EXTRACTION, DISTRIBUTION, CHARACTERIZATION AND BIOACTIVITIES OF PHENOLICS IN MILLET GRAINS

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Extraction, distribution, characterization and bioactivities of phenolics in millet grains

By

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I dedicate this work, to the memory

of my beloved father Gamage Dharmadasa

Abstract

Seven millet varieties grown in Sri Lanka (proso, foxtail, finger (Ravi), finger (local), kodo, and little) and India (pearl) were used in this study. Soluble and bound phenolics obtained by solvent extraction were used to evaluate antioxidant, DNA scission and enzyme inhibitory and antiproliferative activities by several chemical and biological methods. Distribution of phenolics and their antioxidant activity was assessed in dehulled grains and hulls separated by air classification. Phenolic compounds in free, soluble conjugates and insoluble bound fractions were identified and quantified. In addition, accessibility of phenolics in the digestive tract was determined using a simulated *in vitro* digestion model and colonic fermentation. The contents of total phenolics (TPC), total flavonoids (TFC) and proanthocyanidins (PC) as well as scavenging activities of test compounds were evaluated using superoxide, hydroxyl, peroxyl, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, H_2O_2, and singlet oxygen. Different concentrations of extract were used to determine their IC_{50} values. In addition, the ferrous chelating and reducing activities of extracts were measured. The highest phenolic content of soluble phenolics of millet grains was obtained with 70% (v/v) acetone for 25 minutes under reflux conditions. The TPC of soluble and insoluble bound phenolic fractions of millet varieties were 7.2 - 32.4 and 2.2-81.6 μmol ferulic acid eq/g defatted meal, respectively. This demonstrates that bound phenolics are important contributors to antioxidant potential of millets. Kodo millet showed the highest soluble phenolic content followed by finger (local), finger (Ravi), little, foxtail, pearl and proso millets. Millets with dark brown pigmented testa and pericarp possessed a higher soluble phenolic content than those with white or yellow testa and pericarp. The TFC of soluble and insoluble extracts were 1.2-33.7 and 0.3-

4.5 μmol catechin eq (CE)/g defatted meal, respectively. The soluble extracts of finger (local) millet had the highest PC (311.28 μmol CE/g defatted meal). All varieties exhibited effective radical, H_2O_2 and singlet oxygen inhibition, reducing activity as well as ferrous ion chelating power which varied according to the assay employed and the variety used. Several hydroxybenzoic and hydroxycinnamic acids and flavonoids were identified and quantified by HPLC analysis. In general, ferulic and *p*-coumaric were found to be the major phenolic acids followed by protocatechuic, caffeic, sinapic, *p*-hydroxybenzoic, gentisic, and vanillic acids and their contents depended on the variety and the form present in the grain matrix. Flavonoids identified were flavones, flavan-3-ol monomers and dimers, and flavanonols. All seven varieties effectively inhibited oxidation in a pork model system, and stripped corn oil. At a concentration of 0.5 mg/ml all extracts inhibited oxidation by 16-88% in the linoleic acid emulsion system. All phenolic extracts inhibited Cu (II)-induced human LDL and liposome oxidation and DNA scission. Millet extracts at 0.05 mg/mL inhibited LDL cholesterol oxidation by 1-41% and exhibited a dose-dependent inhibition of DNA scission. The antiprolifeative studies showed that kodo millet extracts inhibited HT-29 cells proliferation in the range of 75-100% after 4 days of incubation. The hulls from dehulled millets had higher TPC and antioxidant activity compared to their whole, dehulled and cooked grain counterparts. The phenolic extracts of dehulled millet grains, following *in vitro* enzymic digestion under simulated gastrointestinal conditions and colonic fermentation, showed effective antioxidant activity and were accessible under physiological conditions.

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List of Abbreviations

AAC	antioxidant activity coefficient
AAPH	2,2`-azobis-(2-methylpropionamidine)dihydrochloride
ABTS	2,2´-azinobis-(3-ethylbenzothiazoline-6-sulphonate)
ANOVA	analysis of variance
AQE	aqueous extracts
AUC	area under the kinetic curve
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CD	conjugated dienes
CE	catechin equivalents
CID	collision induced dissociation
CRC	colorectal cancer
DAD	diode array detector
diFA	diferulic acids
DMPO	5,5-dimethyl-1-1-pyrroline-*N*-oxide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DP	degrees of polymerization
DPN	*N,N*- dimethyl *p*-nitrosoaniline
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRSA	DPPH radical scavenging activity
EFM	extracts of colonic fermentation
EGD	extracts of gastric digestion
EGID	extracts of gastrointestinal digestion
EPH	extracts after simulated gastrointestinal pH conditions
EPR	electron paramagnetic resonance
ESI	electrospray ionization
FAE	ferulic acid equivalents
HASA	hypochlorous acid scavenging activity
HBAS	hydroxybenzoic acids and derivatives
HCAS	hydroxycinnamic acids and derivatives

$HOO^{\cdot}$	hydroperoxyl radical
HPLC	high performance liquid chromatography
HPSA	hydrogen peroxide scavenging activity
LDL	low density lipoprotein
MDA	malondialdehyde
MS	mass spectrometry
MS^n	tandem mass spectrometry
Na_3EDTA	ethylenediaminetetraacetic acid trisodium salt
$O_2^{\cdot -}$	superoxide radical
ORAC	oxygen radical absorbance capacity
$^1O_2$	singlet oxygen
$^{\cdot}OH$	hydroxyl radical
PBS	phosphate buffer solution
PC	proanthocyanidins content
PF	protection factor
PG	propyl gallate
$RO^{\cdot}$	alkoxyl radical
$ROO^{\cdot}$	peroxyl radical
ROS	reactive oxygen species
RP	reducing power
SCFA	short chain fatty acids
SCO	stripped corn oil
SOSA	singlet oxygen scavenging activity
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TBHQ	*tert*-butylhydroquinone
TCA	trichloroacetic acid
TEAC	trolox equivalent antioxidant capacity
TFC	total flavonoid content
TPC	total phenolic content
UV	ultra violet
XO	xanthine oxidase

CHAPTER 1

Introduction and overview

1.1 Introduction

Millet is referred to a group of small-grained annual cereal grasses belonging to the family *Poaceae*. Of the total millet produced in the world about 90% is utilized in the developing countries. The global millet production was about 27 million tonnes in 2009 (*1*). Countries in African and Asian continents produced 56 and 41 % of the total world production, respectively. Contribution of millets to cereal production was 1%, but their vital importance as food crops with respect to the agro-ecosystems is significant (*1*). It is estimated that about two thirds of millets produced are consumed as food while the rest is used for planting seeds, animal feed, beer and bird seeds (*2*). Whole grains serve as recognized sources of many health-promoting components such as dietary fibre, vitamins, minerals and phytochemicals; the latter include phenolic compounds.

Plant phenolics are secondary metabolites composed of an aromatic ring bearing one or more hydroxyl groups, possibly together with a number of other substituents. Phenolic compounds contribute to different attributes in foods, including stability against lipid oxidation. Furthermore, phenolic compounds may act as antioxidants to protect against chronic diseases in which oxidative stress is a major contributory factor in their aetiology. The potency of phenolic compounds to act as antioxidants may arise from their ability to donate hydrogen atoms from hydroxyl groups attached to the benzene ring to free radicals and in turn form a resonance stabilized, less reactive phenoxyl radical. Plant phenolic compounds also act as

reducing agents, singlet oxygen quenchers and metal ion chelators (*3*). In addition, some investigations have shown that protective role of phenolics in health and wellness may be attributed to the activation or repression of particular genes via transcription factors (*4, 5*).

There are different classes of phenolic compounds in plants and the most common ones associated with whole grains are phenolic acids and to a lesser extent flavonoids (*3*). In general, phenolic compounds in grains exist in the free, soluble-conjugates and insoluble-bound forms and their proportions vary with the type of grain. Studies have shown that the bran of cereals consist mainly of ferulic acid and its oxidatively coupled products, the diferulates (*6*). The flavonoids reported in cereal grains include flavonol, flavones, flavan -3-ols and anthocyanins (*3*).

1.2 Overview

Cereals are staple foods for many people in the world. Cereal grains contribute a significant amount of energy, protein, selected micronutrients and non-nutrients in the diet of populations all over the world in both developed and developing countries (*7*). Cereal and cereal based food products provide more than 56% of the energy and 50% of the protein consumed worldwide (*7*). Economically, important cereals in the world are maize, rice, wheat, barley, sorghum, millets, oat and rye (*8*).

Table 1.1 Scientific and common names of major types of millets

Scientific name	Common name[a]	Taxonomy (Tribe)
Pennisetum glaucum	Pearl, bajra, cattail, bulrush, candlestick, sanyo, munga, seno	*Paniceae*
Elusine coracana	Finger, ragi, African,bird's foot, rapoko, Hunsa, wimbi, bulo, telebun, koracan, kurakkan	*Eragrostideae*
Setaria italica	Foxtail, Italian, German, Hungarian, Siberian, kangani, navane, thanahal	*Paniceae*
Panicum milliaceum	Proso, common, hog, broom, samai, Russian, panivarigu, panic, maha meneri	*Paniceae*
Panicum sumatrense	Little, blue panic, heen meneri	*Paniceae*
Paspalum scrobiculatum	Kodo, varagu, bastard, ditch, naraka, water couch, Indian paspalum, creeping paspalum, amu	*Paniceae*
Echinochola crus-galli	Barnyard, Japanese, sanwa, sawan, Korean, kweichou	*Paniceae*
Eragrostis tef	Teff, Abyssinian lovegrass	*Eragrostideae*
Digitaria exilis	Fonio, fundi, hungry rice, acha, crabgrass, raishan	*Paniceae*

(Source: Data adapted from *9 and 10).* [a] Underlined common names are used throughout the thesis.

Pearl millet (*Pennisetum glaucum*)

Proso millet (*Panicum miliaceum*)

Kodo millet (*Paspalum scrobiculatum*)

Foxtail millet (*Setaria italica*)

Finger millet (*Eleusine coracana*)

Little millet (*Panicum sumatrense*)

Plate 1.1 Major Millet types

Table 1.2 Nutrient composition of major cereals and millets (per 100g of edible portion at 12% moisture)

Cereal	Energy (kcal)	CHO (g)	Protein (g)	Fat (g)	CF (g)	Calcium (mg)	Iron (mg)	Niacin (mg)
Rice (brown)	362	76.0	7.9	2.7	1.0	33.0	1.8	4.3
Wheat	348	71.0	11.6	2.0	2.0	30.0	3.5	5.1
Maize	358	73.0	9.2	4.6	2.8	26.0	2.7	3.6
Sorghum	329	70.7	10.4	3.1	2.0	25.0	5.4	4.3
Pearl millet	363	67.0	11.8	4.8	2.3	42.0	11.0	2.8
Finger millet	336	72.6	7.7	1.5	3.6	350	3.9	1.1
Foxtail millet	351	63.2	11.2	4.0	6.7	31.0	2.8	3.2
Proso millet	364	63.8	12.5	3.5	5.2	8.0	2.9	4.5
Little millet	329	60.9	9.7	5.2	7.6	17.0	9.3	3.2
Kodo millet	353	66.6	9.8	3.6	5.2	35.0	1.7	2.0

Abbreviations are CHO-carbohydrates; CF-crude fibre

(Source: Data adapted from reference *2*)

1.2.1 Millets

Millet is a generic name that includes several small seeded cereals. They do not belong to a single species or a single genus. The major millet type in terms of world production is pearl millet (*Pennisetum glaucum*) which accounts for about 46% (*11*). A number of other minor millets exist, namely finger, proso, foxtail, kodo, little, Japanese barnyard, fonio, and teff millets. **Plate 1.1** shows some of the major millet species consumed as staple foods in the world and **Table 1.1** presents common names

of millets used in different countries and their taxonomy. In general, they have small kernels and hence are grouped as millets. The word millet is derived from the French word "mille" which means thousand, implying a handful of millets may contain thousands of grains (*10*).

Nutritionally, millets are equivalent to other cereal grains (*2*). The protein content ranges from 7.7 to 11.8% among different millet grains and is comparable with other cereals such as maize, rice, and wheat (**Table 1.2**). Except finger millet, other millet types have higher fat content ranging from 3.5 to 5.2 % compared to other cereals. Millets are rich in iron and phosphorus. In addition, finger millet has a high calcium content of 350 mg/100 g. In general, all millets are high in fibre (*2*).

Millets are important subsistence crops in semi-arid and tropical regions in Asia and Africa due to their resistance to pests and diseases, short growing season, ability of thriving in less fertile soils and productivity under heat and drought conditions (*2*). However, in the USA pearl millet grows as summer grazing and hay crops while proso millet is cultivated for grains in the Great Plains mainly in Dakotas, Colorado and Nebraska (*12*). The significant millet crop in Canada is proso millet cultivated in Canadian prairies (Alberta, Saskatchewan and Manitoba) and in Southwestern Ontario.

The main uses of millet grains in the USA and Canada are for feed and bird seeds. In traditional growing areas in Africa, East-Asia and Indian subcontinent, millets are prepared for consumption in different ways using flour/meal and malt of the grains. Foods prepared from millets vary across continents, countries and regions within the same country and include porridges, steam-cooked products, fermented and

unfermented breads, boiled rice-like products, alcoholic and nonalcoholic beverages and snacks (*13*).

Agro-morphological differentiations have been observed among millet varieties and these are known to be genetically controlled (*14*). The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) has launched several pearl millet improvement programs. The main objective of these programs has been to improve different traits such as flowering time, plant height, and yield components such as panicle numbers, panicle size, and grain size (*14*). Furthermore, sorghum, millets and other grains (INSORMIL) collaborative research support program (CRSP) has been funding several undertakings in developing countries in Africa and central America, and in the United States from 1979. The mission aimed to establish collaborative research as a mechanism to develop human and institutional research capabilities to overcome constraints to sorghum and millet production, marketing, utilization and technology transfer for the mutual benefit of the less developed sorghum and millet producing countries and the United States (*15*).

1.2.2 Whole grain consumption and health

Epidemiological evidences suggest that regular consumption of whole grains and whole grain products is associated with the reduced risk of several age-related chronic diseases (*16-22*). All dietary guidelines have cereals and cereal products as the predominant food group recommending their consumption in the highest proportion in the diet (*23, 24*).

Whole grains are rich sources of fibre, vitamins, minerals and phytochemicals such as phenolics, lignans, β-glucan, inulin, resistant starch, sterols and phytates. The additive and synergistic effects of bioactive phytochemicals in plant based foods are

suggested for the beneficial health outcomes (*25*). Furthermore, phenolic compounds such as ferulic acids and dehydrodiferulates in whole grains may complement those in fruits and vegetables due to their unique presence in cereal grains (*3*). Bound antioxidative phenolics in cereals can survive gastrointestinal digestion to reach the colon intact and may provide protective effect in the colon upon release by microbial fermentation (*26*). This may explain the protective role of whole grains in prevention of colon cancer as revealed by epidemiological studies.

1.2.2.1 Millet grains and health

A limited number of in vivo studies have demonstrated the beneficial role of millet grain consumption in the reduction of diseases associated with oxidative stress. Lakshmi Kumari and Sumathi (*27*) have shown that consumption of finger millet based diets results in significantly lower plasma glucose levels in individuals with non-insulin dependent diabetes mellitus. However, sample size of this study was limited to only six individuals. A protective effect of feeding finger millet and kodo millet whole grains meal against hyperglycaemic status and alloxan-induced oxidative stress in Wistar rats was reported (*28*). The effect of finger millet feeding was also examined on the dermal wound healing process of alloxan induced diabetic rats (*29*). The results showed that finger millet feeding for 4 weeks to the diabetic animals controlled the glucose levels and improved the antioxidant status as well as the wound healing process. In another study, Hegde et al. (*30*) demonstrated that topical application of an aqueous paste of kodo millet and finger millet flour was effective in rat dermal wound healing. Recently, Lee et al. (*31*) reported that foxtail and proso millet grain feeding decreased the triacylglycerol and C-reactive proteins in hyperlipidaemic rats suggesting their potential therapeutic use. Furthermore, Shobana

et al. (*32*) showed that feeding of 20% finger millet seed coat had hypoglycaemic, hypocholesterolaemic, nephroprotective and anti-cataractogenic properties in streptozotocin induced diabetic rats.

1.2.3 Free radicals and antioxidants

A free radical can be defined as a species capable of independent existence that contains one or more unpaired electrons in its outer orbital (*33-35*). Free radicals are generally unstable, but very reactive, thus are capable of initiating a series of damaging chemical changes in biological and food systems. Oxygen, the indispensable element for life, seems to be a paradox due to both beneficial and toxic effects. A small proportion (1-3%) of oxygen we breath is freed from the energy production system of oxidative phosporylation in the mitochondria and forms molecules collectively referred to as reactive oxygen species (ROS) that play a dual role as both toxic and beneficial compounds. In living systems, non-radical molecules generate radicals when they are brought into contact with radicals and transition metal ions such as those of iron and copper which can move electrons (*33*). At low or moderate levels, ROS exert beneficial effects in cellular signaling systems and the body's immune function (*36, 37*). At high concentrations, ROS generate oxidative stress in biological systems and play a key role in the development of degenerative disorders such as cancer, arthritis, autoimmune disorders, type 2 diabetes, cardiovascular and neurodegenerative diseases and aging. Hence, maintainance of the delicate balance of ROS *in vivo* is important for optimum health.

In food systems, oxidation of lipids leads to the development of rancid and off flavours during processing and storage that makes foods less acceptable to the consumers. In addition, nutritional loss due to the destruction of essential fatty acids

and fat soluble vitamins and formation of potentially toxic end products makes them unacceptable.

The antioxidants function in biological and food systems by scavenging free radicals and singlet oxygen, or by stabilizing transition metal ions, hence preventing, delaying or inhibiting degradation induced by free radical reactions (*3*). Antioxidants may act as chain breaking or oxidation prevention compounds. The body has a number of mechanisms to counteract the oxidative stress caused by internal as well as external factors and when they are overwhelmed under pathophysiological conditions, exogenous antioxidants supplied through foods or supplements are necessary. In foods, processing and storage may lead to the destruction of endogenous antioxidative compounds, thus they may be added to products in order to maintain food quality. Antioxidants include many compounds such as minerals, vitamins, proteins, carbohydrates and polyphenolics that are capable of donating hydrogen atoms or electrons to a pro-oxidant.

1.2.4 Phenolic antioxidants

Research shows that whole grains, fruits and vegetables, legumes, and oilseeds contain bioactive phytochemicals that may provide beneficial health effects other than the basic nutrients such as carbohydrates, proteins, fats, minerals and vitamins (*38-45*). Phenolic compounds are one of the most highly diversified groups of phytochemicals found in all plant organs and are therefore an integral part of the human diet.

Phenolics are synthesized as first line defense chemical compounds against infections (*46*), wounding (*47*), nutritional stress (*48*), cold stress (*49*) and visible light (*50*). Furthermore, deficiency of iron, phosphorus and nitrogen and the application of

herbicides can also induce the production of secondary metabolites in plants (*51, 52*). Typically the phenolic profile of a plant food is species specific (*53, 54*). The level of phenolic compounds present in a given species of plant material depends on different factors such as cultivar (*55-58*), environmental conditions (*57*), cultural practices (*59*), post harvest practices (*60*), processing conditions (*61,62*) and storage (*63*).

Phenolic compounds contribute to different attributes in foods such as bitterness, astringency, colour, flavour and stability against lipid oxidation (*3*). The proposed mechanisms of antioxidant activity include free radical scavenging, transition metal ions chelation, reducing peroxide, and stimulation of in vivo antioxidative enzyme activities, among others (*64*). Phenolic compounds also have the ability to bind and precipitate macromolecules such as proteins, carbohydrates and digestive enzymes imparting deleterious nutritional effects (*65*). However, with the knowledge of their ability to act as antioxidants, new efforts are focused to find their association with the prevention of degenerative diseases and the mechanism(s) of actions in biological systems (*66, 67*). In addition, crude extracts of plant materials, rich in phenolics, have drawn the interest of the food industry as additives due to their antioxidant activity against oxidative degradation of lipids in foods.

Plant phenolics are derived from a limited pool of biosynthetic precursors such as pyruvate, acetate, a few amino acids, acetyl CoA and malonyl CoA (*68*) following the pentose phosphate, shikimate, and phenylpropanoid metabolism pathways (*69, 70*). Phenylalanine, and, to a lesser extent, tyrosine are two main amino acids involved in the synthesis of phenolic compounds in plants (*42, 43*). Phenolic compounds most widely occurring in plants, include simple phenolics, phenolic acids, coumarins, flavonoids, stilbenes, tannins, lignans and lignins (*3*).

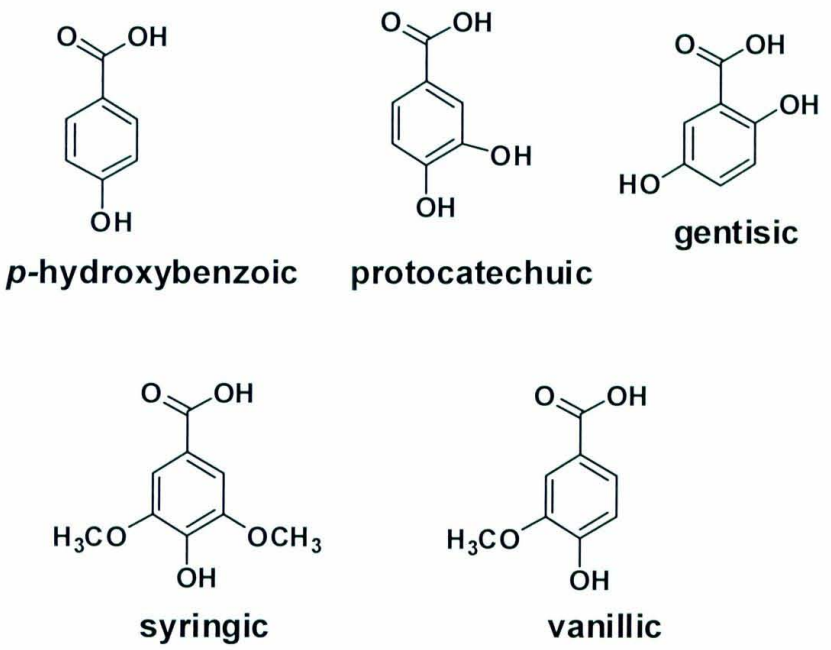


Figure 1.1 Chemical structures of hydroxybenzoic acids

1.2.4.1 Phenolic acids

Two classes of phenolic acids, hydroxybenzoic acids and hydroxycinnamic acids are found in plant materials (*3*). Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, vanillic, syringic, and protocatechuic acids, among others (**Figure 1.1**). The hydroxycinnamic acids include *p*-coumaric, caffeic, ferulic, and sinapic acids **(Figure 1.2**). These latter compounds with a phenyl ring (C_6) and a C_3 side chain are known as phenylpropanoids and serve as precursors for the synthesis of other phenolic compounds. Loss of a two carbon moiety forms the benzoic acids and further decarboxylation of benzoic acid derivatives leads to the formation of simple phenols (*3*).

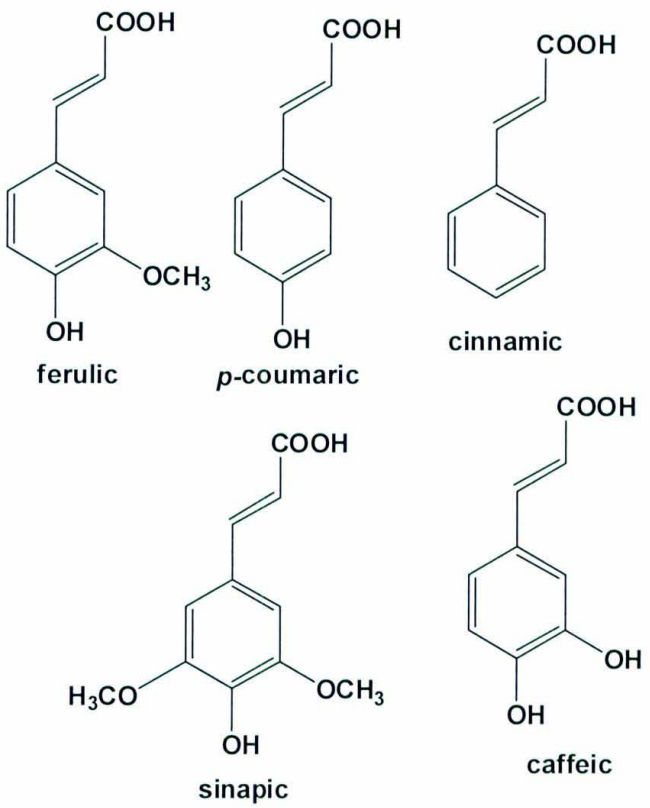


Figure 1.2 Chemical structures of hydroxycinnamic acids

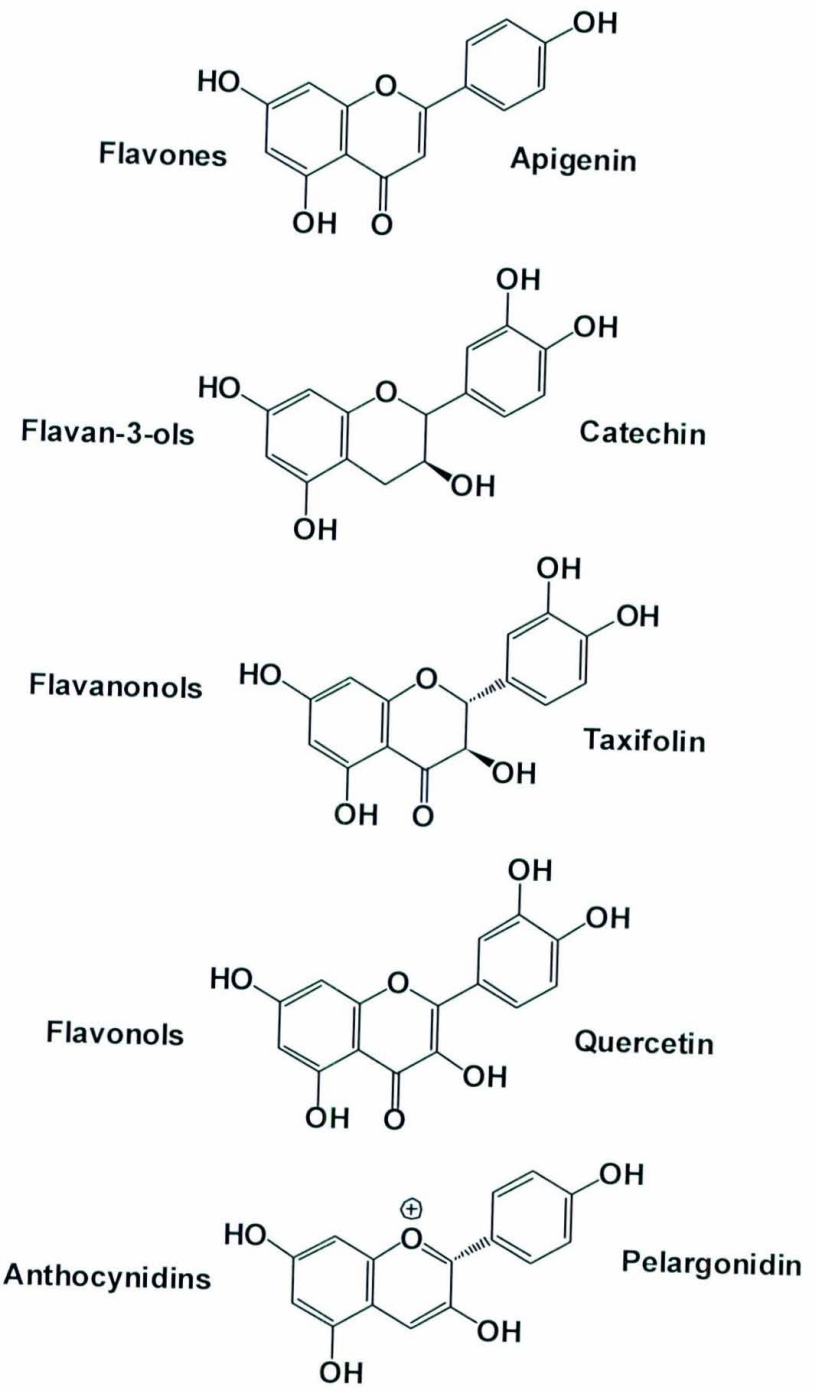


Figure 1.3 Chemical structures of flavonoids

1.2.4.2 Flavonoids

Flavonoids have the diphenylpropane (C_6-C_3-C_6) skeleton and are synthesized by condensation of a phenylpropanoid compound with three molecules of malonyl coenzyme A. This reaction is catalyzed by the enzyme chalcone synthase that leads to the formation of chalcones. The chalcones are subsequently cyclized under acidic conditions to form flavonoids (*3*). There are different subclasses of flavonoids, namely flavones, flavonols, flavanones, flavanonols, isoflavones, flavanols, and anthocyanins (**Figure 1.3**). Flavones and flavonols are present as aglycones in foods. They have similar C ring structures with a double bond at the 2-3 positions. Flavones lack a hydroxyl group at the third position (*3*). **Table 1.3** presents different subgroups of flavonoids and provides examples for compounds in each subgroup.

1.2.4.3 Lignans

Lignans are compounds that comprise of two coupled phenylpropanoid units linked by the central carbons of their side chains. The common plant lignans found in the human diet include secoisolariciresinol, matairesinol, lariciresinol, pinoresinol and syringaresinol (*25*). Secoisolariciresinol, and matairesinol are readily converted to mammalian lignans, enterodiol and enterolactone, respectively, by intestinal microflora in the human gut and they exert strong antioxidant (*71-73*) and estrogenic (*74*) activities.

Table 1.3 Different sub-groups of flavonoids

Flavonoids	Names of compounds
Flavones	Luteolin, apigenin, chrysin, rutin
Flavonols (flavon-3-ols)	Quercetin, kaempferol, myricetin, isorhamnetin
Flavanones	Hesperidin, hesperitin, naringenin, naringin, eriodictyol
Flavanonols	Taxifolin
Isoflavones	Daidzein, genistein and glycitein
Flavanols (flavan-3-ols)	Catechin, gallocatechin, epicatechin, epigallocatechin
Polymeric flavanols (proanthocyanidins)	Procyanidin B1, B2, B3, B4 ; Procyanidin C1, C2
Anthocyanidins	Cyanidin, delphinidin, pelargonidin, petunidin, malvidin
Chalcones	Butein, okanin

1.2.4.4 Lignins

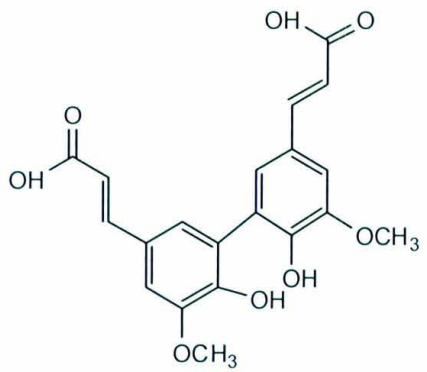
Lignins are formed via polymerization of a mixture of the three monolignols, namely *p*-coumaryl, sinapyl and coniferyl alcohols (*75*). Additional compounds are incorporated into lignin in small quantities. They include coniferaldehyde, sinapaldehyde, dihydroconiferyl alcohol, 5-hydroxyconiferyl alcohol, tyramine ferulate and *p*-hydroxy-3-methoxybenzaldehyde, among others (*76-79*). Lignins are found in whole grain cereals accounting for 3-7% of the bran fraction (*80*).

1.2.4.5 Tannins

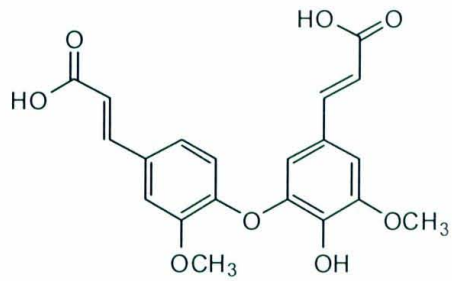
Tannins which are also referred to as proanthocyanidins are composed of a group of compounds with a wide diversity in structure and have the ability to bind and precipitate proteins (*3*). Tannins are classified into three groups, namely condensed tannins, hydrolysable tannins and complex tannins (*81*).

1.2.5 Phenolics in cereals

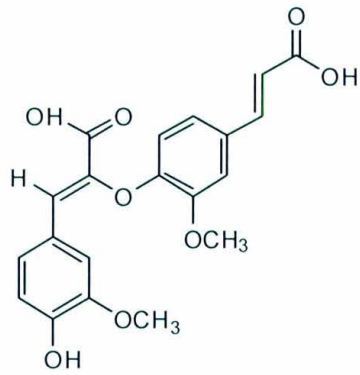
Cereals contain mainly free and conjugated forms of phenolic acids, which include derivatives of hydroxybenzoic and hydroxycinnamic acids (**Figures 1.1 and 1.2**). In addition, several flavonoids, namely anthocynidins, flavanols, flavones, flavanones, chalcones, and aminophenolic compounds are found in cereals (**Figure 1.3**) (*3*). Phenolic acids and flavonoids are found in different parts of the grain and aleurone layer in the highest concentration (*83*). Foods rich in phenolic compounds have been shown to possess antimutagenic, antiglycemic and antioxidative properties (*84, 85*). Several *in vitro* and epidemiological studies have shown that dietary ferulic acid may be important in the prevention of chronic diseases (*42, 86, 87*). In addition, phenolic acids such as caffeic, *p*-coumaric, ferulic and protocatechuic acids have been reported to process antifungal effects (*88, 89*). Furthermore, outer layers of cereal grains are rich sources of diferulic acids (diFA) such as 8-O-4'-diFA, 8,5'-diFA, 5,5'-diFA and 4-O-5' diFA, among others (**Figure 1.4**) (6, *90-92*).



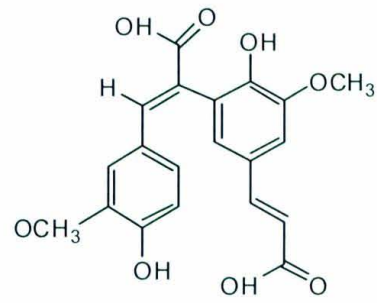
5-5-DiFA



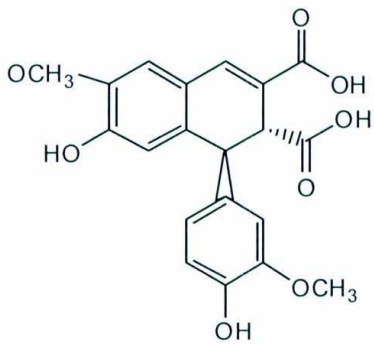
4-O-5-DiFA



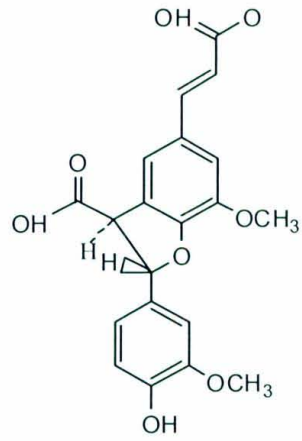
8-*O*-4-DiFA



8-5-noncyclic-DiFA



8-8 cyclic-DiFA



8-5 cyclic-DiFA

Figure 1.4 Chemical structures of ferulic acid cross-linked compounds

Table 1.4 Contents (mg/kg of fresh weight) of phenolic acids in cereal whole grain products

Phenolic acids	Rye[a]	Wheat[a]	Barley[a]	Buck wheat[b]	Oat[c]	Millet[b]	Corn[a]	Brown rice
Caffeic	10	37	1.7	85	3.1	1.1	26	nd
Ferulic	860	890	250	12	250	260	380	240
Sinapic	120	63	11	21	55	nd	57	20
Protocatechuic	9.4	nd	1.6	nd	nd	nd	nd	nd
Vanillc	22	15	7.1	5.3	18	11	4.6	7.8
p-coumaric	41	37	40	15	nd	18	31	76
p-hydroxy benzoic	6.8	7.4	3.1	110	16	3	5.7	15
Syringic	6.7	13	5.0	nd	20	2.1	7.8	nd
DiFA[d]	290	280	130	nd	110	78	89	17
Total	1366	1342	450	248	472	373	601	376

[a]-flour [b]- grits [c]- flakes [d] - DiFA diferulic acids, nd- not detected

(Source: Data adapted from reference *138*)

Table 1.4 presents the phenolic acids and their contents in several whole grain cereals. A unique array of free phenolic compounds along with their glycosides and insoluble bound counterparts, which are associated with polysaccharides in the cell walls, are present in cereal grains (*93*). Phenolic compounds are concentrated in the bran layers and are liable for losses during the separation of seed coat in the milling process (*94*). The content of phenolic compounds varies with the type of cereal (*95*), genotype of the cereal (*96*) and the morphological fraction of the grain (*95, 97*).

1.2.5.1 Phenolics in millets

Limited information is available on phenolic profiles and their contents in different millet varieties and the available data are mainly on finger millets. Phenolic

compounds of finger millets are concentrated in the seed coat and their content in the seed coat and flour fraction were 6.2 and 0.8%, respectively (*98*). Further, it has been shown that the content of phenolics differed depending on the variety of finger millet; the brown varieties contained a higher proportion (1.3-2.3 g %) than did white (0.3-0.5 g %) varieties. Finger millet phenolics extracted with acidic methanol under reflux conditions were stable up to 90°C, but were highly sensitive to pH change. Increase in pH led to the formation of a precipitate which was reversibly solubilized upon increasing acidity (*58*).

