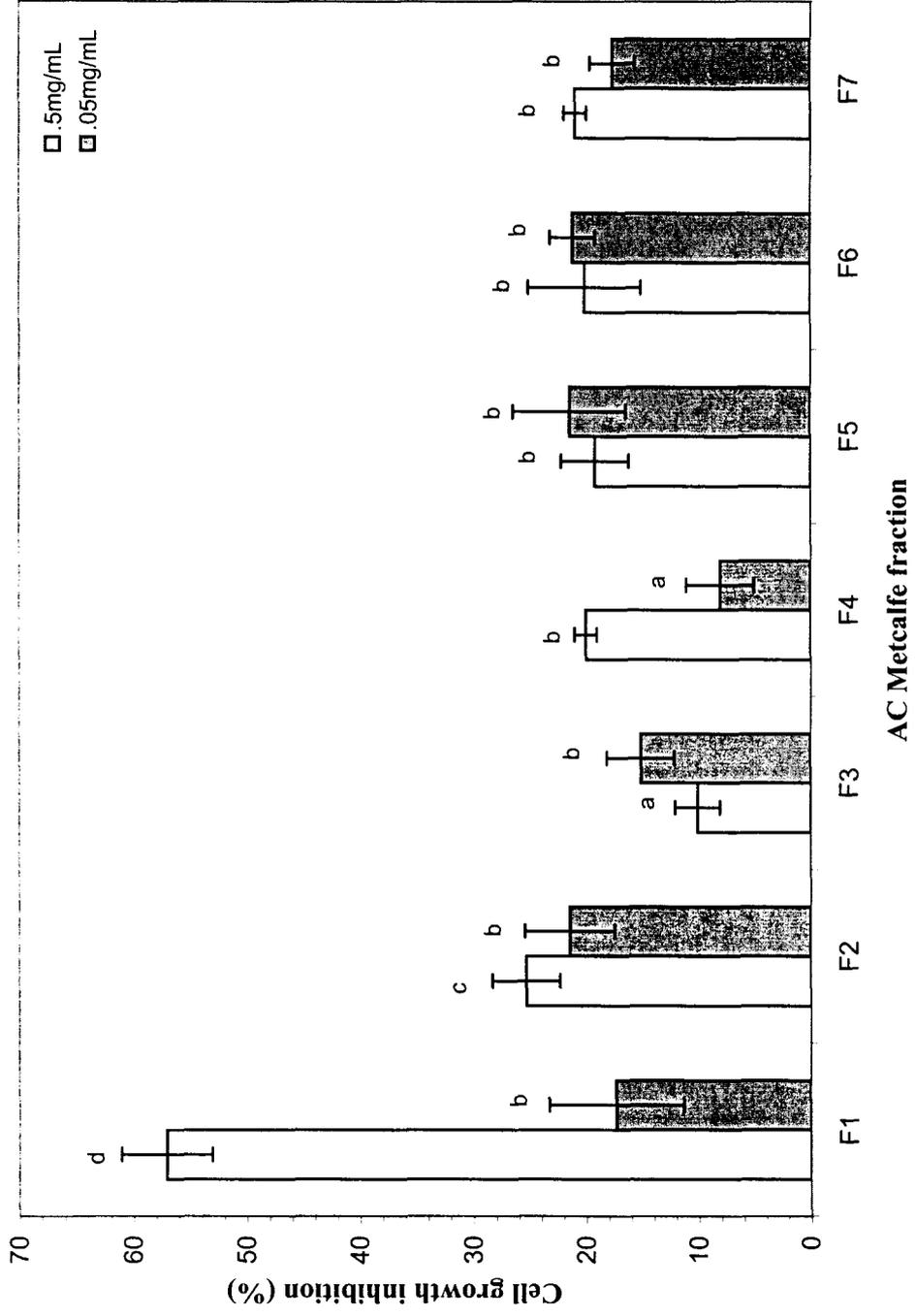
#### **6.4.16 Analysis of phenolic acid content of barley fractions**

Phenolic compounds in barley include anthocyanins, flavonols, phenolic acids, catechins and procyanidins. Phenolic acids, one of the main classes of phenolic compounds, were quantified in this study. **Table 6.10** lists the content of free phenolic acids of the Falcon and AC Metcalfe barley cultivar fractions. IN both cultivars, vanillic, caffeic, *p*-coumaric, and ferulic acids were the major phenolic acids detected while ferulic acid and vanillic acid were the major free phenolic acids. The content of free phenolic acids was significantly ( $p < 0.05$ ) higher in Falcon compared to AC Metcalfe and this correlates with the higher antioxidant activities observed in the TEAC, ORAC<sub>FL</sub>, DPPH and PCL tests. Fractions 1 to 3 contained higher amounts of free phenolic acids while the rest of the fractions contained substantially low amounts. **Table 6.10** lists the content of esterified phenolic acids that were liberated during the alkaline hydrolysis process.

**Figure 6.13** The effect of antioxidative extracts from Falcon fractions on percent inhibition of Caco-2 cancer cells at the end of day 4 at 0.5 mg/mL and 0.05 mg/mL concentrations.



**Figure 6.14** The effect of antioxidative extracts from AC Metcalfe fractions on percent inhibition of Caco-2 cancer cells at the end of day 4 at 0.5mg/mL and 0.05 mg/mL concentrations.



Sinapic acid, which was not identified in the free phenolic acid fraction was identified in the soluble esters after alkaline hydrolysis. Phenolic acids present were esterified with alcohols, phenols and alkaloids and were released into the soluble fraction upon hydrolysis of the material. Generally, alkaline hydrolysis is the method most commonly used for extracting esterified or bound phenolics at room temperature. In F1 of the Falcon cultivar, there was an increase of phenolic acids upon hydrolysis; 4.8 fold for vanillic acid, 5.8 fold for caffeic acid, 6.1 fold for *p*-coumaric acid and 19 fold for ferulic acid while the corresponding values for F1 of AC Metcalfe were 8.0, 20.4, 4.8 and 42 fold, respectively.

There is an increased awareness and interest in the antioxidant behaviour and potential health benefits associated with phenolic acids. Humans consume an estimated amount of 25 mg – 1 g of phenolic acids on a daily basis from fruits, vegetables, grains, tea, coffee and spices, among others (Clifford, 2000). Besides the protective behaviour of phenolic acids, they possess other biological activities.

Caffeic acid that was detected in substantial amounts in barley fractions has been reported to possess antitumour activity against colon cancer (Olthof *et al.*, 2001), selectively blocking biosynthesis of leukotrienes that are directly involved in allergic reactions, asthma and other immunoregulation diseases.

**Table 6.10** Content of free phenolic acids in Falcon and AC Metcalfe barley fractions<sup>1</sup>.

Fraction/Cultivar	Vanillic	Caffeic	<i>p</i> -coumaric	Ferulic
Falcon				
F1	5.86 ± 0.01	3.86 ± 0.26	1.71 ± 0.36	3.57 ± 0.35
F2	5.71 ± 0.12	0.71 ± 0.11	1.14 ± 0.02	4.86 ± 0.24
F3	4.29±0.01	n.d.	1.14 ± 0.11	4.71 ± 0.05
F4	2.86± 0.02	n.d.	0.57 ± 0.01	2.71 ± 0.01
F5	2.00 ± 0.04	n.d.	0.57 ± 0.00	2.00 ± 0.00
F6	1.29± 0.02	n.d.	0.57 ± 0.00	1.71 ± 0.01
F7	0.86 ± 0.00	n.d.	0.23 ± 0.02	1.14 ± 0.01
AC Metcalfe				
F1	3.43 ± 0.01	1.14 ± 0.01	1.14 ± 0.01	2.29 ± 0.25
F2	3.43 ± 0.10	0.71 ± 0.02	0.86 ± 0.01	2.14 ± 0.22
F3	2.14± 0.05	0.29 ± 0.17	0.86 ± 0.00	1.71 ± 0.26
F4	1.86± 0.00	0.29 ± 0.11	0.71 ± 0.01	1.57 ± 0.01
F5	1.43 ± 0.11	n.d.	0.43 ± 0.04	1.43 ± 0.07
F6	1.14 ± 0.00	n.d.	0.42 ± 0.01	1.14 ± 0.01
F7	0.23 ± 0.00	n.d.	n.d.	0.86 ± 0.00

<sup>1</sup>Free phenolic acid content is expressed in  $\mu\text{g}$  per gram defatted material basis.  
n.d.; not determined.

Sinapic acid was not determined in any of the extracts.

**Table 6.11** Content of esterified phenolic acids in Falcon and AC Metcalfe barley fractions<sup>1</sup>.

Fraction/ Cultivar	Vanillic	Caffeic	<i>p</i> -Coumaric	Ferulic	Sinapic
Falcon					
F1	21.62 ± 0.01	18.02 ± 0.26	8.32 ± 0.36	62.79 ± 0.35	36.59 ± 0.08
F2	11.24 ± 0.11	11.72 ± 0.11	4.49 ± 0.02	35.68 ± 0.24	20.81 ± 0.11
F3	7.40 ± 0.00	7.70 ± 0.02	2.03 ± 0.11	18.83 ± 0.05	10.57 ± 0.16
F4	1.90 ± 0.03	2.11 ± 0.01	0.95 ± 0.01	6.80 ± 0.01	3.57 ± 0.14
F5	2.64 ± 0.07	2.58 ± 0.01	1.20 ± 0.00	9.30 ± 0.00	4.78 ± 0.08
F6	1.25 ± 0.02	1.13 ± 0.05	0.56 ± 0.00	3.85 ± 0.01	1.94 ± 0.01
F7	1.12 ± 0.00	1.12 ± 0.07	0.39 ± 0.02	3.40 ± 0.01	1.79 ± 0.00
AC Metcalfe					
F1	24.08 ± 0.04	21.84 ± 0.01	6.44 ± 0.01	89.88 ± 0.25	70.00 ± 0.12
F2	13.08 ± 0.08	14.82 ± 0.02	4.69 ± 0.01	44.47 ± 0.22	29.21 ± 0.15
F3	8.06 ± 0.05	8.15 ± 0.17	2.40 ± 0.00	35.58 ± 0.26	16.49 ± 0.06
F4	5.79 ± 0.09	4.23 ± 0.11	1.86 ± 0.01	26.62 ± 0.01	10.31 ± 0.18
F5	3.60 ± 0.16	2.04 ± 0.11	1.29 ± 0.04	15.85 ± 0.07	4.55 ± 0.00
F6	1.94 ± 0.02	1.20 ± 0.23	0.71 ± 0.01	7.76 ± 0.01	1.94 ± 0.02
F7	0.51 ± 0.00	0.28 ± 0.00	0.15 ± 0.00	0.92 ± 0.00	0.48 ± 0.01

<sup>1</sup>Esterified phenolic acid content is expressed as µg per gram defatted material.

## 6.5 Conclusions

In summary, the antioxidant and anti-radical activities are mainly concentrated in the outer fractions, F1 to F3 (up to 25% of the grain on weight basis) in both Falcon and AC Metcalfe cultivars. Antioxidant activity gradually decreased towards the inner layers. Pearling fraction 1 (approximately outer 9% w/w of kernel weight) of both cultivars contained the highest TPC thus, it rendered the highest antioxidant and antiproliferative activities. Antiproliferative activity of the rest of the fractions was not very consistent. In general, the Falcon cultivar exhibited a higher antioxidant activity than AC Metcalfe in the TEAC, DPPH, and superoxide radical tests. PCL and ORAC<sub>FL</sub> exhibited a similar trend. Phenolic acids identified in Falcon and AC Metcalfe included vanillic, caffeic, *p*-coumaric, ferulic, and sinapic acids. Barley, a cereal that is currently underutilized for human consumption carries antioxidative constituents. Therefore, it is important not to remove the outer layers from the grains during processing as this may lead to substantial loss of phenolic compounds.

## CHAPTER 7

### EFFECT OF HYDROLYSIS ON ANTIOXIDANT ACTIVITY

#### 7.1 Introduction

Cereals contain a myriad of phenolic compounds including benzoic acid and cinnamic acid derivatives, anthocyanins, quinones, flavanols, chalcones, flavones, lignans, proanthocyanidins, and amino-phenolic compounds (Goupy *et al.*, 1999; Shahidi and Naczk, 2004). Polyphenols in barley include anthocyanins, flavonols, phenolic acids, catechins and proanthocyanidins (Goupy *et al.*, 1999). There are more than 50 proanthocyanidins reported in barley. Proanthocyanidins in barley include oligomeric and polymeric flavan-3-ol, (-)-catechin (c), and (-)-gallocatechin (gc). The most abundant proanthocyanidin in barley are dimeric proanthocyanin B3 and procyanidin B3. Major trimers include T1 (gc-gc-c), T2 (gc-c-c), T3 (c-gc-c), and T4 or procyanidins C2 (c-c-c) (Friedrich *et al.*, 2000).

Cereals also contain a wide range of phenolic acids, mainly those belonging to the benzoic acid and cinnamic acid groups. Phenolic acids are different from other phenolic compounds bearing acidic properties due to the presence of a carboxylic acid group. Ferulic acid and *p*-coumaric acid are the major phenolic acids found in many cereals, including barley. A significant proportion of these phenolic acids are known to be linked to lignans and arabinoxylans (Nordkvist *et al.*, 1984). Ferulic acid is highly concentrated in the cell walls of the aleurone layer which is rich in arabinoxylan (McNeil *et al.*, 1975; Maillard and Berset, 1995).

Phenolic compounds in cereal grains can be found in free, soluble conjugate or esterified, and insoluble-bound forms. It is reported that 74 and 69% of total phenolics

present in rice and corn, respectively, are in the insoluble-bound forms (Adom and Liu, 2002). Most of the studies found in the literature have not investigated insoluble-bound phenolic compounds, hence those studies have led to underestimation of the content of phenolic compounds present.

Having both carboxylic acid and hydroxyl groups in their structures, phenolic acids are capable of forming both ester as well as ether linkages with other compounds leading to the formation of linkages with cell wall polysaccharides. Phenolic acids can be esterified with small molecules such as alcohols, other phenolic acids, phenols, and alkaloids, among others (Yu *et al.*, 2001). Non-starch polysaccharides such as xylose, and arabinose units are easily esterified with phenolic acids. Phenolic acids with carboxyl and hydroxyl groups can bind either starch or other polysaccharides through hydrogen bonds, chelation, or covalent bonds (Yu *et al.*, 2001).

Phenolic esters can be hydrolyzed by different means, including alkaline, acidic or enzymatic hydrolysis in order to release insoluble-bound phenolics from the cereal matrix (Yu *et al.*, 2001). Generally, alkaline hydrolysis is the preferred method for extracting esterified or bound phenolics at room temperature and was used in this study

## **7.2 Objectives**

The objectives of this part of the study were to extract free, esterified, and insoluble-bound phenolic compounds from six barley cultivars using alkaline hydrolysis and to determine antioxidative, and antiradical efficacies of the phenolic fractions so obtained.

## 7.3 Materials and methods

### 7.3.1 Materials

Materials were obtained as mentioned in **Section 5.3.1**.