Rao and Muralikrishna (*62*) showed that phenolic acids of finger millet are mainly present in the free form (71%), whereas protocatechuic acid is the major free phenolic acid (45 mg/100g) among gallic, caffeic, vanillic, ferulic and *p*-coumaric acids. In addition, major bound phenolic acids of finger millets were ferulic, caffeic and *p*-coumaric acids, which accounted for 18.6, 1.64, and 1.2 mg/ 100g of flour, respectively, meanwhile trace amounts of protocatechuic and syringic acids were reported. They also reported that the antioxidant activity of finger millet, as determined by the β-carotene-linoleate model system of free phenolic acid extract, was higher than that of bound phenolic acids.

Flavonoids are reported in millets. Hilu *et al.* (*99*) reported eight flavones, namely orientin, isoorientin, vitexin, isovitexin, saponarin, violanthin, lucenin-1, and tricin in the leaves of finger millet. The flavones, glucosylvitexin, glucosylorientin and vitexin were isolated from pearl millet grains (*100*). Some studies have shown that pearl millet flavones are goitrogenic (*100,101*). Sartelet *et al.* (*103*) have reported the presence of apigenin and luteolin in fonio millet grains. Watanabe (*104*) isolated two flavones with antioxidant activity from Japanese barnyard millet grains and these

included luteolin and tricin. Luteolin and its glycosides exhibit health beneficial properties such as antioxidant, antiinflammatory, cancer preventive and antiarrhythmic activities (*105*). Tricin was isolated as one of the antitumour constituents and also exhibited antileukemic activity (*106*). Finger millet is the only millet thus far reported to contain condensed tannins (*107*). Brown finger millets were reported to contain a higher proportion (0.12-3.47% catechin equivalents) of condensed tannins than did white finger millets (0.04-0.06%) (*108*).

In Japanese barnyard millet, Watanabe (*104*) isolated another phenolic compound with antioxidant activity, namely *N*-(*p*-coumaroyl) serotonin. This compound was reported to possess antiinflammatory activity by inhibition of proinflammatory cytokine synthesis from human monocytes *in vitro* (*109*). The literature on the proportion of free and bound phenolic acids in millets is limited (*107*). It has been reported that ferulic, *p*-coumaric, and cinnamic acids are major phenolic acids in millets (*109, 111*) (**Table 1.5).**

1.2.5.2 Effect of processing on phenolic content of cereals

Cereal grains are usually subjected to some type of processing such as soaking, decortication, flaking, grinding, malting, fermentation and heat treatment. Cereal whole grains undergo considerable changes in composition during threshing (separation of grains from stalks), milling, fermentation and baking.

Upon malting the content of free and bound phenolic acids in finger millet changed and the content of bound phenolic acid continuously decreased whereas free phenolic acid content increased upon malting for 96 h. Malting of finger millets for 96 hours decreased the protocatechuic acid content from 45 to 16 mg/100g. However, the content of other free phenolic acids, namely *p*-coumaric, gallic and ferulic acids was

increased upon 96 h of malting by 2-, 4- and 10-fold, respectively (*62*). According to Hag *et al.* (*112*), fermentation and dehulling of pearl millet reduced the total phenol content as determined by Folin-Denis method. The total phenol content of standard cultivar of pearl millet decreased from 304 to 122 mg/100g upon fermention for 14 hours. Dehulling which is a process for removing the outer layers of the grain also reduced the total phenolic content by 22% from its original value (*112*). Opoku *et al.* (*113*) showed that germination of millet (*Pennisetum typhoides*) decreased the levels of tannins from 1.6 to 0.83%. Progressive decortication from 0 to 50% and cooking reduced the concentration of *C*-glycosylflavones (vitexin and orientin) of pearl millets (*114*).

Table 1.5 Phenolic acid composition (µg /mg sample) of millet grains

Phenolic acids	Finger	Pearl	Teff	Foxtail
Protocatechuic	23.1	11.8	25.5	na
Gentisic	61.5	96.3	15	21.5
P-hydroxybenzoic	8.9	22	na	14.6
Vanillic	15.2	16.3	54.8	87.1
Caffeic	16.6	21.3	3.9	10.6
Syringic	7.7	17.3	14.9	93.6
p-Coumaric	56.9	268.2	36.9	213.7
Ferulic	387	679.7	285.9	765.8
Cinnamic	35.1	345.3	46	781.7

na – value not available

(Source: Data adapted from reference *109*)

Processing of sorghum bran into cookies and bread reduced the levels of proanthocyanidins and the effect was more prominent in the high-molecular-weight polymers (*115*). Furthermore, cookies showed a higher retention of proanthocyanidins (42-84%) than that of bread (13-69%). Extrusion of sorghum grain showed an increase in the levels of proanthocyanidins oligomers with DP $\leq$ 4 and decrease in polymers with (degrees of polymerization) DP $\geq$ 6 suggesting possible breakdown of the high-molecular-weight ploymers to the low-molecular-weight components (*115*).

Zielinski *et al.* (*116*) reported that extrusion cooking caused significant changes in the phenolic acids content in the extruded wheat, barley, rye and oats.

There was an increase in all analyzed free and ester-bound phenolic acids, except for sinapic and caffeic acids. Sinapic and caffeic acids were not found in the hydrothermally processed grains. They further showed that the changes of the free phenolic acid contents were more intensive than ester-bound phenolic acids. The results further indicated that hydrothermal processing of cereal grains may liberate phenolic acids and their derivatives from the cell walls and may render high antioxidant potential. Ferulic acid was a predominant phenolic in raw wholegrain as well as in extruded grains (*116*). Furthermore, cooking as well as steaming reduced the tannin content of high tannin sorghum (*117, 118*). Later, Matuschek *et al.* (*119*) reported that cooking, soaking and germination reduced the total phenol content of sorghum udo and finger millet.

1.2.5.3 Bioavailability of phenolics

Bioavailability and bioefficacy of polyphenolics in humans have been reviewed (*120,122*). Very few studies have been carried out on the bioavailability of hydroxycinnamic acids which are abundant in cereals. Esterification to arabinoxylans of the grain cell walls hampered the absorption of ferulic acids in rats (*122, 123*). However, Rondini et al. (*124*) showed that bound ferulic acid from bran is more bioavailable than the pure ferulic acid fed to rats. Furthermore, it has been shown that quercetin was more available when ingested from onions than that as a pure compound in healthy ileostomic subjects (*125*). Later, they suggested that glucose moiety may enhance the absorption of quercetin in onions in humans (*125*). Bourne and Rive-Evans (*126*) reported that urinary excretion of total free ferulic acid and feruloyl glucuronide was 11-25% of that ingested from tomatoes providing

approximately 21-44 mg of ferulic acid. Chen et al. (*127*) have shown that phenolic acids from oats are bioavailable.

1.3 Rationale of the study

Cereals serve as the staple food in many populations in the world due to their high content of carbohydrates. Increasing scientific knowledge on cereals and whole grain products has shown that regular consumption of whole grains helps to lower the risk of cardiovascular disease, type 2 diabetes, gastrointestinal cancers, metabolic syndrome and all-cause mortality (*42, 128,-133*). Although a substantial body of knowledge on phenolic compounds of principal cereals and their antioxidant properties is growing up, the available data on phenolic compounds and their antioxidant properties for different millet varieties are unavailable or fragmentary. In addition, the available literature on the proportion of soluble and bound phenolic acids present in millets is limited and flavonoids present in millet varieties, especially in the bound form, have not been investigated to any great extent. Bound phenolics could be released under the microbial fermentation in the colon and may exert beneficial health effects. Furthermore, the effects of different processing operations on the phenolic content and their antioxidant activities are also limited. The processing byproducts, hulls, of millets have not been studied to any length as a potential source of bioactives.

Millet, being underutilized has more potential to be used in human foods as composite flour with other major cereals such as wheat and rice or legumes with minimum organoleptic effects (*134-136*). In addition, potential use of millet in the development of gluten-free cereal products and beverages has been studied (*137*). A few investigators have shown that millets are good sources of phenolic compounds

among different cereal grains (*138, 139*). Research on phenolic compounds of millets has so far been limited to few millet varieties. Though small-grained cereals are collectively termed as millet, botanically they belong to different plant species. Therefore, the profile of phenolic compounds in millets and their antioxidant properties may be different.

1.4 Objectives

(1) To determine antioxidant properties of soluble and bound phenolic compounds of whole grains of millets;

(2) To identify and quantify soluble and bound phenolic compounds present in the whole grains of millets;

(3) To determine the antioxidant activity of phenolic compounds of millets in biological and food model systems;

(4) To determine the effect of processing (dehulling & cooking) on the antioxidant activity of phenolics of millet grains;

(5) To assess the bioaccessibility of phenolics and their antioxidant activities as affected by simulated enzymatic digestion and colonic fermentation; and

(6) To determine bioactivities of hulls as a processing byproduct.

1.5 Thesis format

The antioxidant and inhibitory activities against reactive oxygen species of whole grain millet soluble and bound phenolics were determined and reported in Chapters 2 (published in *Journal of Agricultural and Food Chemistry* 58: 6706-6714) and 3 (published in *Journal of Agricultural and Food Chemistry* 59: 428-436). The profiles of free, esterified, etherified and insoluble bound phenolics of whole millet grains

were identified and quantified using HPLC and tandem mass spectrometry and presented in Chapter 4 (published in *Journal of Functional Foods,3,144-158*). The bioactivities of millet phenolics, namely enzyme inhibitory, antiproliferative, inhibition of DNA scission and liposome oxidation of phenolics of soluble millet grain extracts are reported in Chapter 5 (published in *Journal of Functional Foods,3, 159-170*). Millet grain soluble extracts were evaluated in several food model systems as sources of natural antioxidants and reported in Chapter 6. The effect of dehulling and cooking of millet grains on their antioxidant activities was determined and presented in Chapter 7. Phenolic compounds extracted from hulls, separated by air classification, were evaluated for their antioxidant, antiproliferative, and inhibition of DNA scission, liposome oxidation, and LDL cholesterol oxidation and reported in Chapter 8. Bioaccessibility of processed millet phenolics was determined *in vitro* by employing a simulated enzymic digestion model and colonic fermentation and presented in Chapter 9. This thesis is comprised of 10 chapters in total with this chapter (Chapter 1) presenting an introduction and overview for the present study. Chapters 2-9 are based on articles already published or submitted, and manuscripts prepared for publication. The original format of the published articles has somewhat been changed to maintain the uniformity of the thesis. Chapter 10 presents an overall summary, conclusion and future direction of the study reported.

1.6 References

1. FAOSTAT, **2011** http:// www. faostat.fao.org. (Accessed on 12 Feburary, 2011).
2. FAO. Sorghum and millets in human nutrition. FAO, Rome, 1**995**.
3. Shahidi, F.; Nazck, M. Phenolics in Food and Nutraceuticals. CRC press, Boca Raton, FL, **2004**, pp 1-82.
4. Lee, J.M.; Calkins, M.J.; Chan, K.M.; Kan, Y.W.; Johnson, J.A. Identification of the NF-E2 related factor 2- dependent genes conferring protection against oxidative stress in primary cortical astrocytres using oligonuleotide microarray analysis. *J. Biol. Chem*. **2003**, 278, 12029-12038.
5. Na, H.K.; Surh, Y.J. Intracellular signaling network as a prime chemopreventive target of (-) epigallocatechin gallate. *Mol. Nutr. Food Res*. **2006**, 50, 152-159.
6. Ralph, J.; Quideau, S.; Grabber, J.H.; Hatfield, R.D. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. J. Chem. Soc. Perkin Trans 1, **1994**, 3485-3498.
7. BNF (British Nutrition Foundation), Nutritional aspects of cereals, BNF, London, **2004**.
8. FAO, Human Nutrition in the Developing World, FAO, Rome, **1997**.
9. Serrna-Saldivar, S.; Rooney, L.W. Structure and chemistry of sorghum and millets. In *Sorghum and Millets: Chemistry and Technology:* Dendy, D.A.V. (eds) American Association of Cereal Chemists, Inc. St. Paul, MN, USA, **1995,** pp 69- 124.
10. Tylor, J.R.N.; Emmambux, M.N. Gluten-free foods and beverages from millets. In: *Gluten-free cereal products & beverages:* Arendt, E.K. & Bello, F.D. (eds) Academic Press, New York, **2008**, pp 119-148.
11. Marathee, J.P. Structure and characteristics of the world millet economy. In *Advances in small millets:* Riley, K.W., Gupta, S.C., Seetharam, A., and Mushonga, J.N. (eds) International Science Publisher, New York, NY, USA, **1994**, pp 159-180.
12. Lyon, D.J.; Burgener, P.A.; Deboer, K.; Harveson, R.M.; Hein, G.L.; Hergert, G.W.; Krall, J.M.; Nielsen, D.C.; Vigil, M.F. Producing and marketing proso millet in the Great Plains. University of Nebraska Extension Circular #EC137, **2008,** Lincoln, NE.

13. Murty, D.S.; Kumar, K.A. Traditional uses of sorghum and millets. In *Sorghum and Millets: Chemistry and Technology:* Dendy, D.A.V. (eds) American Association of Cereal Chemists, Inc. St. Paul, MN, USA, **1995,** pp 185-221.

14. ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). **2009**. ICRISAT West and Central Africa 2008 Research Highlights. Niamey, Niger: ICRISAT. 44pp.

15. INSORMIL Sorghum, millet and other grains CRSP, 2010 Annual Report, **2010.** INTSORMIL Publication 10-01, 168pp.

16. Jacobs, D.R.; Slavin, J.; Marquart, L. Whole grain intake and cancer: a review of literature. *Nutr. Cancer*, **1995**, 22, 221-229.

17. Jacobs, D.R.; Meyer, K.A.; Kushi, L.H.; Folsom, A.R. Whole grain intake may reduce risk of coronary heart disease death in postmenopausal women: The Iowa Women's Health Study. *Am. J. Clin. Nutr*. **1998**, 68, 248-257.

18. Anderson, J.W., Hanna, T.J., Peng, X., Kryscio, R.J. Whole grain foods and heart disease risk. *J. Am. Coll. Nutr.* **2000**, 19, 291S-299S.

19. Liu, S.; Stampfer, M.J.; Hu, F.B.; Giovanucci, E.; Rimm, E.; Manson, J.E.; Hennekens, C.H.; Willett, W.C. Whole grain consumption and risk of coronary heart disease: results from the Nurses' Health study. *Am. J. Clin. Nutr*. **1999,** 70, 412-419.

20. Liu, S.; Manson, J.E.; Stampfer, M.J.; Hu, F.B.; Giovanucci, E.; Colditz, G.A.; Hennekens, C.H.; Willett, W.C. A prospective study of whole grain intake and risk of type 2 diabetes mellitus in US women. *Am. J. Pub. Health*, **2000**, 90, 1409-1415.

21. Meyer, K.A.; Kushi, L.H.; Jacobs, D.R. J.; Slavin, J.; Sellers, T.A.; Folsom, A.R. Carbohydrates, dietary fiber, incident type 2 diabetes mellitus in older women. *Am. J. Clin. Nutr*. **2000**, 71, 921-930.

22. Nicodemus, K.K.; Jacobs, D.R.J.; Folsom, A.R., Whole and refined grain intake and risk of incident postmenopausal breast cancer. *Cancer Causes Control*, **2001**, 12, 917-925.

23. USDA, The 2005 dietary guidelines for Americans. **2005**, www.dietaryguidelines.gov (accessed on May 1, 2008)

24. Canada's Food Guide, **2007**, WWW.healthcanada.gc.ca./foodguide (accessed on May 1, 2008)

25. Liu, R.H. Whole grain phytochemicals and health. *J. Cereal Sci*. **2007**, 46, 207-219.

26. Kroon, P.A.; Faulds, C.B.; Ryden, P.; Robertson, J.A; Williamson, G. Release of covalently bound ferulic acid from fiber in the human colon. *J. Agric. Food Chem.* **1997**, 45, 661-667.

27. Lakshmi Kumari, P.; Sumathi, S. Effect of consumption of finger millet on hyperglycemia in non-insulin dependent diabetes mellitus (NIDDM) subjects. *Plant Foods Hum. Nutr.* **2002**, 57, 205-213.

28. Hegde P.S.; Rajasekaran N.S.; Chandra T.S. Effects of the antioxidant properties of millet species on the oxidative stress and glycemic status in alloxan-induced rats. *Nutr. Res*. **2005**, 25, 1109-1120.

29. Rajasekaran, N.S.; Nithya, M.; Rose, C.; Chandra, T.S. The effect of finger millet feeding on the early responses during the process of wound healing in diabetic rats, *Biochim. Biophys. Acta,* **2004**, 1689, 190-201.

30. Hegde, P.S.; Anitha, B.; Chandra, T.S. In vivo effect of whole grain flour of finger millet (*Eleucine coracana*) and kodo millet (*Paspalum scrobiculatum*) on rat dermal wound healing, *Indian J. Exp. Biol.* **2005**, 43, 254-258.

31. Lee, S.H.; Chung, I.M.; Cha, Y.S.; Park, Y. Millet consumption decreased serum concentration of triglyceride and C-reactive protein but not oxidative status in hyperlipidemic rats. *Nutr. Res*. **2010**, 30, 290-296.

32. Shobana, S.; Sreerama, Y.N.; Malleshi, N.G. Composition and enzyme inhibitory properties of finger millet (*Eleusine coracana* L.) seed coat phenolics: Mode of inhibition of a-glucosidase and pancreatic amylase. *Food Chem*. **2009**, 115, 1268-1273.

33. Halliwell, B.; Murcia, M.A.; Chirico, S.; Aruoma, O.I. Free radicals and antioxidants in food and in vivo: what they do and how they work. *Crit. Rev. Food Sci. Nutr.* **1995**, 35, 7-20.

34. Droge, W. Free radicals in the physiological control of cell function. *Review. Physiol Rev*. **2002**, 82, 47-95.

35. Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C.J.; Telser, J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol. Cell. Biochem*. **2004**, 2676, 37-56.

36. Young, I.; Woodside, J. Antioxidants in health and disease. *J. Clin. Pathol.* **2001**, 54, 176-186.

37. Halliwell, B. Biochemistry of oxidative stress. *Buiocem. Soc. Trans.* **2007**, 35, 1147-1150.

38. Dabrowski, K.J.; Sosulski, F.W. Composition of free and hydrolizable phenolic acids in the flours and hulls of 10 legume species. *J. Agric. Food Chem.* **1984**, 32, 131-133.

39. Kazimierz, J.; Dabrowski, K. J.; Sosulski, F. W. Composition of free and hydrolizable phenolic acids in defatted flours of ten oil seeds. *J. Agric. Food Chem.* **1984**, 32, 128-130.

40. Nazck, M.; Shahidi, F. Phenolic constituents of Rapeseed. In *Plant Polyphenols: Synthesis, Properties, Significance:* Hemingway, R.W. & Laks, P.E. (eds.), Plenum press, NY, **1992,** pp 895-910.

41. Kris-Etherton, P.M.; Yu-Poth, S.; Sabate, J.; Ratcliffe, H.E.; Zhao, G.; Etherton, T.D. Nuts and their bioactive constituents: effects on serum lipids and other factors that affect disease risk. *Am. J. Clin. Nutr.* **1999**, 70 (supplement 3), 504S-511S.

42. Slavin, J.L. Mechanism for the impact of whole grain foods on cancer risk. *J. Am. Coll. Nutr.* **2000**, 19, 300S-307S.

43. Shahidi, F. Antioxidant factors in plant foods and selected oil seeds: Mini review. *BioFactors*, **2000**, 13, 179-185.

44. Shahidi, F. Phytochemicals in oilseeds. In *Phytochemicals in nutrition and health:* CRC press, Boca Raton, Florida, **2002**, pp 139-156.

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

46. Beckman, C.H. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defense responses in plants? *Physiol. Mol. Plant Pathol.* **2000,** 57, 101-110.

47. Hahlbrock, K.; Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. *Plant Mol. Biol.* **1989**, 40, 347-369.

48. Graham, T.L. Flavonoid and isoflavonoid distribution in developing soybean seedling tissue and in seed root exudates. *Plant Physiol.* **1991**, 95, 594-603.

49. Christie, P.J.; Alfenito, M.R.; Walbot, V. Impact of low temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta*, **1991**, 194, 541-549.

50. Beggs, C.J.; Khun, K.; Bocker, R.; Wellmann, E. Phytochrome induced flavonoid biosynthesis in mustard (*Sinapsis alba* L) cotyledons: enzymatic control and differential regulation of anthocyanin and quercetin formation. *Planta*, **1987**, 172, 121-126.

51. Weidner, S.; Amarowicz, R.; Karamac, M.; Fraczek, E. Changes in endogenous phenolic acids during development of *Secale cereale* caryopses and after dehydration treatment of unripe rye grains. *Plant Physiol. Biochem.* **2000**, 38, 595-602.

52. Orsak, M.; Lachman, J.; Vejdova, M.; Pivec, V.; Hhamouz, K. Changes of selected secondary metabolites in potatoes and buckwheat caused by UV, γ- and microwave irradiation. *Rostlinna Vyroba*, **2001**, 47, 493-500.

53. Maillard, M. N.; Berset, C. Evolution of antioxidant activity during kilning. Role of insoluble bound phenolic acids of barley and malt. *J. Agric. Food Chem.* **1995**, 43, 1789-1793.

54. Mekeehen, J.D.; Busch, R.H.; Fulcher, R.G. Evaluation of wheat (*Triticum aestieum* L.) phenolic acids during grain development and their contribution to *Fusarium* resistence. *Agric. Food Chem.* **1999**, 47, 1476-1482.

55. Harukaze, A.; Masatsune, M.; Homma, S. Analysis of free and bound phenolics in rice. *Food Sci. Technol. Res.* **1999**, 5, 74-79.

56. Yu, L.; Haley, S.; Perret, J.; Harris, M.; Wilson, J.; Qian, M. Free radical scavenging properties of wheat extracts. *J. Sci. Food Agric.* **2002,** 50, 1619-1624.

57. Abdel-Aal, E. S. M.; Pierre, H. Composition and stability of anthocyanins in blue grained wheat. *J. Agric. Food Chem.* **2003**, 51, 2174-2180.

58. Chethan, S.; Malleshi, N.G. Finger millet Polyphenols: Optimization of extraction and the effect of pH on their stability. *Food Chem.* **2007**, 105, 862-870.

59. Li, S.; Zhang, Q.H. Advances in the development of functional foods from buckwheat. *Crit. Rev. Food Sci. Nutr.* **2001**, 41, 451-464.

60. Siebenhandl, S.; Grausgruber, H.; Pellegrini, N.; Rio, D.D.; Fogliano, V.; Pernice, R.; Berghofer, E. Phytochemical profile of main antioxidants in different

fractions of purple and blue wheat, and black barley. *J. Agric. Food Chem.* **2007**, 55, 8541-8547.

61. Yang, F.; Basu, T.K.; Ooraikul, B. Studies on germination conditions and antioxidant contents of wheat grain. *Int. J. Food Sci. Nutr.* **2001**, 52, 319-330.

62. Rao, M.V.S.S.T.S.; Muralikrishna, G. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Elucine coracana* Indaf-15). *J. Agric. Food Chem.* **2002**, 50, 889-892.

63. Zhou, K.; Yu, L. Antioxidant properties of bran extracts from Trego wheat grown at different locations, *J. Agric. Food Chem.* **2004**, 52, 1112-1117.

64. Zhou, K.; Laux, J.J.; Yu, L Comparison of Swiss red wheat grain and fractions for their antioxidant properties, *J. Agric. Food Chem.* **2004**, 52, 1118-1123.

65. Lugasi, A.; Hovari, J; Sagi, K.V.; Biro, L. The role of antioxidant phytonutrients in the prevention of diseases. *Acta Biologica Szegediensis*, **2003**, 47, 119-125.

66. Duthie, G.G.; Duthie, S.J.; Kyle, J.A.M. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. *Nutr. Res. Rev.* **2000,** 13, 79-106.

67. Tapiero, H.; Tew, K.D.; Ba, N.; Mathe, G. Polyphenols: Do they play a role in the prevention of human pathologies? *Biomed. Parmacother.* **2002**, 56, 200-207.

68. Robards, K.; Prenzler, P.D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolic compounds and their role in oxidative process in fruits. *Food Chem.* **1999**, 66, 401-436.

69. Ryan, D.; Robards, K. Phenolic compounds in olives. *Analyst*, **1998**, 123, 31R-44R.

70. Randhir, R.; Lin, Y.; Shetty, K. Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pac. J. Clin. Nutr.* **2004**, 13, 295-307.

71. Thompson, L.U.; Robb, P.; Serraino, M.; Cheung, F. Mammalian lignan production from various foods. *Nutr. Cancer* **1991**, 16, 43-52.

72. Wang, C.; Makela, T.; Hase, T.; Adlercreutz, H.; Kurzer, M.S. Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. *J. Steroid Biochem. Mol. Biol.* **1994**, 50, 205-212.

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

74. Niemeyer, H.B; Metzler, M. Differences in the antioxidant activity of plant and mammalian lignans. *J. Food. Eng.* **2003**, 56, 255-256.

75. Lewis, N; Yamamato, E. Lignins: Occurrence, biosynthesis and biodegradation. *Annu. Rev. Plant Physiol.* **1990**, 41, 455-496.

76. Pillonel, C.; Mudler, M.M.; Boon, J.J.; Forster, B.; Binder, A. Involvement of cinnamyl-alcohol dehydrogenase in the control of lignin formation in *Sorghum bicolor* L Monech. *Planta*, **1991**, 185, 538-544.

77. Ralph, J.; Hatfield, R.D.; Piquemal, J.; Yahiaoui, N.; Pean, M.; Lapierre, C.; Boudet, A.M. NMR characterization of altered lignins extracted from tobacco plants down regulated for lignification enzymes cinnamyl alcohol dehydrogenase and cinnamyl-CoA reductase. *Proc. Natl. Acad. Sci.* USA, **1998**, 95, 12803-12808.

78. Ralph, J.; Lapierre, C.; Marita, J.M.; Kim, H.; Lu, F.; Hatfield, R.D.; Ralph, S.; Chapple, C.; Franke, R.; Hemm, M.R.; Van Doorsselaere, J.; Sederoff, R.R.; O'Malley, D.M.; Scott, J.T.; Mackay, J.J.; Yahiaoui, N.; Boudet, A.M.; Pean, M.; Pilate, G.; Jouanin, L.; Boerjan, W. Elucidation of new structures in lignins of CAD- and COMT- deficient plants by NMR. *Phytochemistry*, **2001**, 57, 993-1003.

79. Marita, J.; Vermerris, W.; Ralph, J.; Hatfield, R.D. Variation in the cell wall composition of maize brown midrib mutants. *J. Agric. Food Chem.* **2003**, 51, 1313-1321.

80. Kim, H.; Ralph, J.; Lu, F.; Ralph, S.A.; Boudet, A-M.; MacKay, J.J.; Sederoff, R. R.; Ito, T.; Kawai, S.; Ohashi, H.; Higuchi, T. NMR analysis of lignins in CAD-deficient plants. Part 1. Incorporation of hydroxycinnamaldehydes and hydroxybenzaldehydes into lignins. *Organic Biomol. Chem.* **2003**, 1, 158-281.

81. Fardet, A.; Rock, E.; Remesy, C. Is the *in vitro* antioxidant potential of whole – grain cereals and cereal products well reflected *in vivo*? *J. Cereal Sci.* **2008**, 48, 258-276.

82. Khanbabaee, K.; Van Ree, T. Tannins: classification and definition. *Nat. Prod. Rep.* **2001**, 18, 641-649.

83. Shirley, B.W. Flavonoids in seeds and grains: Physiological function, agronomic importance and the genetics of biosynthesis. *Seed Sci. Res.* **1998**, 8, 415-422.

84. Visioli, F.; Galli, C. Olive oil phenols and their effects on human health. *J. Agric. Food Chem.* **1998**, 46, 429-4296.

85. Benavente-García, O.; Castillo, J.; Lorente, J.; Ortuño, A.; Del Rio, J.A. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chem.* **2000**, 68, 45-462.

86. Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, 56, 317-333.

87. Kikuzaki, H.; Hisamoto, M.; Hirose, K.; Akiyama, K.; Taniguchi, H. Antioxidant properties of ferulic acid and its related compounds. *J. Agric. Food Chem.* **2002**, 50, 2161-2168.

88. Moure, A.; Cruz J.M.; Franco, D.; Domínguez, J.M.; Sineiro J.; Domínguez H.; Núñez, M.J.; Parajó, J.C. Natural antioxidants from residual sources. *Food Chem.* **2001**, 72, 145-171.

89. Dragland, S.; Senoo, H.; Wake, K.; Holte, K.; Blomhoff, R. Several culinary and medicinal herbs are important sources of dietary antioxidants. *J. Nutr.* **2003**, 133, 1286-1290.

90. Garcia-Conesa, M.T.; Puumb, G.W.; Kroon, P.A.; Walace, G.; Williamson, G. Antioxidant properties of ferulic acid dimmers. *Redox Rep.* **1997**, 3, 239-244.

91. Bunzel, M.; Ralph, J.; Bruning, P.; Steinhart, H. Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. *J. Agric. Food Chem*, **2006**, 54, 6409-6418.

92. Hernanz, D.; Nunez, V.; Sancho, A.I.; Faulds, C.B.; Williamson, G.; Bartolome, B.; Gomez-Cordoves, C. Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley. *J. Agric. Food Chem.* **2001**, 49, 4884-4888.

93. Miller, H.E.; Rigelhof, F.; Marquart, L.; Prakash, R.D.A.; Kanter, M. Antioxidant content of whole grain breakfast cereals, fruits and vegetables. *J. Am. Coll. Nutr*. **2000**, 19, 312S-319S.

94. Tian, S.; Nakamura, K.; Kayahara, H. Analysis of phenolic compounds in white rice, brown rice and germinated brown rice. *J. Agric. Food Chem.* **2004**, 52, 4808-4813.

95. Zielinski, H.; Kozlowska, H. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *J. Agric. Food Chem.* **2000**, 48, 2008-2016.

96. Fujita, M.; Takeda, K.; Kohyama, N.; Doi, Y.; Matsunaka, H. Genotypic variation in polyphenol content of barley grain. *Euphytica.* **2002**, 124, 55-58.

97. Saddi, A.; Lempereur, I.; Sharonors, S.M.; Autran, J.C.M.; Manfait, M. Spatial distribution of phenolic materials in Durum wheat grain as probed by Confocal Fluorescence Spectral Imaging. *J. Cereal Sci.* **1998**, 28, 107-114.

98. Chethan, S.; Malleshi, N.G. Finger millet polyphenols: Characterization and their nutraceutical potential. *Am. J. Food Technol.* **2007**, 2, 582-592.

99. Hilu, K.W.; De Wet, J.M.J.; Seigler, D. Flavonoid patterns and systematic in *Elusine*. *Biochem Systematic Ecol.* **1978**, 6, 247-249.

100. Reichert, R.D. The pH-sensitive pigments in pearl millet. *Cereal Chem.* **1979**, 56, 291-294.

101. Gaitan, E.; Lindsay, R. H.; Reichert, R. D.; Ingbar, S. H.; Cooksey, R. C.; Legan, J.; Meydrech, E. F.; Hill, J.; Kubota, K. Antithyroid and goitrogenic effects of millet: role of C- glycosylflavones. *J. Clin. Endocrinol. Metab.* **1989**, 68, 707-714.

102. Birzer, D.M.; Klopfenstein, C.F.; Leipold, H.W. Goiter- causing compounds found in pearl millet. *Nutr. Rep. Int.* **1987**, 36, 131-140.

103. Sartelet, H.; Serghart, S.; Lobstain, A.; Ingenbleek, Y.; Anton, R.; Petitfrere, E.; Aguie-Aguie, G.; Martiny, L.; Haye, B. Flavonoids extracted from *Fonio* millet (*Digitaria exilis*) reveal potent antithyroid properties. *Nutrition*, **1996**, 12, 100-106.

104. Watanabe, M. Antioxidative phenolic compounds from Japanese Barnyard Millet (*Echinochloa utilis*) grains. *J. Agric. Food Chem.* **1999**, 47, 4500-4505.

105. Han, X.; Shen, T.; Lou, H. Dietary polyphenols and their biological significance. *Int. J. Mol. Sci.* **2007**, 8, 950-988.

106. Lee, K.H.; Tagahara, K.; Suzuki, H.; Wu, R.Y.; Harina, M.; Hall, I.H. Antitumor agents, tricin, kaempferol-3-O-β- D-glucopyranoside and (+)-

nortrachelogenin, antileukemic principles from *Wiktroemia indica*. *J. Nat. Pro*. **1981**, 44, 530-535.

107. Dykes, L.; Rooney, L.M. Sorghum and millet phenols and antioxidants. *J. Cereal Sci*. **2006**, 44, 236-251.

108. Ramachandra, G.; Virupaksha, T.K.; Shadaksharaswamy, M. Relationship between tannin levels and in vitro protein digestibility in finger millet (*Elucine corocana* Gaertn.). *J. Agric. Food Chem.* **1977**, 25, 1101-1104.

109. Kawashima, S.; Hayashi, M.; Takii,T.; Kimura, H.; Zhang, H.L.; Nagatsu, A.; Sakakibara, J.; Murata, K.; Oomoto, Y.; Onozaki, K. Serotonin derivative, *N*-(*p*-coumaroyl) serotonin, inhibits the production of TNF-α, IL-1α, IL-1 β and IL-6 by endotoxin-stimulated human blood monocytes. *J. Interferon Cytokine Res.* **1998**, 18, 423-428.

110. McDonough, C.M.; Rooney, L.W.; Earp, C.F. Structural characteristics of *Eleusine coracana* (Finger millet) using scanning electron and fluorescence microscopy. *Food Microstruct.* **1986**, 5, 247-256.

111. McDonough, C.M.; Rooney, L.W. The millets. In *Handbook of Cereal Science and Technology*: Kulp, K., & Ponte, J.G., (eds.), Marcel Dekker, Inc., NY, **2000**, pp 177-201.

112. Hag, M.E.E.; Tinay, A.H.E.; Yousif, N.E. Effect of fermentation and dehulling on starch, total polyphenols, phytic acid content and in vitro protein digestibility of pearl millet. *Food Chem*. **2002**, 77, 193-196.

113. Opoku, A.R.; Ohenhen, S.O.; Ejiofor, N. Nutrient composition of millet (*Pennisetum typhoides*) grains and malts. *J. Agric. Food Chem*. **1981**, 29, 1247-1248.

114. Akingbala, J.O. Effect of processing on flavonoids in millet (*Pennisetum americanum*) flour. *Cereal Chem*. **1991,** 68, 180-183.

115. Awika, J.M.; Dykes, L.; Gu, L.; Rooney, L.W.; Prior, R.L. Processing of sorghum (*Sorghum bicolor*) and sorghum products alters procyanidin oligomer and polymer distribution and content. *J. Agric. Food Chem.* **2003**, 51, 5516-5521.

116. Zielinski, H.; Kozłowska, H.; Lewczuk, B. Bioactive compounds in the cereal grains before and after hydrothermal processing. *Innov. Food Sci. Emerg. Technol.* **2001**, 2, 159-169.

117. Mitaru, B.N.; Rechiert, R.D.; Blair, R. Kinetics of tannin deactivation during anaerobic storage and boiling treatments of high tannin sorghums. *J. Food Sci.* **1984**, 49, 1566-1568.

118. Ekpenyong, T.E. Effect of cooking on polyphenolic content of some Nigerian legumes and cereals. *Nutr. Rep. Intern.* **1985**, 31, 561-565.

119. Matuschek, E.; Towo, E.; Savanberg, U. Oxidation of polyphenols in phytate-reduced high-tannin cereals: Effect on different phenolic groups and on *in vitro* accessible iron. *J. Agric. Food Chem.* **2001**, 49, 5630-5638.

120. Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. 1. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, 81, 230S-242S.

121. Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. 11. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, 81, 243S-255S.

122. Adam, A.; Crespy, V.; Levart-Verny, M-A.; Leenhardt, F.; Leuillet, M.; Demigne, C.; Remesy, C. The bioavailability of ferulic acid is governed primarily by the food matrix rather than its metabolism in intestine and liver in rats. *J. Nutr.* **2002**, 132, 1962-1968.

123. Zhao, Z.; Egashira, Y.; Sanada, H. Ferulic acid is quickly absorbed from rat stomach as the free form and then conjugated mainly in the liver. *J. Nutr.* **2004**, 134, 3083-3088.

124. Rondini, L.; Peyrat-Maillard, M-N.; Fromentin, G.; Durand, P.; Tome, D.; Prost, M.; Berset, C. Bound ferulic acid from bran is more bioavailable than the free compound in rat. *J. Agric. Food Chem.* **2004,** 52, 4338-4343.

125. Hollman, P.C.; de Vries, J.H.M.; van Leeuween, S.D.; Mengelers, M.J.B.; Katan, M.B. Absorption of dietary glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, 62, 1276-1282.

126. Bourne, L.C.; Rive-Evans, C. Bioavailability of ferulic acid. *Biochem. Biophy. Res. Comm.* **1998,** 253, 222-227.

127. Chen, C-Y.; milbury, P.E.; Kwak, H-K.; Collins, W.; Samuel, P.; Blumberg, J.B. Avenanthramides and phenolic acids from oats are bioavailable and act synergistically

with vitamin C to enhance hamster and human LDL resistance to oxidation. *J. Nutr.* **2004**, 134, 1459-1466.

128. Slavin, J.L.; Jacobs, D.; Marquart, L.; Wiemer, K. Grain processing and nutrition. *Crit. Rev. Biotechnol.* **2001**, 21, 49-66.