### 7.3.2 Methods

#### 7.3.2.1 Separation of phenolic fractions using alkaline hydrolysis

The free phenolics, soluble esters and insoluble-bound phenolic acids from AC Metcalfe, Falcon, Phoenix, Tercel, Tyto and Peregrine barley samples were isolated using the procedure explained by Krygier *et al.* (1982) and Naczka and Shahidi (1989). Barley meals (2 g) were extracted six times with a 40 mL mixture of methanol-acetone-water (7:7:6, v/v/v) using a Polytron homogenizer at 10,000 rpm for 4 min. The resulting mixture was centrifuged at 4000xg for 5 min and the supernatants were collected and combined. The solvent mixture was evaporated at 30°C *in vacuo* to approximately 40 mL, followed by extracting six times with diethyl ether at a 1:1 (v/v) solvent-supernatant ratio. The combined extracts were evaporated to dryness *in vacuo* at 30°C in order to obtain free phenolic acid fraction which was subsequently dissolved in methanol. The supernatant (B) (**Figure 7.1**) containing soluble conjugates were subsequently hydrolyzed with 30mL of 4 M NaOH under N<sub>2</sub> for 4h at room temperature. The resultant hydrolysate was acidified to pH 2 using 6M HCl followed by extraction with diethyl ether. The ether extracts were combined and evaporated to dryness *in vacuo* at 30°C and subsequently dissolved in methanol to obtain soluble esters. The leftover meal (A) (**Figure 7.1**) was treated with 20 mL of 4 M NaOH and hydrolyzed for 4h at room temperature under a stream of N<sub>2</sub> acidified to pH 2 with 6 M HCl and centrifuged at 4000 x g for 5 min. The supernatant was extracted with diethyl ether six times. The combined extracts were

evaporated to dryness at 30°C *in vacuo* and subsequently dissolved in methanol to obtain insoluble-bound phenolic fraction.

#### **7.3.2.2 Determination of antioxidant activity of phenolic fractions obtained from barley**

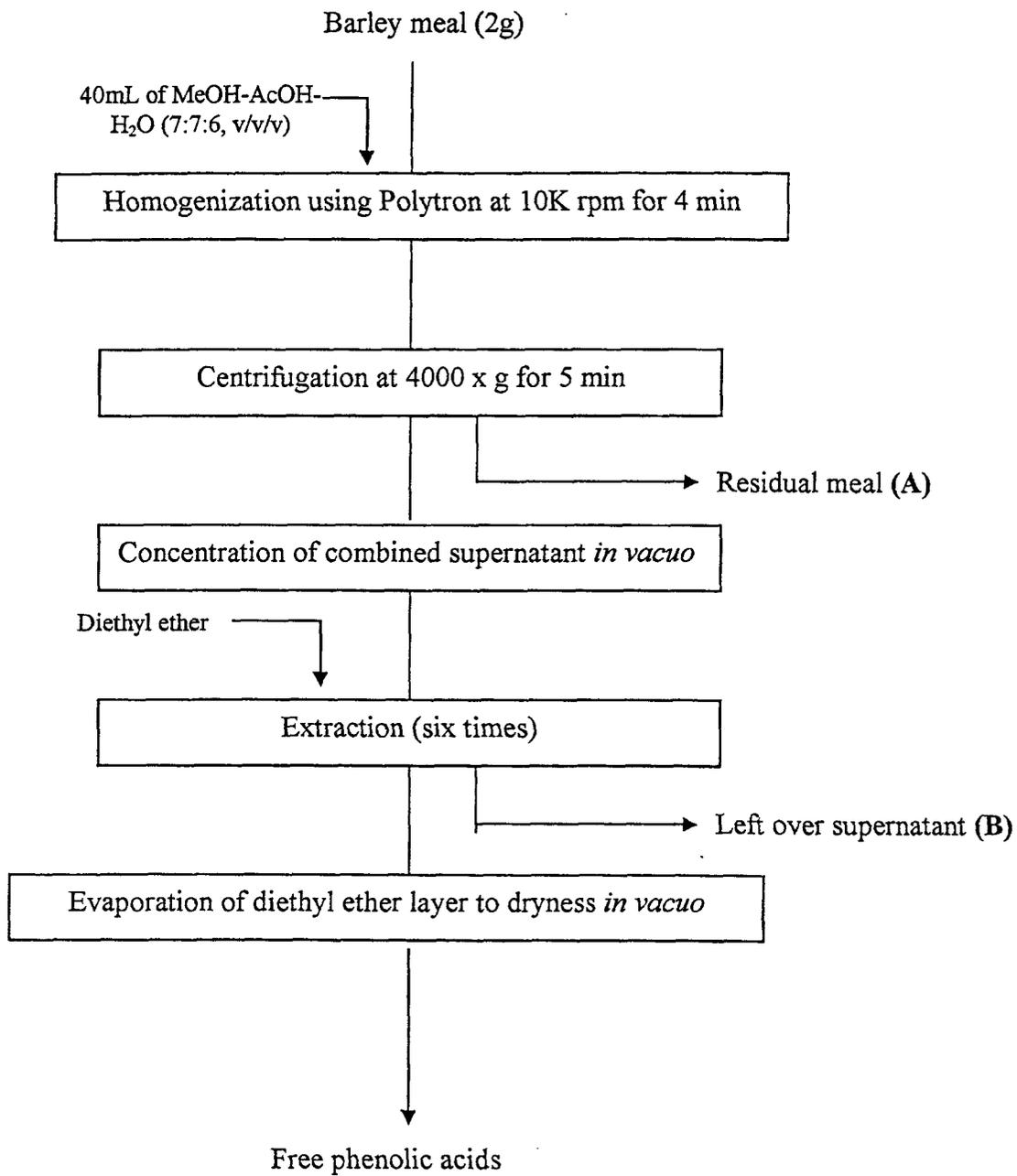
Total phenolic content of the extracts so obtained was determined according to the method explained in **Section 3.2.5**. Antioxidative capacity of the extracts was determined according to the methods explained in **Sections 3.2.6 – 3.2.10**. In this series of experiments, methanolic extract in the liquid form was used in place of lyophilized extracts employed in the previous sections.

### **7.4 Results**

#### **7.4.1 Total phenolic content**

The contents of free, soluble conjugate, and insoluble-bound phenolics of barley extracts are listed in **Table 7.1**. The content of insoluble-bound phenolics was significantly higher than those of soluble conjugate and free phenolic fractions among all barley extracts tested. Free phenolic content ranged from 0.18 to 0.42 mg ferulic acid equivalents per gram defatted material with the highest content in Peregrine extracts while soluble conjugates ranged from 0.42 to 0.81 mg ferulic acid equivalents per gram of defatted material. The insoluble-bound fraction ranged from 2.03 to 3.36 mg ferulic acid equivalents per gram defatted material of Tercel and Peregrine, respectively. The ratio of soluble phenolics (the sum of free and soluble conjugate fractions) to insoluble-bound phenolics ranged from 1: 2.74 to 1: 3.92.

**Figure 7.1** Flow chart for preparation of free, soluble conjugate, and insoluble-bound phenolic fractions of barley samples.



Left over Supernatant (B)



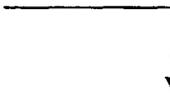
Hydrolysis with 4M NaOH for 4h under N<sub>2</sub>



Acidification with 6M HCl to pH 2



Diethyl ether



Extraction six times



Evaporation of diethyl ether layer to dryness *in vacuo*



Soluble conjugates  
(esterified phenolics)

Left over meal (A)



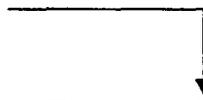
Hydrolysis with 4M NaOH for 4h under N<sub>2</sub>



Acidification with 6M HCl to pH 2



Diethyl ether



Extraction six times



Evaporation of diethyl ether layer to dryness *in vacuo*



Insoluble-bound  
phenolic compounds

**Figure 7.2** depicts the percentage contribution of each of the phenolic fractions towards TPC. The free phenolic content represents contribution from free and soluble conjugate fractions. Soluble conjugate phenolics may be oxidized and therefore contribute towards TPC and antioxidant activity.

Adom and Liu (2002) analyzed corn, wheat, oat and rice cereals and reported that corn had the highest free phenolic content (0.411 mg/g of grain), followed by rice (0.407 mg/g of grain), then wheat (0.368 mg/g of grain), and oat (0.343 mg/ g of grain). The content of insoluble-bound phenolic was significantly higher among all of the above cereals (Adom and Liu, 2002).

It is evident that a significantly high proportion of phenolic content is present in the bound form in cereals. Insoluble-bound phenolic compounds are associated with cell wall materials, especially complex carbohydrates such as arabinoxylan. Gastrointestinal enzymes usually are not capable of releasing phenolics from the complexes of phenolic compounds and complex carbohydrates, hence they pass the upper intestinal tract intact.

However, the bound phenolics that reach the colon potentially be digested by the enzymes produced by colon microflora and as a result some phenolics are released and absorbed by the body. Therefore, the insoluble-bound phenolics in cereals play a significant role in the diet by providing phenolic compounds through colonic digestion (Adom and Liu, 2002).

**Table 7.1** Contents of free, soluble conjugate, and insoluble-bound phenolics of barley cultivars<sup>1</sup>.

Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics	Total phenolic content <sup>2</sup>	Soluble to insoluble-bound phenolics <sup>3</sup> ratio
Falcon	0.34 ± 0.02 <sup>c</sup>	0.63 ± 0.00 <sup>c</sup>	2.66 ± 0.06 <sup>c</sup>	3.63	1: 2.74
AC Metcalfe	0.31 ± 0.01 <sup>c</sup>	0.81 ± 0.01 <sup>d</sup>	3.34 ± 0.01 <sup>e</sup>	4.46	1: 2.98
Tyto	0.26 ± 0.02 <sup>b</sup>	0.52 ± 0.02 <sup>b</sup>	2.78 ± 0.03 <sup>d</sup>	3.56	1: 3.56
Tercel	0.22 ± 0.00 <sup>ab</sup>	0.49 ± 0.01 <sup>ab</sup>	2.16 ± 0.00 <sup>b</sup>	2.64	1: 3.04
Phoenix	0.18 ± 0.00 <sup>a</sup>	0.42 ± 0.00 <sup>a</sup>	2.03 ± 0.01 <sup>a</sup>	2.63	1: 3.38
Peregrine	0.42 ± 0.02 <sup>d</sup>	0.73 ± 0.04 <sup>d</sup>	3.36 ± 0.02 <sup>e</sup>	4.51	1: 3.92

<sup>1</sup>Phenolic contents are expressed as mg ferulic acid equivalents/g defatted material.

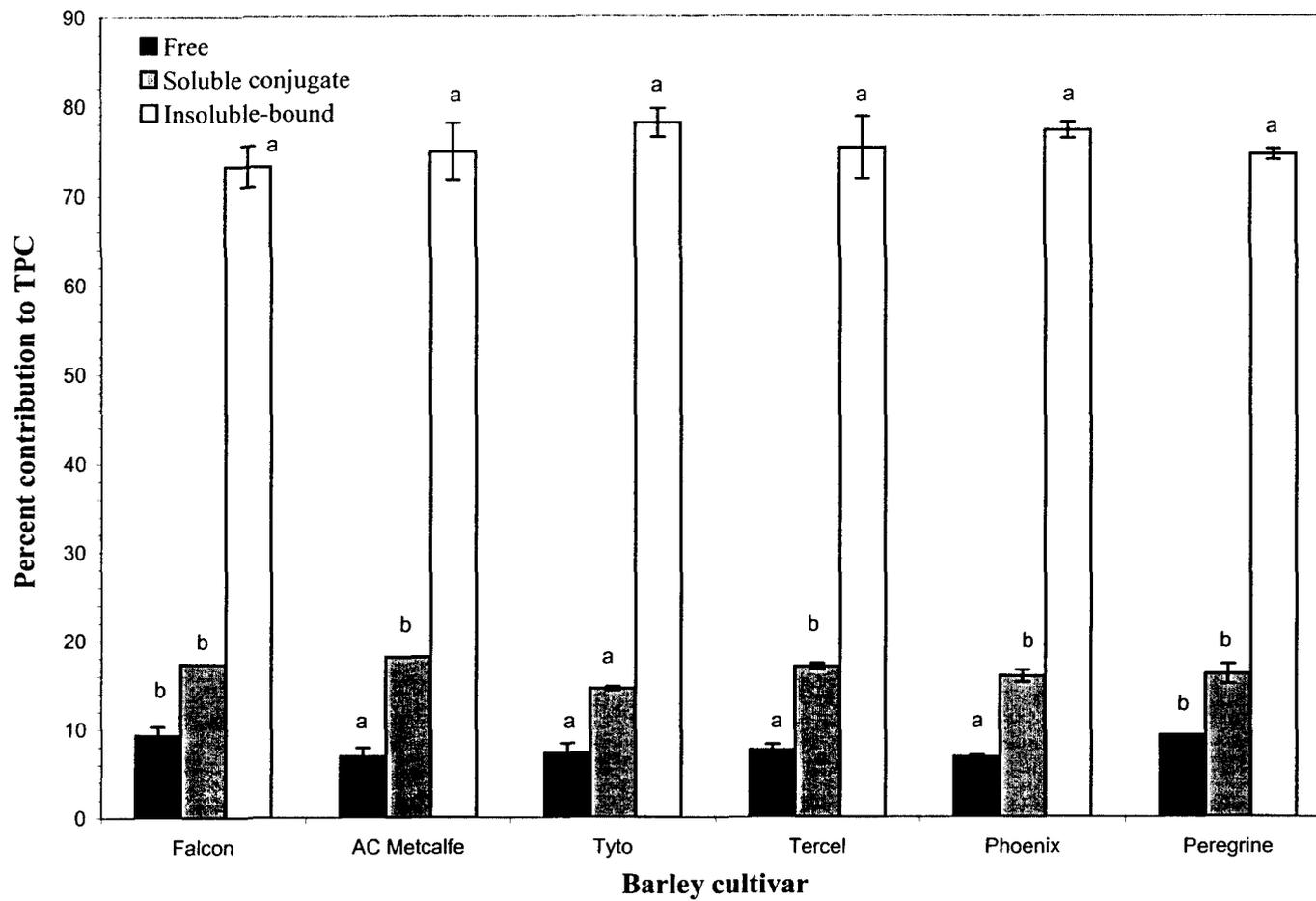
<sup>2</sup>Summation of free, soluble conjugate and insoluble-bound phenolics; expressed as mg ferulic acid equivalents/ g defatted material.

<sup>3</sup>Soluble phenolics represent summation of free and soluble conjugate phenolic fractions.

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

**Figure 7.2** Percentage contribution of free, soluble conjugate, and insoluble-bound phenolic fractions towards the total phenolic content (TPC) of barley extracts.



Researchers have adopted alkaline, acid and enzymatic hydrolysis procedures to release bound phenolics from various materials. In this study, 4 h alkaline hydrolysis was used to release insoluble-bound phenolics from barley samples as reported by Maillard and Berset (1995). However, Adom and Liu (2002) adopted 1h alkaline hydrolysis indicating that 4h hydrolysis may degrade some of the phenolic acids.