129. Slavin, J. Why whole grains are protective: Biological mechanisms. *Proc. Nutr. Soc.* **2003**, 62, 129-134.

130. Liu, S. A prospective study of dietary fiber intake and risk of cardiovascular disease among women. *J. Am. Coll. Cardiol.* **2002**, 39, 49-56.

131. McKeown, N.M. Whole grain intake is favorably associated with metabolic risk factors for type 2 diabetes and cardiovascular disease in the Framingham Offspring Study. *Am. J. Clin. Nutr.* **2002**, 76, 390-398.

132. Jensen, M. K. Intake of whole grains, brans and germ and the risk of coronary heart disease in men. *Am. J. Clin. Nutr.* **2004**, 80, 1492-1499.

133. Seal, C.J. Whole grains and CVD risk. *Proc. Nutr. Soc.* **2006**, 65, 24-34.

134. Eneche, E.H. Biscuit-making potential of millet/pigeon pea flour blends. *Plant Foods Hum. Nutr.* **1999**, 54, 21-27.

135. McWatters, K.H.; Ouedraogo, J.B.; Resurreccion, A.V.A.; Hung, Y.C.; Phillips, R.D. Physical and sensory characteristics of sugar cookies containing mixtures of wheat, fonio (*Digitaria exilis*) and cowpea (*Vigna ungiculata*) flours. *Int. J. Food Sci. Tech.* **2003**, 38, 403-410.

136. Ragaee, S.; Abdel-Aal, E. M. Pasting properties of starch and protein in selected cereals and quality of their food products. *Food Chem.* **2006**, 95, 9-18.

137. Schober, T.J.; O'Brien, C.M.; McCarthy, D.; Darnedde, A.; Arendt, E.K. Influence of gluten-free flour mixes and fat powders on the quality of gluten-free biscuits. *Eur. Food Res. Technol.* **2003**, 216, 369-376.

138. Mattila, P.; Pihlava, J.; Hellstrom, J. Contents of phenolic acids, alkyl-and alkenylresorcinols, and avenanthramides in commercial grain products. *J. Agric. Food Chem.* **2005**, 53, 8290-8295.

139. Choi, Y.; Heon-Sang, J.; Lee, J. Antioxidant activity of methanolic extracts from some grains consumed in Korea. *Food Chem.* **2007**, 103, 130-138.

CHAPTER 2

The content and contribution of insoluble bound phenolics to the antioxidant capacity of millets

2.1 Abstract

Soluble and insoluble bound phenolic extracts of several varieties of millet (kodo, finger, foxtail, proso, pearl and little millets) whole grains were evaluated for their phenolic contents and antioxidative efficacy using trolox equivalent antioxidant capacity (TEAC), reducing power (RP), β-carotene-linoleate model system as well as ferrous chelating activity. In addition, ferulic and *p*-coumaric acids were present in soluble and bound phenolic fractions of millets and their content was determined using high performance liquid chromatography (HPLC) and HPLC-(mass spectrometry) MS. Kodo millet had the highest total phenolic content whereas proso millet possessed the least. All millet varieties showed high antioxidant activities although the order of their efficacy was assay dependent. HPLC analysis of millet phenolic extracts demonstrated that the bound fractions contained more ferulic and *p*-coumaric acids compared to their soluble counterparts. The results of this study showed that soluble as well as bound fractions of millet grains are rich sources of phenolic compounds with antioxidant, metal chelating and reducing power. The potential of whole millets as natural sources of antioxidants depends on the variety used. The importance of insoluble bound fraction of millet as a source of ferulic acid and *p*-coumaric acid was established and their contribution to the total phenolic content must be taken into account in the assessment of antioxidant activity of millets.

2.2 Introduction

Cereals are staple foods for many populations around the world. Epidemiological studies have demonstrated that regular consumption of whole grain cereals and their products can protect against the risk of cardiovascular diseases, type 2 diabetes, gastrointestinal cancers, and a range of other disorders (*1*). In addition to major macronutrients, whole grains contribute significant quantities of micronutrients and non-nutrient phytochemicals, the latter include phenolic compounds, in the human diet. In the past two decades there has been a renewed interest in polyphenols as 'life span essentials' due to their role in maintaining body functions and health throughout the adult and latter phases of life. This is to address concerns about oxidative stress caused by imbalance between antioxidant defense mechanisms and increased production of free radicals is considered to be a leading cause in the development of chronic degenerative diseases. Therefore, consumption of whole grains is recommended in order to achieve optimal health. Canada's food guide recommends 6-7 and 8 daily servings of grain products, particularly whole grains, for females and males, respectively.

The potency of phenolic compounds to act as antioxidants arises from their ability to donate hydrogen atoms via hydroxyl groups on benzene rings to electron deficient free radicals and in turn form a resonance stabilized and less reactive phenoxyl radical. Plant phenolics may also act as reducing agents, singlet oxygen quenchers and metal chelators (*2*). Whole grain cereals are a significant source of phenolic compounds, especially phenolic acids such as ferulic, *p*-coumaric, vanillic, caffeic, syringic and sinapic acids and to a lesser extent flavonoids (*2*). Phenolic compounds in grains exist in the free, soluble conjugates and insoluble bound forms

(*3*). Phenolics are not equally distributed in the grain and a high proportion is found in the outer layers, namely the aleurone layer, testa and pericarp which form the main components in the bran fraction. Studies have shown that cereal brans mainly consist of ferulic acid and its oxidatively coupled products, the diferulic acids. Though insoluble bound phenolics are not readily available for absorption, they can be released under the low pH conditions of the gastrointestinal tract (*4*) and upon colonic fermentation (*5*). Upon release, they can exert a localized effect on the gut lumen or could be absorbed into the blood stream. Therefore, determination of bound phenolics and their antioxidant activity is of paramount importance to the understanding of the health benefits of grains.

Millets are important crops in semi-arid and tropical regions of the world due to their resistance to pests and diseases, short growing season and productivity under heat and drought conditions when major cereals cannot be relied upon to provide sustainable yields. Of the total millet produced in the world about 90% is utilized in the developing countries and about two thirds of millets produced are consumed as food (*6*). Major phenolic acids reported in millets are in general ferulic, and *p*-coumaric acids among others (*7*). However, more is known about finger millet varieties than other millet grains. Phenolic compounds of finger millets are concentrated in the testa and the content of phenolic compounds differs according to millet variety (*8*). Flavonoids so far reported in millets are flavones. Finger millets are reported to contain proanthocyanidins, also known as condensed tannins (*9*).

In addition to antioxidant properties, polyphenols of millets, particularly finger millet, possess other health benefits such as antimicrobial, anti-inflammatory, antiviral, anticancer and anti-platelet aggregation and cataractogenesis inhibitory

activities (*10*). Hegde et al. (*11*) reported that whole grain meals of kodo and finger millets protect against hyperglycemic and alloxan-induced oxidative stress in Wistar rats. Millets are underutilized in many developed countries. There is an immense potential to process millet grains into value added foods and beverages in developing countries. Furthermore, millets, as they do not contain gluten, are good for celiacs.

Although several studies have reported the phenolic compounds of principal cereals and their antioxidant properties there remains an apparent gap in the early literature for such information on different millet varieties. Limited published data indicate that millets are good sources of phenolic compounds among different cereal grains. Millets, though named as a whole group due to the small size of the seeds, belong to a range of different species of the family *Poaceae*. Due to the fact that millets are consumed in several forms they can contribute different nutrients and bioactive compounds to the diet, including phenolic compounds. Therefore, it is necessary to assess phenolic compounds of millets in order to explore and possibly promote their use as functional foods ingredients. The objectives of present study were (a) to investigate the potential antioxidant activity of phenolic compounds in whole grain millet varieties of kodo, finger, foxtail, proso, little and pearl; (b) to determine the contribution of insoluble bound fraction of phenolic compounds to the antioxidant activity; and (c) to determine the content of major phenolic acids (ferulic and *p*-coumaric) present in the grain samples.

2.3 Materials and methods

Seven millet grain samples, namely foxtail (*Setaria italica;* variety ISC 480*)*, proso (*Panicum miliacium;* variety AC 254*)*, finger millet (*Elusine coracana;* varieties Ravi

and local), kodo (*Paspalum scrobiculatum;* a local variety), little millet (*Panicum sumatrense*; a local variety), and pearl millet (*Pennisetum glaucum;* dark green cultivar*)* were used in this study. All grain samples, with the exception of pearl millet, were obtained from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka. Pearl millet (dark green cultivar), grown in India, was obtained through Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India.

Folin Ciocalteu's reagent, ferulic acid, *p*-coumaric acid, vanillin, catechin, aluminium chloride, sodium nitrite, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), sodium chloride, trolox, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, ferrous chloride, ascorbic acid, ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), mono- and dibasic potassium phosphates, Tween 40 (polyoxyethylene sorbitan monopalmitate), *β*-carotene, linoleic acid, butylated hydroxyanisole (BHA), 3-(2-pyridyl)-5-6-diphenyl-1,2,4,-triazine-4,4-disulphonic acid sodium salt (Ferrozine), were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Diethyl ether, ethyl acetate, hexane, acetone, methanol, chloroform, acetonitrile, formic acid, hydrochloric acid, sodium hydroxide and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON).

2.3.1 Sample preparation

Whole millet grains, cleaned using seed cleaners to remove soil and other particles, were ground using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc. Brockville, ON) to obtain a fine powder which passed through mesh 16 (sieve opening 1 mm, Tylor test sieve, Mentor, OH). All samples were defatted by

blending with hexane (1:5 w/v, 5 min for 3 times) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT) at ambient temperature. Defatted samples were vacuum packed in polyethylene pouches and stored at –20°C until used within one week for extraction of phenolics.

2.3.2 Extraction of soluble phenolic compounds

Ultrasonic-assisted extraction procedure was used for soluble phenolic compounds. Defatted meal (5 g) was mixed with 100 mL of 70% (v/v) acetone and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA) and sonicated at the maximum power for 25 min under refluxing conditions. After centrifugation of the resulting slurry for 5 min at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA), the supernatant was collected and extraction was repeated two more times. Combined supernatants were evaporated *in vacuo* at 40°C (Buchi, Flawil, Switzerland) and lyophilized for 72 h at -46°C and 34 x 10^{-3} mbar (Freezone, Model 77530, Labconco Co., Kansas City, MO). Residues of whole grain samples were air dried for 12 h and stored at -20°C until used to extract bound phenolic compounds within a week. During all stages, extracts were protected from light by covering them with aluminium foil. Lyophilized crude phenolic extracts were stored at -20°C until used for further analysis.

2.3.3 Extraction of free and esterified phenolic compounds

Free phenolic acids and those liberated from soluble esters were extracted from the lyophilized crude phenolic extract (*12*). An aqueous suspension of extract (250 mg in 10 mL) was adjusted to pH 2 with 6 M HCl, and free phenolics were extracted 5 times into diethyl ether and ethyl acetate (1:1, v/v). The free phenolic extract was

evaporated to dryness under vacuum at room temperature. The water phase was neutralized to pH 7 with 2 M NaOH and then lyophilized. The resulting residue was dissolved in 10 mL of 2 M NaOH and hydrolyzed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 with 6 M HCl, phenolic acids released from soluble esters were extracted from the hydrolyzates 5 times with diethyl ether and ethyl acetate (1:1, v/v) and evaporated to dryness under vaccum.

2.3.4 Extraction of bound phenolic compounds

The residue of the whole grain sample obtained after extraction of soluble phenolics was hydrolyzed with 2 M NaOH at room temperature for 4 h with stirring under nitrogen. The resulting slurry was acidified to pH 2 with 6 M HCl and extracted 5 times with hexane to remove fatty acids which are released during alkaline hydrolysis. Bound phenolic compounds were extracted five times with diethyl ether and ethyl acetate (1:1, v/v) and subsequently desolventized to dryness at room temperature in a rotary evaporator. Phenolic compounds were reconstituted in 5 mL of HPLC grade methanol and stored at -20°C until used.

2.3.5 Determination of total phenolic content (TPC)

The total phenolic content of each extract was determined using the method described by Singleton and Rossi (*13*) with slight modifications. Briefly the crude extracts of soluble phenolic compounds were dissolved in methanol to obtain a concentration of 2.5 mg/mL. Folin Ciocalteu's reagent (0.5 mL) was added to centrifuge tubes containing 0.5 mL of extract and the contents mixed thoroughly by vortexing. The reaction was neutralized by adding 1 mL of saturated sodium carbonate to each tube, followed by the addition of distilled water (8 mL) and thorough mixing. Tubes were

allowed to stand at room temperature in the dark for 35 min followed by centrifugation for 10 min at 4000 x g. The absorbance of the resulting blue colour supernatant was measured at 725 nm (Model HP 8452A diode array spectrophotometer, Agilent Technologies, Palo Alto, CA) using appropriate blanks for background subtraction. The content of total phenolics in each extract was determined using a standard curve prepared for ferulic acid and expressed as micromoles of ferulic acid equivalents (FAE) per gram of defatted meal.

2.3.6 Determination of total flavonoid content (TFC)

Total flavonoid content was determined using a colourimetric method described by Kim et al. (*14*) with slight modifications. One millilitre of aliquot of the extract, dissolved in methanol (2.5 mg/mL), was mixed with 4 mL of distilled water in a 50 mL centrifuge tube; 0.3 mL of 5% $NaNO_2$ was then added to the tube which was allowed to react for 5 min. Subsequently, 0.3 mL of 10% $AlCl_3$ was added to the reaction mixture and allowed to stand for 1 min. Finally, 2 mL of 1 M NaOH and 2.4 mL of distilled water were added and mixed immediately. Centrifuge tubes were kept in the dark at room temperature for 15 min followed by centrifugation for 5 min at 4000 x g. The absorbance was read at 510 nm against a blank prepared in a similar manner by replacing the extract with distilled water. Total flavonoid content calculated from a standard curve for catechin, was expressed as micromoles catechin equivalents (CE) per gram of defatted meal.

2.3.7 Determination of proanthocyanidins content (PC)

Proanthocyanidins content of crude phenolic extracts of millets were determined colourimetrically as described by Price et al. (*15*). To 1 mL methanolic solution of the

extract, 5 mL of 0.5% vanillin-HCl reagent (0.5% vanillin (w/v) in 4% concentrated HCl in methanol) were added followed by incubation for 20 min at room temperature. A separate blank for each sample was read with 4% HCl in methanol. The absorbance was read at 500 nm and the content of proanthocyanidins was expressed as micromoles CE per gram of defatted meal.

2.3.8 Trolox equivalent antioxidant capacity (TEAC)

The total antioxidant capacity of the millet extracts was determined according to the method explained by van den Berg et al. (*16*) and modified in our laboratory (*12, 17*). TEAC assay is based on the scavenging of long lived 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical anion (ABTS•-). An ABTS•- solution was prepared by mixing 2.5 mM AAPH with 2.0 mM ABTS in 100mM saline phosphate buffer (pH 7.4, 0.15 M NaCl) (PBS). The solution was heated for 16 min at 60°C, protected from light by covering in tinfoil and stored at room temperature. The ABTS•- solution was filtered using medium porosity filter papers (Fisher Scientific Co., Pittsburgh, PA) before mixing with the extracts. A blank was used for each measurement to account for the decrease in the absorbance of the radical solution itself with time. Millet extracts were dissolved in PBS at a concentration of 1 mg/mL and further diluted to fit them within the range of values in the standard curve (6.25-50 µM prepared using trolox). The total antioxidant capacity was measured by mixing 40 µL of the sample with 1960 µL of the ABTS•- solution. Absorbance of the reaction mixture was measured at 734 nm immediately at the point of mixing (t_0) and after 6 min (t_6). The decrease in absorbance at 734 nm after 6 min of addition of trolox and extract was calculated using the following equation: ΔA trolox = (A t_0 trolox – A t_6 trolox) – (A t_0 blank –A t_6 blank) where ΔA is the reduction of

absorbance and A the absorbance at a given time. TEAC values were expressed as micromoles trolox equivalents (TE) per gram of defatted meal.

2.3.9 Reducing power

The reducing power of soluble and bound phenolic extracts of millets was determined according to the method of Oyaiza (*18*). The extracts (1 mL) were mixed with 2.5 mL of a phosphate buffer solution (0.2M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%, w/v) in a centrifuge tube. The mixture was incubated at 50° C for 20 min and 2.5 mL of 10% TCA were added followed by centrifugation at 1750 x g for 10 min. The supernatant (1 mL) was transferred into a tube containing 2.5 mL of deionized water and 0.5 mL of 0.1% (w/v) $FeCl_3$ and the absorbance was read using a spectrophotometer at 700 nm. The standard curve was prepared using ascorbic acid. Increased absorbance of the reaction mixture indicated increased reducing power. The results were expressed as micromoles ascorbic acid equivalents (AAE) per gram of defatted meal.

2.3.10 *β*-carotene-linoleate model system

The antioxidant activity of extracts was evaluated in a β-carotene-linoleate model system as explained by Jayaprakasha et al. (*19*) with some modifications. Briefly 0.5 mL of β-carotene (1 mg/mL) dissolved in chloroform was pipetted into a 50 mL round bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at room temperature, 20 mg of linoleic acid, 200 mg of Tween 40 emulsifier and 50 mL of aerated distilled water were added to the flask with vigorous agitation to form an emulsion. Emulsion was freshly prepared for each experiment. Absorbance measurement was carried out using a microplate reader equipped with a built-in

incubator (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany). Extracts in methanol (20 µL) were manually pipetted into sample wells of a Costar® flat bottom 96 well assay plate (Corning Incoperated, Corning, NY) and injector pump was programmed to inject *β*-carotene-linoleic acid emulsion (200 µL) in each of the well with automatic mixing. The microplate was incubated at 45° C and absorbance was read at 450 nm. Gain adjustment was done to improve the sensitivity of measurements before the start. The microplate reader was programmed to perform additional shaking of the contents in wells before each reading was taken. Readings of samples were recorded immediately at zero time and every 10 min up to 120 min. An equal amount of methanol was used for the control. Blank samples devoid of *β*-carotene were prepared for background subtraction. Butylated hydroxyanisole (BHA) and ferulic acid (200 ppm) in methanol were used as reference standards. Antioxidant activity coefficient (AAC) after 120 min of incubation was calculated using the following equation: $AAC = (Aa_{(120)} - Ac_{(120)}) / (Ac_{(0)} - Ac_{(120)})$ where $Aa_{(120)}$ and $Ac_{(120)}$ are the absorbance values measured at 120 min for the sample and the control, respectively, and $Ac_{(0)}$ is the absorbance value of the control, at 0 min. The results were expressed as AAC per gram of defatted meal.

2.3.11 Ferrous ions chelating activity

The ability of millet phenolic extracts to chelate ferrous ions was measured according to the method described by Dinis et al. (*20*). Different concentrations (0.5-4 mg/mL) of soluble phenolic extracts were used to measure chelating activity of ferrous ions. Briefly, 0.4 mL of extracts in distilled water was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by adding 5 mM ferrozine (0.2 mL) and the total volume was adjusted to 4 mL with distilled water. The mixture was vigorously

shaken and left at room temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. For the control, distilled water was used instead of the extract. Appropriate blanks were prepared with 0.4 mL of the sample and 3.6 mL of distilled water for background subtraction. Different concentrations (0.05-2 mM) of Na_3EDTA were used to prepare the standard curve. The inhibition percentage of ferrozine-ferrous ion complex formation was calculated by the following equation. Metal chelating effect (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. The results were expressed as micromoles EDTA equivalents per gram of defatted meal.

2.3.12 Determination of ferulic and *p*-coumaric acids content: HPLC analysis

Ferulic and *p*-coumaric acids content of free, esterifed and insoluble-bound phenolic fractions of millet grains were determined by HPLC analysis. The RP-HPLC analysis were carried out using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a G1311A quaternary pump, a G1379A degasser and a G1329A ALS automatic sampler, a G1330B ALS Therm, a G1316A Colcom column compartment, a G1315B diode array detector (DAD) and a system controller linked to Chem Station Data handling system (Agilent Technologies, Palo Alto, CA). Separations were conducted with a SUPERLCOSILTM LC-18 column (4.6 × 250 mm, 5 μm; Merck, Darmstad, Germany). The mobile phase consisted of 1% formic acid (eluent A) and methanol-acetonitrile-formic acid (94:5:1; v/v/v) (eluent B). Gradient elution was used as follows; 0 min, 20% B; 10 min, 30% B; 15 min, 40%B; 18 min, 45% B; 20 min, 50% B; 30 min, 70% B and 40 min, 85% B. The flow rate was adjusted to 0.5 mL/min and the detection of compounds was performed at 254,

280 and 320 nm. All samples were filtered through a 0.45 µm PTFE membrane syringe filter (Whatman Inc., Florham Park, NJ) before injection. Ferulic and *p*-coumaric acids were identified by comparing their relative retention times, and UV and ESI-MS spectra with authentic compounds. An external standard method with ferulic and *p*-coumaric acids was used for quantification purposes.

2.3.13 HPLC-ESI-MS analysis

HPLC- MS analysis was performed under the HPLC analytical conditions explained above using Agilent 1100 series capillary liquid chromatography/mass selective detector (LC/MSD) ion trap system in electrospray ionization (ESI) negative ion mode. Complete system control and data evaluation were achieved with Agilent LC/MSD Trap software (Agilent Technologies, Palo Alto, CA). The mass spectrometer was operated in a scan range from 100-700 m/z using drying gas (N_2) temperature 350°, drying gas flow 10 L/min, and nebulizer gas (N_2) pressure 60 psi.

2.3.14 Statistical analysis

All experiments were carried out in triplicates unless otherwise stated and data were reported as mean ± standard deviation. The significance of differences between soluble and bound extracts of millets were determined using Student's t test at $p \leq 0.05$. The differences of mean values among millet varieties was determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p \leq 0.05$ significance level. Correlation analysis was performed between phenolic contents and antioxidant activity of soluble and bound extracts using Pearson and Spearman's correlations, respectively. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL).

2.4 Results and discussion

The fat contents of millet grains generally range from 1.5 to 5 g / 100 g edible portion at 12% moisture level (*6*). Thus, Ground millet grains were defatted to remove lipids and lipid soluble components that may affect phenolic compounds and antioxidant activity assays. Preliminary studies showed that ultrasonic-assisted extraction with 70% acetone under reflux conditions affords high phenolic yield and antioxidant activity. **Table 2.1** presents yields of crude extracts after lyophilization. To the best of our knowledge this study is the first to report on the total phenolic content and antioxidant capacity of insoluble bound phenolic extracts of kodo, proso, foxtail, little and pearl millets as well as the content of soluble and insoluble bound flavonoids in the extracts of millet grains.

2.4.1 Total phenolic content (TPC)

The TPC of soluble and bound fractions of whole grain millets were determined using the Folin Ciocalteu's assay. In this method, under alkaline conditions, phenolic groups are deprotonated leading to the formation of phenolate ions, which reduce the phosphotungstic-phosphomolybdic complex in the Folin Ciocalteu's reagent to a blue colour. The soluble phenolic fraction includes both free and soluble conjugates which are responsible for the *in vitro* antioxidant capacity of the extracts.

Table 2.1 Yield, total phenolic (TPC) and flavonoid (TFC) contents of soluble and bound phenolic extracts of whole millets

Millet type	Yield[a]	TPC[b]		TFC[c]	
		Soluble	Bound	Soluble	Bound
Kodo	28 ± 0.5e	32.39 ± 0.93a	81.64 ± 0.15a	33.71 ± 0.73a	4.53 ± 0.14a
Finger (local)	43 ± 0.1c	31.39 ± 1.22a	3.20 ± 0.19d	7.86 ± 0.33b	0.36 ± 0.05c
Finger (Ravi)	42 ± 0.4c	21.16 ± 0.31b	3.83 ± 0.18d	7.01 ± 0.06b	1.05 ± 0.06b
Foxtail	49 ± 0.8b	10.79 ± 0.82c	11.59 ± 0.23b	1.26 ± 0.03c	0.47 ± 0.09c
Little	62 ± 0.4a	12.67 ± 0.33c	9.64 ± 0.28c	1.59 ± 0.17c	0.40 ± 0.06c
Pearl	59 ± 0.1a	8.63 ± 0.38d	9.14 ± 0.17c	1.67 ± 0.01c	0.28 ± 0.10c
Proso	32 ± 0.1d	7.19 ± 0.12d	2.21 ± 0.01e	1.18 ± 0.07c	0.44 ± 0.05c

[a] Yield of soluble phenolic extract expressed as mg /g of defatted meal .

[b] Expressed as μmol ferulic acid equiv /g defatted meal.

[c] Expressed as μmol catechin equiv /g defatted meal.

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

Total phenolic contents of soluble and insoluble bound fractions of different millet varieties ranged from 7.19 ± 0.12 to 32.39 ± 1.22 and from 2.21 ± 0.01 to 81.64 ± 0.15 μmol FAE /g defatted meal, respectively (**Table 2.1**). Similar to other cereals (*21-27*) influence of genotype on the content of phenolic compounds was observed in this study. Kodo millet showed the highest soluble phenolic content followed by

finger (local), finger (Ravi), little, foxtail, pearl and proso millets. In general, phenolic content of millets obtained in the present study were higher than those noted in other cereals such as barley and wheat. According to Madhujith and Shahidi (*24*) the TPC of different barley cultivars ranged from 0.35 to 6.13 µmol FAE /g defatted meal. Beta et al. (*26*) reported that the TPC of mostly hull-less barley cultivars ranged from 13.8 to 20.3 µmol FAE /g barley. Phenolic contents of whole grains of soft and hard wheats were 3.96 and 6.65 µmol FAE /g defatted meal, respectively (*27*). In the present study, millets with dark brown pigmented testa and pericarp (kodo and two finger millet varieties) possessed a higher phenolic content of soluble phenolic fractions than those with white or yellow testa and pericarp (pearl, proso, foxtail, and little millets), in agreement with findings of others (*28*).

The comparison between soluble and insoluble bound phenolic contents showed an inconsistency in their trend among different millet types. Kodo millet exhibited 2.5 times higher phenolic content in bound phenolic extract compared to its soluble phenolic counterpart. A higher phenolic content for bound phenolic extracts of whole grains of corn, wheat, oats, rice and barley than their soluble counterparts was documented in the literature (*17, 22, 29*). However, foxtail and pearl millets had similar ($p > 0.05$) soluble and bound phenolic contents. On the other hand, the TPC of bound extracts of finger (local), finger (Ravi), little and proso millets were lower ($p < 0.05$) than those of their corresponding soluble phenolic counterparts. In agreement with this finding, some studies have reported a lower phenolic content for bound phenolic extracts for whole grains of finger millet and buckwheat than their soluble counterparts (*8, 30*). The variety of cereals may account for differences in the results obtained as well as possible variations in the extraction conditions employed in the

present study. Extraction of phenolic compounds may vary due to their chemical nature, extraction method employed, sample particle size, ratio of sample to solvent, extraction time, pH and temperature. Ultrasonic-assisted extraction employed in the present study is a simple alternative to conventional extraction methods. Passage of ultrasonic waves produces acoustic cavitations in the solvent and the mechanical effect exerted by ultrasonic waves allows greater penetration of the solvent into the sample matrix and increases the contact surface area between the solid and the liquid phase. This facilitates the diffusing of the solute from solid phase to the solvent. Furthermore, in addition to phenolics other compounds such as simple carbohydrates and/or amino acids may be present in the crude extracts and could interfere with determinations of TPC by Folin Ciocalteu's assay used, leading to discrepancies of the results obtained in the present work (*21*).

2.4.2 Total flavonoids content (TFC)

Flavonoids are the most studied group of phenolic compounds and are known to possess antioxidant, anticancer, antiallergic, antiinflammatory, antineuro-inflammatory and gastroprotective properties. Total flavonoids content was quantified using the chelating power of flavonoids with aluminium (III). Flavonoids form a pink coloured complex with aluminium (III) through the 4-keto and neighbouring hydroxyl groups or through adjacent hydroxyl groups in the B ring.

Among soluble extracts of different millet varieties studied, kodo millet had the highest TFC followed by finger (local), finger (Ravi), pearl, little, foxtail and proso millets as shown in **Table 2.1**. The TFC of soluble extracts ranged from 1.18 ± 0.07 to 33.71 ± 0.73 µmol CE /g defatted meal. The soluble extracts of little, foxtail, pearl and proso millets did not show any significant ($p > 0.05$) difference among

varieties and in general, were 4-7 and 20-29 fold lower than that of two finger millet varieties and kodo millet, respectively. As noted in TPC, varieties with dark pigmented testa and pericarp showed a higher TFC than those with white or yellow pigmented testa. Recently, Shen *et al.* (*31*) also reported that white rice had a lower mean flavonoid content compared to those of red rice and black rice. The TFC varied significantly ($p < 0.05$) between soluble and bound fractions for all millet varieties tested in this study. In general all soluble extracts had higher TFC than their corresponding bound extracts. Bound phenolic extract of kodo had the highest (4.53 ± 0.14 μmol CE /g defatted meal) TFC whereas pearl millet showed the least (0.28 ± 0.10 μmol CE /g defatted meal). In contrast to the results obtained in this study, Adom and Liu (*34*) reported that soluble extracts of corn, wheat, oats and rice contained a lesser TFC than those of their bound counterparts. The TFC reported in the soluble extracts of oats, rice, corn and wheat were 0.45, 0.33, 0.16 and 0.09 μmol CE/g of grain, respectively, whereas TFC for corresponding bound extracts were 0.71, 0.6, 1.52, and 1.15 μmol CE/g of grain, respectively (*22*). Adom *et al.* (*32*), using different varieties of wheat, further showed that bound TFC was higher than that of soluble TFC. In general, values of TFC reported in the present study for different millet varieties were higher than those reported for other cereals, namely corn, wheat, oats and rice (*22, 32*). This study reported for the first time the TFC of soluble and insoluble bound phenolic extracts of millets as determined using a colourimetric method. Limited information is so far available on the quantification and characterization of millet flavonoids and their identification is being further perused.

2.4.3 Proanthocyanidin content (PC)

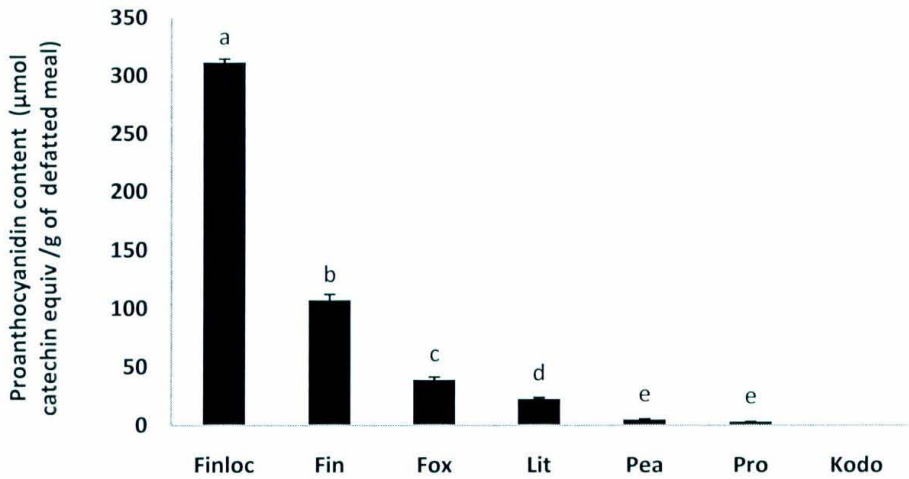


Figure 2.1 Proanthocyanidins content of whole millet varieties. Finloc, finger (local); Fin, finger (Ravi); Fox, foxtail; Lit, little; Pea, pearl; Pro, proso; Kodo, kodo millets. Bars with different letters are significantly different ($p<0.05$).

Proanthocyanidin are oligomeric or polymeric flavonoids consisting of flavan-3-ol units. They are biologically active and when present in sufficient quantities may lower the nutritional value and biological availability of proteins and minerals (*33*). Several *in vivo* assays have demonstrated their antiinflammatory, antiviral, antibacterial and antioxidant properties. **Figure 2.1** shows the PC expressed as µmol CE/g defatted meal. Among the millet varieties used in this study, finger (local) millet had the highest PC (311.3 ± 3.0 µmol CE/g defatted meal) followed by finger (Ravi), foxtail, little, pearl and proso millets. Low amounts of PC were detected in proso and pearl millets. Proanthocyanidins were not detected in kodo millet. The values reported for millets were higher than those for barley.

Tannin contents reported for barley showed a range of 0.21- 0.20 μmol CE/g barley (*26*). The vanillin test used in this study is quite specific for flavanols (monomers and polymers) and dihydrochalcones with a single bond at the 2, 3 positions and free meta-oriented hydroxyl groups on the B ring (*2*). Condensed tannins are generally more potent antioxidants than their corresponding monomers. These have so far been reported only in finger millet varieties. Nevertheless, in the present study all millet varieties, except kodo, had a positive reaction with vanillin reagent and in addition to the two finger millet varieties foxtail and little millets also showed a considerable quantity of proanthocyanidins of 39 and 23 μmol CE / g defatted meal, respectively (**Figure 2.1**). The solvent system (70% acetone) used in this study could enhance the extraction of proanthocyanidins. Chavan et al. (*33*) reported that PC of 70% acetone extract of beach pea was 11 times higher than that of 70% methanol extract. However, previous studies have documented that a number of compounds other than condensed tannins such as eriodictyol and luteoforol may give a positive response in the vanillin reaction. Therefore, it is required to confirm availability of proanthocyanidins using specific tests such as chlorox bleach test (*34*).

Table 2.2 Trolox equivalent antioxidant activity (TEAC) and reducing power (RP) of soluble and bound phenolic extracts of whole millets

Millet type	TEAC[a]		RP[b]	
	Soluble	Bound	Soluble	Bound
Kodo	41.68 ± 0.24a	86.13 ± 2.60a	18.79 ± 0.34a	29.33 ± 2.60a
Finger (local)	12.37 ± 0.08b	6.77 ± 0.90d	26.75 ± 0.36b	7.10 ± 0.90b
Foxtail	11.14 ± 0.68b	40.61 ± 4.66b	5.02 ± 0.01c	8.25 ± 4.66c
Proso	6.73 ± 0.73c	11.14 ± 0.55d	3.64 ± 0.10d	2.96 ± 0.55d
Finger (Ravi)	6.29 ± 0.33c	5.03 ± 0.73d	17.06 ± 0.35e	6.30 ± 1.35e
Pearl	4.15 ± 0.24d	6.77 ± 1.37d	6.69 ± 0.17f	11.29 ± 1.37f
Little	3.70 ± 0.73d	18.34 ± 0.17c	3.93 ± 0.07d	4.17 ± 0.17g

[a] Expressed as μmol trolox equiv /g of defatted meal.

[b] Expressed as μmol ascorbic acid equiv /g of defatted meal.

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

2.4.4 Trolox equivalent antioxidant capacity (TEAC)

The present analysis shows that antioxidant activities of millet were different among varieties and between soluble and bound fractions of the same grain. The TEAC assay is widely used to assess antioxidant capacity of different biological matrices.

The ability of antioxidant compounds to reduce the ABTS radical anion to its nonradical form is compared with that of trolox, which is a water soluble analogue of α-tocopherol. In this study, TEAC test is performed in an aqueous buffer, thus only water-soluble compounds are measured. In the modified TEAC assay (*16*) which was used in the present work ABTS radical anions with a characteristic blue-green colour

were pre-generated by heating ABTS with the thermo-labile azo compound AAPH before addition of the extracts. As shown in previous studies (*16*) some compounds show a biphasic reaction pattern that includes fast and slow reactions in the TEAC assay. Therefore, TEAC values depend on the time point used to read the absorbance (*16*). In the present study TEAC at 6 min was chosen as it includes more of the slow reaction as many antioxidants also demonstrate a slow reaction as well with ABTS radical anion (*24, 27*).

Table 2.2 shows the TEAC of soluble and bound phenolics of millets. The TEAC value ranged from 3.70 ± 0.73 to 41.48 ± 0.24 μmol TE /g defatted meal for soluble phenolics. Soluble extract of kodo millet showed 4 to 10 times higher TEAC than those of other six millet varieties tested in the present study. Except for two finger millet varieties, bound extracts of all other varieties examined showed higher ($p < 0.05$) TEAC values than those of their soluble counterparts. Bound phenolic extracts of kodo millet (86.13 ± 2.6 μmol TE /g defatted meal) showed the highest TEAC that was 17 fold higher than that of finger (Ravi) millet (5.03 ± 0.73 μmol TE /g defatted meal), which had the least.

The TEAC for different barley cultivars ranged from 3.74 to 6.82 μmol TE /g defatted meal (*24*) and those for whole grains of wheat were 4.24 and 4.99 μmol TE /g defatted meal for soft and hard wheat, respectively (*27*). According to Yu et al. (*35*) TEAC of hard winter wheat varieties ranged from 1.08 to 1.91 μmol TE /g grain. In general, results of the present study had higher TEAC values for both soluble and bound extracts than those reported for barley and wheat suggesting their potential as important sources of natural antioxidants. The TEAC of soluble extracts of whole grain millets associated with TPC ($r^2 = 0.696$; $p < 0.01$) and TFC ($r^2 = 0.965$; $p <$

0.01). Furthermore, TEAC of bound phenolic extracts positively and significantly correlated with their corresponding TPC ($r^2 = 0.705$; $p < 0.01$). Many previous studies have reported a significant correlation between phenolic content and TEAC of the extracts of cereals (*27*). Although soluble extracts of kodo and finger (local) millets showed similar TPC, finger (local) millet had 3.4 times lower TEAC value than that of kodo millet. This demonstrates that the contents of phenolics alone may not sufficiently explain the observed antioxidant activity of plant phenolic extracts which are mixtures of different compounds with variable activities in test systems employed in the determination of antioxidant activity of samples. However, ABTS radical anion is a synthetic organic radical which is not relevant in biological and food systems. Thus, interpretation of TEAC of millet phenolic extracts in relation to such systems could be difficult. Therefore determination of inhibitory activity of millet phenolics on peroxyl and hydroxyl radicals and other reactive oxygen species is underway and will be communicated separately.