#### **7.4.2 Total antioxidant capacity (TAC) as measured by Trolox equivalent antioxidant capacity (TEAC)**

Total antioxidant activity of free, soluble conjugates and insoluble-bound phenolic fractions is listed in **Table 7.2**. It is evident that TAC follows a similar trend as TPC among all the barley extracts tested. Total antioxidant activity of free, soluble conjugate and insoluble-bound phenolic fractions ranged from 1.89 to 3.11, 1.77 to 3.98, and 7.44 to 9.88  $\mu\text{mol Trolox/ g}$  defatted material, respectively. The highest TAC was rendered by Peregrine while the lowest efficacy was rendered by Tyto extracts. The TEAC value of a compound represents the concentration of Trolox that has the same antioxidant capacity as the compound or a mixture of compounds of interest (van den Berg, 1999). Thus, the TEAC value may be considered as a stoichiometric number related to TEAC, for Trolox value of 1. **Figure 7.3** depicts the percentage contribution of each of the phenolic fractions towards the total antioxidant activity. Insoluble-bound phenol fraction contributed the highest proportion towards TAC, followed by soluble conjugate and free phenolics.

**Table 7.2** Total antioxidant capacity (TAC) of free, soluble conjugate, and insoluble-bound phenolic fractions of barley cultivars as measured by Trolox equivalent antioxidant capacity (TEAC)<sup>1</sup>.

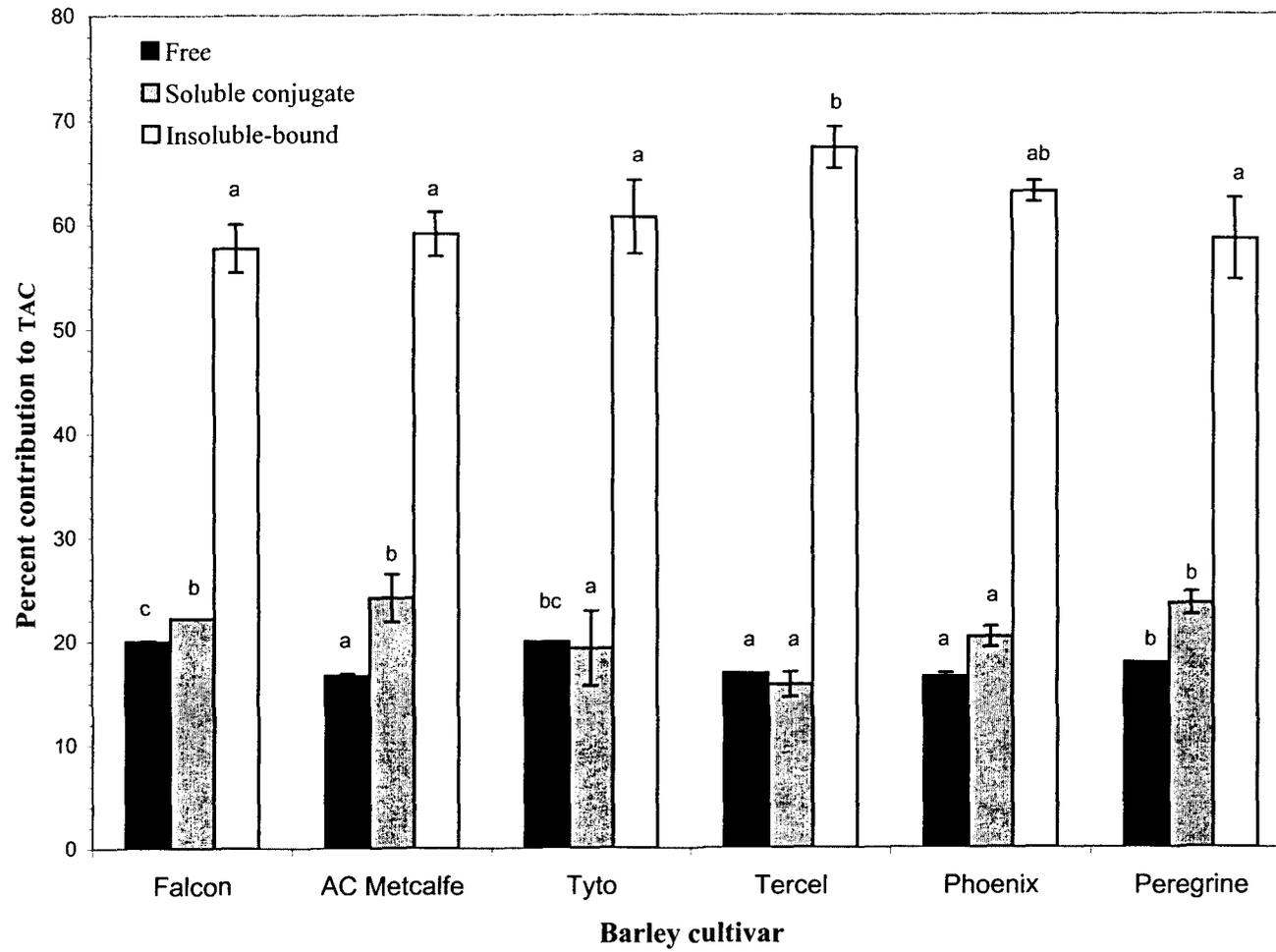
Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics
Falcon	3.11 ± 0.02 <sup>c</sup>	3.46 ± 0.05 <sup>c</sup>	8.98 ± 0.06 <sup>c</sup>
AC Metcalfe	2.67 ± 0.31 <sup>cd</sup>	3.87 ± 0.16 <sup>d</sup>	9.45 ± 0.17 <sup>cd</sup>
Tyto	2.45 ± 0.02 <sup>bc</sup>	2.37 ± 0.22 <sup>b</sup>	7.44 ± 0.23 <sup>a</sup>
Tercel	1.89 ± 0.05 <sup>a</sup>	1.77 ± 0.08 <sup>a</sup>	7.53 ± 0.19 <sup>a</sup>
Phoenix	2.16 ± 0.09 <sup>ab</sup>	2.66 ± 0.04 <sup>b</sup>	8.23 ± 0.06 <sup>b</sup>
Peregrine	3.02 ± 0.02 <sup>dc</sup>	3.98 ± 0.09 <sup>d</sup>	9.88 ± 0.26 <sup>d</sup>

<sup>1</sup>TEAC values are expressed as mg  $\mu$ mol Trolox/g defatted material.

Results are means of three determinations  $\pm$  standard deviation.

Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

**Figure 7.3** Percentage contribution of free, soluble conjugate, and insoluble-bound phenolic fractions towards the total antioxidant capacity (TAC) as measured by Trolox equivalent antioxidant capacity (TEAC) of barley.



#### 7.4.3 DPPH radical scavenging capacity of phenolic fractions of barley

DPPH radical scavenging capacity of free, soluble conjugate and insoluble-bound phenolic fractions from six barley cultivars is listed in **Table 7.3**. DPPH radical scavenging capacity of free phenolic fraction ranged from 1.76 to 2.56  $\mu\text{mol}$  ferulic acid equivalents per gram defatted material with the highest efficacy rendered by Phoenix extract. The corresponding values for soluble conjugate and insoluble-bound fractions were 1.99 to 2.98 and 3.95 to 5.62  $\mu\text{mol}$  ferulic acid equivalents per gram defatted material, respectively.

In the DPPH assay, antioxidants reduce  $\text{DPPH}^\bullet$ , one of the few stable organic nitrogen radicals that has an absorption maximum at 515 nm. One of the advantages of using  $\text{DPPH}^\bullet$  is that it does not require preparation of the radical before the assay. The DPPH assay is based on the measurement of reducing the ability of antioxidants toward  $\text{DPPH}^\bullet$ , which can be monitored by measuring the decrease in absorbance spectrophotometrically or by electron paramagnetic resonance signal (Prior *et al.*, 2005).

#### 7.4.4 Oxygen radical absorbance capacity ( $\text{ORAC}_{\text{FL}}$ ) of phenolic fractions from barley.

Oxygen radical absorbance capacity of free, soluble conjugate, and insoluble-bound phenolic fractions is listed in **Table 7.4**.  $\text{ORAC}_{\text{FL}}$  values of free, soluble conjugate, and insoluble-bound phenolic fractions were in the range of 5.99 - 8.45, 11.78 - 18.88, and 22.13 - 34.67, respectively. As observed in the TAC and DPPH radical scavenging sections, the contribution of the insoluble-bound fraction towards the total  $\text{ORAC}_{\text{FL}}$  is significantly higher.

**Table 7.3** DPPH radical scavenging capacity of free, soluble conjugate, and insoluble-bound phenolic fractions of barley cultivars<sup>1</sup>.

Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics
Falcon	2.20 ± 0.02 <sup>e</sup>	2.76 ± 0.00 <sup>e</sup>	4.56 ± 0.06 <sup>d</sup>
AC Metcalfe	1.89 ± 0.01 <sup>b</sup>	2.23 ± 0.01 <sup>c</sup>	3.95 ± 0.01 <sup>a</sup>
Tyto	2.11 ± 0.02 <sup>d</sup>	1.99 ± 0.02 <sup>a</sup>	4.76 ± 0.03 <sup>c</sup>
Tercel	1.76 ± 0.00 <sup>a</sup>	2.10 ± 0.01 <sup>b</sup>	4.12 ± 0.00 <sup>b</sup>
Phoenix	2.56 ± 0.00 <sup>f</sup>	2.44 ± 0.00 <sup>d</sup>	4.22 ± 0.01 <sup>b</sup>
Peregrine	1.98 ± 0.02 <sup>c</sup>	2.98 ± 0.04 <sup>f</sup>	5.62 ± 0.02 <sup>e</sup>

<sup>1</sup>DPPH radical scavenging capacity values are expressed as  $\mu\text{mol Trolox equivalents/g}$  defatted material. Results are means of three determinations  $\pm$  standard deviation. Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

**Table 7.4** Oxygen radical absorbance capacity (ORAC<sub>FL</sub>) of free, soluble conjugate, and insoluble-bound phenolics fractions of barley cultivars <sup>1</sup>.

Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics
Falcon	6.10 ± 0.03 <sup>b</sup>	15.92 ± 0.20 <sup>d</sup>	29.43 ± 0.06 <sup>c</sup>
AC Metcalfe	7.44 ± 0.00 <sup>e</sup>	14.67 ± 0.07 <sup>c</sup>	25.87 ± 0.09 <sup>b</sup>
Tyto	8.45 ± 0.02 <sup>f</sup>	12.88 ± 0.17 <sup>b</sup>	32.75 ± 0.79 <sup>d</sup>
Tercel	6.54 ± 0.03 <sup>c</sup>	16.43 ± .12 <sup>e</sup>	22.13 ± 1.22 <sup>a</sup>
Phoenix	5.99 ± 0.04 <sup>a</sup>	11.78 ± 0.05 <sup>a</sup>	23.98 ± 0.12 <sup>ab</sup>
Peregrine	7.11 ± 0.02 <sup>d</sup>	18.88 ± 0.04 <sup>f</sup>	34.67 ± 1.99 <sup>d</sup>

<sup>1</sup> ORAC values are expressed as  $\mu\text{mol Trolox equivalents/g}$  defatted material.

Results are means of three determinations  $\pm$  standard deviation.

Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

**Figure 7.4** illustrates the percentage contribution of each of the phenolic fractions towards total ORAC<sub>FL</sub>. Peregrine exhibited the highest total ORAC<sub>FL</sub> followed by Tyto, Falcon, AC Metcalfe, Phoenix, and Tercel.

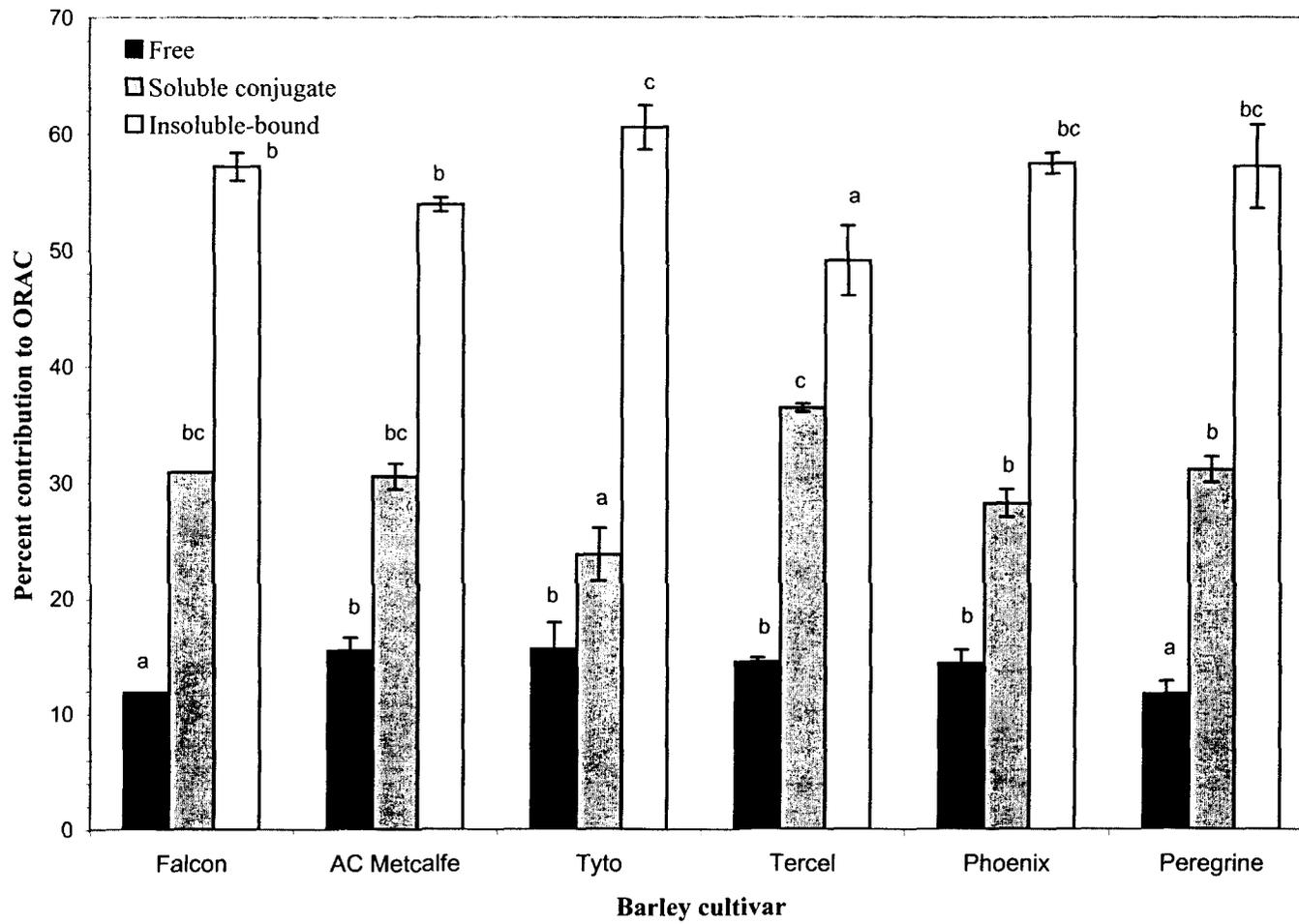
#### **7.4.5 Inhibition of LDL cholesterol oxidation by phenolic fractions from barley.**

Percentage inhibition of oxidation of LDL cholesterol by free, soluble conjugate, and insoluble-bound phenolic fractions at a final concentration of 16  $\mu\text{g}/\text{mL}$  is listed in **Table 7.5**. Percentage inhibition of oxidation of LDL cholesterol was in the range of 5.33 - 12.32, 14.33 - 29.87, and 42.99 - 72.32 for free, soluble conjugate and insoluble-bound phenolic fractions, respectively. As expected, insoluble-bound phenolic fraction rendered the highest antioxidant activity towards inhibition of LDL oxidation followed by soluble conjugates. Conjugated diene is often used as an indicator of the level of peroxidation of LDL in antioxidant studies. The inhibition of the LDL oxidation of the extracts was expressed as percentage inhibition based on the CD value after 100 min of incubation.