2.4.5 Reducing power (RP)

Compounds with reducing power (RP) are capable of donating electrons thus reducing the oxidized intermediates of peroxidation by acting as antioxidants. Reductants in the extracts reduce ferric/ferricyanide complex to the ferrous form. Millet extracts tested in the present study exhibited a considerable RP thereby acting as effective reductones. Reducing power of soluble phenolic extracts of millets ranged from 3.64 ± 0.1 to 26.75 ± 0.36 µmol AAE/g defatted meal (**Table 2.2**). Soluble phenolics of finger (local) millet had the highest RP whereas proso millet showed the least.

Reducing power of bound phenolics ranged from 2.96 ± 0.55 to 29.33 ± 2.6 µmol AAE/g defatted meal. Bound phenolics of the two finger millet varieties and

proso millet exhibited a lower RP than their soluble counterparts. Reducing power of bound phenolic extracts showed a significant ($p < 0.05$) difference from that of soluble phenolic extracts except for little millet. In the present study, bound extracts of kodo, foxtail and pearl millets showed generally 1.6 times higher RP than that of their soluble counterparts. In agreement with other assays used in this study RP of dark brown pigmented varieties was quite higher than other varieties investigated. Reducing power of soluble phenolic extracts positively associated with TPC ($r^2 = 0.93$; $p < 0.01$), TFC ($r^2 = 0.551$; $p < 0.01$) and PC ($r^2 = 0.773$; $p < 0.01$) whereas only TPC ($r^2 = 0.669$; $p < 0.01$) of the bound extracts positively correlated with their corresponding RP. These results suggest that phenolic compounds of millet extracts serve as a viable source of electron donors.

2.4.6 *β*-Carotene-linoleate model system

In *β*-carotene-linoleate aqueous emulsion system, heat induced oxidation results in the formation of free radicals which are formed by abstracting a hydrogen atom from the active bis-allylic methylene group of linoleic acid in C-11 between two double bonds. Free radicals attack *β*-carotene causing the molecule to lose its conjugation, resulting in the loss of the characteristic yellow-orange colour of the molecule. Phenolic compounds protect the *β*-carotene from bleaching by reacting with linoleate free radical. Thus, in this system antioxidant potential of millet extracts is rendered in an aqueous medium, which is more relevant to foods and biological systems.

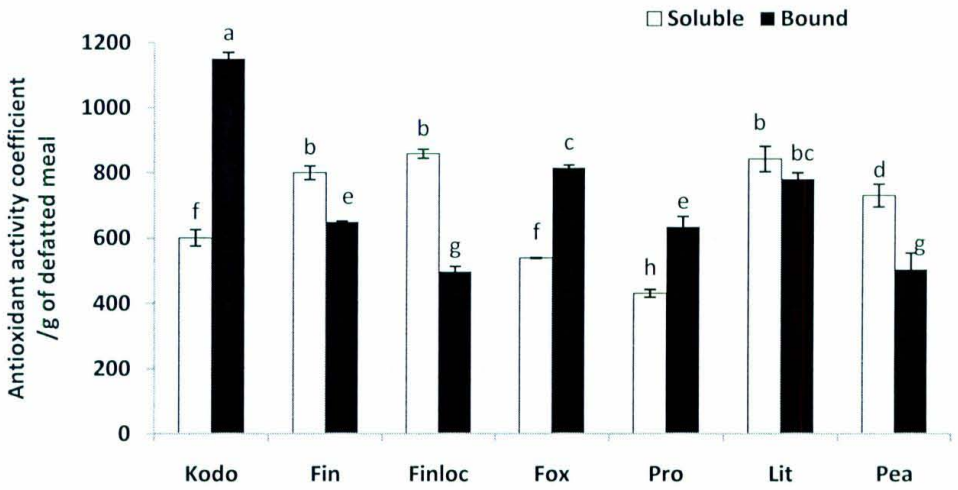


Figure 2.2 Antioxidant activity of soluble and bound phenolic extracts of whole millets in β-carotene-linoleate model system. Kodo, kodo ; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters are significantly different ($p<0.05$). The letter 'a' represents the highest value.

Antioxidant activity coefficients of soluble and bound phenolic extracts of millets are presented in **Figure 2.2**. Soluble extracts of the two finger millet varieties and little millet showed high AAC followed by pearl, kodo, foxtail and proso millets. Bound extracts of kodo showed the highest AAC among others. The antioxidative efficacy of bound extracts was in the order of kodo > foxtail > little > finger (Ravi) > proso > pearl > finger (local). Except for little millet, all other varieties tested demonstrated a significant ($p < 0.05$) difference in β-carotene bleaching, between soluble and bound phenolic extracts. **Figure 2.3** shows the change of corrected absorbance of β-carotene retained with time in the presence of soluble extracts of phenolic compounds and reference standards, ferulic acid and a synthetic antioxidant,

BHA. BHA showed greater antioxidant activity than ferulic acid and all soluble phenolic extracts. This was also observed for all bound extracts (data not shown). Synthetic antioxidants have been reported to contain high antioxidant activity in emulsion systems. Kodo and two finger millet varieties studied exhibited a higher antioxidant activity than ferulic acid whereas foxtail, proso, little and pearl millets soluble extracts had a lower activity at 200 ppm.

Both soluble and bound phenolic extracts showed high potential antioxidant activity against linoleate and any other free radicals formed within the system. Rao and Muralikrishna (*8*) reported high AAC in soluble extracts than corresponding bound extracts in finger millet varieties as noted in the present study. The observed activity of millet extracts can be attributed to their phenolic content and the profile of phenolic compounds present therein. Antioxidant activity coefficient of bound phenolic extracts was positively and significantly associated with TPC ($r^2 = 0.73$; $p < 0.01$) and TFC ($r^2 = 0.616$; $p < 0.01$). However, AAC of soluble phenolic extracts of millet showed a significant correlation only with PC ($r^2 = 0.562$; $p < 0.01$) whereas TPC and TFC were insignificant ($p>0.05$). Based on the results shown here it can be speculated that besides the content, composition of phenolics and other factors may also play a role in the antioxidant activity of millet phenolics in this system.

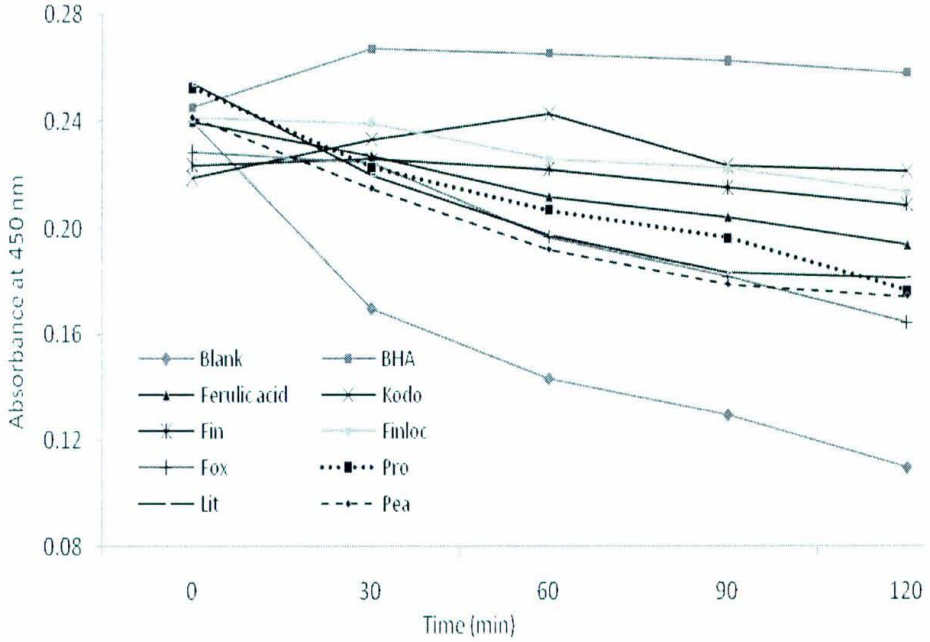


Figure 2.3 Absorbance of retained *β*-carotene at the presence of soluble phenolic extracts of whole millets and reference standards in *β*-carotene-linoleate model system. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets.

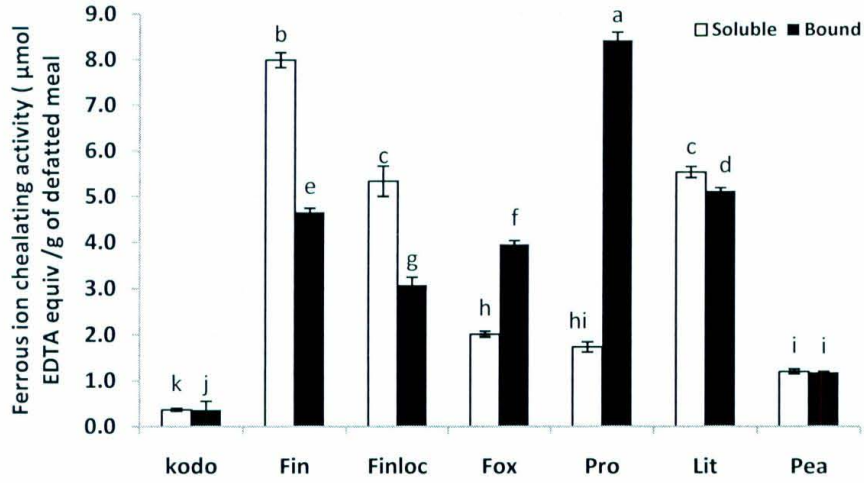


Figure 2.4 Ferrous ion chelating activity (µmol EDTA equiv/g defatted meal) of soluble and bound phenolic extracts of whole millet varieties. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters are significantly different ($p<0.05$). The letter 'a' represents the highest value.

2.4.7 Ferrous ion chelating activity

Ferrous ion is a key transition metal ion responsible for initiation of peroxidation in foods and biological systems. In the body ferrous ions contribute to the generation of hydroxyl radicals via Fenton's reaction and lead to the destruction of biomolecules, thus causing disease conditions and aging. Chelating agents reduce the concentration of metal ions available for catalyzing peroxidation and thus are known to serve as effective secondary antioxidants. In this assay, ferrous ions form a complex with ferrozine and the intensity of the purple colour of the complex decreases in the presence of chelating agents.

Figure 2.4 shows the ferrous ion chelating activity of soluble and bound phenolic extracts of millets. Ferrous ion chelating activity of soluble phenolic extracts ranged from 0.37 ± 0.03 to 7.99 ± 0.16 μmol EDTA equivalents/g defatted meal. Soluble extracts of kodo millet had the lowest ferrous ion chelating activity whereas finger (Ravi) millet showed the highest. The results of this study indicate that phenolic extracts of millets may serve as a potential source of chelating agents inhibiting radical mediated chain reactions.

Millet varieties, except kodo millet, used in this study exhibited superior ferrous ion chelating activity than a number of other cereals. Ferrous ion chelating activity of soluble extracts of barley varieties ranged from 1.1 to 2.1 μmol EDTA equivalents/g defatted meal (24) whereas soluble extracts of soft and hard wheat whole grains were 2.4 and 2.5 μmol EDTA equivalents/g defatted meal, respectively (*27*). The chelating capacities of hard winter varieties ranged from 6.5 to 18.8 μmol EDTA equivalents/g extract (*35*). In this study, the ferrous ion chelating activity of soluble millet extracts decreased in the order of finger (Ravi) > little > finger (local) > foxtail > proso > pearl > kodo (**Figure 2.4**). Interestingly, ferrous ion chelating activity of soluble extracts of millets did not show a significant ($p > 0.05$) correlation with either TPC or TFC, but demonstrated a significant positive correlation with PC ($r^2 = 0.551$; $p < 0.01$). Proanthocyanidins form stable complexes with metal ions acting as an effective ferrous ion chelator.

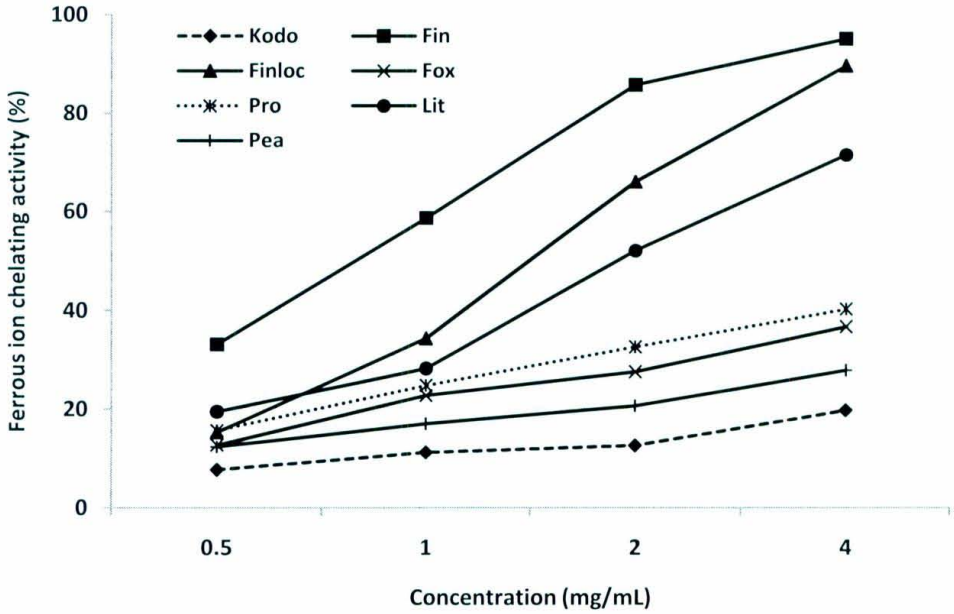


Figure 2.5 Ferrous ion chelating activity (%) of soluble phenolic extracts of whole millet varieties at different concentrations. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets.

Thus, soluble extracts of two finger millet varieties which were rich sources of PC showed a higher ferrous ion chelating activity than that of the kodo millet despite the fact that it has a high TPC, similar to that of finger millets. Consistent with the present findings, lack of correlation between phenolic contents and ferrous ion chelating activity was reported in several studies (*7,20*). These results further suggest that factors other than phenolic content affect the ferrous chelating activity of millet extracts. As shown in **Figure 2.5**, soluble phenolic extracts of millets had a dose dependent ferrous chelating activity. For all tested concentrations of soluble extracts, finger (Ravi) millet exhibited a higher ferrous ion chelating activity than others whereas kodo millet showed the least.

In this study, ferrous ion chelating activity of bound phenolic extracts ranged from 0.85 ± 0.19 to 8.42 ± 0.18 μmol EDTA equivalents/g defatted meal. Proso millet, which contained the lowest TPC showed the highest activity among bound phenolic extracts. As suggested by other authors this could be due to the presence of non phenolic antioxidant compounds such as phytic acid (*4*). Phytic acid is found in varying concentrations in cereals, legumes, nuts and oilseeds and serve as a potential antioxidant by virtue of forming iron-phytate chelate. Extraction conditions employed for bound fractions in the present study may release phytates thus contributing to high ferrous chelation activity. Except for pearl millet, all other varieties exhibited significant differences in ferrous ion chelating activity between soluble and bound extracts. Bound extracts showed a negative correlation with TPC ($r^2 = 0.53$; $p < 0.05$) and an insignificant negative correlation with TFC ($r^2 = 0.09$; $p>0.05$).

2.4.8 Ferulic and *p*-coumaric acids contents

In general, ferulic and *p*-coumaric acids are reported as the major hydroxycinnamic acids in cereals (*24, 27, 36, 37*). **Table 2.3** presents the contents of ferulic and *p*-coumaric acids of free, esterified and insoluble bound phenolic fractions of different millet varieties. Pearl millet had the highest free ferulic acid content (22.37 ± 1.16 μg/g defatted meal) which was at least 3 times higher ($p < 0.05$) than that of other millet varieties tested. The esterified ferulic acid content ranged from 8.47 ± 0.44 to 196.18 ± 5.77 μg/g defatted meal. Kodo millet had the highest total soluble ferulic acid content with a high contribution from esterified fraction. Total soluble ferulic acid contents of all millet varieties used in this study were lower ($p < 0.05$) than those of their bound counterparts.

Little millet had the highest total soluble *p*-coumaric acid content among all millet varieties tested. Total soluble *p*-coumaric acid content ranged from 1.22 to 109.62 μg/g defatted meal. Except for pearl millet, bound phenolic fractions of all other millet varieties showed higher ($p < 0.05$) *p*-coumaric acid contents than their soluble counterparts. Proso millet contained the highest level of *p*-coumaric acid among the bound phenolic extracts whereas pearl millet had the least (**Table 2.3**). In addition, bound phenolic extracts of little, foxtail and kodo millets contained 44, 40 and 32 times higher *p*-coumaric acid levels, respectively, than that of pearl millet.

The HPLC analysis revealed that the content of each phenolic acid in the bound fraction was higher than that in the soluble fraction of all millet varieties examined in this study. In the soluble fractions of millets ferulic acid was the most abundant hydroxycinnamate except for little millet and the ratios of ferulic acid to *p*-coumaric acid content of kodo, finger (Ravi), finger (local), foxtail, proso, little and pearl millets were 11.6, 4.0, 15.8, 1.32, 2.0, 0.9 and 3.9, respectively. However, in the insoluble bound fraction, ferulic acid was dominant only in kodo, finger (Ravi), finger (local), and pearl millets and the ratios of ferulic acid to *p*-coumaric acid of kodo, finger (Ravi), finger (local), foxtail, proso, little and pearl millets were 2.47, 9.35, 6.84, 0.70, 0.29, 0.19 and 30.89, respectively. Thus, the findings of the present study lend further support to an earlier study by Rao and Muralikrishna (*37*) who showed that ferulic and *p*-coumaric acids were the major bound phenolics present in finger millet. In this study the bound phenolic fraction accounted for 64-96 and 50-99% of total ferulic acid and *p*-coumaric acid contents of millet grains, respectively. These findings are similar to those reported in previous studies for other cereals such as rice and wheat (*36, 38*).

Table 2.3 Content (μg/g of defatted meal) of ferulic and *p*-coumaric acids in free, esterified and insoluble bound phenolic extracts of whole millets

Millet type	Free	Esterified	Soluble[a]	Bound
		Ferulic acid		
Kodo	5.79 ± 0.07bc	196.18 ± 5.77a	201.97 ± 5.70a	1685.04 ± 1.00a
Finger (Ravi)	3.94 ± 0.05cd	8.47 ± 0.44e	12.40 ± 0.49e	325.54 ± 7.47d
Finger (local)	2.61 ± 0.99d	16.20 ± 0.10e	18.81 ± 0.89 e	263.85 ± 2.27e
Foxtail	3.46 ± 0.31cd	93.14 ± 0.47b	96.60 ± 0.15b	592.92 ± 3.33c
Proso	4.40 ± 0.48cd	28.95 ± 3.26d	33.35 ± 3.74d	325.50 ± 1.34d
Little	7.50 ± 0.53b	94.09 ± 0.80b	101.58 ± 0.27b	178.82 ± 7.89f
Pearl	22.37 ± 1.16a	60.14 ± 0.26c	82.51 ± 1.42c	638.91 ± 2.86b
		***p*-Coumaric acid**		
Kodo	4.84 ± 0.11c	14.31 ± 0.57c	19.15 ± 0.46c	679.52 ± 6.33d
Finger (Ravi)	1.31 ± 0.08d	1.44 ± 0.05d	2.75 ± 0.14d	34.81 ± 1.28ef
Finger (local)	0.89 ± 0.16d	0.33 ± 0.01d	1.22 ± 0.15d	38.59 ± 0.12e
Foxtail	19.11 ± 0.01b	54.18 ± 0.70b	73.29 ± 0.69b	842.61 ± 1.51c
Proso	0.48 ± 0.09d	15.86 ± 0.88c	16.34 ± 0.96 c	1139.06 ± 1.18a
Little	39.30 ± 1.18a	70.31 ± 2.68a	109.62 ± 3.87a	917.64 ± 8.22b
Pearl	3.80 ± 0.06c	16.96 ± 0.11c	20.77 ± 0.18c	20.68 ± 0.04f

[a] Sum of free and esterified phenolic fractions.

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

According to Zhao et al. (*39*) ferulic acid can be absorbed from rat stomach in its free form and is likely to be metabolized into conjugated ferulic acid in the liver.

Moreover, excretion of ferulic acid in its sulphate and glucuronide forms in human subjects has been observed, thus suggesting its absorption and metabolism in the body (*40*). This further demonstrates the importance of including bound phenolic fraction of grains in assessing the antioxidant activity of millets. Bound phenolics in cereal grains, in general hydroxycinnamates such as ferulic and *p*-coumaric acids are linked via an ester bond to the arabinoxylans in the plant cell wall. Andrearsen et al. (*41*) reported that gastrointestinal esterase from intestinal mucosa and microflora (both human and rat) can release ferulic and diferulic acids from cereal bran. The results of the present study therefore suggest that bound phenolic compounds of millet with high antioxidant activity may also exert their health benefits locally in the colon upon their release by colonic fermentation.

The overall effect of millet phenolic extracts as effective antioxidants appears to depend on a number of factors other than the phenolic content. In this study, when comparing soluble and bound extracts of the same variety, it was generally noted that for some varieties (kodo, finger and pearl millets) high phenolic content in either bound or soluble fraction determines the antioxidant efficacy of the extracts in different antioxidant assays. Thus, the insoluble bound fraction of kodo millet and soluble fractions of the two finger millet varieties demonstrated stronger antioxidant efficacy when compared to their soluble and insoluble bound counterparts, respectively. However, though proso and little millets had higher soluble phenolic contents, their bound phenolic counterparts showed a higher antioxidant activity. It is noteworthy that insoluble bound fractions of proso and little millets had a lower ferulic acid to *p*-coumaric acid ratio of 0.29 and 0.19, respectively, compared to those of their soluble counterparts of 2.0 and 0.92, respectively. It appears that *p*-coumaric

acid is contributing more, compared to ferulic acid to the antioxidant capacity of bound extracts of these two millet varieties, in addition to other phenolics that may be present. These results further suggest that compounds in the extract may exert their antioxidant activity individually as well synergistically. Hence, further phenolic profile analysis is in progress which is necessary to understand the relationship between composition and antioxidant properties of extracts. Furthermore, investigation of the millet phenolics as potential natural antioxidants in several food and biphasic model systems and biological substrates are currently underway.

2.5 Conclusion

This study demonstrated a wide variation in the phenolic contents and antioxidants capacity in whole millet grains. Varietal differences existed in the contents of phenolics as well as antioxidant capacities between soluble and insoluble bound phenolic fractions. Significant contribution from both fractions was noted for the total antioxidant capacity of whole grains of millets as assessed by *in vitro* antioxidant activity assays. However, their potential activity as natural antioxidants depends on the quantity as well as the type of phenolic constituents that need to be fully identified in order to explain the antioxidant capacity of the extracts. A considerable amount of ferulic and *p*-coumaric acids were found in the bound form in whole grains. Thus, contribution of bound phenolic fraction should be taken into account in the assessment of the antioxidant activity of such extracts. The knowledge generated from this study may help to exploit the use of millets, among other cereals, as a nutraceutical ingredient and to promote their use in disease risk reduction and overall health.

2.6 References

1. McKeown, N.M. Whole grain intake is favorably associated with metabolic risk factors for type 2 diabetes and cardiovascular disease in the Framingham Offspring Study. *Am. J. Clin. Nutr.* **2002**, 76, 390-398.

2. Shahidi, F.; Naczk, M. Phenolics in Food and Nutraceuticals. CRC press, Boca Raton, FL, **2004**, pp 1-82.

3. Naczk, M.; Shahidi, F. The effect of methanol-ammonia-water treatment on the content of phenolic acids of canola. *Food Chem.* **1989**, 31, 159-164.

4. Liyana-Pathirana, C.; Shahidi, F. Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions. *J. Agric. Food Chem.* **2005**, 53, 2433-2440.

5. Kroon, P.A.; Faulds, C.B.; Ryden, P.; Robertson, J.A; Williamson, G. Release of covalently bound ferulic acid from fiber in the human colon. *J. Agric. Food Chem.* **1997**, 45, 661-667.

6. Food and Agriculture Organization of the United Nations (FAO) Introduction. In *Sorghum and millets in human nutrition*, FAO Food and Nutrition series, No. 27, FAO, Rome, Italy, **1995**, pp 1-12.

7. McDonough, C.M.; Rooney, L.W. The millets. In *Handbook of Cereal Science and Technology*: Kulp, K. & Ponte, J.G. (eds), Marcel Dekker, Inc., NY, **2000**, pp 177-201.

8. Rao, M.V.S.S.T.S.; Muralikrishna, G. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Elucine coracana* Indaf-15). *J. Sci. Food Agric.* **2002**, 50, 889-892.

9. Dykes, L.; Rooney, L.M. Sorghum and millet phenols and antioxidants. *J. Cereal Sci.* **2006**, 44, 236-251.

10. Viswanath V.; Urooj, A.; Malleshi, N.G. Evaluation of antioxidant and antimicrobial properties of finger millet (*Elusine coracana*). *Food Chem.* **2009**, 114, 340-346.

11. Hegde P.S.; Rajasekaran N.S.; Chandra T.S. Effects of the antioxidant properties of millet species on the oxidative stress and glycemic status in alloxan-induced rats. *Nutr. Res.* **2005**, 25, 1109-1120.

12. Madhujith, T.; Izydorczyk, M.; Shahidi, F. Antioxidant properties of pearled barley fractions. *J. Agric. Food Chem.* **2006**, 54, 3283-3289.

13. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, 16, 144-158.

14. Kim, D.; Jeong, S.W.; Lee, C.Y. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem.* **2003**, 81, 321-326.

15. Price, M.L.; Van Scoyoc, S.; Butler, L.G. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *J. Agric. Food Chem.* **1978**, 26, 1214-1217.

16. van den Berg, R.; Haenen, G.R.M.M.; van den Berg, H.; Bast, A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurement of mixtures. *Food Chem.* **1999**, 66, 511-517.

17. Liyana-Pathirana, C.; Shahidi, F. Importance of insoluble-bound phenolics to antioxidant properties of wheat. *J. Agric. Food Chem.* **2006**, 54, 1256-1264.

18. Oyaizu, M. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* **1986**, 44, 307-315.

19. Jayaprakasha, G.K.; Singh, R.P.; Sakariah, K.K. Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro. *Food Chem.* **2001**, 73, 285-290.

20. Dinis T.C.P.; Madeira, V.M.C.; Almeida, L.M. Action of phenolic derivates (acetoaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* **1994**, 315, 161-169.

21. Zielinski, H.; Kozlowska, H. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *J. Agric. Food Chem.* **2000**, 48, 2008-2016.

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

23. Yu, L.; Haley, S.; Perret, J.; Harris, M.; Wilson, J.; Qian, M. Free radical scavenging properties of wheat extracts. *J. Agric. Food Chem.* **2002**, 50, 1619-1624.

24. Madhujith, T.; Shahidi, F. Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *J. Agric. Food Chem.* **2006**, 54, 8048-8057.

25. Mpofu, A.; Sapirstein, H.D.; Beta, T. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *J. Agric. Food Chem.* **2006**, 54, 1265-1270.

26. Beta, T.; Naing S.K.; Nam, S.; Mpofu A.; Therrien, M. Antioxidant activity in relationship to phenolic content of diverse food barley genotypes. In *Antioxidant measurement & applications*: ACS symposium series 956; Shahidi, F.; Ho C-T., Eds.; American Chemical Society, Washington DC, **2007**, pp 242-254.

27. Liyana-Pathirana, C.; Shahidi, F. Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chem.* **2007**, 101, 1151-1157.

28. Sripriya G.; Chandrasekaran, K.; Murty, V.S.; Chandra, T.S. ESR spectroscopic studies on free radical quenching action of finger millet (*Elusine coracana*) . *Food Chem.* **1996**, 57, 537-540.

29. Madhujith, T.; Shahidi, F. Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. *Food Chem.* **2009**, 117, 615-620.

30. Hung, P.V.; Morita, N. Distribution of phenolic compounds in the graded flours milled from whole buckwheat grains and their antioxidant capacities. *Food Chem.* **2008**, 109, 325-331.

31. Shen, Y.; Jin, L.; Xiao, P.; Bao, J. Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain colour, size and weight. *J. Cereal Sci.* **2009**, 49, 106-111.

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

33. Chavan, U.D.; Shahidi, F.; Naczk, M. Extraction of condensed tannins from beach pea *Lathyrus maritmus* L.) as affected by different solvents. *Food Chem.* **2001**, 75, 509-512.

34. Earp, C.E.; Akinbala, J.O.; Ring, S.H.; Roony, L.W. Evaluation of several methods to determine tannin in sorghum with varying kernel characteristics. *Cereal Chem.* **1981**, 58, 234-238.

35. Yu, L.; Haley, S.; Perret, J.; Harris, M. Antioxidant properties of hard winter wheat extracts. *Food Chem.* **2002**, 78, 457-461.

36. Harukaze, A.; Murata, M.; Homma, S. Analyses of free and bound phenolics in rice. *Food Sci. Technol. Res.* **1999**, 5, 74-79.

37. Rao, M.V.S.S.T.S.; Muralikrishna, G. Non-starch polysaccharides and bound phenolic acids from native and malted finger millet (Ragi, *Elucine coracana* Indaf-15). *Food Chem.* **2001**, 72, 187-192.

38. Okarter, N.; Liu, C.; Sorrells, M.E.; Liu, R.H. Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. *Food Chem.* **2010**, 119, 249-257.

39. Zhao, Z.; Egashira, Y.; Sanada, H. Ferulic acid is quickly absorbed from rat stomach as the free form and then conjugated mainly in the liver. *J. Nutr.* **2004**, 134, 3083-3088.

40. Virgili, F.; Pagana, G.; Bourne, L.; Rimbach, G.; Natella, F.; Rice-Evans, C.; Packer, L. Ferulic acid excretion as a marker of consumption of a French maritime pine (*Pinus maritime*) bark extract. *Free Radical Biol. Med.* **2000,** 28, 1249-1256.

41. Andreasen, M.F.; Kroon, P.A.; Williamson, G.; Garcia-Conesa, M.R. Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radical Biol. Med.* **2001**, 31, 304-301.

CHAPTER 3

Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species

3.1 Abstract

Oxidative stress, caused by reactive oxygen species (ROS), is responsible for modulating several pathological conditions and aging. Soluble and bound phenolic extracts of commonly consumed millets, namely kodo, finger (Ravi), finger (local), foxtail, proso, little and pearl were investigated for their phenolic content and inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and ROS, namely hydroxyl radical, peroxyl radical, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$). Inhibition of DPPH and hydroxyl radicals was detrmined using electron paramagnetic resonance (EPR) spectroscopy. The peroxyl radical inhibitory activity was measured using oxygen radical absorbance capacity (ORAC) assay. The H_2O_2, HOCl and $^1O_2$ scavenging was evaluated using colourimetric methods. In addition major hydroxycinnamic acids were identified and quantified using high performance liquid chromatography (HPLC) and HPLC- (mass spectrometry) MS. All millet varieties displayed effective radical and ROS inhibition activities which generally positively correlated with phenolic contents, except for hydroxyl radical. HPLC analysis revealed the presence of ferulic and *p*-coumaric acids as major hydroxycinnamic acids in phenolic extract and attributed to the observed effects. Bound extracts of millet contributed 38-99% to ROS scavenging, depending on the variety and the test system employed. Hence bound phenolics must be included when evaluating antioxidant activity of millets and other cereals.

3.2 Introduction

Reactive oxygen species (ROS) are generated ubiquitously in the human body from either endogenous or exogenous sources. Within the cell, ROS can be generated in mitochondrial and microsomal electron transport systems, soluble oxidase enzyme systems and during phagocyte activation (*1*). Exogenous ROS generate from air and water pollutants, cigarette smoke, organic solvents, heavy metals, certain drugs and radiation, among others (*2*). Free radicals are highly reactive species with one or more unpaired electrons in their last orbital. Oxygen free radicals, important in biological systems include superoxide ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and hydroperoxyl ($HOO^{\cdot}$) radicals whereas major non-radical reactive species are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite (ONOO), nitric oxide (NO) and singlet oxygen ($^1O_2$).

ROS which include both free radicals and non-radicals may play a dual role either as toxic or beneficial compounds depending on their concentration in biological systems. Oxygen radicals at low or moderate levels impart beneficial roles such as signal transduction, activation of host immunity system, and gene transcription, among others (*3,4*). On the other hand, excessive generation of ROS causes oxidative stress, a deleterious process leading to the oxidation of biomolecules such as proteins, lipids, carbohydrates and DNA (*5*). Oxidative stress is known to play a major role in the development of several chronic ailments such as different types of cancer, cardiovascular diseases, arthritis, diabetes, autoimmune and neurodegenerative disorders and aging. Thus, external sources of antioxidants are required to prevent oxidative damage in the human body once internal antioxidant defense systems such as either enzymes (superoxide dismutase, catalase and glutathione peroxidase) or

other relevant compounds (lipoic acid, uric acid, ascorbic acid, α-tocopherol and glutathione) are overwhelmed.

Accumulating epidemiological evidences have suggested that consumption of fruits, vegetables, cereals and legumes exerts protective effects against the occurrence of a multitude of disease states and phenolics, which form a considerable part of dietary phytochemicals, may partly attribute to these health benefits (*6*). Phenolics and polyphenolic compounds act as antioxidants by inhibiting generation of ROS, directly scavenging ROS, chelating transition metal ions and by acting as reducing agents (*7*). Phenolic compounds are converted to phenoxyl radicals by donating hydrogen atoms or electrons in the process of scavenging of free radicals. Antioxidant efficiency of a phenolic compound is related to its molecular structure, and the number of hydroxyl groups present. In addition, the degree of stabilization of the phenoxyl radical is affected by conjugation and resonance effects in which substituent groups in the aromatic ring play a major role.

Cereals serve as a good source of natural antioxidants. Several recent studies have reported the antioxidant potency of phenolic extracts from barley, wheat, rice, oat, rye, and millet (*8-12*). Millets are underutilized in the western world, but are among the cereals of primary economic importance in the African and Asian countries. Millets are highly nutritious among cereals and as they do not contain gluten, there is a good prospect for their use in developing of gluten-free foods and beverages for patients with gluten sensitivity. Few studies have reported that millets are rich sources of phenolic compounds but this information is mainly confined to finger millet. The predominant phenolic compounds identified in finger millets were phenolic acids, flavones and proanthocyanidins.

In vivo studies have reported the beneficial role of millets on blood glucose and cholesterol regulation (*13, 14*) as well as wound healing (*15,16*) in diabetic rats. Hedge et al. (*17*) have demonstrated that diets containing whole grains of kodo and finger millets can protect against hyperglycemic and alloxan-induced oxidative stress in Wistar rats. Furthermore, it has been shown that rats fed on kodo millet enriched diet had a greater reduction in blood glucose and cholesterol levels compared to those fed on finger millet, thus suggesting the protective role of antioxidative compounds such as phenolics and phytates present in millets, among other constituents. In addition, Shobana et al. (*18*) showed that phenolic extracts from finger millet coat were responsible for inhibiting enzymes such as alpha-glucosidase and pancreatic amylase, thus indicating their therapeutic potential for managing postprandial hyperglycemia. Therefore, the prospects for developing millet-based specialty foods should be considered for health promotion and wellness.

A close scrutiny of the literature shows few studies that report on the ability of millet phenolics for scavenging 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), DPPH and hydroxyl radicals (*11,19-21*). Unlike ABTS and DPPH radicals which are less relevant to biological systems, ROS are commonly found in living tissues and no information is available on the scavenging properties of hydrogen peroxide, hypochlorous acid, and singlet oxygen by phenolic compounds in different millet varieties. The objectives of the present study were (a) to investigate the antiradical and ROS scavenging properties of soluble and insoluble bound phenolic extracts present in different whole grain millet varieties, namely kodo, finger, foxtail, proso, little and pearl and (b) to determine phenolic contents in extracts of millet

whole grains in order to explore the relationship between their phenolic contents and antiradical activities.

3.3 Materials and methods

Seven millet varieties, namely foxtail (*Setaria italica)*, proso (*Panicum miliacium)*, two finger millet (*Elusine coracana*) varieties (Ravi and local), kodo (*Paspalum scrobiculatum*), little millet (*Panicum sumatrense*) and pearl millet (*Pennisetum glaucum*) were used in this study. Pearl millet (dark green cultivar), grown in India, was obtained through Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India. Other grain samples were obtained from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka.

Folin Ciocalteu's reagent, ferulic acid, *p*-coumaric acid, catechin, aluminium chloride, sodium nitrite, 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), sodium chloride, trolox, mono- and dibasic potassium phosphates, mono- and dibasic sodium phosphates, 2,2-diphenyl-1-picrylhydrazyl (DPPH), H_2O_2, $FeSO_4$, 5,5-dimethyl-1-1-pyrroline-*N*-oxide (DMPO), fluorescein, *N,N*- dimethyl *p*-nitrosoaniline (DPN), histidine, sodium hypochlorite, sulphuric acid, taurine, potassium iodide were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Diethyl ether, ethyl acetate, hexane, acetone, methanol, acetonitrile, formic acid, hydrochloric acid, sodium hydroxide and sodium carbonate were procured from Fisher Scientific Ltd. (Ottawa, ON).

3.3.1 Sample preparation

Whole millet grains, free from soil and other particles, were ground to obtain a fine powder using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada

Inc. Brockville, ON) and the powder passed through mesh 16 (sieve opening 1 mm, Tylor test sieve, Mentor, OH). Ground millet samples were blended with hexane (1:5, w/v, 5 min for 3 times) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT) at ambient temperature to remove lipids and lipid-soluble components. Defatted samples were air dried at room temperature for 12 h before packing in polyethylene pouches under vacuum and stored at –20°C until used within one week for further analysis.

3.3.2 Extraction of soluble phenolic compounds

Soluble phenolic extracts were obtained using ultrasonic-assisted extraction with 70% (v/v) acetone under refluxing conditions (*22*). Residues of whole grain samples were air dried for 12 h and stored at -20°C and then used within a week to extract bound phenolic compounds within a week. During all stages, extracts were minimally exposed to light by covering them with aluminium foil. Lyophilized crude phenolic extracts were stored at -20°C until used for further analysis.

3.3.3 Extraction of bound phenolic compounds

The residue of the whole grain sample left after extraction of soluble phenolics was used to extract bound phenolics by alkaline hydrolysis which was performed with 2 M NaOH at room temperature for 4 h with stirring under nitrogen (*22*). Phenolic compounds extracted were reconstituted in 5 mL of HPLC grade methanol and stored at -20°C until used.

3.3.4 Determination of total phenolic content (TPC)

The total phenolic content of each extract was determined using the method described by Singleton and Rossi (*23*) with slight modifications as explained previously (*22*). The content of total phenolics in each extract was determined using a standard curve prepared for ferulic acid and expressed as micromoles of ferulic acid equivalents (FAE) per gram of the grain (dw).

3.3.5 Determination of total flavonoid content (TFC)

Total flavonoid content was determined using a colourimetric method described by Kim et al. (*24*) and Chandrasekara and Shahidi (*22*). A standard curve prepared with catechin was used to calculate total flavonoid content. The TFC was expressed as micromoles of catechin equivalents (CE) per gram of the grain (dw).

3.3.6 HPLC analysis of major hydroxycinnamic acids

Major hydroxycinnamic acid content of soluble and insoluble bound phenolic fractions of millet grains were tentatively identified and quantified by HPLC and further confirmed using HPLC-MS as explained previously (*22*). The results were expressed as microgram per gram of the grain (dw).

3.3.7 DPPH radical scavenging activity

The effect of extracts on the scavenging of DPPH radicals was determined according to the method adapted from Madhujith and Shahidi (*10*) with slight modifications. The phenolic extracts (0.5 mL) of different concentrations in methanol (0.062-2.5 mg/mL) were added to 2 mL of methanolic solution of DPPH (0.19 mM), vortexed and allowed to stand at room temperature in the dark. The mixture was passed to the

sample cavity of Bruker E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker E-scan, Bruker Biospin Co. Billercia, MA) and the spectrum was recorded after 10 min. Methanol was used as the control in place of extract and different concentrations (0.025-0.3 mg/mL) of ferulic acid in methanol were used for constructing the standard curve. The parameters of Bruker E-scan EPR spectrometer were set as follows; 5.02 x 10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.53 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 (%) = {(EPR signal intensity for the control – EPR signal intensity for the sample)/ EPR signal intensity for the control} x 100. The DPPH radical scavenging activity was expressed as micromoles of ferulic acid equivalents (FAE) per gram of the grain (dw).

3.3.8 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was determined according to the method explained by Madhujith and Shahidi (*10*) with slight modifications. Extracts of soluble and bound phenolic compounds of millets were dissolved in deionized water and diluted appropriately. Extracts (100 µL) were mixed with 100 µL of H_2O_2, (10 Mm), and 200 µL of DMPO (17.6 mM) and 100 µL of $FeSO_4$ (0.1 mM). After 1 min the mixtures were introduced into the sample cavity of EPR spectrometer and the spectrum was recorded. Deionized water was used as the control in place of the extract. Ferulic acid dissolved in deionized water was used to prepare the standard curve (0.05-2.0 mg/mL). Hydroxyl radical scavenging capacities of the extracts were

calculated using the following equation. Hydroxyl radical scavenging capacity (%) = {(EPR signal intensity for the control – EPR signal intensity for the sample)/ EPR signal intensity for the control} x 100. The hydroxyl radical scavenging activity of the extracts was expressed as micromoles of ferulic acid equivalents (FAE) per gram of the grain (dw).

3.3.9 Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity measures antioxidant scavenging activity of test compounds against peroxyl radical generated by AAPH. The ORAC assay was based on the method explained by Madhujith and Shahidi (*25*). The samples, standards and other reactants were prepared in 75 mM phosphate buffer (pH 7.0) and the final reaction mixture (295 µL) contained 200 µL (0.11 µM) of fluorescein as oxidizable substrate, 20 µL of extract or trolox and 75 µL of AAPH (63.4 mM). The reaction was carried out in a Costar® 3695 flat bottom 96 well black microplates (Corning Incoperated, Corning, NY). Determination of ORAC was carried out using a plate reader equipped with a built-in incubator (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany). Fluorescein (200 µL) was manually pipeted into the wells containing the extract or standards (20 µL) followed by incubation for 15 min at 37°C. The injector pump was programmed to inject APPH at the end of incubation during the first cycle. The plate was automatically shaken for 4 s after each addition and the microplate reader was programmed to perform additional shaking of the contents in wells before each reading was taken. A gain adjustment was performed before the beginning of the measurements to optimize the maximum sensitivity, by manually pipetting 200 µL of fluorescein into wells. Fluorescence recorded every

minute for 25 cycles and each cycle was 210s. A control (phosphate buffer, fluorescein and AAPH) and different concentrations of Trolox (6.25 -50 μM) as the standard were performed in each assay. All reactions mixtures were prepared in duplicate and three independent runs were performed for each sample. ORAC values of extracts were expressed as micromoles Trolox equivalents (TE) per gram of the grain (dw) using the standard curve calculated for each experiment.

3.3.10 Hydrogen peroxide (H_2O_2) scavenging activity

The efficacy of phenolic extracts in scavenging H_2O_2 was determined according to the method explained by Wettasinghe and Shahidi (*26*). The reaction was carried out in 45 mM sodium phosphate buffer (pH 7.4). The extracts dissolved in distilled water (0.4 mL) were added to 0.6 mL of H_2O_2 (40 mM). Total reaction volume was brought up to 2 mL with sodium phosphate buffer. After incubating for 40 min at 30° C the absorbance was measured at 230 nm. Blanks were run for each sample replacing H_2O_2 with buffer for background correction. A standard curve was prepared with ferulic acid. Percentage H_2O_2 scavenging activity of millet phenolic extracts was calculated using the following equation. H_2O_2 scavenging activity (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. The results were expressed as micromoles FAE per gram of the grain (dw).

3.3.11 Singlet oxygen inhibition

The ability of millet phenolic extracts to inhibit singlet oxygen was measured according to a spectrophotometric method explained by Maldonado et al. (*27*). The chemical solutions and phenolic extracts were prepared in 45 mM sodium phosphate buffer (pH 7.4). The reaction mixture consisted of 0.4 mL phenolic extract, 0.5 mL

DPN (200 µM), 0.2 mL histidine (100 mM), 0.2 mL sodium hypochlorite (100 mM), and 0.2 mL H_2O_2 (100 mM) and the total volume was made to 2 mL with sodium phosphate buffer. The absorbance of the reaction mixture was measured at 440 nm after incubation for 40 min at 30° C. Blanks were run for each sample with 0.4 mL of extract and phosphate buffer. A control was prepared replacing phenolic extract with sodium phosphate buffer. Different concentrations of ferulic acid were used to establish the standard curve. The percentage inhibition of singlet oxygen was calculated as follows. Singlet oxygen inhibition activity (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. The results were expressed as micromoles FAE per gram of the grain (dw).

3.3.12 Hypochlorous acid (HOCl) scavenging capacity

The method of Weiss et al. (*28*) was adapted, with slight modification, for determining the HOCl scavenging activity of soluble and insoluble bound millet phenolics. HOCl was prepared by adjusting the pH of a 1% (v/v) NaOCl solution to 6.2 with 1% (v/v) sulphuric acid. The concentration of the HOCl solution was determined spectrophotometrically at 235 nm using the molar extinction coefficient of 100 M^{-1} cm^{-1} for HOCl. The HOCl solution was prepared freshly each day. The extracts and ferulic acid were dissolved in 50 mM phosphate saline buffer solution (pH 7.4) to obtain different concentrations. The reaction mixture for measuring the scavenging of HOCl contained 100 µL taurine (150 mM), 100 µL of the extract, 100 µL HOCl and phosphate saline buffer in a final volume of 1 mL. After incubating for 10 min at room temperature, 10 µL of potassium iodide (2 M) were added to the reaction mixture. Blanks with extracts and phosphate saline buffer were run for each sample for background correction. The absorbance of the yellow colour reaction mixture was

measured at 350 nm. The percentage inhibition of HOCl by millet phenolic extracts was calculated as follows. HOCl inhibition activity (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. A standard curve was prepared using different concentrations of ferulic acid and the results were expressed as micromoles FAE per gram of the grain (dw).

3.3.13 Statistical analysis

All experiments were carried out in triplicates and data were reported as mean ± standard deviation. The Student's t test at $p \leq 0.05$ was used to determine significance of differences between soluble and bound extracts of millets. The differences of mean values among millet varieties was determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p \leq 0.05$ significance level. Correlation analysis was performed between phenolic contents and ROS scavenging activity of soluble and bound extracts using Pearson and Spearman's correlations, respectively. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL).

3.4 Results and discussion

The present study investigated the antiradical potential of phenolic extracts from whole millet grains against synthetic DPPH radical and several ROS which are important in biological systems. To the best of our knowledge this study reports for the first time the scavenging properties of different physiologically important ROS in soluble and insoluble bound phenolic extracts of all millet grains examined here with some exceptions for which DPPH radical scavenging effects were reported only for their soluble phenolic fractions.

3.4.1 Total phenolic content (TPC)

The TPC of soluble and insoluble bound phenolic extracts in whole millet grains ranged from 4.87 to 25.55 and from 1.51 to 62.50 μmol FAE/g of the grain, respectively (**Figure 3.1A**). Finger (local) and kodo millets showed a higher soluble phenolic content compared to other varieties tested. Furthermore, TPC of soluble extracts did not significantly ($p > 0.05$) differ between each pair of kodo and finger (local) millets, foxtail and little millets and proso and pearl millets. The order of TPC of insoluble bound extracts of the millet varieties were kodo > foxtail > little > pearl > finger (Ravi) = finger (local) > proso. The insoluble bound TPC was not significantly different between the two finger millet varieties. Furthermore, bound extracts of kodo millet had 2.5 times higher ($p < 0.05$) phenolic content while finger (Ravi), finger (local), proso and little millets showed 5.6, 10, 3 and 1.3 times lesser ($p < 0.05$) TPC than that of soluble extracts, respectively. The results of this study clearly showed that the distribution of TPC between soluble and bound phenolic extracts may vary depending on the variety as shown for different cereal grains (*29, 30*). In this study one commonly consumed variety of each millet species was used to determine the phenolic content and their antioxidant activities, except for finger millet that two varieties of Ravi and local were used. The results showed that phenolic contents as well as their antioxidant activities differed even between two varieties belonging to the same millet species, thus demonstrating the importance of selecting several varieties from each species for their ROS scavenging activities.

3.4.2 Total flavonoid content (TFC)

The TFC of soluble and bound phenolic fractions ranged from 25.80 to 0.80 and from 3.46 to 0.20 µmol CE/g of the grain (**Figure 3.1B**). Kodo millet soluble phenolic extracts showed the highest TFC in all varieties tested and TFC of soluble phenolic extracts were significantly higher ($p < 0.05$) than that of their bound counterparts. TPC ($r^2 = 0.537$; $p < 0.01$) of soluble extracts positively and significantly associated with TFC whereas bound extracts showed an insignificant association ($r^2 = 0.142$; $p > 0.05$) suggesting their poor contribution to the TPC of bound extracts.

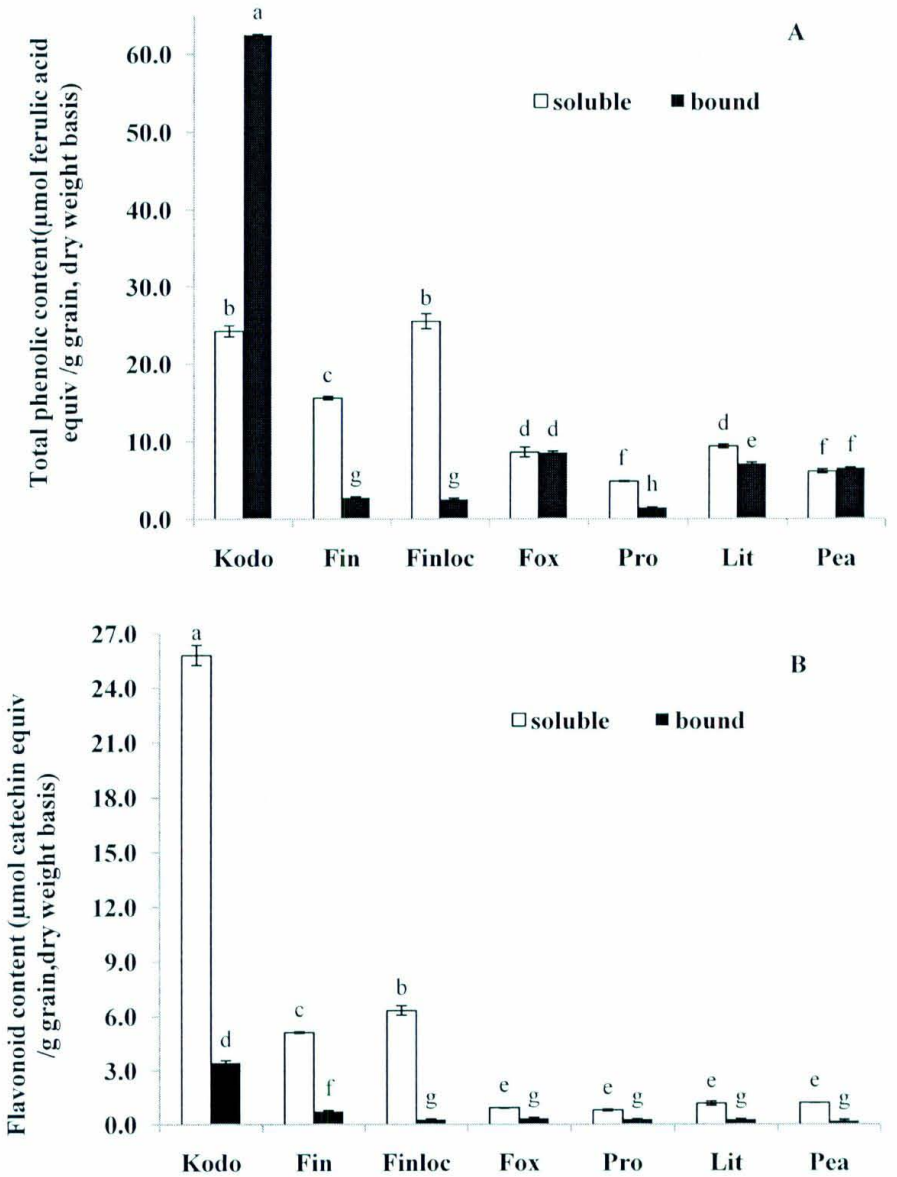


Figure 3.1 Total phenolic content (1A) and flavonoid content (1B) of soluble and bound phenolic extracts of whole millets. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters have significantly different ($p < 0.05$) mean values. The letter 'a' represents the highest value.

Table 3.1 Ferulic and p-coumaric acid contents (μg/g dry weight of the grain) in soluble and bound phenolic extracts of whole millets

Millet type	Ferulic acid			*p*-Coumaric acid		
	Soluble [a]	Bound [a]	Total [b]	Soluble [a]	Bound [a]	Total [b]
Kodo	154.6 ± 4.4a	1290.0 ± 0.8a	1444.6 ± 3.6a	14.7 ± 0.4c	520.2 ± 4.9d	534.9 ± 5.2d
Finger (Ravi)	9.1 ± 0.4f	237.7 ± 5.5d	246.7 ± 5.8d	2.0 ± 0.1d	25.4 ± 0.9ef	27.4 ± 1.0e
Finger (local)	15.1 ± 0.7e	212.0 ± 1.8f	227.1 ± 2.5e	1.0 ± 0.1d	31.0 ± 0.1e	32.0 ± 0.1e
Foxtail	71.6 ± 0.1b	439.5 ± 2.5c	511.1 ± 2.6c	54.3 ± 0.5b	624.6 ± 1.1c	678.9 ± 1.6c
Proso	22.8 ± 2.6d	222.5 ± 0.9e	245.3 ± 1.7d	11.2 ± 0.7c	778.5 ± 0.8a	789.6 ± 1.5a
Little	75.0 ± 0.2b	132.1 ± 5.8g	207.1 ± 5.6f	81.0 ± 2.9a	677.9 ± 6.1b	758.9 ± 8.9b
Pearl	59.4 ± 1.0c	460.0 ± 2.1b	519.4 ± 1.0b	15.0 ± 0.1c	14.9 ± 0.1f	29.8 ± 0.1e

[a] Values in each column having the same letter are not significantly different ($p > 0.05$). [b] Sum of soluble and bound phenolic extracts.

3.4.3 Hydroxycinnamic acid content

Table 1 presents the two major hydroxycinnamic acids, ferulic and *p*- coumaric acid contents of whole grain of millets. The ferulic acid content of soluble and bound phenolic extracts of whole millet ranged from 9.1 to 154.6 and 132.1 to 1290.0 µg/g of the grain, respectively. Kodo millet showed the highest content of total ferulic acid content which was 7 times higher than that of little millet which exhibited the least. In general bound phenolic fraction contained higher ($p < 0.05$) content of ferulic and *p*-coumaric acids compared to their soluble counterparts. The contribution of bound phenolics to the total ferulic and *p*-coumaric acid contents of different millet varieties ranged from 64 to 96 and from 50 to 98%, respectively. When compared the total ferulic and *p*-coumaric acids contents it is noteworthy that kodo, finger and pearl millets had a higher total ferulic acid and a lower *p*-coumaric acid contents than foxtail, proso and little millets suggesting the varietal differences of individual phenolic compounds though several small grain cereals are considered as a group referring to millet. This also emphasized the fact that using of botanical names of millets when they are used in antioxidant activity studies to compare with other cereals such as rice, wheat, barley, oats and rye.

3.4.5 DPPH radical scavenging activity (DRSA)

DPPH radical is a synthetic organic radical which is widely used to evaluate free radical scavenging properties of antioxidative compounds. DPPH is a more stable radical compared to the highly reactive and transient peroxyl and hydroxyl radicals that are involved in lipid peroxidation and tissue injury in biological systems.

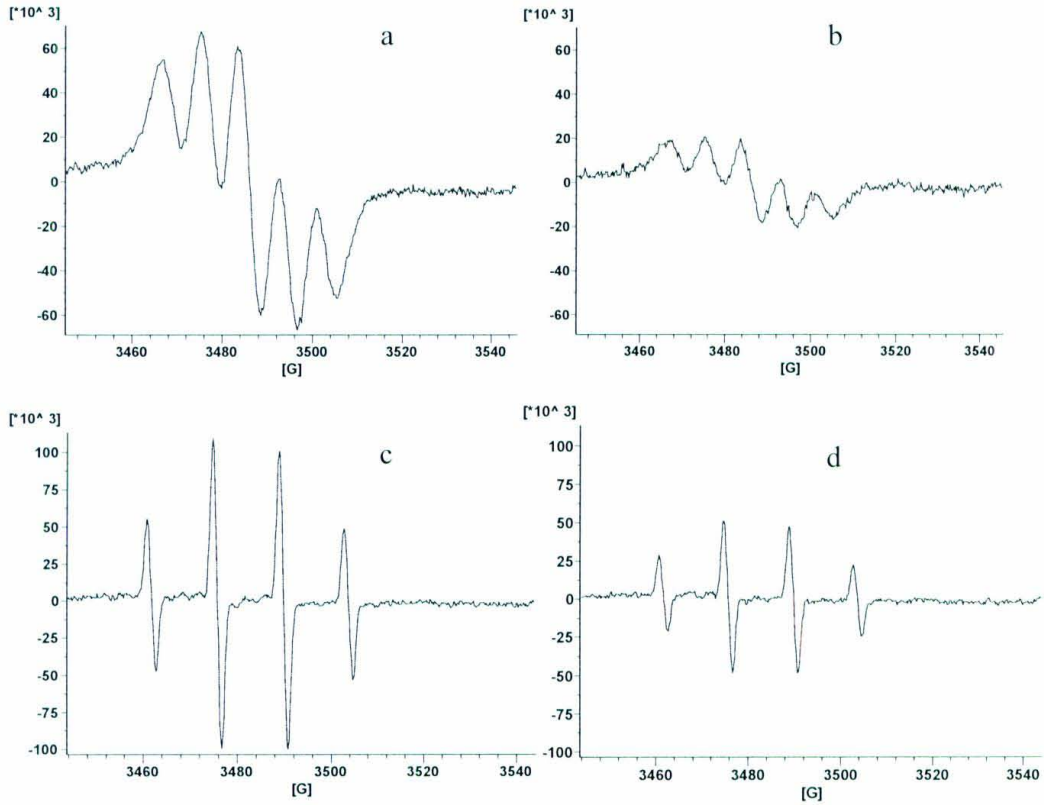


Figure 3.2 Electron Paramagenetic Resonance signals of DPPH radicals alone (a), DPPH radicals in the presence of kodo extract at 0.5 mg/mL (b), DMPO-OH adduct alone (c), and DMPO-OH adduct in the presence of kodo extract at 1.25 mg/mL (d).

The DRSA assay is based on the ability of antioxidant compounds to donate a hydrogen atom or an electron to the stable DPPH radical to convert it to the non-radical form, which occurs slowly on the electron transfer process (*31*). In the present study EPR spectroscopy was used to detect the signal intensity of DPPH radicals left upon reacting with phenolic compounds in the test system. **Figure 3.2 (A and B)** show representative EPR spectra of DPPH radicals alone and in the presence of

soluble phenolic extracts of kodo millet which showed the highest and lowest signal intensities respectively.

Table 3.2 DPPH radical scavenging activity (DPPH) and Oxygen Radical Absorbance Capacity (ORAC) of soluble and bound phenolic extracts of whole millets

Millet type	DPPH[a]		ORAC[b]	
	Soluble	Bound	Soluble	Bound
Kodo	25.69 ± 0.34c	17.38 ± 0.02a	74.25 ± 0.10b	606.88 ± 5.94a
Finger (Ravi)	29.43 ± 0.79b	5.84 ± 0.51d	71.90 ± 1.70b	44.32 ± 4.18g
Finger (local)	54.40 ± 0.56a	6.93 ± 0.53c	115.05 ± 3.63a	85.24 ± 4.84f
Foxtail	4.54 ± 0.38f,g	5.36 ± 0.03d,e	42.34 ± 4.12d	202.64 ± 6.66b
Proso	3.72 ± 0.04f	2.77 ± 0.01g	23.93 ± 2.38f	90.86 ± 4.81e,f
Little	7.59 ± 0.24e	4.19 ± 0.05f	64.46 ± 0.54c	172.39 ± 6.37c
Pearl	9.95 ± 0.88d	7.14 ± 0.04b,c	41.73 ± 3.17d,e	125.44 ± 4.77d

[a] Expressed as µmol ferulic acid equiv /g grain (dry weight basis).

[b] Expressed as µmol ferulic acid equiv /g grain (dry weight basis).

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

The DRSA of soluble and bound phenolic extracts ranged from 54.40 to 3.72 and from 17.38 to 2.77 μmol FAE/g of the grain, respectively (**Table 3.2**). In general soluble phenolic extracts of millet had higher DRSA compared to those of their bound counterparts and the differences were significant ($p < 0.05$), except for foxtail millet. According to Madhujith et al. (*32*) the DRSA of two barley cultivars (Falcon and AC Metcalfe) ranged from 69.30 to 0.08 μmol FAE/g of defatted meal for different pearling fractions. These results added more evidence to the values obtained for soluble millet fractions in the present study. Among soluble extracts two finger millet varieties and kodo millet had 7 to 15 fold higher DRSA compared to proso millet which showed the least activity. The high DRSA reported in soluble extracts of finger and kodo millets can be attributed to the high content of phenolics and their constituent compounds such as tannin and flavonoids. Yokozawa et al. (*33*) showed that tannins and flavonoids were effective DPPH radical scavengers.

The trend of DRSA among different millet varieties used in the present work is in agreement with the previous studies reported for millet. Sripriya et al. (*15*) showed that finger millet had superior DRSA compared to foxtail and pearl millets and other cereals such as wheat, rice, and sorghum. Hegde and Chandra (*14*) reported that kodo millet had a higher DPPH radical scavenging potential compared to foxtail, little and finger and barnyard millets using an EPR method. In disagreement with present results, Choi et al. (*13*) reported a lower DRSA which was 16 % at a concentration of 4 mg/mL of the soluble crude extracts of foxtail and proso millet than those reported in this study. However, in the present work DRSA of crude soluble extracts at the same concentration of foxtail and proso millets were 42 and 72%, respectively. This

difference could be attributed to several factors including variation in the growing environment of millets, genotype and different solvents and methods used in the extraction of phenolics. According to Mpofu et al. (*34*) growing environment and genotype affect the phenolic content and the antioxidant activity of cereals such as wheat. In addition, solvent and method of extraction employed may result in different antioxidant activities of the same plant material.

In this study, the DRSA of soluble extracts positively and significantly associated with the TPC ($r^2 = 0.882$; $p < 0.01$) and TFC ($r^2 = 0.868$; $p < 0.01$). Similarly, TPC ($r^2 = 0.192$; $p < 0.01$) of the bound extracts positively correlated with their corresponding DRSA. The present analyses lend further support to those reported by several other authors; a significant correlation exists between TPC and DRSA for the phenolic extracts of cereals such as wheat, barley, rye, sorghum, pearl millet, oat and buckwheat (*8,11*) indicating the major contribution of phenolics to DRSA. However, in contrast to the results obtained in this study, Yu et al. (*9*) did not observe a significant correlation between TPC and DRSA for the hard winter varieties of wheat.

3.4.6 Oxygen radical absorbance capacity

In the ORAC assay peroxyl radicals are generated by thermal decomposition of the azo compound AAPH. Fluorescein (FL) is employed as the probe and the fluorescence decay indicates its reaction with peroxyl radical. In the presence of antioxidative compounds FL decay is inhibited and their intensity can be measured at 485 nm excitation and 525 nm emission. The protective effect of phenolic extracts against peroxyl radicals is calculated from net integrated area under the kinetic curve (AUC) and compared to that of the trolox, a water soluble analogue of α-tocopherol.

The results in this study demonstrated that both soluble and bound phenolic extracts scavenge peroxyl radicals effectively though the trend of scavenging between soluble and bound extracts differed among varieties tested in this study (**Table 3.2**). The ORAC values of soluble and bound millet extracts ranged from 23.9 to 115.1 and from 44.3 to 606.9 μmol TE/ g of the grain, respectively. The ability of millet soluble fractions to scavenge peroxyl radicals was in the order of finger (local)> kodo> finger (Ravi)> little> foxtail> pearl> proso millets. Kodo bound phenolic extracts showed the highest peroxyl radical scavenging activity followed by foxtail, little, pearl, proso, finger (local) and finger (Ravi).

Interestingly, in the present study millet phenolic extracts exhibited superior ORAC values than those of other cereals such as barley and wheat reported by other authors (*12, 25, 30, 35*). This could be attributed to the high phenolic content in whole millet grains. Madhujith and Shahidi (*25*) showed that ORAC values of whole barley extracts from six different cultivars ranged from 11 to 19 μmol TE/g of defatted meal. In another study they further showed that ORAC values of insoluble bound extracts of barley cultivars tested ranged from 22 to 35 μmol TE/g of defatted meal indicating their higher ORAC values compared to that of soluble extracts (*30*). Liyana-Pathirana and Shahidi (*12*) showed that bound phenolic extracts of soft and hard wheat whole grains had nearly 6 fold higher ORAC values than those of soluble phenolic extracts. Furthermore, Okarter et al. (*35*) reported that ORAC values of soluble and bound phenolic extracts of whole wheat ranged from 19.6 to 37.5 and from 31.9 to 59.5 μmol TE/ g of the grain (dw), respectively, and these values were within the range of those obtained for different whole millet soluble and bound phenolic extracts in this work.

In the present study ORAC values of soluble extracts positively and strongly correlated with TPC ($r^2 = 0.886$; $p < 0.01$) and insignificantly with TFC ($r^2 = 0.153$; $p > 0.05$) suggesting their contribution in scavenging peroxyl radicals in the aqueous medium employed in the present work. However, ORAC values of bound extracts associated positively and strongly with TPC ($r^2 = 0.856$; $p < 0.01$) whereas it showed an insignificant and poor correlation with TFC ($r^2 = 0.195$; $p > 0.05$).

The ORAC assay is based on the hydrogen atom transfer reaction and thus hydrogen donating ability of millet phenolics to scavenge peroxyl radical is demonstrated in this study. Peroxyl radicals are intermediate species generated during oxidation of membrane lipids. Thus, scavenging efficacy of peroxyl radicals by phenolic extracts investigated in this study shed light on using millet as a source of natural antioxidants to manage disease conditions in which ROS play a major role in their etiology.

3.4.7 Hydroxyl radical scavenging activity (HRSA)

Hydroxyl radical ($^{\cdot}OH$) is extremely reactive oxygen centered radical, which generates another radical by reacting with biomolecules such as proteins, DNA and lipids. In addition its significant role as an initiator of lipid peroxidation is well documented. The HRSA of soluble and bound millet phenolic extracts are presented in **Table 3.3**. In the present study hydroxyl radicals were prepared through the Fenton reaction in which ferrous sulphate reacts with H_2O_2 to produce hydroxyl radicals ($Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + {}^{\cdot}OH + {}^{-}OH$). As the resulting hydroxyl radicals are short lived, a spin trap, DMPO, is used to make them stable for detection by EPR spectrometry as DMPO-HO radical adduct (**Figure 3.2, c and d**).

In the present work, the HRSA of soluble and bound phenolic extracts of millet ranged from 53.7 to 17.6 and 1375.9 to 58.2 μmol FAE/g of the grain, respectively. Bound phenolic extracts of kodo, finger (Ravi), finger (local), foxtail, proso, little and pearl millets showed 8.5, 9, 15, 8, 81, 34 and 1.5 times higher ($p < 0.05$) HRSA, respectively, than their soluble phenolic counterparts. The contribution of bound fraction to the total HRSA of millet varieties ranged from 57 to 99% suggesting the necessity of their inclusion in the analysis of radical scavenging activities.

However, in contrast to the trend observed for the HRSA, soluble phenolic extracts showed higher DRSA compared to bound counterparts of millets in this study. These differences could be due to the existing differences in the molecular size and stereoselectivity of hydroxyl and DPPH radicals in their reaction with antioxidant compounds. In addition, composition of phenolic compounds in bound and soluble millet extracts and their solubility in the test medium used for two assays may also affect their capacity to act as antioxidants (*36, 37*).

Furthermore, in HRSA assay, the phenolic extracts were added to the assay mixture before starting the Fenton reaction. This could partly allow the extracts to suppress hydroxyl radical formation by chelating ferrous ion which is required for the Fenton reaction. In a previous study it was demonstrated that millet phenolic extracts exhibit a wide range of ferrous chelating activity (*22*). Nevertheless, in the present work soluble extracts of kodo millet exhibited a high HRSA though it possessed a low ferrous chelating activity. Thus, other experimental models such as neutrophil or monocyte myeloperoxidase-dependent hydroxyl radical formation systems (*38*) are warranted to understand the HRSA of millet phenolics.

Table 3.3 Hydroxyl radical scavenging activity (OH) and singlet oxygen quenching capacity of soluble and bound phenolic extracts of whole millets

Millet type	OH[a]		Singlet oxygen [b]	
	Soluble	Bound	Soluble	Bound
Kodo	43.18 ± 5.17b	365.24 ± 7.41d	19.19 ± 0.47d	65.76 ± 1.65a
Finger (Ravi)	17.58 ± 0.01g	149.82 ± 6.56f	11.24 ± 1.29e	10.24 ± 1.23de
Finger (local)	53.65 ± 2.99a	790.71 ± 7.01c	7.82 ± 0.07f	14.88 ± 1.21c
Foxtail	28.18 ± 3.18e	229.74 ± 6.17e	27.86 ± 1.58bc	8.65 ± 0.34e
Proso	17.76 ± 0.92fg	1375.9 ± 6.20a	20.27 ± 1.62d	0.55 ± 0.21g
Little	33.12 ± 2.48de	1110.2 ± 71.2b	30.46 ± 0.83b	1.40 ± 0.07fg
Pearl	42.92 ± 0.73bc	58.21 ± 0.34g	55.50 ± 0.44a	22.77 ± 0.09b

[a] Expressed as µmol ferulic acid equiv /g grain (dry weight basis).

[b] Expressed as µmol ferulic acid equiv /g grain (dry weight basis).

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

The correlation analysis showed that HRSA of soluble phenolic extracts positively associated with TPC ($r^2 = 0.574$; $p < 0.01$) whereas bound extracts negatively and insignificantly associated with TPC and TFC suggesting contribution of other factors that may be present in the extracts other than the phenolic compounds. These results further indicate that phenolic content, as assessed by Folin Ciocalteu's reagent may not explain the radical scavenging activity of plant phenolic extracts but

composition, structural features of the phenolics and other antioxidative compounds as well as possible synergistic interactions present must also be taken into consideration.

3.4.8 Singlet oxygen scavenging activity (SOSA)

Singlet oxygen is a highly energetic molecule and induces a unique oxidation process by directly reacting with electron-rich double bonds without forming free radical intermediates in foods and biological systems. The association of singlet oxygen with a number of pathological conditions such as pigmentation, cataract, skin aging, cancer and Parkinson's disease is documented (*39*). They are also reported to be involved in cholesterol oxidation (*40*) and DNA damage (*41*). The rate of singlet oxygen oxidation is greater than that of atmospheric triplet oxygen due to the low activation energy needed for chemical reactions and it has relatively long half-life in aqueous solutions (*42*). Therefore, it is important to study inhibitory effects of phenolics on singlet oxygen.

In the present study, singlet oxygen was generated through the reaction between sodium hypochlorite and H_2O_2. DPN and histidine were used as a selective scavenger and a selective acceptor of singlet oxygen, respectively, and the assay estimates the percentage inhibition in the production of singlet oxygen. The SOSA of soluble and bound phenolic extracts of millet ranged from 7.82 to 55.50 and from 0.55 to 65.76 µmol FAE/g of the grain, respectively (**Table 3.3**). Soluble extracts of pearl, little, proso, foxtail, finger (Ravi) and kodo millets showed 7, 4, 2.6, 3.6, 1.4 and 2.4 times higher SOSA, respectively, compared to finger (local) millet. Except for finger (Ravi) millet, all other millet varieties tested showed significant ($p < 0.5$) differences of SOSA between soluble and bound phenolic extracts. Furthermore, bound extracts of proso millet had the least SOSA which was 2.5 to 120 fold less than those of other

bound millet extracts examined in this study. It is interesting to note that bound extracts of millet varieties with dark pigmented pericarp and testa (kodo and finger millets) showed higher singlet oxygen scavenging activity whereas those with light coloured pericarp and testa (foxtail, proso, little and pearl millets) had lesser activity than their soluble counterparts. Thus, results obtained in this study lend further support to the fact that antioxidant efficacy of plant phenolic extracts may vary depending on the variety and the *in vitro* test system employed for the evaluation of antioxidant activity, among others.

Furthermore, in the present work SOSA of soluble extracts showed a significant negative association with TPC ($r^2 = 0.438$; $p < 0.01$) and insignificant association with TFC ($r^2 = 0.031$; $p > 0.05$). However, bound extracts exhibited a strong positive association with TPC ($r^2 = 0.939$; $p < 0.01$) and TFC ($r^2 = 0.920$; $p < 0.01$) indicating the contribution of phenolic compounds present in the extracts for governing the SOSA observed. Previous studies have reported the singlet oxygen scavenging properties of phenolic compounds such as quercetin and tea catechins (*43, 44*). It is noteworthy that finger (local) millet soluble extracts which had the highest radical scavenging activity against DPPH, peroxyl and hydroxyl radicals demonstrated the lowest SOSA in the present study suggesting the different mechanisms which might be involved in the respective test systems.

3.4.9 Hydrogen peroxide scavenging activity (HPSA)

Hydrogen peroxide is a non-radical oxidizing species in biological systems and has the ability to penetrate cell membranes and cause DNA damage, inactivate some enzymes by oxidation of thiol groups and initiate lipid peroxidation. Activated

phagocytes generate H_2O_2 as a means of killing the invading microorganisms in the body. In addition, several oxidase enzyme systems also generate H_2O_2.