#### **7.4.6 Inhibition of peroxy radical-induced supercoiled plasmid DNA scission by phenolic fractions.**

The percentage inhibition offered by free, soluble conjugate, and insoluble-bound phenolic fractions of barley extracts at 1 mg/mL concentration were in the range of 8.87 - 12.56%, 15.97 - 25.32%, and 82.34 - 96.66%, respectively. The inhibitory effect was tested at 0.4 to 1.2 mg/mL, however, 1mg/mL concentration was in calculating the inhibitory effect.

**Figure 7.4** Percentage contribution of free, soluble conjugate, and insoluble-bound phenolic fractions towards the oxygen radical absorbance capacity (ORAC<sub>FL</sub>) of barley.



**Table 7.5** Percentage inhibition of Cu(II)-induced human LDL oxidation by free, soluble conjugated, and insoluble-bound phenolic fractions from barley.

Barley cultivar	Free phenolics	Soluble conjugated phenolics	Insoluble-bound phenolics
Falcon	10.32 ± 0.44 <sup>cd</sup>	17.89 ± 0.42 <sup>b</sup>	58.00 ± 4.77 <sup>c</sup>
AC Metcalfe	7.64 ± 1.32 <sup>b</sup>	14.33 ± 0.11 <sup>a</sup>	43.99 ± 2.98 <sup>a</sup>
Tyto	8.97 ± 0.76 <sup>bc</sup>	18.87 ± 1.07 <sup>c</sup>	42.99 ± 1.67 <sup>a</sup>
Tercel	6.97 ± 0.32 <sup>ab</sup>	17.66 ± 2.71 <sup>b</sup>	53.45 ± 4.22 <sup>b</sup>
Phoenix	5.33 ± 0.14 <sup>a</sup>	21.78 ± 1.59 <sup>d</sup>	66.12 ± 2.55 <sup>d</sup>
Peregrine	12.32 ± 0.92 <sup>d</sup>	29.87 ± 0.54 <sup>e</sup>	72.32 ± 6.23 <sup>e</sup>

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different (p>0.05).

**Table 7.6** Percentage inhibition of peroxy radical-induced supercoiled strand scission of PBR322 DNA by free, soluble conjugated, and insoluble-bound phenolic fractions from barley.

Barley cultivar	Free phenolics	Soluble conjugated phenolics	Insoluble-bound phenolics
Falcon	11.89 ± 1.32 <sup>bc</sup>	22.33 ± 1.61 <sup>cd</sup>	87.95 ± 3.45 <sup>abc</sup>
AC Metcalfe	9.22 ± 0.99 <sup>ab</sup>	17.78 ± 2.22 <sup>ab</sup>	96.66 ± 5.76 <sup>c</sup>
Tyto	8.87 ± 0.87 <sup>a</sup>	15.97 ± 0.01 <sup>a</sup>	82.34 ± 3.12 <sup>ab</sup>
Tercel	12.33 ± 1.21 <sup>c</sup>	19.94 ± 0.65 <sup>bc</sup>	84.34 ± 2.80 <sup>ab</sup>
Phoenix	10.00 ± 1.11 <sup>abc</sup>	24.45 ± 1.40 <sup>d</sup>	78.98 ± 2.55 <sup>a</sup>
Peregrine	12.56 ± 0.67 <sup>c</sup>	25.32 ± 0.09 <sup>d</sup>	92.23 ± 4.88 <sup>bc</sup>

Results are means of three determinations ± standard deviation.

Values in each column having the same superscript are not significantly different ( $p > 0.05$ ).

Peregrine exhibited the highest inhibition against DNA scission while Phoenix exhibited the lowest inhibition of 78.2% at 4mg/mL. It is important to note that no pro-oxidant activity was observed at any level for all the extracts, and all extracts showed a similar pattern of concentration dependence in protecting supercoiled plasmid DNA.

## **7.5 Conclusions**

It was determined that the major proportion of phenolic compounds is present in barley kernel as the insoluble-bound form. Insoluble-bound phenolic compounds contribute most followed by soluble conjugates toward TPC of barley. This finding is comparable with those reported in the literature for barley and other cereals such as wheat and rye. The antioxidant and antiradical activities of insoluble-bound phenolic fraction, in general, was higher than those of soluble conjugates and free phenolic fractions.

## CHAPTER 8

### SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

#### 8.1 Summary and conclusions

The optimum conditions for extraction of antioxidative compounds from barley, as determined by response surface methodology (RSM), were 80% aqueous methanol at 60°C for 40 min. The optimum parameters determined by RSM agreed with the experimental extractions.

Of the six whole barley kernel (Falcon, AC Metcalfe, Tyto, Tercel, Phoenix, and Peregrine) extracts tested, Peregrine yielded the highest total phenolic content (TPC) followed by AC Metcalfe and Falcon. TPC of Tercel and Phoenix were significantly lower compared to the other extracts. Whole kernel extracts of the six barley cultivars showed strong antioxidant and antiradical activities and they were comparable to those of wheat and rye reported in the literature (Zielinski and Kozłowska, 2000). In general, Peregrine kernel extract exhibited the highest antioxidant activity followed by Falcon and AC Metcalfe as determined by several *in vitro* assays employed in the second phase of the study. This might have been attributable to the higher total phenolic content (TPC) in these three extracts. TPC and TEAC showed a strong correlation, however, TPC did not show a good correlation with DPPH radical scavenging capacity, oxygen radical absorbance capacity (ORAC<sub>FL</sub>) and hydroxyl radical absorbance capacity (HORAC<sub>FL</sub>). The antioxidant efficacies measured by different assays produced different trends, although Peregrine, Falcon, and AC Metcalfe extracts exhibited the highest activity in most of the assays. Differential chemical composition of the barley extracts as well as the differential mechanisms of action of antioxidants in different assays might be responsible

for the observed differences in their reported activities. Peregrine, AC Metcalfe, and Falcon yielded the highest activity as determined by PCL. Peregrine and Falcon extracts exhibited the highest reducing power among the whole barley kernel extracts tested. In general, the activity of water-soluble antioxidative compounds was higher than their lipid-soluble counterparts among whole barley kernel extracts tested using photochemiluminescence (PCL). Tyto extract showed the highest  $ORAC_{FL}$  as well as  $ORAC_{FL}/TPC$  ratio. Although Peregrine exhibited the highest antioxidant activity, in general, the  $ORAC_{FL}/TPC$  ratio of Peregrine was moderate among the extracts tested. The correlation between  $ORAC_{FL}$  and  $HORAC_{FL}$  was poor and the different mechanisms involved in the two assays may explain the poor correlation observed. Metal chelation activity of barley cultivars, in general, was poor compared to many other foodstuffs, however, it was similar to the metal chelation ability of other cereals such as wheat. Falcon, Peregrine, and AC Metcalfe exhibited the highest metal chelation activity among the extracts tested while Tyto and Tercel extracts showed the lowest chelation. Whole kernel extracts tested showed strong activity in scavenging superoxide radical and hydrogen peroxide. Superoxide radical scavenging efficacy of the extracts was more or less in the same order of magnitude, however, substantial variation was observed among the extracts in their hydrogen peroxide scavenging efficacies. Peregrine and AC Metcalfe extracts exhibited the highest hydrogen peroxide scavenging activity while the other extracts showed a moderate activity. The activity of Peregrine towards scavenging of hydrogen peroxide was comparable to the efficacy shown by the reference antioxidant, ferulic acid.

In the bulk stripped corn oil model system barley extracts reduced the formation of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) by 10-31% and 27-44%, respectively, at the end of day 7 of incubation. In a  $\beta$ -carotene-linoleate model system whole kernel extracts rendered 63 to 76% protection against bleaching of  $\beta$ -carotene. In an accelerated oxidative study using Rancimat<sup>®</sup>, whole kernel extracts rendered a protection factor of 1.31 to 1.59 while the reference antioxidant, ferulic acid showed prooxidant activity (protection factor of 0.97). Whole kernel extracts exhibited strong activity in neutralizing DPPH and hydroxyl radicals as determined by electron paramagnetic resonance (EPR). The IC<sub>50</sub> value, which represents the concentration of extract needed to yield 50% of initial radical content was from 1.51-3.20 and 2.20 - 9.65, respectively, for DPPH and hydroxyl radicals. The IC<sub>50</sub> values for hydroxyl radical were higher than IC<sub>50</sub> values for DPPH radical, thus indicating that the scavenging efficacy against the latter was stronger. The tested barley cultivars also exhibited substantial protection against copper-induced LDL oxidation. Whole kernel extracts at 2 mg/mL concentration rendered 20 - 34% inhibition at the end of the incubation period. In early incubation stages, all extracts, except Peregrine, exhibited a prooxidant activity, however, they showed strong antioxidant activity during the latter stages of incubation. Whole kernel extracts exhibited strong activity against peroxy and hydroxyl radical-induced nicking of supercoiled double strand DNA. Barley extracts at 4 mg/mL concentration rendered 78 - 92% and 53 - 64%, respectively, protection against peroxy and hydroxyl radical induced-nicking. The protection rendered by the barley extracts was comparable to that exhibited by ferulic acid.

In a cell line study, whole kernel extracts displayed strong antiproliferative efficacy against Caco-2 adenocarcinoma cell proliferation. The effects of the extracts were not obvious during the day 1 of the study, however, the extracts rendered strong antiproliferative activity at the end of day 4 in a concentration dependent manner. Whole kernel extracts rendered 29 to 51% inhibition at 0.5 mg/mL concentration while 0.05 mg/mL concentration did not exhibit substantial activity.

In the third phase of the study, the distribution of antioxidants, in barley kernel was investigated by pearling Falcon and AC Metcalfe grains into seven fractions in a layer-wise fashion up to 50% of the kernel weight. Antioxidants were mainly concentrated in the outermost layers in both barley types. In general, the results of most of the *in vitro* assays indicated that the antioxidant efficacy gradually diminished from outer layers to inner layers. This was explained by the presence of bran, especially the aleurone layers in the first few fractions (F1 to F3), which were highly rich in phenolic compounds. The inner layers contained mainly endosperm, which did not contain high concentrations of phenolic compounds. Fraction 1 (F1), the outermost layer of both Falcon and AC Metcalfe cultivars yielded the highest TPC. Total phenolic content dropped by 62 and 42%, respectively, from F1 to F2. A similar trend was observed with TAC as well. TAC of F1 was 131 times higher than that of F7 with Falcon while F1 of AC Metcalfe was 81 times higher than that of F7. Removal of F1 from the barley kernel caused a 61 and 50% reduction in TAC, respectively, in Falcon and AC Metcalfe cultivars. TAC and DPPH radical scavenging capacity showed a strong correlation for all fractions tested. Reducing power of F1 was approximately 12 times higher than that of pearled grain in both Falcon and AC Metcalfe cultivars.

A PCL study revealed that the activity of water-soluble antioxidants was generally higher than that of their lipid-soluble counterparts. This was obvious in F1 and F2 of both cultivars, however, this trend was inconsistent among the rest of the fractions. The contents of water-soluble and lipid-soluble antioxidants were reasonably well correlated ( $r^2 = 0.79$ ) with each other while TEAC and water-soluble antioxidant activity were strongly correlated ( $r^2 = 0.99$ ) in the Falcon cultivar. However, the lipid-soluble antioxidant content was not well correlated with TEAC in both cultivars. The  $ORAC_{FL}$  value of F7 of the Falcon cultivar was 21 fold lower than that of F1 while the corresponding value for AC Metcalfe was 29. The overall average  $ORAC_{FL}$  value of Falcon was significantly ( $p < 0.05$ ) higher than that of AC Metcalfe.  $ORAC_{FL}$  values and antioxidant activity, as measured by PCL, were strongly correlated with each other ( $ORAC_{FL}$  and water soluble antioxidant activity:  $r^2 = 0.98$ ,  $ORAC_{FL}$  and lipid soluble counterparts;  $r^2 = 0.95$  for Falcon cultivar). The  $HORAC_{FL}$  of fraction 1 of Falcon and AC Metcalfe cultivars was approximately 15 and 12 times higher than that of the respective whole grain extracts. The dilution effect of the outer layers with the endosperm contents, which do not contain a substantial amount of antioxidative constituents yielded poor  $HORAC_{FL}$  values in whole grain extracts of both cultivars. Removal of F1 of Falcon and AC Metcalfe barley resulted in a 48% loss of  $HORAC_{FL}$  activity. Removal of F2 and F3 of the Falcon cultivar rendered 68 and 81% of  $HORAC_{FL}$  activity, respectively. The corresponding values for the AC Metcalfe cultivar were at 67 and 83%. Metal chelation activity of F1 of the Falcon extract was approximately 67 fold higher than that of F7 while the metal chelation capacity of F1 of AC Metcalfe was approximately 26 fold higher than that of F7. Metal chelation capacity gradually decreased from F1 to F7 in

extracts of both Falcon and AC Metcalfe cultivars while pearled grain did not render any measurable activity. The EPR study revealed that IC50 values for DPPH and hydroxyl radical were substantially low for F1-F5, indicating strong antiradical activity. However, extracts of F6, F7 and pearled grain did not exhibit any measurable activity at the concentrations used. Barley fractions also exhibited strong activity against inhibition of LDL cholesterol oxidation as well as peroxy and hydroxyl radical-induced DNA nicking.

In a Caco-2 colorectal cancer cell line study, Falcon barley fractions rendered 14 to 75% protection against cell proliferation while AC Metcalfe fractions rendered 21 to 57% protection. HPLC analysis revealed the presence of ferulic, vanillic, caffeic and *p*-coumaric acids in extracts of different barley fractions.

In the last phase of the study, it was revealed that a major proportion of phenolic compounds in barley kernel were present in the insoluble-bound form. Insoluble-bound phenolic compounds were most dominant followed by soluble conjugates in the TPC of barley. This finding lends further support to the literature reports for barley and other cereals such as wheat and rye. The antioxidant and antiradical activities of insoluble-bound phenolic fraction, in general, were higher than those of corresponding soluble conjugates and free phenolic fractions

## **8.2 Future directions**

While chemical studies and *in vitro* models are important in assessing the antioxidative potential, it is important to evaluate the extracts in *in vivo* systems as well. There is still controversy whether similar *in vitro* effects are attainable *in vivo* owing to the limited knowledge of absorption, bioavailability, and the mechanisms involved *in*

*vivo*. It is still unclear if the antioxidative compounds remain in the human body for sufficient time in an effective chemical form (Karakaya, 2004). Therefore, evaluation of the antioxidative extracts *in vivo* will provide a better insight into their efficiency in health promotion and disease risk reduction.

It is also important to evaluate the possible changes in antioxidant efficacy during thermal processing including roasting, cooking, and baking. Some reports indicate that thermal processing such as kilning may contribute to enhancement of antioxidant activity in barley (Maillard and Berset, 1995), however, there is little information available in the literature on which to base any final conclusion. Furthermore, it is important to study the effect of germination in possible changes on antioxidant activity of barley seeds.

## PUBLICATIONS ORIGINATED FROM THE STUDY

**Madhujith, T.** and Shahidi, F. Antioxidant and antiproliferative effects of barley (*Hordeum vulgare* L.) Submitted to *J. Pharm. Biol.*

**Madhujith, T.** and Shahidi, F. (2007) Antioxidative and Antiproliferative Properties of Selected Barley Cultivars and their Potential of Inhibition of Copper Induced LDL Cholesterol Oxidation. *J. Agric. Food Chem. In press* (Published on-line).

**Madhujith, T.** and Shahidi, F. (2006) Optimization of extracting antioxidative constituents of six barley cultivars and their antioxidant properties *J. Agric. Food Chem.*, 54, 8048-8057.

**Madhujith, T.,** Izydorczyk, M. and Shahidi, F. (2006) Antioxidant potential of pearled barley (*Hordium* species.) *J. Agric. Food Chem.* 54, 33283-3289.

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## **APPENDIX**

**Table A- 5.1** Effect of whole barley extracts on formation of conjugated dienes in a bulk stripped corn oil model system stored at 60°C.

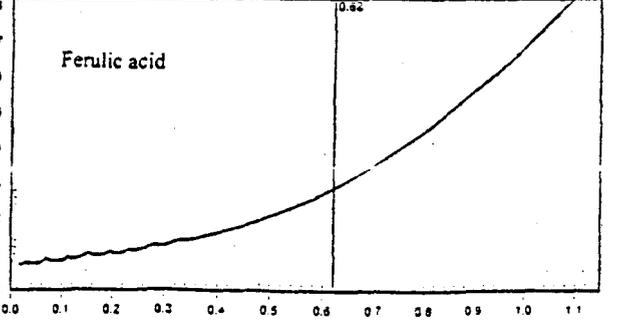
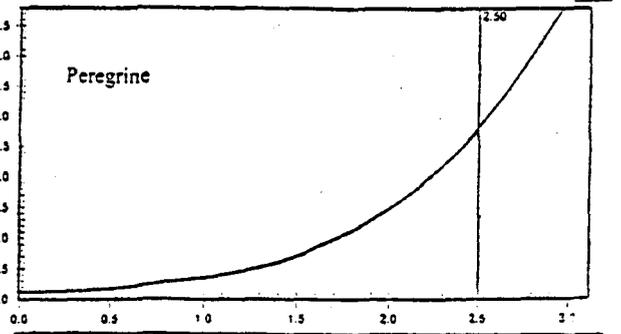
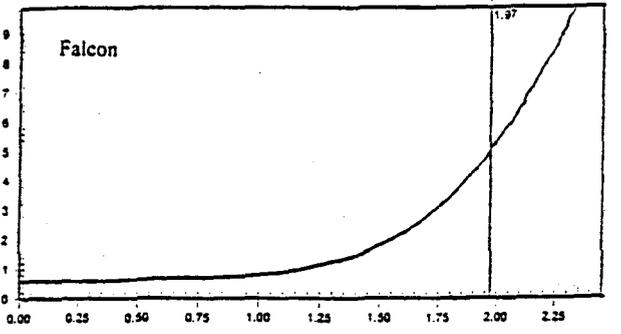
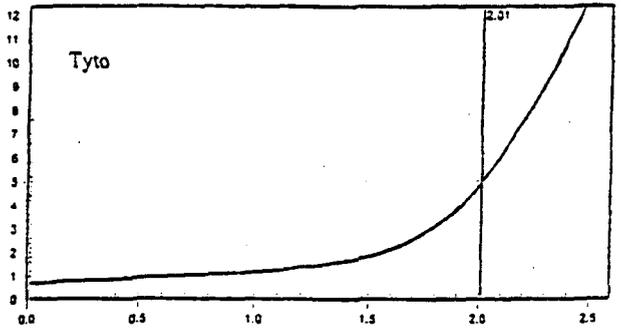
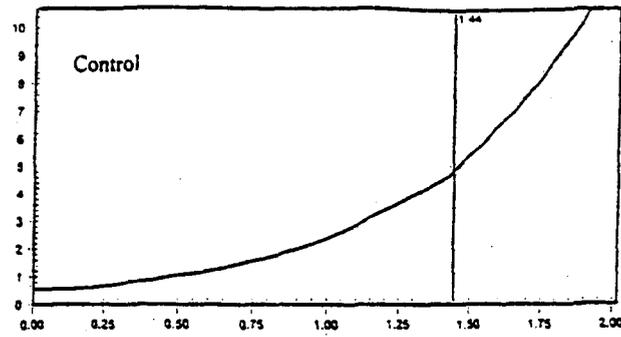
Sample identity	Conjugated diene				
	Day 0	Day 1	Day 3	Day 5	Day 7
Falcon	2.40 ± 0.02	2.77 ± 0.01	3.45 ± 0.00	5.03 ± 0.29	6.69 ± 0.01
ACMetcalf	2.40 ± 0.02	3.10 ± 0.08	4.09 ± 0.00	5.85 ± 0.06	6.31 ± 0.02
Tyto	2.40 ± 0.02	2.69 ± 0.03	3.30 ± 0.01	4.62 ± 0.00	5.96 ± 0.01
Tercel	2.40 ± 0.02	3.18 ± 0.02	3.78 ± 0.01	5.93 ± 0.12	6.17 ± 0.03
Phoenix	2.40 ± 0.02	3.40 ± 0.01	3.91 ± 0.04	5.75 ± 0.13	8.15 ± 0.01
Peregrine	2.40 ± 0.02	2.83 ± 0.01	3.16 ± 0.06	4.94 ± 0.02	5.82 ± 0.02
Ferulic acid	2.40 ± 0.02	4.54 ± 0.02	7.51 ± 0.01	13.07 ± 0.11	18.74 ± 0.04
Blank	2.40 ± 0.02	4.03 ± 0.03	4.20 ± 0.01	6.69 ± 0.07	8.50 ± 0.03

**Table A-5.2** Effect of whole barley extracts on formation of TBARS in a bulk stripped corn oil model system stored at 60°C.

Sample identity	TBARS ( $\mu\text{mol malonaldehyde/kg oil}$ )				
	Day 0	Day 1	Day 3	Day 5	Day 7
Falcon	136.38 $\pm$ 1.50	121.97 $\pm$ 8.21	179.80 $\pm$ 0.50	309.50 $\pm$ 4.27	398.06 $\pm$ 6.18
ACMetcalfé	136.38 $\pm$ 1.50	189.01 $\pm$ 9.65	219.90 $\pm$ 6.26	280.74 $\pm$ 39.3	328.57 $\pm$ 1.11
Tyto	136.38 $\pm$ 1.50	158.37 $\pm$ 8.68	198.75 $\pm$ 1.29	236.72 $\pm$ 7.65	325.73 $\pm$ 19.5
Tercel	136.38 $\pm$ 1.50	168.49 $\pm$ 18.1	293.78 $\pm$ 2.74	336.99 $\pm$ 28.3	421.70 $\pm$ 0.54
Phoenix	136.38 $\pm$ 1.50	147.84 $\pm$ 0.08	283.01 $\pm$ 5.01	281.96 $\pm$ 8.63	407.47 $\pm$ 2.26
Peregrine	136.38 $\pm$ 1.50	154.15 $\pm$ 1.77	188.83 $\pm$ 8.32	281.28 $\pm$ 19.2	329.83 $\pm$ 22.5
Ferulic acid	136.38 $\pm$ 1.50	309.98 $\pm$ 42.5	397.51 $\pm$ 1.50	482.17 $\pm$ 4.90	621.04 $\pm$ 11.4
Blank	136.38 $\pm$ 1.50	144.65 $\pm$ 2.45	266.57 $\pm$ 2.55	422.24 $\pm$ 1.91	495.38 $\pm$ 2.9

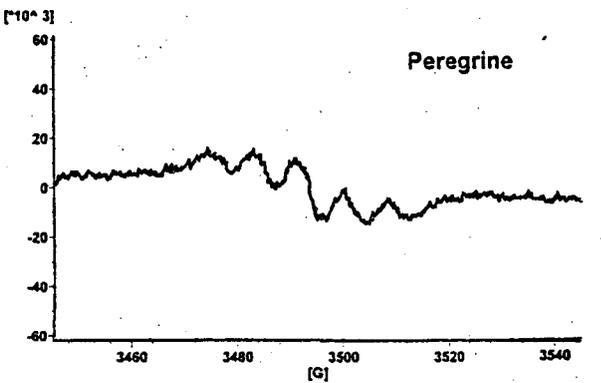
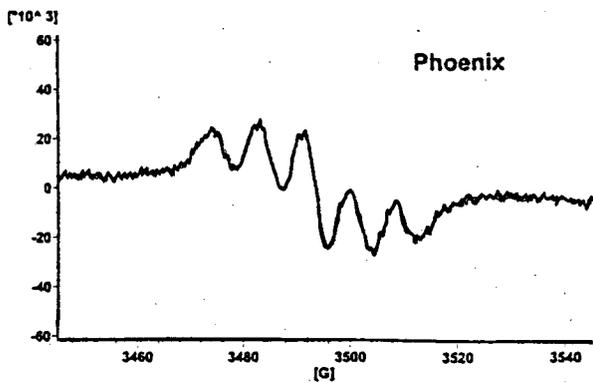
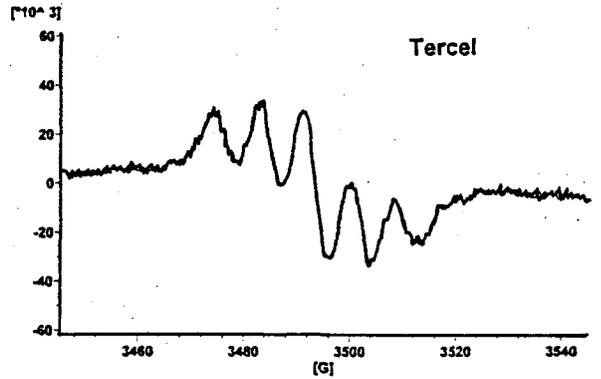
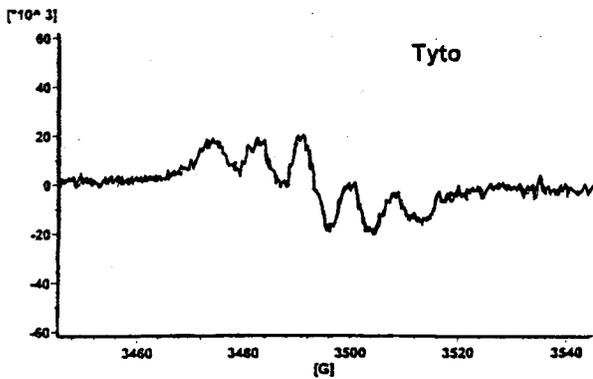
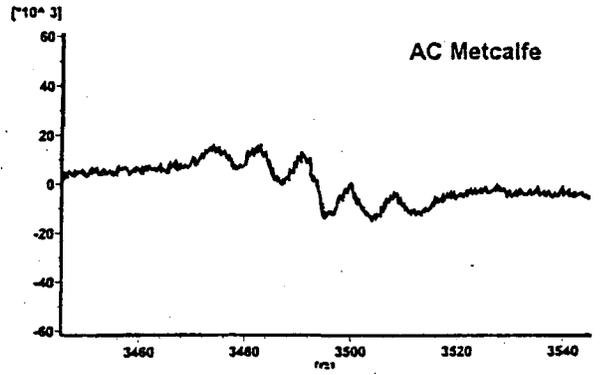
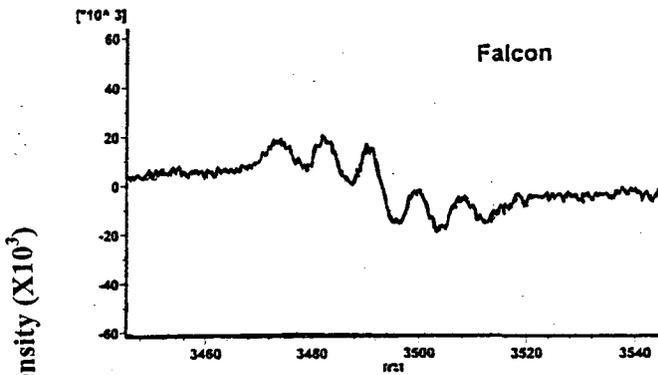
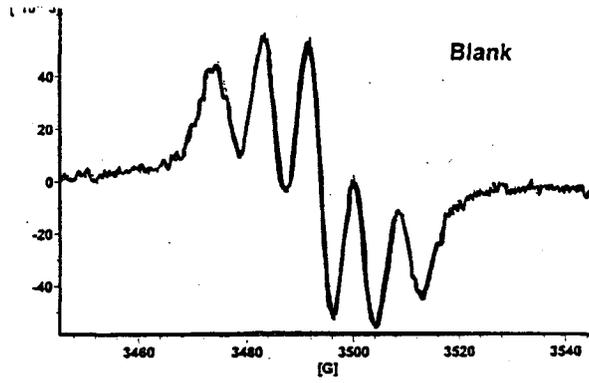
**Figure A-5.3** Representative plots illustrating the change of conductivity of the solution in collection vessels of Rancimat® resulted from accelerated oxidation of 3 g of stripped corn oil in the presence of 60 mg of whole kernel barley extracts.