As shown in **Table 3.4** all soluble and bound millet phenolic extracts tested in the present study demonstrated H_2O_2 scavenging activity indicating their potential to donate electrons to H_2O_2 thus neutralizing it to water. Among the millet varieties tested soluble extracts of kodo, proso, and little millets significantly ($p < 0.05$) differed from their bound counterparts. Kodo and proso bound extracts had 6 and 1.3 fold higher H_2O_2 scavenging activity, respectively, whereas that of little millet showed 1.5 fold lesser activity compared to their soluble counterparts. These results suggest the differences in the composition of the extracts and structural features of compounds which determine their electron donating capacity to the H_2O_2 molecule may govern the degree of scavenging activity of the extracts tested in the present study.

Though H_2O_2 itself is not a highly reactive molecule in the body it can be converted into more toxic species such as singlet oxygen and hydroxyl radicals by reacting with possibly ferrous and cupric ions in the cells leading to the oxidative stress. In addition, high level of H_2O_2 in the body may attack energy producing systems by inactivating glycolytic enzymes such as glyceraldehydes-3-phosphate dehydrogenase (*45*). Hence, HPSA by natural antioxidant sources is important for protection of biological systems.

In the present analysis it was found that HPSA of soluble extracts was not associated with TPC ($r^2 = 0.031$; $p > 0.05$) and TFC ($r^2 = 0.071$; $p > 0.05$). Furthermore, TPC had a significant positive association ($r^2 = 0.663$; $p < 0.01$) with bound extracts whereas association of TFC was insignificant ($r^2 = 0.024$; $p > 0.05$). Earlier, several authors have demonstrated the HPSA of plant phenolic extracts (*26,*

46). Wettasinghe and Shahidi (*26*) showed that crude extracts of borage and evening primrose meals had a potent HPSA. Wang and Jiao (*46*) also reported the potent scavenging activity of different berry extracts against H_2O_2.

Table 3.4 Hydrogen peroxide scavenging activity (H_2O_2) and hypochlorous acid (HOCl) scavenging activity of soluble and bound phenolic extracts of whole millets

Millet type	H_2O_2[a]		HOCl[b]	
	Soluble	Bound	Soluble	Bound
Kodo	21.76 ± 0.39f	133.72 ± 3.69a	35.67 ± 0.44a	106.65 ± 2.00a
Finger (Ravi)	28.41 ± 0.90e	28.04 ± 0.64e	13.59 ± 2.86c	15.41 ± 0.50c
Finger (local)	32.36 ± 0.56d	32.68 ± 0.81d	29.75 ± 1.17b	4.27 ± 0.25f
Foxtail	32.99 ± 0.49cd	33.70 ± 1.23cd	1.70 ± 0.24e	26.56 ± 1.20b
Proso	19.70 ± 0.11f	25.97 ± 0.49eg	10.60 ± 0.28cd	2.36 ± 0.65fg
Little	39.62 ± 1.21a	27.48 ± 0.15ef	39.83 ± 1.92a	14.21 ± 0.22ce
Pearl	37.59 ± 1.12ab	35.28 ± 1.00bd	38.01 ± 2.46a	16.09 ± 0.78cd

[a] Expressed as μmol ferulic acid equiv /g grain (dry weight basis).

[b] Expressed as μmol ferulic acid equiv /g grain (dry weight basis).

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

3.4.10 Hypochlorous acid scavenging activity (HASA)

Table 3.4 presents the HASA of soluble and bound phenolic extracts of different millet varieties. The HASA of soluble and bound extracts ranged from 1.70 to 39.83 and from 2.36 to 106.65 μmol FAE/g of grain, respectively. The HASA of soluble extracts was in the order of little > pearl > kodo > finger (local) > finger (Ravi) > proso > foxtail millets. Except for finger (Ravi) millet, HASA of all millet soluble fractions examined in this study significantly differed from that of their bound counterparts. It is noteworthy that bound extracts of kodo and foxtail exhibited 3 and 15 times higher ($p < 0.05$) HASA, respectively, than their corresponding soluble extracts. HOCl is generated in the body through neutrophil myeloperoxidase which catalyses the oxidation of chloride ions by H_2O_2. HOCl is a potent chlorinating and oxidizing agent which can attack biological substrates, degrade structural proteins, inactivate plasma protease inhibitors, activate neutrophil collagenase and inactivate the antioxidant enzyme catalase through the breakdown of heme prosthetic group (*47*).

In the present study HOCl was generated by the addition of 1% sulphuric acid to sodium hypochlorite to pH 6.2 along with taurine, a β amino acid which is used as a compound capable of reacting with HOCl to form a stable taurine chloramine derivative. Addition of antioxidant compounds inhibit taurine chloramine formation by virtue of reacting with HOCl. Taurine chloramine was quantified using its ability to oxidize iodide to iodine which produced a yellow colour, absorbance of which was read at 350 nm. HOCl is known for its ability to destroy invading microorganisms in the body. However, HOCl also contributes to oxidative damage of healthy human tissues in pathological conditions such as atherosclerosis, inflammatory bowel disease and rheumatoid arthritis.

In this work HASA of soluble extracts was not associated with TPC (r^2 = 0.037; p > 0.05) and TFC (r^2 = 0.171; p > 0.05). The HASA of bound extracts positively correlated with TPC (r^2 = 0.748; p < 0.01) as well as TFC (r^2 = 0.275; p < 0.01) indicating the potential of millet phenolics as a natural source of antioxidants. In agreement with the present analysis, Ramful et al. (*48*) also showed a positive association between TPC and HASA of flavedo extracts of citrus fruits. Hydroxycinnamic acids such as caffeic, sinapic, chlorogenic, ferulic and *p*-coumaric acids reported to possess hypochlorite scavenging activity (*49*). Similarly, Soobrattee et al. (*50*) also demonstrated HASA of several phenolic compounds including ferulic and *p*-coumaric acids. In this study we showed that both soluble and bound extracts of millets contained a considerable amount of hydroxycinnamic acids, namely ferulic and *p*-coumaric acids which may partly explain the HASA exhibited by the millet phenolic extracts.

3.5 Conclusion

The results presented here have shown that the whole grains of millet varieties tested in this study demonstrate *in vitro* antioxidant potency though the order of scavenging activity of each millet extract towards DPPH radical and different ROS does not follow a similar pattern. It has been shown that both soluble and bound extracts of millet have antioxidant potential and hence need to be considered when used in antioxidant activity assays. In general there is a strong and positive association between phenolic content and scavenging of DPPH radical and ROS except for hydroxyl radical inhibition. It can be speculated that phenolic compounds present in the extracts may exert their antioxidant activity individually as well as synergistically

and identification of these compounds is in progress. Evidences from several studies suggest that ROS generated during cellular metabolism and oxidation of lipids and proteins play a major causative role in the occurrence of cancer and cardiovascular diseases, among others. There is a widespread interest in finding natural sources of antioxidants which can be used for managing several pathological conditions and maintenance of health. Thus, millets as neglected cereals have a great potential for the formulation of specialty functional foods for those with gluten intolerance and beyond.

3.6 References

1. Kehrer, J.P. Free radicals as mediators of tissue injury and disease. *Crit. Rev. Toxicol.* **1993**, 23, 21-48.
2. Valko, M.; Rhodes, C.J.; Moncol, J.; Izakovic, M. Free radical, metals and antioxidants in oxidative stress-induced cancer. Mini-review. *Chem Biol interact.* **2006**, 160, 1-40.
3. Zheng, M.; Storz, G. Redox sensing by prokaryotic transcription factors. *Biochem. Pharmacol.* **2000**, 59, 1-6
4. Droge, W. Free radicals in the physiological control of cell function. *Review. Physiol. Rev.* **2002**, 39, 47-95.
5. Darley-Usmar, V.; Halliwell, B. Blood Radicals: Reactive nitrogen species, reactive oxygen species, transition metal ions and the vascular system. *Pharmaceutical Res.* **1996**, 13, 649–662.
6. Halliwell, B.; Rafter, J.; Jenner, A. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *Am J Clin Nutr.* **2005**, 81, 268S-276S.
7. Shahidi, F.; Naczk, M. Phenolics in Food and Nutraceuticals. CRC press, Boca Raton, FL, **2004**, pp 1-82.

8. Zielinski, H.; Kozlowska, H. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *J. Agric. Food Chem.* **2000**, 48, 2008-2016.

9. Yu, L.; Haley, S.; Perret, J.; Harris, M.; Wilson, J.; Qian, M. Free radical scavenging properties of wheat extracts. *J. Agric. Food Chem.* **2002**, 50, 1619-1624.

10. Madhujith, T.; Shahidi, F. Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *J. Agric. Food Chem.* **2006**, 54, 8048-8057.

11. Ragaee, S.; El-Sayed, M.A.; Noaman, M. Antioxidant activity and nutrient composition of selected cereals for food use. *Food Chem.* **2006**, 98, 32-38.

12. Liyana-Pathirana, C.; Shahidi, F. Importance of insoluble-bound phenolics to antioxidant properties of wheat. *J. Agric. Food Chem.* **2006**, 54, 1256-1264.

13. Mani, U.V.; Prabhu, B.M.; Damle, S.S; Mani, I. Glycaemic index of some commonly consumed foods in western India, *Asia Pac. J. Clin. Nutr.* **1993**, 111-114.

14. Kurup, P.G.; Krishnamurthy, S. Glycemic response and lipemic index of rice, raggi and tapioca as compared to wheat diet in human. *Indian J. Exp. Biol.* **1993**, 31, 291-293.

15. Rajasekaran, N.S.; Nithya, M.; Rose, C.; Chandra, T.S. The effect of finger millet feeding on the early responses during the process of wound healing in diabetic rats, *Biochim. Biophys. Acta.* **2004**, 1689,190-201.

16. Hegde, P.S.; Anitha, B.; Chandra, T.S. In vivo effect of whole grain flour of finger millet (*Eleucine coracana*) and kodo millet (*Paspalum scrobiculatum*) on rat dermal wound healing. *Indian J. Exp. Biol.* **2005**, 43, 254-258.

17. Hegde P.S.; Rajasekaran N.S.; Chandra T.S.; Effects of the antioxidant properties of millet species on the oxidative stress and glycemic status in alloxan-induced rats. *Nutr. Res.* **2005**, 25, 1109-1120.

18. Shobana, S.; Sreerama, Y.N.; Malleshi, N.G. Composition and enzyme inhibitory properties of finger millet (*Eleusine coracana* L.) seed coat phenolics: Mode of inhibition of a-glucosidase and pancreatic amylase. *Food Chem.* **2009**, 115, 1268-1273.

19. Choi, Y.; Jeong H-S.; Lee, J. Antioxidant activity of methanolic extracts from some grains consumed in Korea. *Food Chem.* **2007**, 103, 130-138.

20. Hegde P.S.; Chandra, T.S. ESR spectroscopic study reveals higher free radical quenching potential in kodo millet (*Paspalum scrobiculatum*) compared to other millets. *Food Chem.* **2005**, 92, 177-182.

21. Sripriya G.; Chandrasekaran, K.; Murty, V.S.; Chandra, T.S. ESR spectroscopic studies on free radical quenching action of finger millet (*Elusine coracana*). *Food Chem.* **1996**, 57, 537-540.

22. Chandrasekara A.; Shahidi F. The content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *J. Agric. Food Chem.* **2010**, 58, 6706-6714.

23. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, 16, 144-158.

24. Kim, D.; Jeong, S.W.; Lee, C.Y. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem.* **2003**, 81, 321-326.

25. Madhujith, T.; Shahidi, F. Antioxidative and antiproliferative properties of selected barley (*Hordeum vulgarae* L.) Cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. *J. Agric. Food Chem.* **2007**, 55, 5018-5024.

26. Wettasinghe, M.; Shahidi, F. Scavenging of reactive oxygen species and DPPH free radicals by extracts of borage and evening primrose. *Food Chem.* **2000**, 70, 17-26.

27. Maldonado, P.D.; Rivero-Cruz, I.; Mata, R.; Pedraza-Chaverr, J. Antioxidant activity of A-type proanthocyanidins from *Geranium niveum* (Geraniaceae). *J. Agric. Food Chem.* **2005**, 53, 1996-2001.

28. Weiss, S.J.; Klein, R.; Slivka, A.; Wei, M. Chlorination of taurine by human neutrophils, evidence for hypochlorous acid generation. *J. Clin. Invest.* **1982**, 70, 598-607.

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

30. Madhujith, T.; Shahidi, F. Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. *Food Chem.* **2009**, 117, 615-620.

31. Moon, J-K.; Shibamoto, T. Antioxidant assays for plant and food components. *J. Agric. Food Chem.* **2009**, 57, 1655-1666.

32. Madhujith, T.; Izydorczyk, M.; Shahidi, F. Antioxidant properties of pearled barley fractions. *J. Agric. Food Chem.* **2006**, 54, 3283-3289.

33. Yokozawa, T.; Chen, C.P.; Dong, E.; Tanaka, T.: Nonaka, G.; Nishioka, I. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl *Radical. Biochem. Pharmacol.* **1998**, 56, 213–222.

34. Mpofu, A.; Sapirstein, H.D.; Beta, T. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *J. Agric. Food Chem.* **2006**, 54, 1265-1270.

35. Okarter, N.; Liu, C.; Sorrells, M.E.; Liu, R.H. Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. *Food Chem.* **2010**, 119, 249-257.

36. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of free radical method to evaluate antioxidant activity. *Lebensm. -Wiss u. Technol.* **1995**, 28, 25-30.

37. Chen, Z.Y.; Chan, P.T.; Ho, K.Y.; Fung, K.P.; Wang, J. Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. *Chem. Phys. Lipids* **1996**, 79, 157-163.

38. Ramos, C.L.; Pou, S.; Britigan, B.E.; Cohen, M.S.; Rosen, G.M. Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. *J. Biol. Chem.* **1992**, 267, 8307-8312.

39. Meucci, E.; Mordente, A.; Martorana, G.E. Metal-catalyzed oxidation of human serum albumin: conformational and functional changes. Implications in protein aging. *J. Biol. Chem.* **1991**, 266, 4692-4699.

40. Stief, T.W. The physiology and pharmacology of singlet oxygen. *Med. Hypoth.* **2003**, 60, 567-572.

41. Imlay, J.A.; Chin, S.M.; Linn, S. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* **1988**, 240, 640-642.

42. Min, D.B.; Boff, J.M. Chemistry and reaction of singlet oxygen in foods. *Comp. Rev. Food Sci. Food Safety* **2002**, 1, 58-72.

43. Tournaire, C.; Croux, S.; Maurette, M-T.; Beck, I.; Hocquaux, M.; Braun, A.M.; Oliveros, E. Antioxidant activity of flavonoids: Efficiency of singlet oxygen ($^1\Delta_g$) quenching. *J. Photochem. Photobiol. B.* **1993**, 19, 205-215.

44. Guo, Q.; Zaho, B.; Shen, S.; Hou, J.; Hu, J.; Xin, W. ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers. *Biochim. Biophys. Acta* **1999**, 1427, 13-23.

45. Aruoma, O.I. Free radicals, oxidative stress and antioxidants in human health and disease. *JAOCS*, **1998**, 752, 199–212.

46. Wang, S.Y.; Jiao, H. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. *J. Agric. Food Chem.* **2000**, 48, 5677-5684.

47. Hazell, L.J.; Davies, M.J.; Stocker, R. Secondary radicals derived from chloramines of apolipoprotein B-100 contribute to HOCl induced lipid peroxidation of low-density lipoproteins. *Biochem. J.* **1999**, 339, 489-495.

48. Ramful, D.; Bahorun, T.; Bourdon, E.; Tarnus, E., Aruoma, O.I. Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: potential prophylactic ingredients for functional foods application. *Toxicology* **2010**, 278, 75-87.

49. Firuzi, O.; Giansanti, L.; Vento, R.; Seibert, C.; Petrucci, R.; Marrosu, G.; Agostino, R.; Saso, L. Hypochlorite scavenging activity of hydroxycinnamic acids evaluated by rapid microplate method based on the measurement of chloramines. *J. Phar. Pharmacol.* **2003**, 55, 1021-1027.

50. Soorbrattee, M.A.; Neergheen, V.S.; Luximon-Ramma, A.; Aruoma, O.I.; Bahorun, T. Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. *Mutation Res.* **2005**, 579, 200-213.

CHAPTER 4

Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n

4.1 Abstract

Over 50 phenolic compounds belonging to several classes, namely, phenolic acids and their derivatives, dehydrodiferulates and dehydrotriferulates, flavan-3-ol monomers and dimers, flavonols, flavones and flavanonols in four phenolics fractions of several whole millet grains (kodo, finger (Ravi), finger (local), foxtail, proso, little and pearl millets) were positively or tentatively identified using high performance liquid chromatography (HPLC) and HPLC- (tandem mass spectrometry) MS^n. Total phenolic content (TPC) was determined colourimetrically using Folin-Ciocalteu reagent. Antioxidant and antiradical activities of phenolic fractions were estimated using oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and singlet oxygen scavenging activity. In addition, antioxidant activities of phenolic fractions were determined in a β-carotene linoleate emulsion system. In general, hydroxycinnamic acids and their derivatives were the main contributors (87-98%) to the TPC of insoluble bound phenolic fraction of millet varieties examined. Flavonoids were predominantly present in the free phenolic fractions of two finger millet varieties and included catechin, epicatechin, gallocatechin, epigallocatechin, and procyanidin dimers B1 and B2 among others. Thus millet grains may serve as a viable functional food ingredient and a source of natural antioxidants.

4.2 Introduction

Regular consumption of whole grains, fruits, and vegetables coupled with adequate physical activity impart beneficial health outcome by reducing the occurrence of a number of age-related diseases such as diabetes, cardiovascular diseases and some types of cancer. It has been reported that unique phytochemical composition of cereal grains complements those in fruits and vegetables when consumed together (*1*). For instance, ferulic acid and diferulates are mainly found in cereal grains and their abundance is more prominent in the insoluble bound fraction (*1*). As a major component in the base of food guide pyramid, cereals, especially whole grains, are emphasized to contribute to disease risk reduction and overall health and wellness. Major world cereals are wheat, corn, and rice whereas oat, barley, sorghum, millet, rye and buckwheat are among minor cereal grains.

Millets are small seeded grains with different varieties which belong to several plant taxonomic groups and are produced in tropical and semi-arid regions of the world. At present, millets are not placed as a single important commodity in the North American food basket but their importance as an ingredient in multigrain and gluten-free cereal products is highlighted. However, millets serve as a major food component in many African and Asian populations, especifically among non-affluent segments in their respective societies.

Cereal phenolics which exist in several forms in the grain have been reported to render antioxidative and antiproliferative effects and are responsible for the control of cholesterol oxidation in *in vitro* systems (*2-4*). Phenolic compounds are secondary plant metabolites and their type and content in the grains may depend on a number of factors such as the type of cereal, variety, part of the grain, climatic conditions, and

cultivation practices, among others (*1*). Phenolics which are extractable into aqueous or aqueous-organic solvent mixtures are generally referred to as soluble phenolics and these include phenolic compounds existing in the free, non-conjugated form as well as phenolic compounds conjugated to soluble carbohydrates by ester (esterified) and ether (etherified) bonds (*1*). Furthermore, the left over residue after extraction of the soluble phenolics can be used to obtain insoluble bound phenolic compounds, especially hydroxycinnamic acids which are mainly esterified to the sugar residues of polysaccharides providing cross-linking between cell wall polymers (*5*). They also form ether bonds and C-C linkages with lignins (*6*). Some studies have shown that conjugated and insoluble bound phenolics may be released at the variable alkaline and acidic gastro-intestinal conditions and under colonic fermentation and may impart health benefits even at the local sites such as intestinal epithelium and beyond after absorption (*7*).

In general, polyphenolics possess highly diverse compounds with variable bioactivity and bioavailability. Health benefits imparted by cereal phenolics may be a result of additive and synergistic effects of multiple compounds present in the grains thus their identification is important. Few studies have reported on the phenolic acid content and antioxidant activities of free, soluble conjugate and bound phenolic fractions of cereal grains such as rice, oats, wheat, corn and barley (*2, 4, 8-10).* The antioxidant activity of soluble and bound phenolics of millets and the identity of major hydroxycinnamic acids, namely ferulic and *p*-coumaric acids were earlier reported (*11-13*). However, information on the phenolic profile in the free, esterified, etherified and insoluble bound fractions of whole millet grains and their antioxidant activities are limited and reported only for finger millets (*11, 14*).

The objectives of this study were (a) to identify and quantify the content of phenolic compounds of whole millet grains belonging to different varieties, namely kodo, finger (Ravi), finger (local), foxtail, proso, little and pearl millets and (b) to determine the antioxidant activity of phenolics in free, esterified and etherified soluble fractions as well as insoluble bound fractions of millet grains.

4.3 Materials and methods

Seven millet grain samples, namely foxtail (*Setaria italica)*, proso (*Panicum miliacium)*, two finger millet (*Elusine coracana*) varieties (Ravi and local), kodo (*Paspalum scrobiculatum*), and little millet (*Panicum sumatrense*) were obtained from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka, and used in this study. Pearl millet (*Pennisetum glaucum*, dark green cultivar), grown in India, was obtained through Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India. The grains from both sources were harvested in 2007.

Folin Ciocalteu's reagent, *t*-ferulic, *p*-coumaric, *p*-hydroxybenzoic , protocatechuic, gentisic, sinapic, vanillic, caffeic, cholorgenic, cinnamic, gallic, and syringic acids, vanillin, catechin, epicatechin, epigallocatechin, kaempherol, taxifolin, quercetin, apegenin, myricetin, 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox, mono- and dibasic potassium phosphates, mono- and dibasic sodium phosphates, histidine, sodium hypochlorite, *N,N*- dimethyl-*p*-nitrosoaniline (DPN), H_2O_2, Tween 40 (polyoxyethylene sorbitan monopalmitate), *β*-carotene, linoleic acid, and fluorescein were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Diethyl ether, ethyl acetate, hexane, acetone, methanol, chloroform, acetonitrile, formic acid, hydrochloric acid,

sodium hydroxide and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada).

4.3.1 Sample preparation

Whole cleaned millet grains were ground using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc. Brockville, ON, Canada) to obtain a fine powder which passed through mesh 16 (sieve opening 1 mm, Tylor test sieve, Mentor, OH, USA). Ground grain samples were defatted by blending with hexane (1:5 w/v, 5 min for 3 times) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA) to remove interfering lipids at ambient temperature. Defatted samples were vacuum packed in polyethylene pouches and stored at –20°C until used within one week for extraction of phenolics.

4.3.2 Extraction of phenolic compounds

Defatted meal (2 g) was mixed with 40 mL of 70% (v/v) acetone and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) and sonicated at the maximum power for 25 min under refluxing conditions to extract soluble phenolics. After centrifugation of the resulting slurry for 5 min at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA), the supernatant was collected and extraction was repeated once more. The solid residues of whole grain samples were used to extract insoluble bound phenolic compounds. Supernatants were combined and evaporated to a watery residue (50 mL) which was adjusted to pH 2 with 6 M HCl. Free, non-conjugated phenolics were extracted 5 times into diethyl ether and ethyl acetate (1:1, v/v). The organic phase was evaporated to dryness under vacuum at room temperature. The

water phase was used for alkali treatment to release esterified phenolics. During all stages, extracts were protected from light by covering the containers with aluminium foil. To the water phase 2 M NaOH (20 mL) was added and the content hydrolyzed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 with 6 M HCl, phenolic acids cleaved from soluble esters were extracted from the hydrolyzates as described above followed by evaporation to dryness under vacuum and reconstitution in 2 mL of methanol. The remaining water phase was used for acid hydrolysis in order to cleave glycosidic (etherified) bonds. Briefly, 20 mL of 1 M HCl was added to the water phase and heated in 95^{0}C water for 45 min. When the mixture was cooled to room temperature released phenolics were extracted 5 times with diethyl ether (1:1, v/v) and evaporated to dryness under vacum.

The solid residue of the whole grain sample obtained after extraction of soluble phenolics was hydrolyzed with 4 M NaOH (40 mL) at room temperature for 4 h while stirring under nitrogen. The resulting slurry was acidified to pH 2 with 6 M HCl and extracted 5 times with diethyl ether and ethyl acetate (1:1, v/v) and subsequently desolventized at room temperature in a rotary evaporator. The fractions of phenolic compounds so obtained were reconstituted in HPLC grade methanol and stored at -20°C until used for further analysis. All four extractions of each sample were carried out in duplicate, completed within the same day and separately analyzed by HPLC.

4.3.3 Determination of total phenolic content (TPC)

The total phenolic content of each phenolic fraction was determined using the method described by Singleton and Rossi (*15*) with slight modifications as explained

previously (*12*). Phenolic extracts (1 mL contains phenolic compounds extracted from 1 g of defatted meal) were diluted in methanol (20x) to obtain a concentration of 50 µL of the original extract solution per mL of the diluted solution. The content of total phenolics in each extract was determined using a standard curve prepared for ferulic acid and expressed as micromoles of ferulic acid equivalents (FAE) per gram of defatted meal.

4.3.4 Singlet oxygen inhibition

The ability of millet phenolic extracts to inhibit singlet oxygen was measured using a spectrophotometric method previously explained by Chandrasekara and Shahidi (*13*). The percentage inhibition of singlet oxygen was calculated as follows. Singlet oxygen inhibition activity (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. The results were expressed as micromoles of FAE per gram of defatted meal.

4.3.5 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed as previously explained by Chandrasekara and Shahidi (*13*). The extracts were diluted in 75 mM phosphate buffer (pH 7.0). Determination of ORAC was carried out using a plate reader equipped with a built-in incubator (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany). ORAC values of extracts were expressed as micromoles of trolox equivalents (TE) per gram of defatted meal.

4.3.6 DPPH radical scavenging capacity assay using electron paramagnetic resonance (EPR) spectroscopy

The effect of extracts on the scavenging of DPPH radicals was determined according to the method explained by Chandrasekara and Shahidi (*13*). The phenolic extracts (0.5 mL) were used at a concentration of 50 µL/mL in methanol (1 mL of diluted solution contain phenolics extracted from 50 µg of defatted meal). A Bruker E-scan EPR spectrometer (Bruker E-scan, Bruker Biospin Co. Billercia, MA, USA) was used to record the spectrum. The DPPH radical scavenging activity was expressed as micromoles of FAE per gram of defatted meal.

4.3.7 Antioxidant activity in *β*-carotene/linoleate model system

The antioxidant activity of extracts was evaluated in a *β*-carotene/linoleate model system as explained by Chandrasekara and Shahidi (*12*). Absorbance measurement was carried out using a microplate reader equipped with a built-in incubator (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany). Antioxidant activity coefficient (AAC) after 120 min of incubation was calculated using the following equation; $AAC = (A_{a(120)} - A_{c(120)}) / (A_{c(0)} - A_{c(120)})$ where $A_{a(120)}$ and $A_{c(120)}$ are the absorbance values measured at 120 min for the sample and the control, respectively, and $A_{c(0)}$ is the absorbance value of the control, at 0 min. The results were expressed as AAC per gram of defatted meal.

4.3.8 HPLC-DAD-ESI-MS^n analysis

Phenolic composition of free and hydrolyzed phenolic fractions of millet grains were determined by HPLC analysis. The RP-HPLC analysis were carried out using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped

with a G1311A quaternary pump, a G1379A degasser and a G1329A ALS automatic sampler, a G1330B ALS Therm, a G1316A Colcom column compartment, a G1315B diode array detector (DAD) and a system controller linked to Chem Station Data handling system (Agilent Technologies, Palo Alto, CA, USA). Separations were conducted with a SUPERLCOSIL^{TM} LC-18 column (4.6 × 250 mm, 5 μm; Merck, Darmstad, Germany). The mobile phase consisted of 1% formic acid (eluent A) and methanol/acetonitrile/formic acid (94:5:1; v/v/v) (eluent B). Gradient elution was used as follows; 0 min, 20% B; 10 min, 30% B; 15 min, 40%B; 18 min, 45% B; 20 min, 50% B; 30 min, 70% B and 40 min, 85% B. The flow rate was adjusted to 0.5 mL/min and the detection of compounds was performed at 254 and 280 nm. All samples were filtered through a 0.45 μm PTFE membrane syringe filter (Whatman Inc., Florham Park, NJ, USA) before injection.

HPLC-MS analysis was performed under the HPLC analytical conditions explained above using an Agilent 1100 series capillary liquid chromatography/mass selective detector (LC/MSD) ion trap system in electrospray ionization (ESI) negative ion mode. Complete system control and data evaluation were achieved with Agilent LC/MSD Trap software (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer was operated in a scan range from m/z 100-800: smart parameter setting using a drying gas (N_2) temperature of 350° C, drying gas flow 10 L/min, and nebulizer gas (N_2) pressure of 60 psi. In addition to detection of the deprotonated molecular ions, collision induced dissociation (CID) was performed in the MS^3 in which the resulting product ions were used for the identification of compounds.

Phenolic acids, namely gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, gentisic, chlorogenic, caffeic, *trans*-cinnamic, *trans*-ferulic, *cis*-ferulic, *p*-coumaric,

and sinapic acids and vanillin were identified by comparing their relative retention times (RT), and UV and ESI-MS spectra with authentic compounds. *Cis*-ferulic acid was prepared by exposing a known solution of *trans*-ferulic acid to UV light at 254 nm for 3h. Flavonoids used as analytical standards included catechin, epigallocatechin, epicatechin, taxifolin, myricetin, kaempherol, quercetin, and apigenin. Other compounds present in the extracts were tentatively identified using ESI-MS^n and UV spectral data and literature and quantified as equivalents of the closely related available standards. The external standard method in which reference compounds were chromatographed under similar chromatographic conditions separately from samples was used for quantification purposes. Calibration graphs were prepared by plotting concentrations in mg/mL against peak area for 22 standard compounds. The regression coefficients of these graphs ranged from 0.99 to 1.0. Methyl vanillate, protocatechualdehyde and *p*-hydroxybenzaldehyde were quantified using vanillic, protocatechuic, and *p*-hydroxybenzoic acid equivalents, respectively, at 254 nm. Sinapic, *trans*-ferulic, *p*-coumaric, chlorogenic, and caffeic acids were used to quantify their respective derivatives at 280 nm. Gallocatechin was quantified using (-)-epigallocatechin and (+)-catechin was employed for determination of procyanidin dimers at 280 nm. Apigenin derivatives, vitexin, isovitexin, and tricin were quantified as apegenin equivalents at 280 nm. Myricetin derivatives were quantified as myricetin equivalents at 254 nm. The lowest level of detection of a phenolic compound was 100 ng/mL. The results were expressed as µg / g defatted meal.

4.3.9 Statistical analysis

All experiments were carried out in triplicates unless otherwise stated and data were reported as mean ± standard deviation. The differences of mean values among

different millet phenolic fractions was determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p \leq 0.05$ significance level. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 Total phenolic content (TPC) and antioxidant activities

Total phenolic content, as determined by Folin Ciocalteu's reagent, and antioxidant activities of four different phenolic fractions of whole grain millets are presented in **Table 4.1.** Insoluble bound fractions of kodo, foxtail, proso, little and pearl millets had significantly ($P \leq 0.05$) higher TPC than total of free, esterified and etherified fractions which represented the soluble phenolic fraction whereas two finger millet varieties showed the reverse trend. Furthermore, these results were in agreement with those obtained by HPLC analysis in this study (**Tables 4.2-6**). The TPC of free, esterified, etherified and insoluble bound fractions of millet grains were in the range of 0.55-16.2, 0.25-2.02, 0.32-3.94 and 3.2-81.6 µmol FAE /g defatted meal, respectively. Varietal differences of TPC were observed in each phenolic fraction and kodo millet showed the highest TPC in free, esterified and insoluble bound fractions whereas pearl millet had the highest TPC in the etherified fraction, among others. These results suggest that the content of phenolics conjugated by esterification or etherification may vary depending on the millet variety. The TPC in soluble esterified fraction of different barley varieties ranged from 2.2 to 4.2 µmol FAE/g of defatted meal and were less than corresponding insoluble bound fraction as observed in the present study (*4*).

The antioxidant activity as assessed by oxygen radical absorbance capacity (ORAC) was high in the insoluble bound fraction of respective millet varieties with high TPC. Similarly, insoluble bound fractions of two finger millet varieties showed significantly ($P \leq 0.05$) lower ORAC than that of free phenolic fraction in agreement with their lower TPC (**Table 4.1**). In general, ORAC did not differ significantly ($P \leq 0.05$) between esterified and etherified phenolic fractions of millet varieties tested in this study except for proso millet in which etherified fraction had 1.8 times higher ORAC compared to that of its esterified counterpart (**Table 4.1**).

Table 4.1 Total phenolic content (TPC) and antioxidant activities (AOA) of different phenolic fractions of millet grains

Millet type	Free	Esterified	Etherified	Insoluble bound
Total phenolic content (µmol FAE /g defatted meal)				
Kodo	16.2 ± 0.5a	2.02 ± 0.1b	1.55 ± 0.1b	81.6 ± 0.2c
Finger (Ravi)	9.67 ± 0.4a	0.25 ± 0.1b	1.88 ± 0.2c	3.83 ± 0.1d
Finger (local)	10.3 ± 0.4a	0.41 ± 0.1b	2.15 ± 0.1c	3.20 ± 0.2d
Foxtail	4.49 ± 0.2a	0.37 ± 0.1b	0.32 ± 0.1b	11.6 ± 0.2c
Proso	0.55 ± 0.1a	0.70 ± 0.1a	2.05 ± 0.1b	2.21 ± 0.1c
Little	5.77 ± 0.7a	1.37 ± 0.2b	2.48 ± 0.1c	9.64 ± 0.3d
Pearl	1.27 ± 0.2a	1.82 ± 0.2b	3.94 ± 0.1c	9.14 ±0.2d
Oxygen radical absorbance capacity (µmol trolox equiv /g defatted meal)				
Kodo	86.25 ± 2.4a	26.10 ± 0.2b	33.37 ± 2.5b	792.7 ± 7.8c
Finger (Ravi)	111.3 ± 1.0a	20.64 ± 1.7b	37.13 ±1.2b	95.27 ± 4.2c
Finger (local)	110.5 ± 1.6a	22.83 ± 0.1b	39.23 ± 0.5b	103.0 ± 4.0a
Foxtail	88.36 ± 4.4a	23.26 ± 0.6b	32.19 ± 3.1b	277.5 ± 15c
Proso	69.40 ± 4.9a	24.49 ±1.3b	43.22 ± 1.6c	132.9 ± 7.0d
Little	91.28 ± 5.9a	25.12 ± 0.8b	42.80 ± 3.4b	244.5 ± 10c
Pearl	59.01 ± 8.6a	26.72 ± 0.2b	45.78 ± 1.3ab	165.5 ± 4.8c
DPPH radical scavenging activity (µmol FAE /g defatted meal)				
Kodo	11.2 ± 0.1a	2.81 ± 0.1b	1.95 ± 0.1c	22.7 ± 0.1d
Finger (Ravi)	8.93 ± 0.2a	2.32 ± 0.3b	1.54 ± 0.1b	7.97 ± 0.7a
Finger (local)	8.43 ± 0.1a	1.63 ± 0.2b	1.97 ± 0.2b	8.65 ± 0.7a

Table 4.1 Continued…

Foxtail	2.86 ± 0.2a	1.52 ± 0.1b	0.41 ± 0.1c	7.25 ± 0.1d
Proso	0.70 ± 0.1a	1.19 ± 0.2b	1.48 ± 0.2b	4.06 ± 0.1c
Little	2.55 ± 0.1a	1.97 ± 0.1b	1.73 ± 0.2b	5.58 ± 0.2c
Pearl	0.64 ± 0.1a	1.97 ± 0.1b	2.24 ± 0.1c	9.96 ± 0.1d
Singlet oxygen scavenging capacity (µmol FAE /g defatted meal)				
Kodo	12.6 ± 0.2a	7.58 ± 0.7b	2.71 ± 0.2c	83.4 ± 2.3d
Finger (Ravi)	3.74 ± 0.3a	3.56 ± 0.4a	1.72 ± 0.2a	16.7 ±1.6b
Finger (local)	6.62 ± 0.1a	5.52 ± 0.4a	2.58 ± 0.3b	21.1 ± 2.7c
Foxtail	7.94 ± 0.5a	5.23 ± 0.1a	1.91 ± 0.1b	15.39 ± 2.9c
Proso	17.7 ± 0.3a	5.19 ± 0.9b	1.38 ± 0.1c	3.51 ± 0.9b
Little	15.7 ± 0.4a	3.89 ± 0.3b	2.73 ± 0.7b	4.70 ± 0.9c
Pearl	14.1 ± 0.1a	1.43 ± 0.3b	2.87 ± 0.2c	33.2 ± 0.2d
Antioxidant activity in *β*-carotene/linoleate emulsion (AAC/ g defatted meal)				
Kodo	1130 ± 15a	124 ± 2b	146 ± 6b	1152 ± 19a
Finger (Ravi)	707 ± 4a	144 ± 3b	154 ± 13b	645 ± 5c
Finger (local)	802 ± 37a	72 ± 1b	45 ± 11b	497 ± 16c
Foxtail	707 ± 16a	12 ± 1b	38 ± 7b	815 ± 9c
Proso	825 ± 8a	12 ± 7b	62 ± 29b	634 ± 32c
Little	665 ± 60a	72 ± 1b	68 ± 1b	781 ± 19c
Pearl	601 ± 37a	96 ± 1b	180 ± 11c	504 ± 50d

Abbreviations are FAE-Ferulic acid equivalents; AAC-Antioxidant activity coefficient.