Conductivity of solution in collection vessel ( $\mu\text{S}/\text{cm}$ )



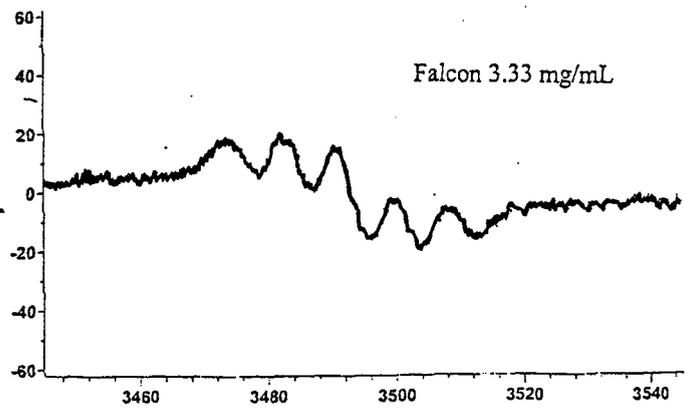
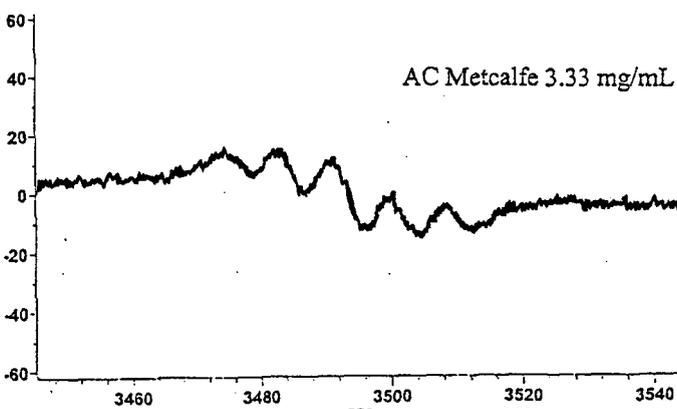
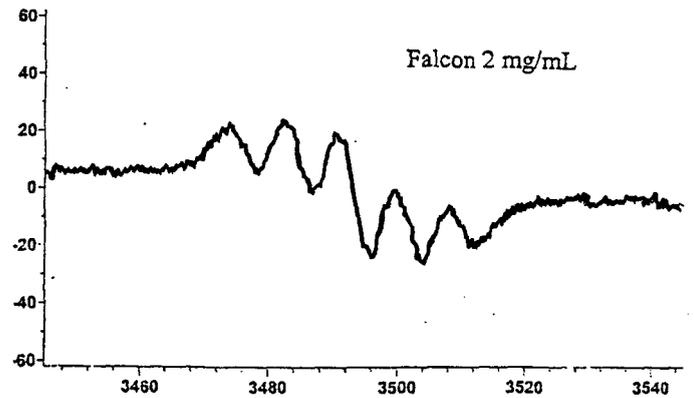
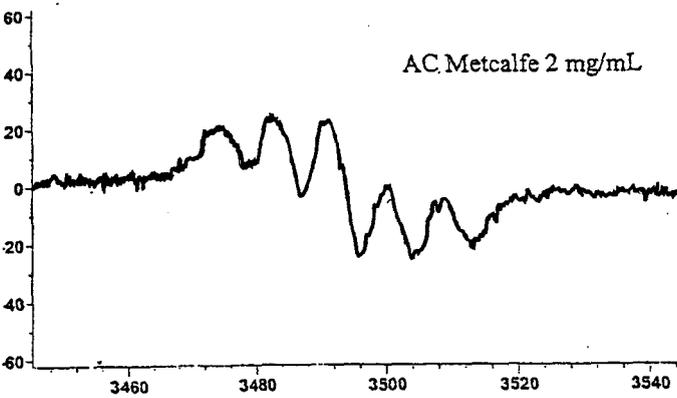
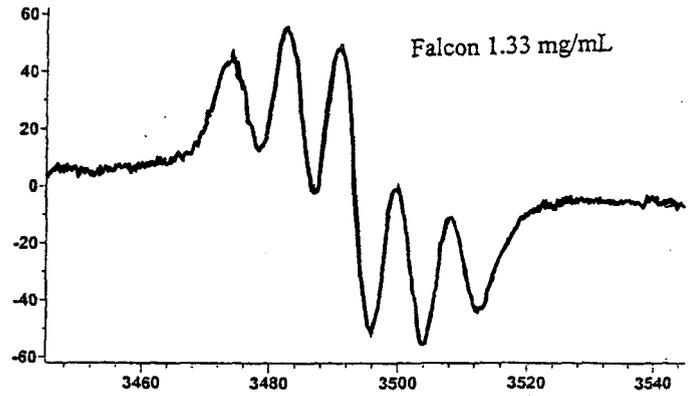
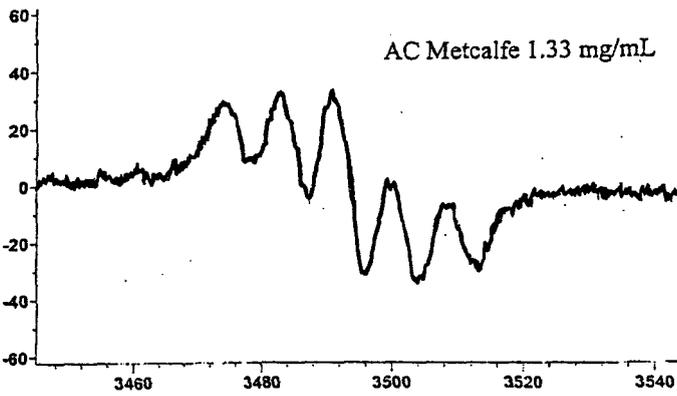
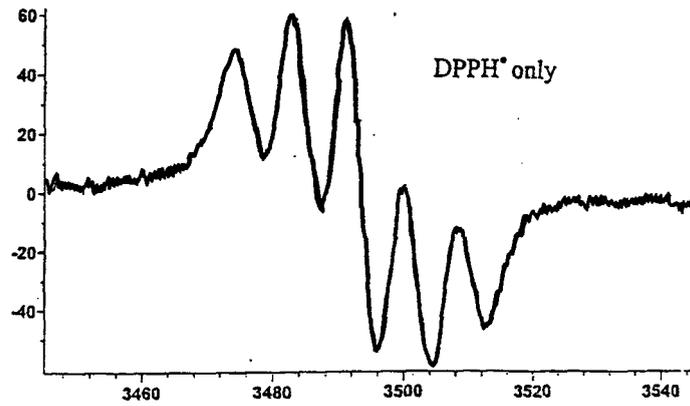
Oxidation period (h)

**Figure A-5.4** EPR spectra obtained for DPPH<sup>•</sup> in the presence of whole kernel extracts at 2.67 mg/mL concentration.



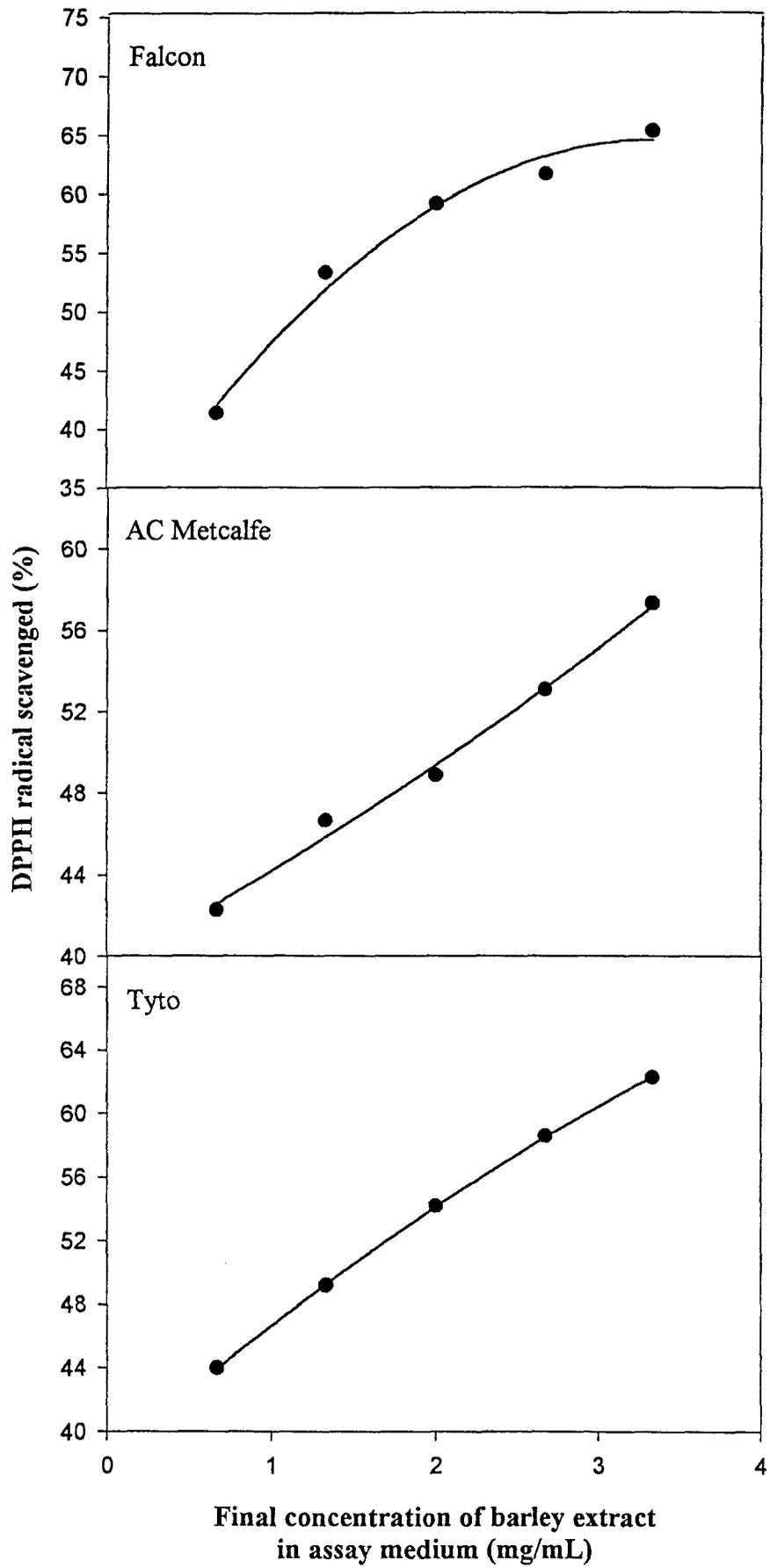
Magnetic field (G)

**Figure A-5.5** EPR spectra obtained for DPPH<sup>•</sup> in the presence of whole kernel Falcon and AC Metcalfe extracts at various concentrations.

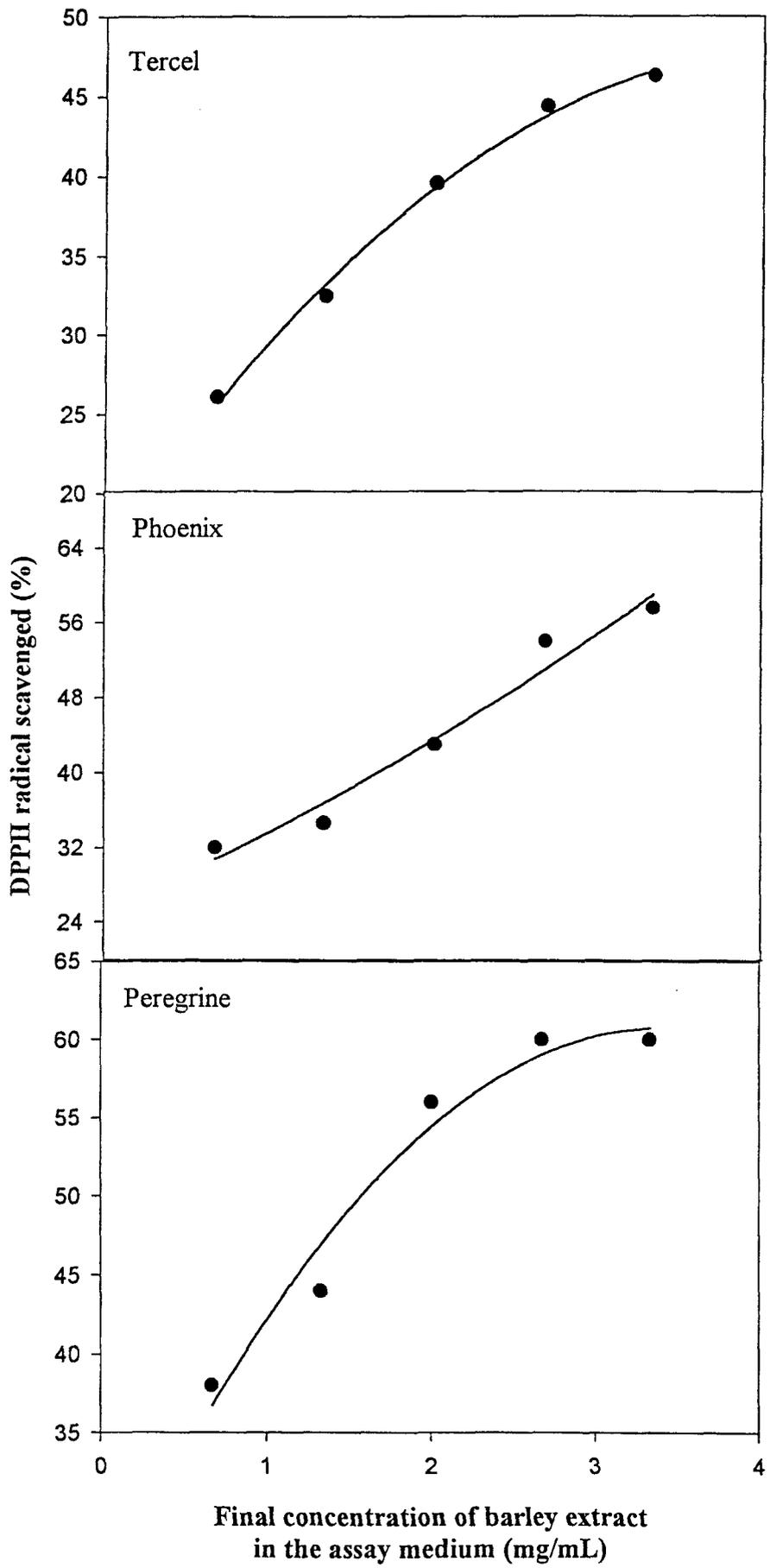


Magnetic field (G)

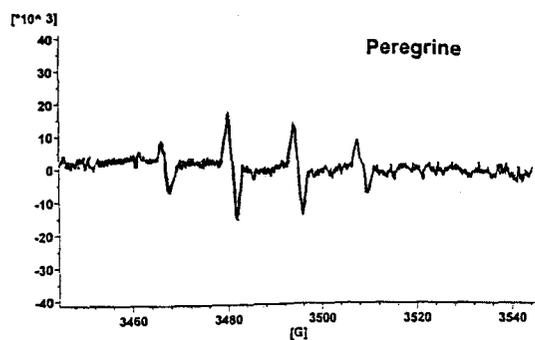
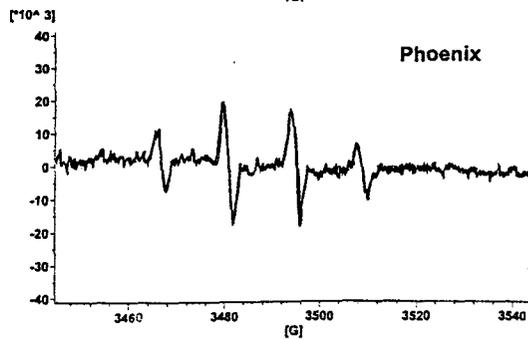
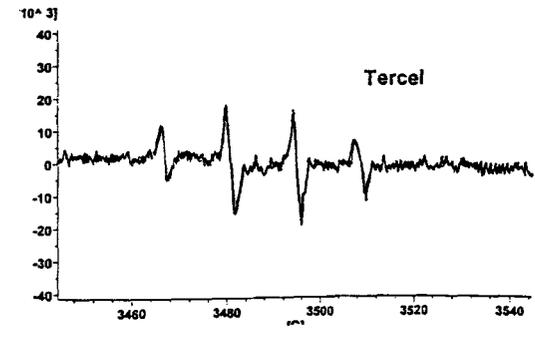
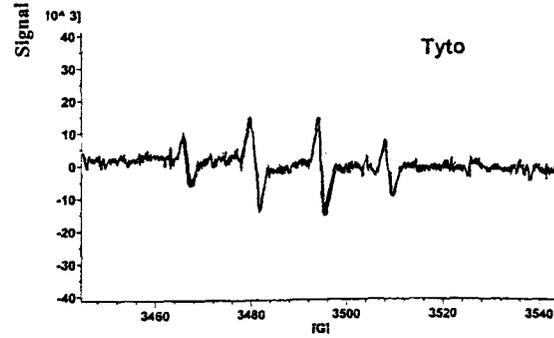
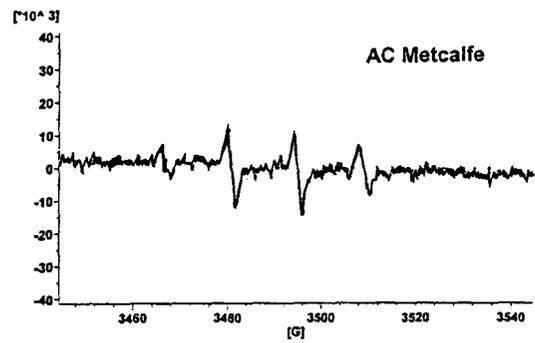
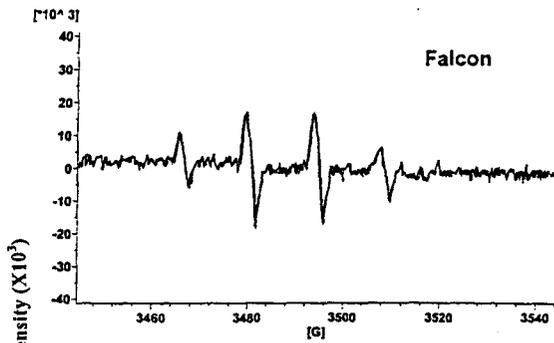
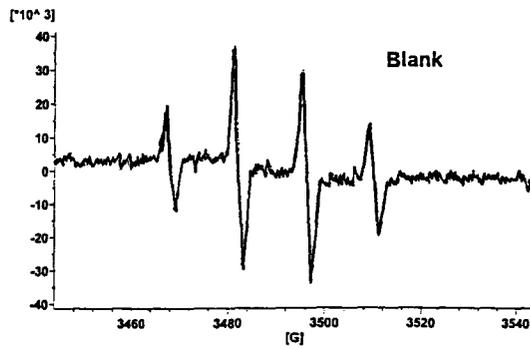
**Figure A-5.6** Concentration dependence of DPPH<sup>•</sup> scavenging by Falcon, AC Metcalfe, and Tyto whole kernel extracts determined by EPR spectrometry.



**Figure A- 5.7** Concentration dependence of DPPH<sup>•</sup> scavenging by Tercel, Phoenix, and Peregrine whole kernel extracts determined by EPR spectrometry.



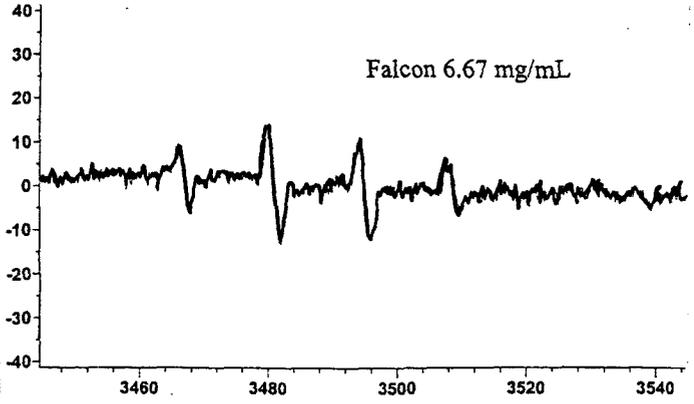
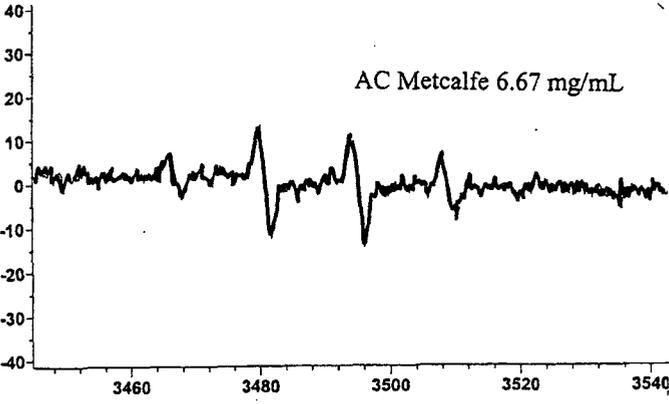
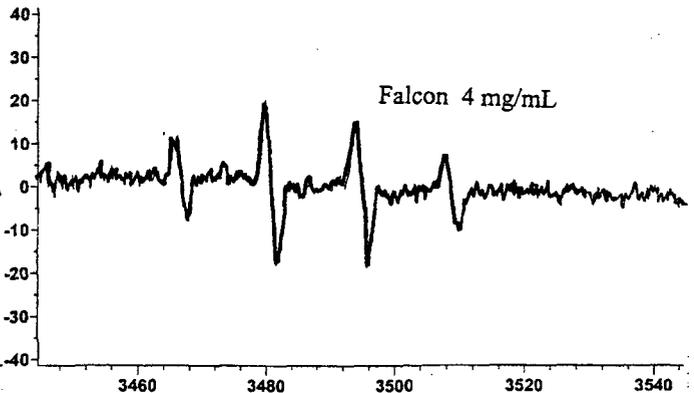
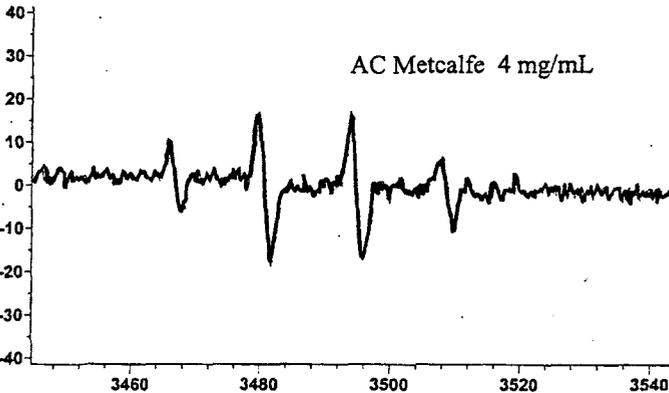
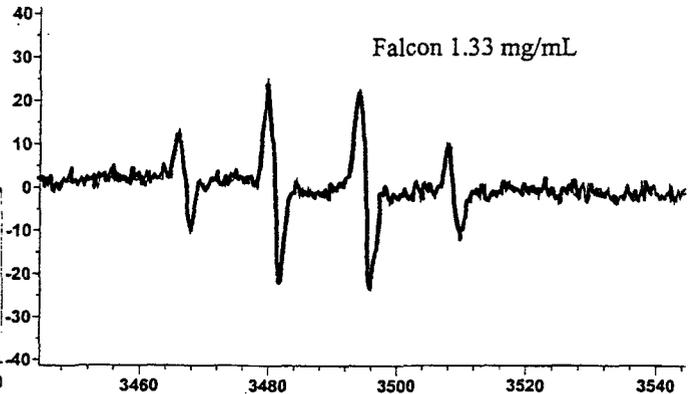
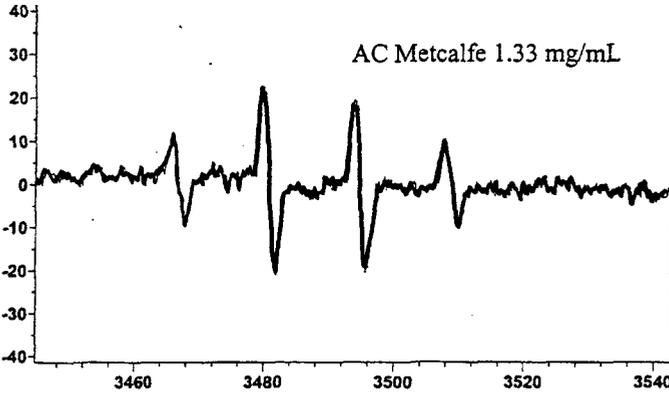
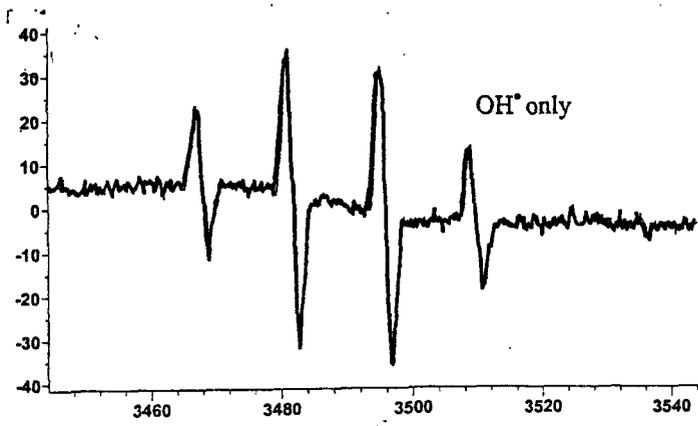
**Figure A-5.8** EPR spectra obtained for  $\cdot\text{OH}$  in the presence of whole kernel extracts at 6.67 mg/mL concentration.



Magnetic field (G)

**Figure A-5.9**

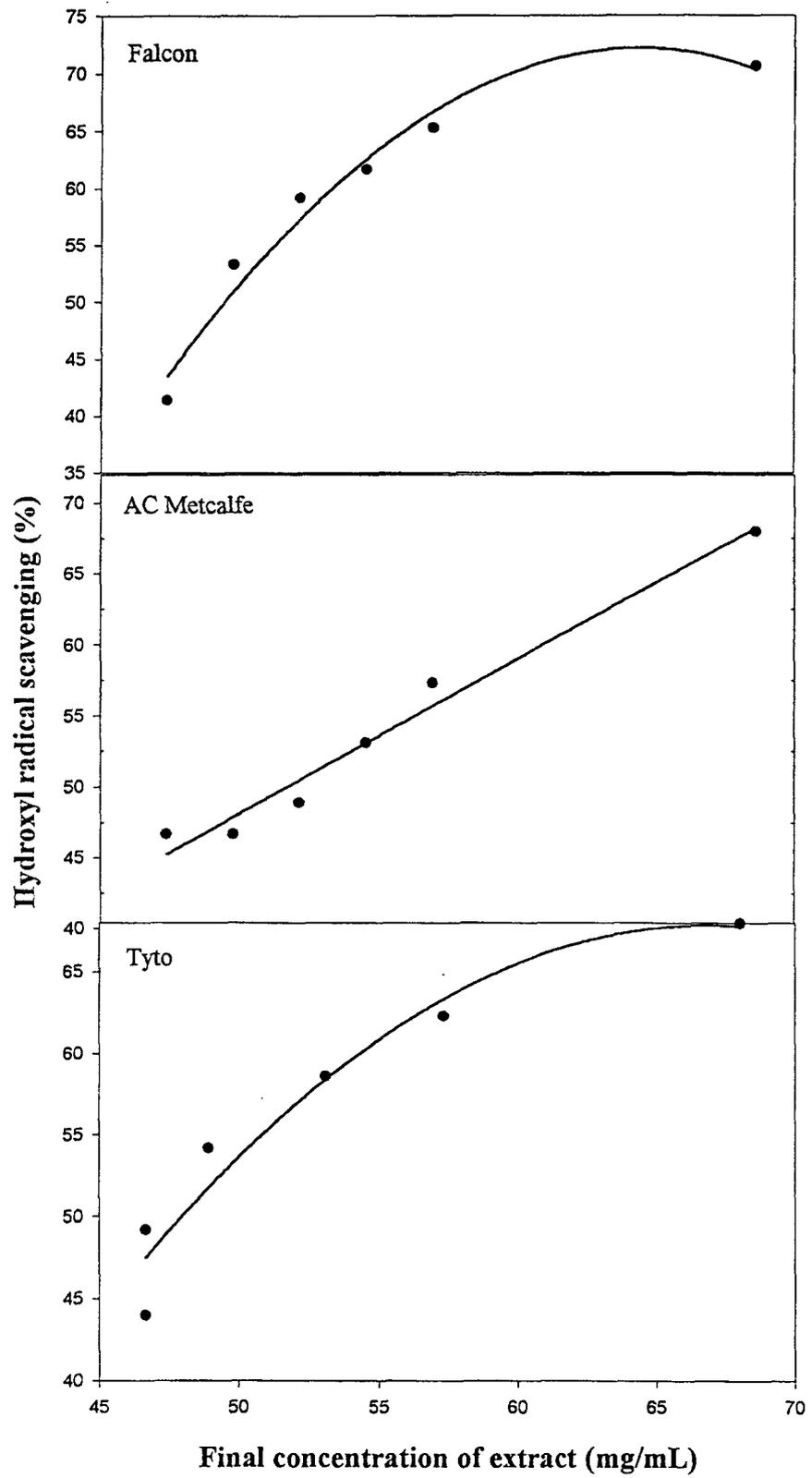
EPR spectra obtained for  $\cdot\text{OH}$  in the presence of whole kernel Falcon and AC Metcalfe extracts at various concentrations.