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

In accordance with the TPC determined in this study, DPPH radical scavenging activity of phenolic fractions with higher TPC of millet grains was higher. The results clearly showed that varietal effect was present for DPPH radical scavenging activity and kodo millet had the highest value which ranged from 1.95 to 22.7 FAE μmol/g defatted meal. Meanwhile, singlet oxygen scavenging capacity ranges for free, esterified, etherified and insoluble bound fractions were 3.74-17.7, 1.43-7.58, 1.38-2.87 and 3.51-83.4 μmol FAE /g defatted meal, respectively. In agreement with other *in vitro* antioxidant test systems used, insoluble bound phenolic fractions of kodo, foxtail and pearl millets showed 7, 2, and 2.4 times higher singlet oxygen scavenging capacity, respectively, than their free phenolic fractions.

Antioxidant activity determined in a *β*-carotene/linoleate emulsion system showed that free and insoluble bound phenolic fractions of each variety had higher AAC/g defatted meal than that of esterified and etherified fractions in accordance with their TPC. Kodo millet showed the highest antioxidant activity in the *β*-carotene/linoleate emulsion system, among others. In agreement with results obtained by Rao and Muralikrishna (*14*), free phenolic fraction had higher AAC/g defatted meal than that of insoluble bound phenolics of finger millet in this study. This could be attributed to the high total phenolic content as well as flavonoids such as catechin, gallocatechin, epicatechin, and procyanidine dimer contents detected in the free fraction of finger millet (**Table 4.3**).

Table 4.2 Individual phenolic compounds identified in millet grains

Peak No	Phenolic compounds	Molecular weight	$[M-H]^-$ (m/z)	ESI negative ions (m/z)
Hydroxybenzoic acids and derivatives				
1	Gallic acid[a]	170	169	125
2	Methyl vanillate	182	181	149, 166
4	Protocatechuic acid[a]	154	153	109
8	Protocatechuic aldehyde	138	137	163
14	*p*-Hydroxybenzoic acid[a]	138	137	93, 125
16	Gentisic acid[a]	154	153	329
19	Vanilllic acid[a]	168	167	123
21	Syringic acid[a]	198	197	153
22	*p*-Hydroxybenzaldehyde	122	121	116, 144,
23	Vannilin[a]	152	151	136,
Hydroxycinnamic acids and derivatives				
3	Sinapaldehyde	208	207	192
6	Sinapoyl glycoside	386	385	164, 223,247, 265
7	Feruloyl hexose	356	355	137, 193, 311
10	Chlorogenic acid[a]	354	353	191,
13	5-Hydroxy feruloyl hexose	372	371	193, 209, 283,
20	Caffeic acid[a]	180	179	135, 167
24	Caffeoyl hexose	342	341	135,161, 179
25	*p*-Coumaryl pentose	296	295	119, 163, 235
26	Cinnamic acid isomer	148	147	119, 135

Table 4.2 Continued...

29	*p*-Coumaric acid[a]	164	163	119, 139
30	Sinapic acid[a]	224	223	179
31	*trans*-Ferulic acid[a]	194	193	135
32	*cis*-Ferulic acid[a]	194	193	135
38	*p*-Coumaric truxilic acid	328	327	163, 289
39	Ferulic truxilic acid	388	387	193, 237
40	TriFA1	578	577	193, 237, 533
41	8,5'-benzo DiFA	386	385	193, 341
43	TriFA 2	578	577	193, 369, 385
45	8,8'-aryl DiFA	386	385	193
47	8,5'-DiFA	386	385	119, 193, 313
49	CAD 1			193,301, 353
50	DiFA 4	386	385	
51	5,5'-DiFA	386	385	113,193, 313
52	Tri FA 3	578	577	137, 341
53	CAD 2			195, 309, 353
54	TriFA 4	578	577	177, 371
Flavonoids				
5	Catechin[a]	290	289	245
9	Taxifolin-*O*-hexoside	466	465	137, 303, 325
11	Gallocatechin	306	305	289
12	Taxifolin-*O*-pentoside	436	435	137, 259, 303, 337

Table 4.2 Continued...

15	Procyanidin dimer B1	578	577	289, 425, 451
17	Epigallocatechin[a]	306	305	151, 179, 247, 287
18	Epicatechin[a]	290	289	245
27	6-*C*-glucosyl-8-*C*-rhamnosyl apigenin	578	577	297,353,383, 457,487, 559
28	Taxifolin[a]	304	303	125, 285
33	Vitexin (Apigenin-8-*C*-glu)	432	431	269, 283,311, 341
34	Luteolin 7-*O*-glucoside	448	447	121, 177, 285, 313, 381
35	Isovitexin (Apigenin-6-*C*-glu)	432	431	269, 311, 341, 353
36	Tricin	330	329	178, 207
37	Myricetin hexoside	480	479	241, 317, 359
42	Myricetin pentoside	450	449	190, 317, 427
44	Myricetin[a]	318	317	151, 179
46	Kaempherol hexoside	448	447	257, 285, 327,401,
48	Quercetin-*O*-pentoside	434	433	301, 371, 407
55	Procyanidin dimer B2	578	577	289, 407, 425
56	Quercetin[a]	302	301	121, 179
57	6-*C*-glucosyl-8-*C*-arabinosyl apigenin	564	563	353,383,413,443, 473, 545
58	6,8-di-*C*-glucosyl apigenin	594	593	353, 383, 473, 503, 575
59	Apigenin	270	269	269

[a] Identification of the compound was confirmed by the authentic standard.

Abbreviations are DiFA - Diferulic acids; TriFA – Triferulic acids; CAD-Chlorogenic acid derivative

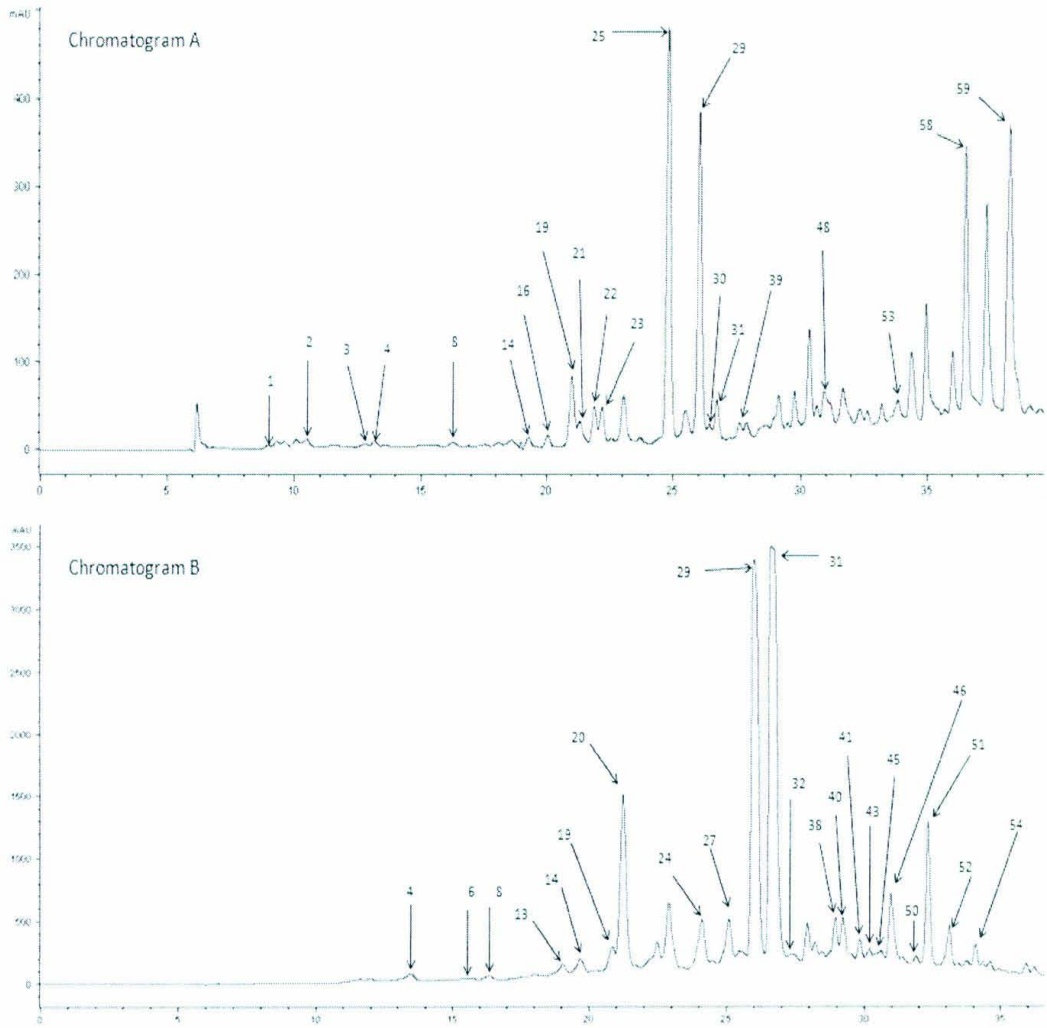


Figure 4.1 Representative HPLC chromatograms of free phenolic fraction of foxtail (A), and insoluble bound fraction of kodo (B) millets. Choice of phenolic type and varieties was based on displaying a maximum number of peaks representing phenolic compounds.

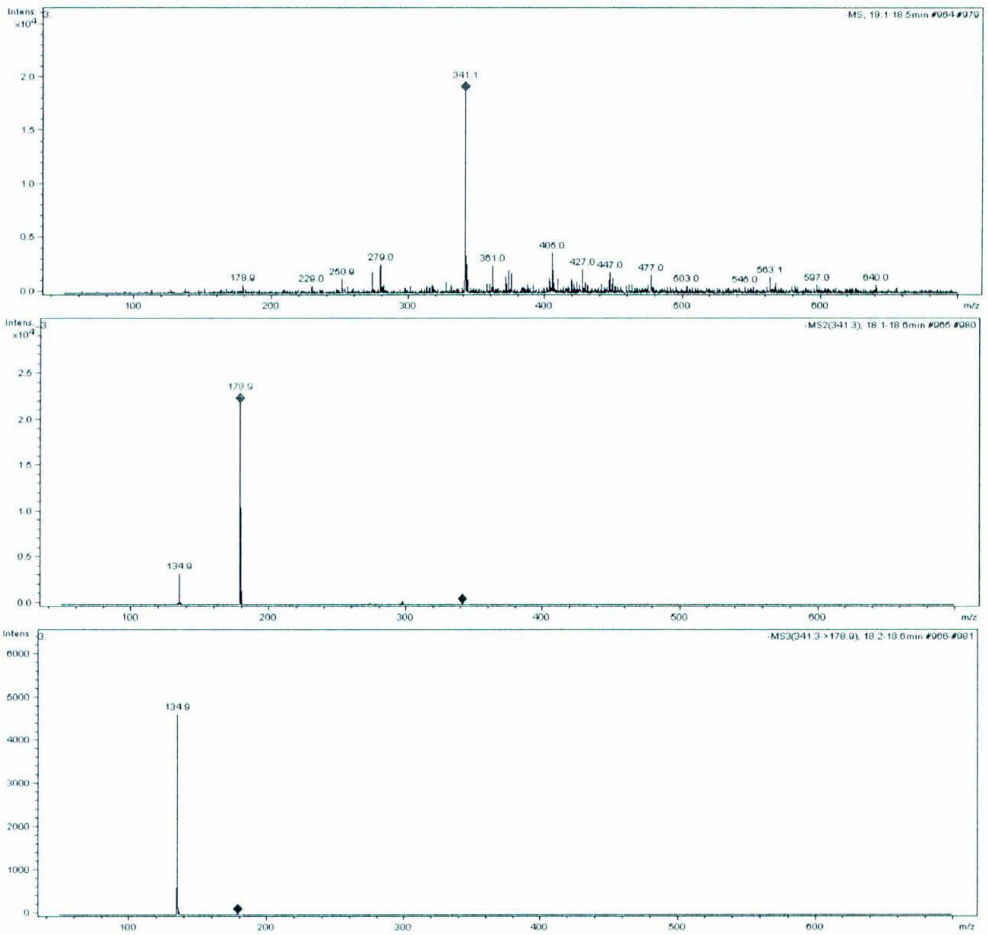


Figure 4.2 Representative mass spectra of caffeoyl hexose (peak 24), tentatively identified in kodo and pearl millet grains by tandem mass spectrometry.

4.4.2 Characterization of phenolic compounds by HPLC-DAD-ESI-MS^3

Table 4.2 presents the mass spectral data and identification results for peaks in the representative chromatograms of different phenolic fractions of millet grains (**Figure 4.1**). The compounds were numbered according to their order of elution from the lowest to the highest retention times (RT). In this study a total of 59 phenolic compounds were characterized directly in the crude phenolic extracts of millet grains

without using additional steps of purification. Twenty one of these compounds were unambiguously identified by comparing RT, and UV absorption and further confirmed using MS spectral data of authentic standards. Other peaks were tentatively identified on the basis of UV spectra, MS^3 characteristics and information available in the literature. Coupling of HPLC with tandem mass spectrometry has served as an efficient analytical tool for identification and structural elucidation of a number of natural compounds in crude plant extracts. In mass spectral analysis ESI negative ion mode was selected as it was able to provide detailed structural information through collision induced dissociation. Phenolic compounds identified in millets belonged to hydroxybenzoic and hydroxycinnamic acids as well as flavonoids. To the best of our knowledge this is the first report of the phenolic profiles and the antioxidant activities of different phenolic fractions obtained from whole millet grains.

4.4.3 Hydroxybenzoic acids and their derivatives

Several hydroxybenzoic acids, namely gallic (1), protocatechuic (4), *p*-hydroxybenzoic (14), gentisic (16), vanillic (19), and syringic (21) acids were positively identified in whole millet grains. Protocatechuldehyde (8), *p*-hydroxybenzaldehyde (22) and a methyl ester of vanillic acid (2) were tentatively identified. The MS spectra generated for hydroxybenzoic acids showed loss of CO_2 giving $[M-H-44]^-$ as a characteristic ion. Thus, ions at m/z 125, 109, 93, 123 and 153 were detected for gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and syringic acids, respectively (**Table 4.2**). The MS spectra generated for peaks 8 and 22 gave deprotonated molecule $[M-H]^-$ at m/z 137 and 121, respectively, corresponding to compounds, protocatechualdehyde and *p*-hydroxybenzaldehyde, respectively. These identifications were supported by MS data and UV spectra in the literature (*16-18*).

The MS spectra of methyl vanillate (2) in the ESI negative ion mode showed a deprotonated molecule $[M-H]^-$ at m/z 181, although the most intense ion was at m/z 166 due to the loss of a methyl group $[M-H-15]^-$ (*17*). Furthermore, vanillin (23) was positively identified by comparison with the RT and UV and MS spectral data of its reference compound.

4.4.4 Hydroxycinnamic acids and their derivatives

Hydroxycinnamic acids positively identified were chlorogenic (10), caffeic (20), *trans*-cinnamic isomer (26), *p*-coumaric (29), sinapic (30), *trans*-ferulic (31) and *cis*-ferulic (32) acids by comparison of their RT and UV and MS spectral data with those of the available standards. The mass spectrum of peak 3 gave a deprotonated molecule $[M-H]^-$ at m/z 207 and a main ion $[M-H-CH_3]^-$ at m/z 192 via the loss of a methyl group suggesting it to be sinapaldehyde (*17*).

Several glycosides of hydroxycinnamic acids were detected in the millet phenolic extracts. Peaks 6, 7, 13, 24, were tentatively identified as hexoses of sinapic, ferulic, 5-hydroxyferulic, and caffeic acids, respectively. The deprotonated molecule ion $[M-H]^-$ of peaks 6, 7, 13, 24, were at m/z 385, 355, 371 and 341, respectively, and the MS^2 fragmentation of these peaks indicated $[M-H-162]^-$ ions which showed the loss of a hexose (**Figure 4.2**). Similar MS fragmentation data were observed in previous studies (*19, 20*). Peak 25 showed $[M-H]^-$ ion at m/z 295 and MS^2 fragmentation ion $[M-H-132]^-$ at m/z 163 which was generated via the loss of a pentose. In agreement with the literature, the compound associated with peak 25 was tentatively identified as a *p*-coumaryl pentose (*21*). Peaks 49 and 53 showed m/z 353 as major fragmentation ion at MS^2 spectra and exhibited UV spectra with similar characteristics to cholorogenic acid, which showed double broad peaks between 260

and 340 nm. Thus, peaks 49 and 53 were tentatively identified as chlorogenic acid derivatives 1 and 2, respectively, comparing their MS spectral data with the literature (*19, 20*).

4.4.5 Dimers and trimers of ferulic acids

Photochemically induced cyclodimerization and radical, oxidative coupling via the action of peroxidases are mechanisms in which hydroxycinnamic acid esters produce dimers and dehydrodimers, respectively. They act as cross-linking agents of polysaccharide chains in plant cell walls (*22*). Alkaline hydrolysis of plant materials released these compounds (*23, 24*). In this study, a number of these compounds were detected in the insoluble bound phenolic fraction of millet grains. It has been reported that antioxidant activities of diferulates are greater than those of monomer compounds and depend on their structure (*25*). This may explain the highest antioxidant activity observed in the insoluble bound phenolic fraction of kodo millet which possessed 12% of their hydroxycinnamic acids and derivatives as dimers or trimers of ferulic acids.

Peaks 38 and 39 were tentatively identified as cyclodimeric phenolic acids of *trans-p*-coumaric acid and *trans*-ferulic acids, respectively. In profiling of peak 38, MS spectrum showed a major deprotonated molecular ion at $[M-H]^-$ at 327 which coincided with m/z of 328. The MS^2 and MS^3 fragmentations showed major molecular ions at m/z 163 and m/z 119, respectively, corresponding to molecular ions of *p*-coumaric acid suggesting *p*-coumaric truxillic acid. Similarly, peak 39 was tentatively identified as *trans*-ferulic truxilic acid (*18*). Truxillic acids have previously been reported as cell wall materials in plants belonging to *Poaceae* (*26*).

Peaks 41, 45, 47, 50 and 51 exhibited an m/z signal at 385 as a major fragment ion $[M-H]^-$ which corresponded to the molecular mass (386) of ferulic acid dehydrodimers. The MS^2 fragmentation showed a prominent ion at m/z 193 and the UV spectra of corresponding peaks showed characteristics of ferulic acid related structures which confirmed their identity as a ferulic acid derivative, in agreement with findings of Hernanz et al. (*27*). Considering both UV spectra and MS^3 data, peaks 41, 45, 47, 50 and 51 were tentatively identified as ferulic acid dehydrodimers (DiFA). A number of DiFAs such as 8,8' aryl DiFA, 8,8' DiFA, 8,5' DiFA, 8-O-4' DiFA, 8,5' benzo DiFA and 5,5' DiFA was previously reported in the insoluble bound fractions of cereals such as wild rice, wheat, rye, barley, oat, and maize (*7,23,28*). In this study peaks 41, 45, 47 and 51 were assigned 8,5' benzo DiFA (benzofuran form), 8,8 aryl DiFA (aryltetralin form), 8,5'-DiFA and 5,5'-DiFA, respectively, based on the UV absorption spectra reported by Waldron et al. (*18*). Peak 50 was also attributed to a DiFA type compound although its final structure was not confirmed.

The UV spectra of peaks 40, 43, 52 and 54 demonstrated similar characters to ferulic acid structures with broad double peaks between 240 and 340 nm. The deprotonated molecular ion $[M-H]^-$ appeared as base peak ion at m/z 577 and corresponded to molecular weight of 578 indicating a dehydrotriferulic acid (TriFA) structure as previously explained (*29*). However no attempts were made in this study to assign individual chemical structures to different TriFAs found in millet grains.

4.4.6 Flavonoids

Several compounds belonging to different classes of flavonoids, namely flavan-3-ol (monomers, and dimers), flavonols and their glycosides, flavones and flavanonol were positively or tentatively identified in millet phenolic extracts. In general flavonoids

are conjugated with sugars and occur as glycosides of *O*- or *C*- forms, but they also exist as free aglycones (*30*). However, it has been shown that in major cereals flavones-*C*-glycosides are predominantly synthesized (*31*).

Compounds associated with peaks 4, 11, 17 and 18 were positively identified as flavan-3-ol monomers by comparing RT, UV and mass spectral data with those of authentic standards and were catechin, gallocatechin, epigallocatechin and epicatechin, respectively. Peaks 15 and 55 showed deprotonated molecular ions $[M-H]^-$ at m/z 577 suggesting a 578 molecular ion that indicates flavan-3-ol dimmer (*21*). Furthermore, MS^2 spectra of these two peaks showed an ion at m/z 289 as a major fragmentation corresponding to catechin or epicatechin. Therefore, peaks 15 and 55 were tentatively identified as procyanidin dimers B1 and B2, respectively. In addition to m/z 289, other main fragments obtained in MS^2 spectra of these two peaks included 425 and 407 which were consistent with previously published literature data for these two compounds (*17, 32-34*).

The mass spectrum of peak 28 showed a deprotonated base peak ion at m/z 303 and MS^2 fragmentation showed a major ion at 285. By comparing RT and UV and mass spectra characteristics, peak 28 was positively identified as taxifolin, a flavanonol. Furthermore, on the basis of the ion fragments in MS^3, peaks 9 and 12 were tentatively identified as a hexoside and a pentoside of taxifolin, respectively. Deprotonated molecular ions $[M-H]^-$ for peaks 9 and 12 were at m/z 465 and 435, respectively. In addition, deprotonated molecular ion of taxifolin at m/z 303 appeared as a main fragment ion in the MS^2 spectra for both 9 and 12 peaks corresponding to $[M-H-162]^-$ and $[M-H-132]^-$ ions, respectively.

Peaks 33 and 35 showed the same deprotonated molecular ions $[M-H]^-$ at m/z 431 suggesting the possibility of them being an isomer pair. Furthermore, both mass spectra showed the typical fragmentation of *C*-glycosides, the loss of 90 and 120 Da. The MS^3 spectrum of peak 33 yielded fragment ions at m/z 341 ($[M-H-90]^-$) and 311 ($[M-H-120]^-$) and in comparison with the literature data it was identified as 8-*C*-glucosyl apigenin (vitexin) (*35*). Similarly, peak 35 was identified as 6-C-glucosyl apigenin (isovitexin) (*36*). Vitexin and isovitexin were earlier reported in pearl millet grain flour (*37*). However, in this study they were only detected in kodo millet. The content of vitexin and isovitexin in pearl millet sample used in this study may not be sufficiently high to be detected under the experimental conditions employed.

The mass spectrum of peak 34 exhibited a deprotonated molecular ion $[M-H]^-$ at m/z 447 corresponding to the molecular weight of 448 for the compound. The resulting MS^2 fragmentation of 447 showed an ion at m/z 285 indicated loss of 162 from the molecular ion at m/z 447 corresponding to the deprotonated aglycone. Thus, peak 34 was tentatively identified as a luteolin 7-*O*-glucoside (*36*). Luteolin was earlier reported in fonio and Japanese barnyard millet grains (*38,39*).

Peak 36 was tentatively identified as tricin, a flavone, and the mass spectrum of respective peak gave a deprotonated molecule ion $[M-H]^-$ at m/z 329 (*40*). Tricin detected in this study in finger millet varieties, was earlier detected in Japanese barnyard millet and brown rice grains (*39, 41*). Tricin is reported to possess anticlonogenic/antiproliferative properties in human derived immortalized and tumourigenic breast and colon cancer cells (*41*).

Peak 44 was positively identified as a flavonol, myricetin, by comparing its RT, UV and mass spectral data with those of authentic standard. UV spectra of peaks

37 and 42 showed maximum absorbance at 262 and 356 nm, respectively, and their deprotonated base molecular ions $[M-H]^-$ were at m/z 479 and 449, respectively. Furthermore, MS^2 spectra of those two peaks exhibited m/z at 317 as a predominant ion corresponding to an aglycone $[M-H]^-$ ion of myricetin. The difference between molecular mass and aglycone mass suggested that peaks 37 and 42 were a hexoside and a pentoside, respectively. Based on these information and comparison with the literature data, peaks 37 and 42 were tentatively identified as a myricetin hexoside and a myricetin pentoside, respectively (*42,43*). Similarly, peaks 46 and 48 were tentatively identified as a kaempherol hexoside and a quercetin-*O*-pentoside, respectively. The MS^3 analysis of peak 46 showed that deprotonated base ion of the glycoside at m/z 447 with maximum absorbance at 255 and 350 nm and the ion at m/z 285 corresponding to the deprotonated aglycone of kaempherol (*44*). Since glucose is the most common sugar present, peak 46 was suggested to be kaempherol 3-*O*-glucoside (*45*). In this study kaempherol derivative was reported in the esterified fractions of kodo, foxtail and proso millet grains. In agreement with the present results, Viswanath et al. (*46*) reported that kaempherol was not detected among other phenolics in finger millet grains. The same behaviour was detected for peak 48, quercetin-*O*-pentoside (m/z 433→ m/z 301) (*36, 44*). In addition, peak 56 was positively identified as quercetin based on RT and UV and mass spectral characteristics of the standard.

Several flavone derivatives were identified in millet grains. Peak 59 was identified as apigenin and positively confirmed by comparison with RT and the MS^3 spectra of its authentic standard. According to Ferreres et al. (*47*) presence of ions at m/z 353 and 383 indicated that the aglycone was apigenin. Thus, peaks 27, 57, and 58 were

tentatively identified as derivatives of apigenin by their UV and tandem mass spectra and the literature data (*47*). Peak 27 was tentatively identified as 6-*C*-glucosyl-8-*C*-rhamnosyl apigenin due to the characteristic losses of 18, 90 and 120 Da for *C*-glycosides (MS, $[M-H]^-$ 577; MS^2, $[M-H-18]^-$ 559, $[M-H-90]^-$ 487, $[M-H-120]^-$, 457). Peak 57 showed ions at m/z 545, 473, and 443 due to the loss of 18, 90 and 120 Da fragments, respectively. Furthermore, deprotonated molecular ion, $[M-H]^-$ at m/z 563 suggested a hexosyl-pentosyl apigenin (apigenin 270 + hexose 162 + pentose 132). Considering the high relative intensity of $[M-H-120]^-$ ion, peak 57 was assigned to be 6-*C*-glucosyl-8-*C*-arabinosyl apigenin (*47*). Similarly, peak 58 gave $[M-H]^-$ at m/z 593 and its MS^2 spectrum produced ions at $[M-H-18]^-$ 575, $[M-H-90]^-$ 503, $[M-H-120]^-$ 473 and $[M-H-210]^-$ 383 which coincided with those reported previously and was tentatively identified as 6,8-di-*C*-glucosyl apigenin (vicenin-2) (*28, 35*). Apigenin derivatives were previously reported in several wild rice varieties (*28*) and in fonio millet (*Digitaria exilis*) (*38*). Some of the major peaks were not even tentitatively identified due to the lack of information available on deprotonated molecular ions and fragment ions obtained by tandem mass spectrometry. Further work is needed for the isolation of these compounds and structure elucidation by complementary analytical tools such as nuclear magnetic resonance (NMR).

Table 4.3 The content of free phenolic compounds (µg/g defatted meal) in soluble fraction of millet grains

Peak	Kodo	Finger (Ravi)	Finger (local)	Foxtail	Proso	Little	Pearl
Hydroxybenzoic acids and derivatives							
1	1.80±0.4	4.98±0.4	3.59±0.7	4.49±0.3	3.81±0.2	2.13±0.4	-
2	-	-	-	1.36±0.1	0.58±0.2	5.11±0.1	2.30±0.1
4	6.84±0.4	50.8±3.6	33.9±7.5	1.55±0.1	0.63±0.1	-	0.47±0.1
8	2.82±1.2	-	-	1.14±0.1	1.02±0.1	-	1.81±0.1
14	1.41±0.1	-	-	4.62±0.6	15.5±0.1	3.73±0.1	2.12±0.4
16	-	-	-	16.8±1.2	8.31±0.8	-	-
19	25.4±2.1	-	-	29.5±1.2	4.39±0.5	15.1±0.6	1.36±0.1
21	-	-	-	3.54±0.3	-	9.34± .1	-
22	-	6.44±0.7	4.61±0.1	2.19±0.1	-	-	-
23	11.2±1.1	-	-	7.37±1.1	2.07±0.5	2.23±0.3	1.20±0.1
TOT	49.5	62.2	42.1	55.8	28.0	37.6	9.3
Hydroxycinnamic acids and derivatives							
3	-	-	-	-	-	5.69±0.9	2.56±0.2
10	3.56±0.2	-	-	-	19.1±1.1	-	-
20	4.11±0.7	-	-	-	37.3±6.8	-	4.88±1.3
25	10.1±0.9	-	-	49.6±0.1	0.79±0.1	21.8±0.1	3.26±0.1
26	37.4±1.3	-	-	-	-	-	-
29	8.65±0.2	2.10±0.1	1.45±0.3	3.88±0.1	1.75±0.1	63.4±1.9	4.04±0.1
30	-	-	-	-	3.24 ±0.1	18.1±2.0	-
31	10.3±0.1	9.37±0.1	7.67±0.1	67.3±2.8	18.1±0.9	27.8±2.0	22.1±1.1

Table 4.3 Continued...

32	-	-	-	16.9±2.6	-	5.24±0.1	14.4±0.9
49	-	-	-	-	-	27.8±0.5	-
53	-	-	-	7.91±0.1	-	3.25±0.1	-
TOT	74.1	11.5	9.1	171	88.6	173	51.2
Flavonoids							
5	-	1611±87	1218±82	6.94±0.4	-	-	-
9	-	24.3±1.4	16.9±0.1	-	-	-	-
11	-	58.7±2.3	54.2±7.8	-	-	-	-
15	-	6.25±0.3	17.8±0.9	-	-	-	-
18	-	134±9.1	114±3.2	-	-	-	-
28	-	8.33±1.4	6.30±0.1	-	-	-	-
33	6.60±0.8	3.71±0.5	1.54±0.5	-	-	-	-
34	46.2±1.9	-	-	-	-	-	-
35	7.39±0.3	5.16±0.4	4.10±0.4	-	-	-	-
37	-	13.4±0.1	7.25±0.1	-	-	-	-
44	-	11.7±1.4	7.87±1.5	-	-	-	-
48	55.5±0.2	8.23±0.8	9.02±0.6	20.4±1.5	-	-	-
55	-	10.5±2.4	6.84±1.0	-	-	-	-
57	61.8±0.3	-	-	16.5±0.7	-	39.6±0.1	2.94±0.2
59	1.52±0.3	-	-	125±2.6	1.9±0.3	9.08±0.3	4.13±0.3
TOT	179	1896	1464	169	1.9	48.7	7.1

Abbreviations are TOT-total for individual class of phenolics

Table 4.4 The content of phenolic compounds (µg/g defatted meal) in soluble fraction yielded from alkaline hydrolysis (esterified) of millet grains

Peak	Kodo	Finger (Ravi)	Finger (local)	Foxtail	Proso	Little	Pearl
Hydroxybenzoic acids and derivatives							
1	-	-	-	-	2.34±0.7	-	5.35±0.6
2	-	-	-	1.11±0.1	-	13.9±0.5	28.3±0.1
4	15.3±0.9	15.2±1.3	18.5±0.1	-	0.45±0.1	-	-
8	1.72±0.1			-	-	-	17.7±0.4
14	12.7±0.9	2.31±0.3	2.67±0.1	7.65±0.4	41.6±1.6	5.47±0.1	24.3±2.1
19	2.97±0.1	-	-	54.4±3.2	66.5±1.5	45.6±0.4	-
21	136±4.3	15.1±0.3	22.1±2.4	13.8±2.6	6.25±0.2	12.9±0.2	-
TOT	169	32.6	43.2	77.0	117	77.8	75.7
Hydroxycinnamic acids and derivatives							
3	23.9±0.6	4.02±1.3	11.4±0.3	16.3±0.1	50.6±2.6	9.79±0.3	58.6±5.3
7	-	-	-	-	4.05±0.8	-	-
13	36.6±7.6	-	-	-	-	-	-
20	42.2±3.1	2.08±0.4	2.52±0.5	1.86±0.5	4.15±1.2	-	21.0±1.1
24	39.2±0.9	-	-	-	-	-	-
29	25.6±1.0	2.85±0.1	3.02±0.4	90.3±1.2	42.3±1.8	95.4±2.3	17.3±0.3
30	53.2±2.2	0.84±0.1	2.52±0.1	16.2±3.0	15.6±1.6	37.4±2.6	-
31	350±10	16.4±0.9	26.6±2.0	155±0.8	88.4±4.9	126±1.1	61.1±1.2
32	26.7±0.7	-	-	16.9±3.3	15.9±2.0	16.7±1.9	12.6±0.8
TOT	598	26.2	40.1	297	221	286	171

Table 4.4 Continued...

Flavonoids							
5	-	78.1±2.8	77.5±0.5	-	-	-	-
9	-	17.2±0.2	12.0±1.2	-	-	-	-
12	-	2.30±0.6	2.63±0.3	-	-	-	-
17	-	45.5±4.3	17.3±2.1	-	-	-	-
36	-	2.33±0.3	3.86±0.1	-	-	-	-
37	-	2.84±0.2	3.09±0.9	-	-	-	43.5±0.8
42	-	7.98±1.2	13.2±0.7	-	-	-	-
44	-	0.68±0.2	1.12±0.2	-	-	-	6.76±0.2
46	60.6±4.5	-	-	1.27±0.3	30.7±2.4	-	-
58	9.21±0.3	-	-	42.6±0.2	2.57±0.6	34.7±1.6	-
59	-	-	-	34.9±0.1	0.43±0.1	3.16±0.1	-
TOT	69.7	157	130	78.7	33.7	37.9	50.3

Abbreviations are TOT-total for individual class of phenolics

4.4.7 Quantification of phenolic compounds in millet grains

The contents of phenolic compounds as quantified by HPLC are presented in **Tables 4.3** to **4.6**. The results showed that the content and type of phenolic compounds varied depending on the millet variety as well as phenolic fraction considered. However, as expected the two finger millet varieties tested showed essentially the same phenolic compounds in each fraction though the contents differed between two varieties which confirm the fact that phenolic content is influenced by the variety. Flavonoids constituted a substantial amount of total phenolic compounds detected in the free, esterified and etherified phenolic fractions of the two finger millet varieties tested and

contributed 96, 72, and 59%, respectively, of the total phenolic content (**Tables 4.3, 4.4 and 4.5,** respectively). The highest flavonoid level was detected in the free phenolic fractions of the two finger millet varieties, of which 84% was attributed to catechin (**Table 4.3**). Antioxidant activity of catechins has been extensively studied due to their contribution to the antioxidant properties of tea beverages *(1)*. In accordance with the present results phenolic acids such as gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric, ferulic, and cinnamic acids and a flavonoid, quercetin, were reported in the crude extract of finger millet (*48*). As observed in previous studies, and confirmed in this study, soluble phenolic fraction which composed of free, esterified and etherified fractions, of two finger millet varieties showed higher TPC and antioxidant activity than that of insoluble bound phenolics in several *in vitro* systems (*12,13*).

In general the content of hydroxycinnamic acids and derivatives (HCAS) was dominant in the free and esterified fractions of foxtail, proso, little and pearl millets and their contribution ranged 43-75, and 58-71%, respectively. Hydroxybenzoic acids and derivatives (HBAS) were high in etherified fractions of foxtail, proso and little millets whereas HCAS were found to make the highest (85%) contribution to the TPC in the etherified fraction of pearl millets. Furthermore, the highest contribution to the TPC in the free, esterified and etherified fractions of kodo millet was flavonoids (59%), HCAS (71%) and HBAS (82%), respectively (**Tables 4.3, 4.4, and 4.5,** respectively). The results of the present study further demonstrated the importance of profiling the phenolic content to understand the behaviour of antioxidant activity of crude millet grain extracts.

Table 4.5 The content of phenolic compounds (µg/g defatted meal) in soluble fraction yielded from acid hydrolysis (etherified) of millet grains

Peak	Kodo	Finger (Ravi)	Finger (local)	Foxtail	Proso	Little	Pearl
Hydroxybenzoic acids and derivatives							
4	8.73±0.4	5.55±0.7	3.76±0.3	-	1.23±0.1	-	1.13±0.1
8	0.56±0.4	8.60±0.3	5.42±0.5	-	-	-	-
14	6.62±0.1	2.32±0.4	3.43±0.2	3.92±0.1	13.6±0.1	11.6±0.5	21.5±0.1
19	29.6±0.3	-	-	12.7±0.5	11.9±0.3	54.9±1.1	7.55±0.7
21	4.65±0.2	10.0±1.6	5.32±0.5	-	-	1.21±0.1	6.26±0.2
TOT	50.2	26.5	17.9	16.6	26.7	67.7	36.4
Hydroxycinnamic acids and derivatives							
20	2.24±0.3	2.85±0.2	4.55±0.1	2.42±0.1	3.69±0.3	11.9±0.3	4.53±0.8
24	-	-	-	-	-	-	65.5±0.1
29	1.31±0.1	0.50±0.3	0.44±0.1	0.75±0.1	2.93±0.1	2.83±0.2	17.0±0.6
30	-	-	-	-	-	-	12.9±0.3
31	5.17±0.2	1.35±0.6	2.59±0.1	2.65±0.2	5.63±0.2	15.6±0.2	92.7±0.9
32	1.15±0.2	2.11±0.4	3.55±0.1	4.16±1.3	3.45±0.2	12.8±0.2	46.8±0.3
TOT	9.87	6.81	11.1	9.99	15.7	43.2	239
Flavonoids							
5	-	36.5±3.7	43.7±1.7	-	-	-	-
34	-	10.6±1.8	1.08±1.0	-	-	-	-
37	-	-	-	-	1.78±0.1	-	2.23±0.1
44	-	-	-	-	16.3±0.6	-	3.25±0.2
59	0.63±0.1	-	-	-	-	-	-
TOT	0.63	47.1	44.8	-	18.0	-	5.48

Abbreviations are TOT-total for individual class of phenolics

Table 4.6 The content of phenolic compounds (µg/g defatted meal) in insoluble bound fraction yielded from alkaline hydrolysis

Peak	Kodo	Finger (Ravi)	Finger (local)	Foxtail	Proso	Little	Pearl
Hydroxybenzoic acids and derivatives							
2	-	-	-	-	-	12.0±0.8	19.8±0.1
4	39.7±6.5	48.2±0.4	55.4±0.1	10.2±3.7	69.7±0.8	48.8±1.9	-
8	10.7±1.3	5.02±0.8	4.90±0.1	-	4.08±1.9	-	4.56±0.9
14	10.5±2.0	1.70±0.3	1.81±0.1	5.63±0.7	55.4±4.9	11.8±0.7	-
19	40.1±7.3	-	-	22.1±0.4	85.8±1.4	46.8±5.3	7.08±0.3
TOT	101	54.9	62.1	38.0	215	119	31.5
Hydroxycinnamic acids and derivatives							
6	16.9±1.0	-	-	-	19.0±1.7	-	19.8±4.2
13	50.5±4.5	-	-	-	-	-	11.4±0.3
20	276±31	11.0±0.9	19.3±1.9	34.0±0.8	294±1.0	19.0±1.2	-
24	149±1.3	-	-	-	-	-	21.9±0.3
29	767±89	36.0±0.9	48.1±8.4	848±5.8	1188±70	924±1.0	53.5±0.9
31	1844±173	331±0.4	394±24	631±5.9	332±21	185±1.5	637±30
32	100±5.3	65.3±9.2	65.9±2.7	101±5.9	18.6±4.2	58.1±0.8	81.5±7.5
38	35.9±0.6	-	-	-	-	-	2.58±0.7
40	47.1±6.2	-	-	38.2±3.8	6.60±1.0	10.4±1.1	-
41	12.2±1.7	-	-	8.58±1.0	-	-	2.10±0.6
43	10.2±2.5	-	-	-	-	-	-
45	94.8±15	-	-	19.6±1.5	-	7.51±0.2	-

Table 4.6 Continued...