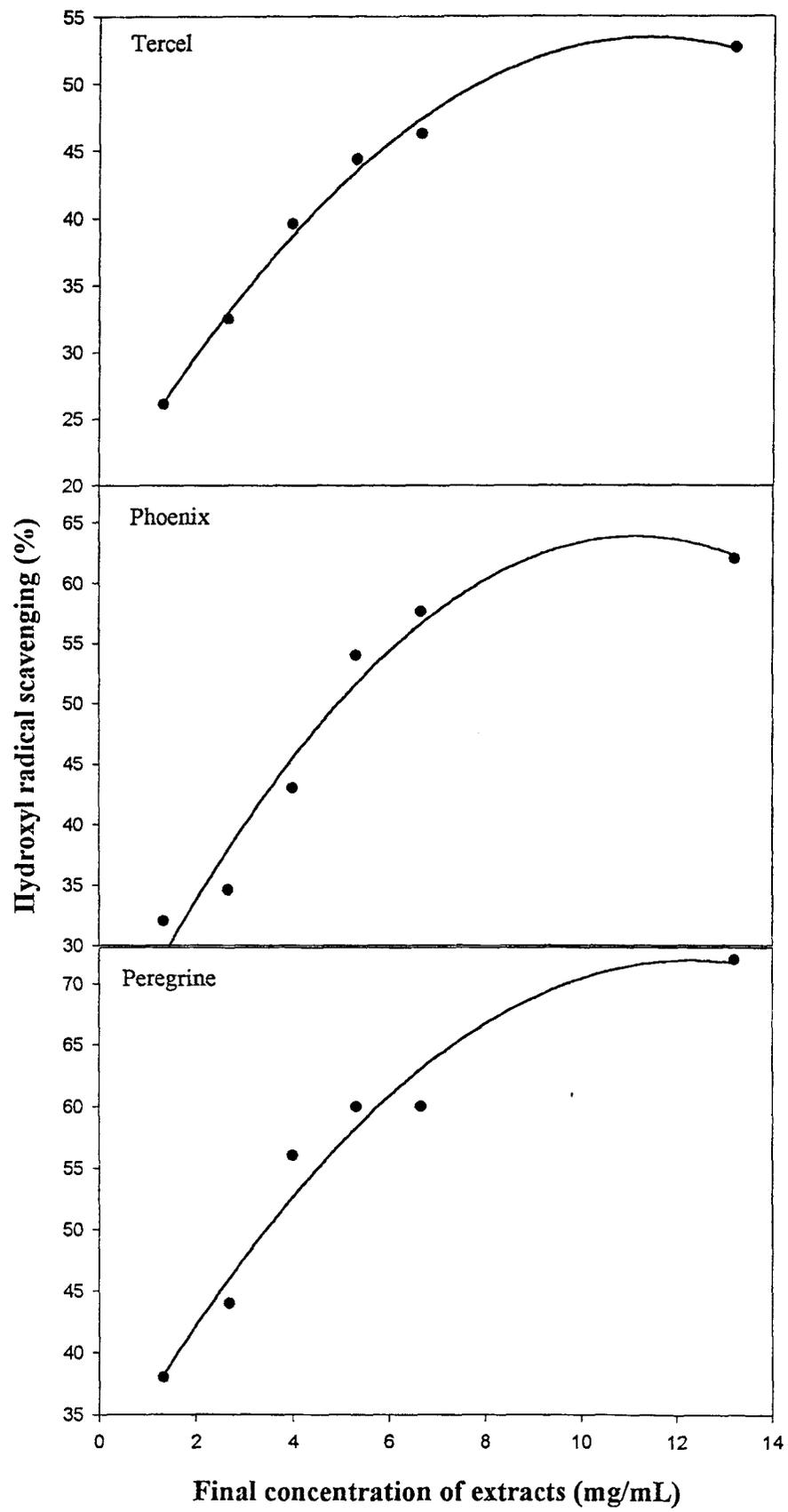
Signal intensity ( $\times 10^3$ )

Magnetic field (G)

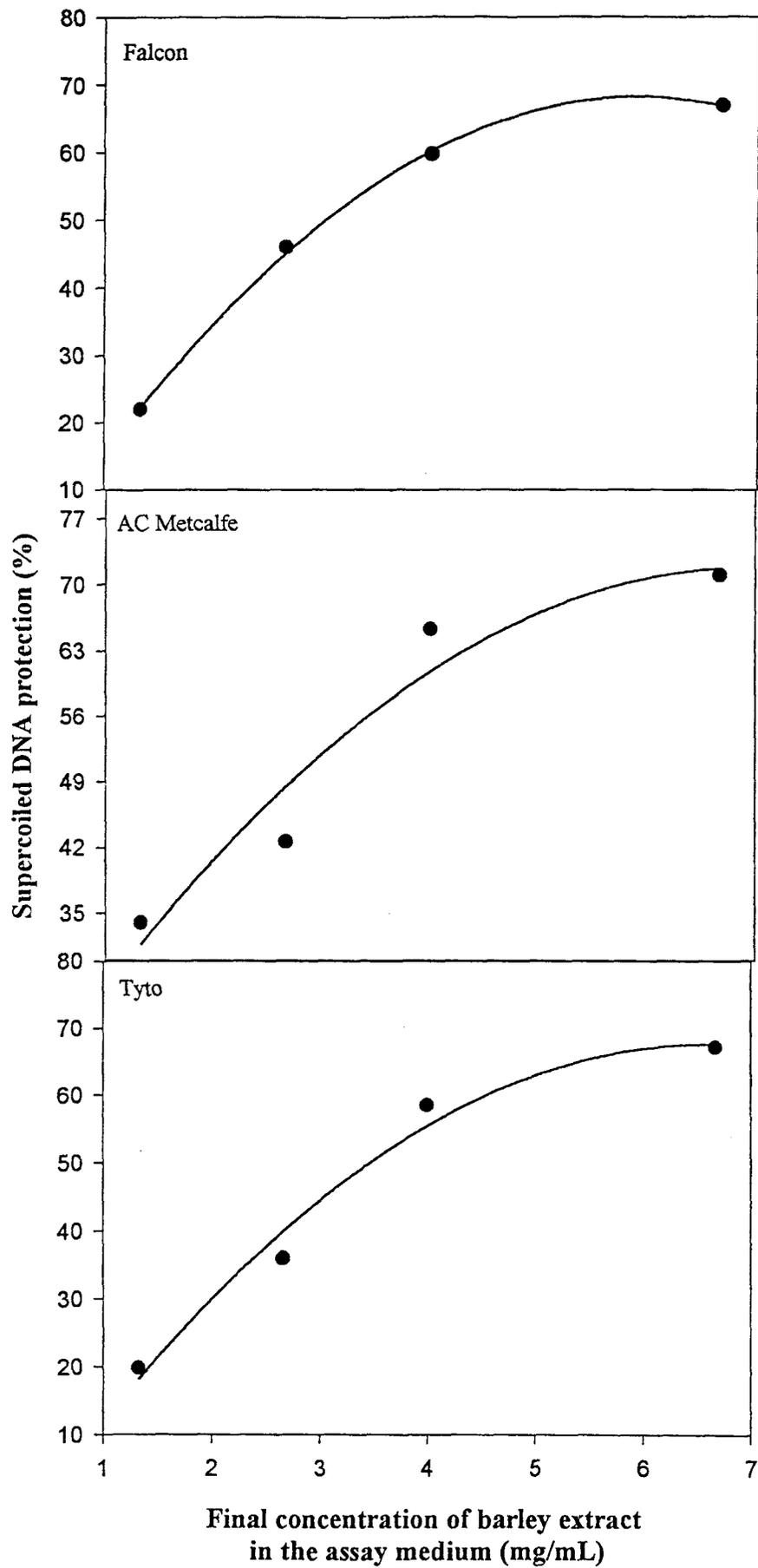
**Figure A-5.10** Concentration dependence of  $\cdot\text{OH}$  scavenging by Falcon, AC Metcalfe, and Tyto whole kernel extracts as determined by EPR spectrometry.



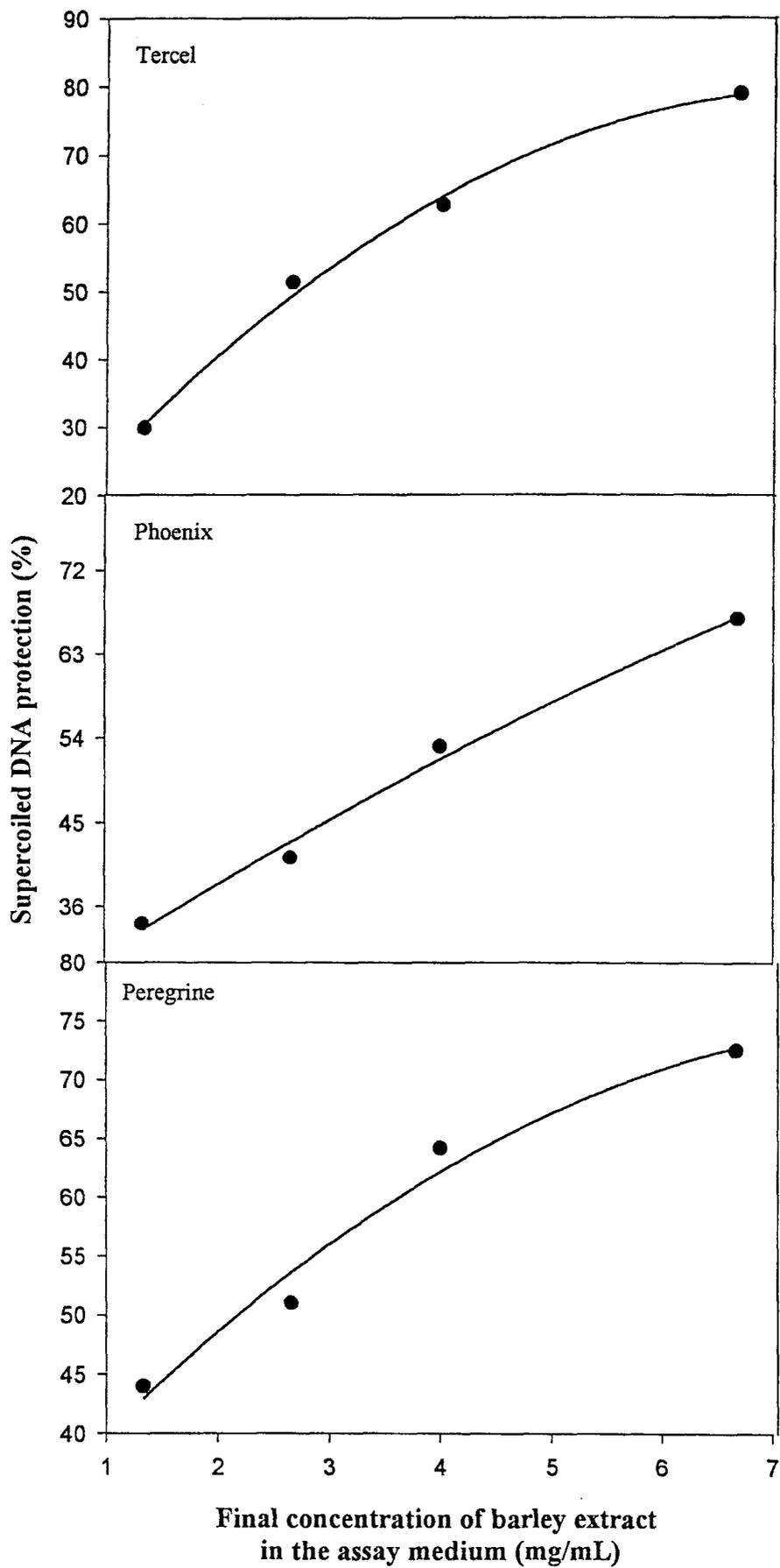
**Figure A-5.11** Concentration dependence of  $\cdot\text{OH}$  scavenging by Tercel, Phoenix, and Peregrine whole kernel extracts determined by EPR spectrometry.



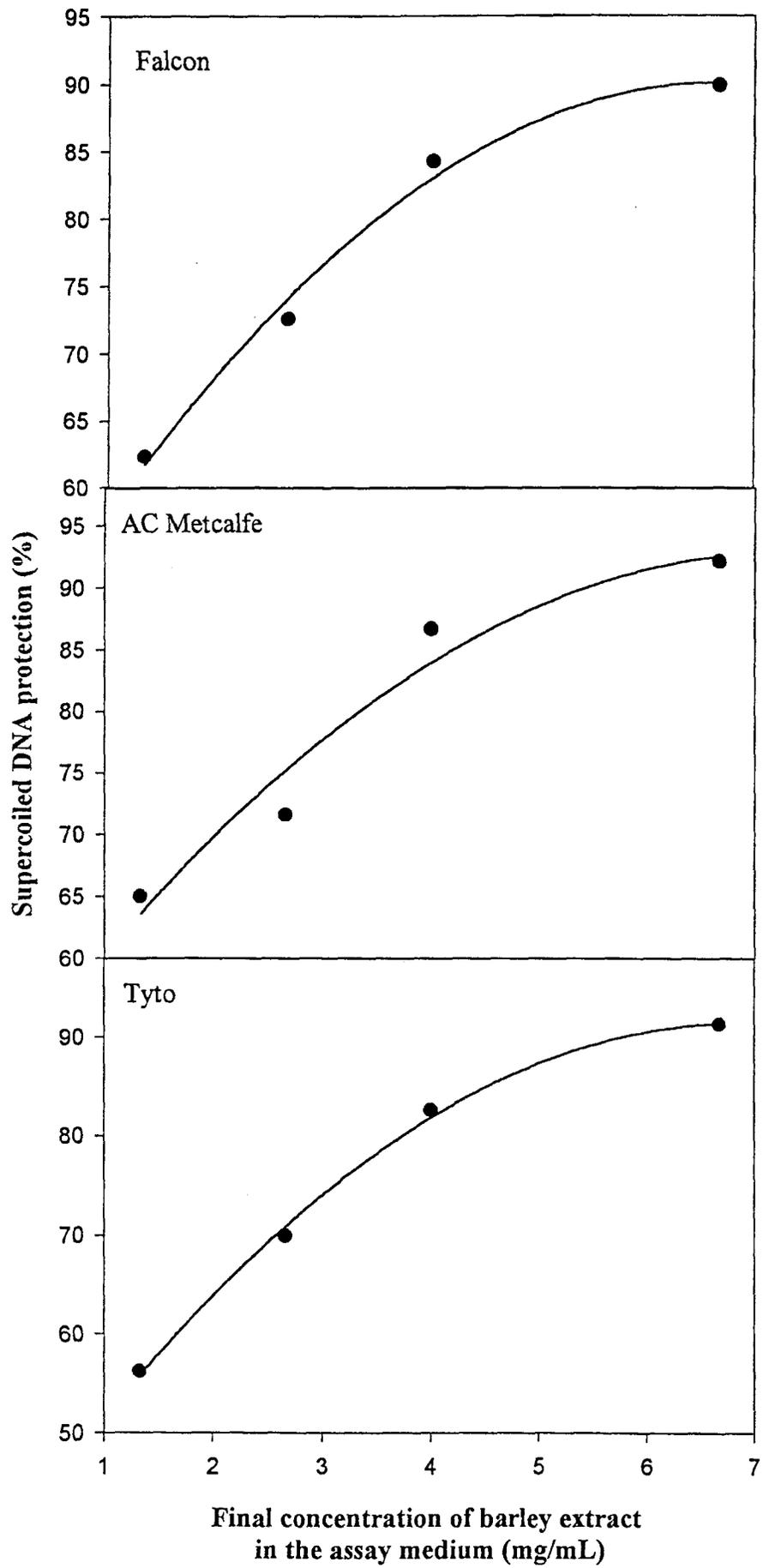
**Figure A-5.12** Concentration dependence of protection capacity (%) of supercoiled DNA against peroxy radical-induced nicking by whole kernel extracts of Falcon, AC Metcalfe, and Tyto.



**Figure A-5.13** Concentration dependence of protection capacity (%) of supercoiled DNA against peroxy radical-induced nicking by whole kernel extracts of Tercel, Phoenix, and Peregrine.



**Figure A-5.14** Concentration dependence of protection capacity (%) of supercoiled DNA against hydroxyl radical-induced nicking by whole kernel extracts of Falcon, AC Metcalfe, and Tyto.



**Figure A-5.15** Concentration dependence of protection capacity (%) of supercoiled DNA against hydroxyl radical-induced nicking by whole kernel extracts of Tercel, Phoenix, and Peregrine.