47	-	5.65±0.4	9.87±1.7	-	-	5.34±0.1	11.0±1.2
50	41.9±3.0	-	-	-	-	15.7±0.4	42.6±2.7
51	173±17.5	11.8±0.6	10.7±2.8	62.2±1.9	5.44±2.1	4.24±0.2	57.0±9.4
52	44.8±2.6	4.90±0.2	5.67±2.2	18.3±6.5	2.67±0.1	12.4±0.1	30.5±4.9
54	25.4±7.0	-	-	7.90±3.7	-	-	-
TOT	3687	468	556	1769	1867	1242	971
Flavonoids							
27	124±12	-	-	-	-	-	7.75±0.5
46	8.06±0.4	-	-	33.5±3.1	-	-	-
56	-	1.73±0.3	2.37±0.5	-	-	-	-
TOT	132	13.3	1.73	2.37	33.5	-	7.75

Abbreviations are TOT-total for individual class of phenolics

In contrast to the variations observed in the soluble fractions, insoluble bound fractions of all millet varieties tested in this study, distinctly showed a high total content of HCAS and values ranged from 468 to 3687 µg/ g defatted meal (**Table 4.6**). The contribution of HCAS in total phenolic content of insoluble bound fractions of millets ranged from 87 to 98 %. Furthermore, depending on the variety *p*-coumaric acid or *trans*-ferulic acid were the main contributors to the total HCAS. The hydroxycinnamic acids are reported to exhibit effective *in vitro* as well as *in vivo* antioxidant activities (*49*). The contribution of *trans*-ferulic acid in insoluble bound fraction of kodo, finger (Ravi), finger (local) and pearl millets were 50, 70, 70, and 65 %, respectively. On the other hand, *p*-coumaric acid, the major hydroxycinnamic acid in the insoluble bound fractions of foxtail, proso and little millets contributed 48, 64 and 74% to the total HCAS (**Table 4.6**). In agreement with the present results, Rao and Muralikrishna (*11*) reported that ferulic, caffeic and *p*-coumaric acids were the

major bound phenolics in finger millet. In addition to monomers of ferulic acids, dehydrodiferulates were also reported previously in proso millet grits (*50*).

4.5 Conclusions

The results of this study indicated that in general insoluble bound form of phenolics attached to the cell wall material of millet grains, is a major contributor to the total phenolic content of the grain. The main classes of phenolic compounds identified in whole millet grains were hydroxybenzoic acids, hydroxycinnamic acids and flavonoids. The contents and distribution of phenolic compounds varied among fractions as well as the genotype. The use of HPLC coupled with UV-DAD and ESI-tandem MS allowed detection and identification of over 50 compounds directly from the crude extract without a need for any additional purification steps, thus serving as a rapid analytical procedure. Profiling of phenolic compounds of millets and determination of their contents and antioxidant activities may advance the understanding of this underutilized cereal as a valuable functional food ingredient and as a source of antioxidants in health promotion.

4.6 References

1. Shahidi, F.; Naczk, M. Phenolics in Food and Nutraceuticals. CRC press, Boca Raton, FL, **2004**, pp 1-82.

2. Liyana-Pathirana, C.; Shahidi, F. Importance of insoluble-bound phenolics to antioxidant properties of wheat. *J. Agric. Food Chem.* **2006**, 54, 1256-1264.

3. Madhujith, T.; Shahidi, F. Antioxidative and antiproliferative properties of selected barley (*Hordeum vulgarae* L.) cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. *J. Agric. Food Chem,* **2007,** 55, 5018-5024.

4. Madhujith, T.; Shahidi, F. Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. *Food Chem.* **2009,** 117, 615-620.

5. Ishii, T.; Hiroi, T. Linkage of phenolic acids to cell-wall polysaccharides of bamboo shoot. *Carbohydrate Res.* **1990**, 206, 297-310.

6. Grabber, J.H.; Ralph, J.; Hatfield, R.D. Cross-linking of maize walls by ferulate dimerization and incorporation into lignin *J. Agric. Food Chem.* **2000**, 48, 6106-6113.

7. Andreasen, M.F.; Kroon, P.A.; Williamson, G.; Garcia-Conesa, M.R. Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radic. Biol. Med.* **2001**, 31, 304-314.

8. Sosulski, F.; Krygier, K.; Hogge, L. Free, esterified and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. *J. Agric. Food Chem.* **1982**, 30, 337-340.

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

10. Dvorakova, M.; Guido, L.F.; Dostalek, P.; Skulilova, Z.; Moreira, M.M.; Barros, A.A. Antioxidant properties of free, soluble ester and insoluble –bound phenolic compounds in different barley varieties and corresponding malts. *J. Inst. Brew.* **2008**, 114, 27-33.

11. Rao, M.V.S.S.T.S.; Muralikrishna, G. Non-starch polysaccharides and bound phenolic acids from native and malted finger millet (Ragi, *Elucine coracana* Indaf-15). *Food Chem.* **2001**, 72, 187-192.

12. Chandrasekara, A.; Shahidi, F. The content of insoluble bound phenolics in millets and their contribution to antioxidant capacity *J. Agric. Food Chem.* **2010,** 58, 6706-6714.

13. Chandrasekara, A; Shahidi, F. Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. *J. Agric. Food Chem.* **2011**, 59, 428-436.

14. Rao, M.V.S.S.T.S.; Muralikrishna, G. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Elucine coracana* Indaf-15). *J. Agric. Food Chem.* **2002**, 50, 889-892.

15. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, 16, 144-158.

16. Perez-Magarino, S.; Revilla, I.; Gonzalez-SanJose, M.L.; Beltran, S. Various applications of liquid chromatography-mass spectrometry to the analysis of phenolic compounds. *J. Chromatogr. A.* **1999**, 847, 75-81.

17. Sanz, M.; Cadahia, E.; Esteruelas, E.; Munoz, A.M.; de Siomn, B.F.; Hernandez, T.; Estrella, I. Phenolic compounds in cherry (*Prunus avium*) Heart wood with a view to their use in cooperage. *J. Agric. Food Chem.* **2010**, 58, 4907-4914.

18. Waldron, K.W.; Parr, A.J.; Ng, A.; Ralph, J. Cell wall esterified phenolic dimers: identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochem. Anal.* **1996**, 7, 305-312.

19. Sandhu, A.K.; Gu, L. Antioxidant capacity, phenolic content and profiling of phenolic compounds in seeds, skin and pulp of *Vitis rotundifolia* (Muscadine grapes) as determined by HPLC-DAD-ESI-MS^n. *J. Agric. Food Chem.* **2010**, 58, 4681-4692.

20. Harbaum, B.; Hubbermann, E.M.; Wolff, C.; Herges, R.; Zhu, Z.; Schwarz, K. Identification of flavonoids and hydroxycinnamic acids in pak choi varieties (Brassica campestris L. ssp. Chinensis var. communis) by HPLC-ESI-MS^n and NMR and their quantification by HPLC-DAD. *J. Agric. Food Chem.* **2007**, 55, 8251-8260.

21. Verardo, V.; Bonoli M.; Marconi, E.; Caboni, M.F. Distribution of bound hydroxycinnamic aids and their glycosyl esters in barley (*Hordeum vulgare* L.) air classified flour: Comparative study between reversed phase-high performance chromatography-mass spectrometry (RP-HPLC/MS) and spectrophotometric analysis. *J. Agric. Food Chem.* **2008**, 56, 11900-11905.

22. Ralph, J.; Quideau, S.; Grabber, J.H.; Hatfield, R.D. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J. Chem. Soc. Perkin Trans 1.* **1994**, 3485-3498.

23. Renger, A.; Steinhart, H. Ferulic acid dehydrodimers as structural elements in cereal dietary fibre. *European Food Res. Technol.* **2000**, 211, 422-428.

24. Andreasen, M. F.; Christensen, L.P.; Meyer, A.S.; Hansen, A. Content of phenolic acids and ferulic acid dehydrodimers in 17 rye (*Secale cereale* L.) varieties. *J. Agric. Food Chem.* **2000**, 48, 2837-2842.

25. Garcia-Conesa, M.T.; Puumb, G.W.; Kroon, P. A.; Walace, G.; Williamson, G. Antioxidant properties of ferulic acid dimers. *Redox Rep.* **1997**, 3, 239-244.

26. Ford, C.W.; Hartely, R.D. GC/MS characterization of cyclodimers from p-coumaric and ferulic acids by photodimerization-A possible factor influencing cell wall biodegradability. *J. Sci. Food Agric.* **1989**, 46, 301-310.

27. Hernanz, E.; Nunez, V.; Sancho, A.I.; Faulds, C.B.; Williamson, G.; Bartolome, C.; Gomez-Cordoves, C. Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley. *J. Agric. Food Chem.* **2001**, 49, 4884-4888.

28. Qiu, Y.; Liu, Q.; Beta, T. Antioxidant properties of commercial wild rice and analysis of soluble and insoluble phenolic acids. *Food Chem.* **2010**, 121, 140-147.

29. Bunzel, M.; Ralph, J.; Bruning, P.; Steinhart, H. Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. *J. Agric. Food Chem.* **2006**, 54, 6409-6418.

30. Stobiecki, M. Application of mass spectrometry for identification and structural studies of flavonoid glycosides. *Phytochemistry.* **2000**, 54, 237-256.

31. Brazier-Hicks, M.; Evans, K.M.; Gershater, M.C.; Puschmann, H.; Steel, P.G.; Edwards, R. The C-glycosylation of flavonoids in cereals. *J. Biol. Chem.* **2009**, 284, 17926-17934.

32. Ruiz, D.; Egda, J.; Gil, M.I.; Tomas-barberan, F.A. Characterization and quantification of phenolic compounds in new apricot (*Prunus armeniaca* L.) varieties. *J. Agric. Food Chem.* **2005**, 53, 9544-9552.

33. Friedrich, W.; Eberhardt, A.; Galensa, R. Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *Eur. Food Res. Technol.* **2000**, 211, 56-64.

34. Sun, J.; Liang, F.; Bin, Y.; Li, P. Duan, C. Screening non-colored phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries. *Molecules.* **2007,** 12, 679-693.

35. Han, J.; Ye, M.; Qiao, X.; Xu, M.; Wang, B.; Guo, D. Characterization of phenolic compounds in the Chinese herbal drug *Artemisia annua* by liquid chromatography coupled to electrospray ionization mass spectrometry. *J. Pharm. Biomed. Anal.* **2008**, 47, 516-525.

36. Sanchez-Rabaneda, F.; Jauregui, O.; Casals, I.; Andres-Lacueva, C.; Izquierdo-Pulido, M.; Lamuela-Raventos, R.M. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *J. Mass Spec.* **2003,** 38, 35-42.

37. Reichert, R.D. The pH-sensitive pigments in pearl millet. *Cereal Chem.* **1979**, 56, 291-294.

38. Soyer P.; Blanc F.; Vissuzaine, C.; Marmuse, J-P.; Menu, Y.; Sartelet, H.; Serghat, S.; Lobstein, A.S.; Ingenbleek, Y.; Anton, R.; Petitfrere, E; Aguie-Aguie, G.; Martiny, L.; Haye, B. Flavonoids extracted from fonio millet (*Digitaria exillis*) reveal potent anti-thyroid properties. *Nutrition.* **1996,** 12, 100-106.

39. Watanabe, M. Antioxidative phenolic compounds from Japanese Barnyard Millet (*Echinochloa utilis*) grains. *J. Agric. Food Chem.* **1999**, 47, 4500-4505.

40. Estiarte, M.; Penuelas, J; Canigueral, S.; Casals, I. A reverse-phase HPLC method for tricin separation from wheat leaves. *Cereal Chem.* **1997**, 74, 495-496.

41. Hudson, E.A.; Dinh, P.A.; Kokubun, T.; Simmonds, M.S.J.; Gescher, A. Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiol. Biomarkers Prev.* **2000**, 9, 1163-1170.

42. Lu, J-L.; Wang, D-M.; Shi, X-G.; Yang, D-P.; Zheng, X-Q.; Ye, C-X. Determination of purine alkaloids and catechins in different parts of Camellia assamica var. kucha by HPLC-DAD/ESI-MS/MS. *J. Sci. Food Agric.* **2009**, 89, 2024-2029.

43. de Brito, E.S.; de Araujo, C.P.; Lin, L., Harnly, J. Determination of the flavonoid components of cashew apple (*Annacardium occidentale*) by LC-DAD-ESI/MS. *Food Chem.* **2007**, 105, 1112-1118.

44. Cai, Y-Z.; Xing, J.; Sun, M.; Zhan, Z-Q.; Corke, H. Phenolic antioxidants (hydrolizable tannins, flavonols and anthocyanins) identified by LC-ESI-MS and MALDI-QIT-TOF MS from *Rosa chinensis* flowers. *J. Agric. Food Chem.* **2005**, 53, 9940-9948.

45. Cuyckens, F.; Claeys, M. Mass spectrometry in the structural analysis of flavonoid. *J. Mass Spec.* **2004**, 39, 1-15.

46. Viswanath, V.; Urooj, A.; Malleshi, N.G. Evaluation of antioxidant and antimicrobial properties of finger millet (*Eleusine coracana*). *Food Chem.* **2009**, 114, 340-346.

47. Ferreres, F.; Silva, B.M.; Andrade, P.B.; Seabia, N.A.; Ferreira,N.A. Approach to the study of C-glycosyl flavones by ion trap HPLC-PAD-ESI/MS/MS: application to seeds of quince (*Cydonia oblonga*). *Phytochem. Anal.* **2003**, 14, 352-359.

48. Chethan, S.; Dharmesh, S.M.; Malleshi, N.G. Inhibition of aldose reductase from cataracted eye lenses by finger millet (*Eleusine coracana*) polyphenols. *Bioorg. Med. Chem.* **2008**, 16, 10085-10090.

49. Shahidi F.; Chandrasekara A. Hydroxycinnamates and their *in vitro* and *in vivo* antioxidant activities. *Phytochem. Rev.* **2008**, 9, 147-170.

50. Mattila, P.; Pihlava, J-M.; Hellstrom, J. Contents of phenolic acids, alkyl-and alkenylresorcinols, and avenanthramides in commercial grain products. *J. Agric. Food Chem.* **2005**, *53*, 8290-8295.

CHAPTER 5

Antiproliferative potential and DNA scission inhibitory activity of phenolics from whole millet grains

5.1 Abstract

Phenolic extracts from seven millet varieties (kodo, finger (Ravi), finger (local) proso, foxtail, little and pearl) were evaluated for their total phenolic content (TPC), total flavonoid content (TFC), ferrous ion chelating activity and singlet oxygen scavenging capacity. Furthermore, the potency of millet phenolic extracts in the inhibition of peroxyl and hydroxyl radical induced supercoiled DNA scission, as well as xanthine oxidase and their antiprolifeative activities against HT-29 cells were studied. In addition, lipid peroxidation inhibition of extracts was examined in a liposome system. The TPC and TFC ranged from 146 to 1156 μmol ferulic acid equivalents and 25 to 1203 μmol catechin equivalents per g crude extract, respectively. All varieties employed in this study exhibited a notable inhibition of lipid peroxidation in liposomes, singlet oxygen quenching and DNA scission inhibition to varying degrees. At the end of day 4 millet extracts inhibited cell proliferation in the range of 28-100%. Thus, the present study demonstrates that millet phenolics may be effective in the prevention of cancer initiation and progression *in vitro*. Millet grains may therefore serve as a nutraceutical and functional food ingredient in health promotion and disease risk reduction.

5.2 Introduction

Phenolics and polyphenolics are diverse groups of secondary metabolites found in a wide range of plant foods. Phenolics may exert their beneficial effects directly by scavenging free radicals, chelating metal ions, quenching singlet oxygen and other reactive oxygen species (ROS) or indirectly by inhibiting ROS producing enzymes such as myeloperoxidase, lipoxygenase, cyclooxygenase and xanthine oxidase. Phenolic compounds have traditionally been considered as 'antinutrients' due to their action in reducing the digestibility of proteins, and interfering with mineral absorption in the intestine. Nonetheless, with the expanding knowledge of their beneficial role in the health and disease, phenolics are now regarded as 'lifespan essentials'.

Free radicals are continuously produced in the body as a part of the normal aerobic respiration and substrate oxidation and when uncontrolled, damage to vital biomolecules such as carbohydrates, lipids, proteins and DNA leading to clinical manifestations of age-related diseases (*1*). Free radical mediated oxidation of membrane lipids has been considered as one of the primary events in the cellular damage. In addition, it has been shown that lipid peroxidation may be a causative factor in carcinogenesis through the action of secondary oxidation products such as malondialdehyde (*2, 3*). Oxidative stress caused by excessive production of ROS plays a role in the pathogenesis of a number of age related diseases such as cancer, cardio and vascular disorders, metabolic syndrome, neurodegenerative diseases and ophthalmologic disorders (*4*). Thus, regulation of oxidative stress is of much importance in ameliorating such diseases, by supplying exogenous antioxidants that can minimize the effects of stimulators that produce ROS in the body.

Inhibition or slowing down of the rapid multiplication of malignant cells is a part of cancer therapy and may also reduce the progression of cancer. There is a widespread scientific interest in natural food components that prevent DNA damage and retard the proliferation of cancer cells which are important in disease management. DNA scission inhibition activity of cereal phenolics has been reported in several publications on wheat and barley grains, thus demonstrating their ability to prevent oxidative damage in supercoiled plasmid pBR DNA (*5, 6*).

Cancer is a predominant health concern in the developed countries and is among the rapidly emerging diseases as a public health problem in the developing countries which adds to health costs in dealing with malnutrition, at the stage of nutrition transition (*7*). Colorectal cancer accounts for the second highest cancer deaths in North America (*8*). In addition, inhibition of Caco-2 colon cancer cell proliferation has been reported for cereal grain phenolics (*5, 6*). Furthermore, grains contain a larger proportion of their phenolics in the insoluble bound form. These bound phenolics are released during colonic fermentation and hence contribute to the beneficial health effects associated with them. Several studies have discussed the importance of inclusion of bound phenolic fraction in determinations of total phenolic contents and the antioxidant activities of cereal grains (*5, 9, 10, 11*).

Millets have been staple foods in the semi-arid regions of Africa and Asia for centuries and grown as a subsistence crop by a multitude of small-holder farmers in many countries of these two continents. Millets have predominantly been used for household consumption and local trade, in areas where they are produced, hence limiting their international market potential. Millets are well known in western countries as 'bird seed' but are also used in multigrain products although specific type

used is not listed on the label. In addition, there is a good potential for using millets in gluten-free foods and beverages for individuals suffering from celiac disease, an autoimmune disorder, which could be treated following a gluten-free diet. Moreover, depending on the type, millets possess different tastes upon cooking, thus, add variety to the diet in addition to their contribution to health and wellness.

Antioxidant and health promoting properties of millets have sparsely been reported, mainly for finger millets. Chethan et al. (*12*) reported that phenolic compounds such as phenolic acids and quercetin from finger millet grains prevent cataractogenesis by inhibiting aldose reductase activity in human lenses. Antioxidant and antimicrobial properties of millet polyphenolics have also been reported (*13*). Using a rat model, *in vivo* studies have revealed that finger millets are effective against diabetes due to their hypoglycaemic, hypocholesterolaemic, nephroprotective and anti-cataractogenic properties (*14*). However, there is a gap in knowledge about the ability of millet grain phenolics in preventing DNA scission and proliferation of malignant cells and their antioxidant activities in liposome systems. Thus, the objectives of this study were to determine the antioxidant activity of millet phenolics in DNA and liposomes as well as evaluating the antiproliferative activity of phenolic extracts in an adenocarcinoma cell line. In addition, ferrous chelating activity, and singlet oxygen inhibition of different concentrations of millet extracts were assessed in order to understand the antioxidant activity of extracts in biological systems. To the best of our knowledge this is the first report on DNA scission, xanthine oxidase and liposome oxidation inhibitory and antiproliferative activities of millet grain phenolics that sheds light on their use as a nutraceutical and a functional food ingredient in the therapy of diseases associated with oxidative stress.

5.3 Materials and methods

Seven millet grain samples, namely foxtail (*Setaria italica;* variety ISC 480*)*, proso (*Panicum miliacium;* variety AC 254*)*, finger millet (*Elusine coracana;* varieties Ravi and local), kodo (*Paspalum scrobiculatum;* a local variety), little millet (*Panicum sumatrense*; a local variety), and pearl millet (*Pennisetum glaucum;* dark green cultivar*)* harvested in 2007 crop year were used in this study. All grain samples, with the exception of pearl millet, were obtained from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka. Pearl millet, grown in India, was obtained through Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India.

Folin Ciocalteu's reagent, ferulic acid, catechin, aluminium chloride, sodium nitrite, 2,2′-azobis-(2-ethylpropionamidine) dihydrochloride (AAPH), sodium chloride, ferrous chloride, *N,N*- dimethyl-*p*-nitrosoaniline (DPN), histidine, sodium hypochlorite, hydrogen peroxide, ferrous sulphate, mono- and dibasic sodium phosphates, 3-(2-pyridyl)-5-6-diphenyl-1,2,4,-triazine-4,4-disulphonic acid sodium salt (ferrozine), ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), L-α-phosphatidylcholine type XVI-E, xanthine oxidase, xanthine, pBR 322 plasmid DNA, agarose, tris acetate, bromophenol blue, xylene cyanol, and glycerol, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Hexane, acetone, methanol, choloroform, hydrochloric acid, sodium hydroxide and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada). SYBR safe gel stain was purchased from Probes Invitrogen (Eugene, OR, USA). Biological grade dimethyl sulphoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). McCoy's 5A medium was purchased from Invitrogen Co. (Carlsbad, CA,

USA) and HT-29 cells were purchased from American Type Culture Collection (Rockville, MD, USA).

5.3.1 Sample preparation

Whole millet grains were cleaned to remove soil and other particles and subsequently ground using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc. Brockville, ON, Canada) in order to obtain a fine powder which passed through mesh 16 sieve (1 mm opening, Tyler test sieve, Mentor, OH, USA). All samples were defatted by blending with hexane (1:5 w/v, 5 min, 3 times) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA) at ambient temperature. Defatted samples were vacuum packed in polyethylene pouches and stored at –20°C until used within one week for extraction of phenolics.

5.3.2 Preparation of crude phenolic extracts

An ultrasonic-assisted procedure was used for the extraction of soluble phenolic compounds under reflux conditions. Defatted meal (5 g) was mixed with 100 mL of 70% (v/v) acetone and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) and sonicated at the maximum power for 25 min. After centrifugation of the resulting slurry for 5 min at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA), the supernatant was collected and extraction was repeated two more times. Combined supernatants were evaporated *in vacuo* at 40°C (Buchi, Flawil, Switzerland) and lyophilized for 72 h at -46°C and 34 x 10^{-3} mbar (Freezone, Model 77530, Labconco

Co., Kansas City, MO, USA). Lyophilized crude phenolic extracts were stored at -20°C until used for further analysis.

5.3.3 Determination of total phenolic content (TPC)

The total phenolic content of each extract was determined according to Singleton and Rossi (*15*) with slight modifications as explained previously (*10*). The content of total phenolics in each extract was determined using a standard curve prepared for ferulic acid and expressed as micromoles of ferulic acid equivalents (FAE) per gram of crude extract.

5.3.4 Determination of total flavonoid content (TFC)

Total flavonoid content was determined using a colourimetric method described by Kim et al. (*16*) and Chandrasekara and Shahidi (*10*). A standard curve prepared with catechin was used to calculate total flavonoid content. The TFC was expressed as micromoles of catechin equivalents (CE) per gram of crude extract.

5.3.5 Ferrous ion chelating activity

The ability of millet phenolic extracts to chelate ferrous ion was measured according to the method described by Dinis et al. (*17*) as previously reported (*10*). Different concentrations of soluble phenolic extracts (0.5 to 8 mg/mL) were used to measure chelating activity of ferrous ions to determine the concentration that inhibits 50% of chelating activity (IC_{50}). Briefly, 0.4 mL of extracts in distilled water was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by adding 5 mM ferrozine (0.2 mL) and the total volume was adjusted to 4 mL with distilled water. The mixture was vigorously shaken and left at room temperature for 10 min. The

absorbance of the reaction mixture was measured at 562 nm. For the control, distilled water was used instead of the extract. Appropriate blanks were prepared with 0.4 mL of the sample and 3.6 mL of distilled water for background subtraction. The inhibition percentage of ferrozine-ferrous ion complex formation was calculated by using the following equation. Metal chelating effect (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. The results were expressed as IC_{50} values (mg/mL).

5.3.6 Singlet oxygen inhibition

The ability of millet phenolic extracts to inhibit singlet oxygen was measured according to a spectrophotometric method explained by Maldonado et al. (*18*) as previously reported (*11*). In brief, the chemical solutions and different concentrations of phenolic extracts (0.0625 to 5 mg/mL) were prepared in 45 mM sodium phosphate buffer (pH 7.4). The reaction mixture consisted of 0.4 mL phenolic extract, 0.5 mL DPN (200 μM), 0.2 mL histidine (100 mM), 0.2 mL sodium hypochlorite (100 mM), and 0.2 mL H_2O_2 (100 mM) and the total volume was made to 2 mL with sodium phosphate buffer. The absorbance of the reaction mixture was measured at 440 nm after incubation for 40 min at 30^0 C. Blanks were run for each sample with 0.4 mL of extract and phosphate buffer. A control was prepared replacing phenolic extract with sodium phosphate buffer. The percentage inhibition of singlet oxygen was calculated as follows. Singlet oxygen inhibition activity (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100.

5.3.7 Supercoiled plasmid DNA strand scission inhibition

Inhibition activity of millet phenolics against supercoiled strand DNA scission induced by peroxyl and hydroxyl radicals was evaluated according to a previously described method with slight modifications (*19, 20*). Supercoiled plasmid DNA (pBR 322 from *Escherichia coli* RRI) was dissolved at a concentration of 50 µg/mL in 0.5 M, pH 7.4 phosphate buffer solution (PBS). Soluble millet phenolic extracts (0.25 and 0.5 mg/mL) were prepared in PBS. In an Eppendorf tube (500 µL), 2 µL of a solution of supercoiled plasmid DNA, PBS, phenolic extract, H_2O_2 (1 mM) and $FeSO_4$ (0.5 mM) were added in the order stated to determine the inhibitory activity of millet extracts against hydroxyl radical induced DNA strand scission. The mixture was incubated at 37°C for 1 h in the dark (*19*). The loading dye (2 µL), consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol in distilled water, was added to the reaction mixture at the end of the incubation period.

In another experiment, inhibitory effect of millet extracts against peroxyl radical induced DNA scission was investigated. In this, AAPH was dissolved in PBS in order to attain a final concentration of 9 mM which was then mixed with DNA and the extracts to a final volume of 10 µL. A control with DNA alone and a blank devoid of phenolic extracts were prepared with each set of phenolic extracts tested.

The samples were electrophoresed using a 0.7% (w/v) agarose gel prepared in Tris-acetic acid-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5). SYBR safe was added at a concentration of 100 µL/L of TAE buffer as a gel stain. Submarine gel electrophoresis was run at 60 V for 5 h using a model B1A horizontal mini gel electrophoresis system (Owl Separation systems Inc., Portsmonth, NH, USA) and a model 300V power supply (WMR International Inc., West Chester, PA, USA) at room

temperature in TAE buffer. The bands were visualized under transillumination of UV light using AlphaImager™ gel documentation system (Cell Biosciences, Santa Clara, CA, USA). The images were analyzed using Chemilmager 4400 software (Cell Biosciences, Santa Clara, CA, USA) to quantify DNA scission. The protective effect of millet phenolic extracts was calculated using retention percentage of the normalized supercoiled DNA as given below. DNA retention % = (Intensity of supercoiled DNA with the oxidative radical and extract / Intensity of supercoiled DNA in control) X 100.

5.3.8 Human colon adenocarcinoma cell proliferation inhibition

A colorectal cancer (CRC) cell line, HT-29 was used to study their proliferation inhibition according to the method described by Wang et al. (*21*). The HT-29 cells were propagated in T-150T flasks in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) albumin and 1% antibiotic/antimycotic. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 (*22, 23*). The cells were plated at 2500 cells per well in a 96-well microplate and incubated for 24 h at 37°C. Soluble millet phenolic extracts dissolved in DMSO were introduced into the wells containing cell culture media to obtain final concentrations of 0.1 mg/mL and 0.5 mg/mL. The control contained cell culture media and DMSO. Both treatment levels and the control contained a final concentration of 0.99 % DMSO. The live cells on each of the wells were studied using an ATP-Lite 1 step kit (PerkinElmer, Shelton, CT, USA) which produces luminescence proportionately to the amount of ATP present. Consequently, the amount of ATP is in proportion to the number of living cells. Luminescence readings were taken using a Victor multi-well plate reader

(PerkinElmer, Shelton, CT, USA) immediately prior to the treatment and 4, 24, 48, 72 and 96 h afterwards. The treatment and control media were replaced every 24 h up to 96 h during the incubation period. The antiproliferative effects of millet phenolics against HT-29 cells were expressed as percent inhibition calculated against the control.

5.3.9 Inhibition of liposome oxidation induced by UVA light

The liposome suspension was prepared according to a method previously described by Huang and Frankel (*24*). Briefly, 20 mg of α-phosphatidylcholine (PC) were dissolved in chloroform in a round bottom flask and the solvent was evaporated in a rotary evaporator *in vacuo* at room temperature. After removing traces of solvent by nitrogen flush, 10 mL of 10 mM phosphate buffered saline (PBS) (pH 7.4, 150 mM NaCl) were added. The mixture was vortexed and sonicated for 1 and 3 min, respectively, before making the PC mixture to a final concentration of 0.5 mg/mL in PBS. To evaluate the inhibition of liposome oxidation, 0.8 mL of PC and 0.1 mL of extract (1 mg/mL) were mixed in an Eppendorf tube (1.5 mL) and total volume was made to 1 mL with PBS. The control was prepared with PC mixture and PBS. The mixtures were incubated for 5 min at 37^0 C before exposing to UV light for 60 min (Fahlman & Krol, 2009) using a Spectroline ® UV lamp (364 nm, UVA) (Model ENF-240 C, Spectronics Co., Westbury, NY, USA) at room temperature. At the end of incubation conjugated diene hydroperoxides of a 100 µL of sample diluted to 1 mL with methanol formed were measured spectrophotometrically (Model HP 8452A diode array spectrophotometer, Agilent Technologies, Palo Alto, CA, USA) at 234 nm. The results were expressed as percentage inhibition of oxidation.

5.3.10 Xanthine oxidase inhibition

Xanthine oxidase inhibition activity of millet phenolic extracts was determined according to the method explained by Noro et al. (*25*). Xanthine oxidase (XO), extracts and ferulic acid were dissolved in 45 mM phosphate buffer solution (pH 7.4). The assay mixture consisted of 0.1 mL of extract solution, 0.3 mL of buffer and 0.1 mL of XO (0.5 units/mL). The mixture was pre-incubated at 25^0C for 15 min and the reaction was started by adding 0.2 mL of 0.15 mM xanthine solution. After incubation for 30 min at 25^0C, 0.3 mL of 1 M HCl was added to stop the reaction and the absorbance was read at 290 nm. A blank was run for each sample similarly but HCl was mixed before the addition of XO solution to the assay mixture. Xanthine oxidase inhibitory activity was expressed as percentage inhibition, calculated as {1-(Absorbance of the sample / Absorbance of the control)} x 100.

5.3.11 Statistical analysis

All experiments were carried out in triplicates unless otherwise stated and data were reported as mean ± standard deviation. The significance of differences between two concentrations of millet extracts were determined using Student's t test at $p \leq 0.05$. The differences of mean values among millet varieties was determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p \leq 0.05$ significance level. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

5.4 Results and Discussion

The DNA scission inhibition, antiproliferative activities and xanthine oxidase inhibitory activities of phenolics extracted from several millet grains were examined.

In addition, antioxidant activity of millet phenolics against liposome peroxidation induced by UVA light was reported. Millets used in the present study consisted of different testa colours, and grain shapes and sizes. The weight of thousand grains of kodo, finger (Ravi), finger (local), foxtail, proso, little and pearl millets were 5.2, 2.1, 2.9, 3.1, 5.7, 0.4, and 10.6 g respectively. As the results are given per gram of crude extract or as percentage inhibition depending on the assay system employed, yields of crude phenolic extracts of millet varieties used in this study were calculated and presented in **Table 5.1**. Values so obtained ranged from 21 to 46 mg/g grain on a dry weight basis.

Table 5.1 Yield, total phenolic (TPC) and flavonoid (TFC) contents of phenolic extracts of whole millets

Millet type	Yield (mg/g of grain,dry weight)	TPC (µmol ferulic acid equiv /g crude extract)	TFC (µmol catechin equiv /g crude extract)
Kodo	21 ± 0.4	1157 ± 33. 3 a	1203 ± 26.1 a
Finger (Ravi)	30 ± 3.0	503.8 ± 7.48 c	166.8 ± 1.46 b
Finger (local)	35 ± 0.9	730.1 ± 28.3 b	182.9 ± 7.55 b
Foxtail	40 ± 6.0	215.9 ± 16.7 d	25.15 ± 0.68 c
Proso	22 ± 0.7	231.9 ± 3.86 d	37.94 ± 2.35 c
Little	46 ± 3.0	204.4 ± 5.36 d	25.66 ± 2.81 c
Pearl	28 ± 7.0	146.3 ± 6.41 e	28.26 ± 0.18 c

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

5.4.1 Total phenolic content (TPC)

Table 5.1 presents the TPC of different millet varieties employed in this study as determined by the Folin Ciocalteu's reagent. The TPC of millet extracts ranged from 146.3 to 1156.6 µmol FAE per gram of crude lyophilized extract. Kodo millet had the highest TPC followed by finger (local), finger, proso, foxtail, little and pearl millets, thus establishing varietal effect on the TPC. Varieties such as kodo and finger millet with reddish brown or dark brown seed coats showed a higher TPC than grains such as foxtail, proso, little and pearl millets with light yellow and cream coloured seed coats. In general, phenolic compounds are concentrated in the outer layers, namely hull (pericarp), seed coat (testa) and aleuron layer of cereal grains. It may be worth pointing out that in preparation of small grains such as finger millets for foods generally seed coat which is attached tightly to the aleuron layer is not removed but only the outermost hull, thus preserving much of phenolics present in the grain. In a previous study phenolic compounds present in whole millet grains were characterized and quantified using HPLC and HPLC/MS and these belonged mainly to the hydroxybenzoic, and hydroxycinnamic acids as well as flavonoids (*26*).

5.4.2 Total flavonoid content (TFC)

Flavonoids exhibit various biological activities such as antiallergenic, antiviral, anti-inflammatory, and vasodilating actions apart from their superior antioxidant activities. In general, cereal grains have less flavonoids compared to fruits, vegetables and specific beverages such as red wine, tea and coffee, (*27*). However, recent analysis of flavonoid content in different millet types has shown that the contribution of

flavonoids, especially those in the free form, to the total phenolic content should not be overlooked in certain varieties such as finger and kodo millets (*26*).

The TFC of different millet varieties ranged from 25.1 to 1203.8 µmol CE /g crude extract. Kodo millet extract had a significantly ($p \leq 0.05$) higher flavonoid content compared to foxtail millet which showed the lowest (Table 5.1). The decreasing order of flavonoid content in crude phenolic extracts of millet varieties tested in this study was kodo > Finger (local) > Finger (Ravi) > proso > pearl > little > pearl > foxtail. Results of a previous study showed that the composition of flavonoids was different between kodo and finger millet varieties (*26*). Flavonoids in kodo millet were predominantly composed of flavones (apigenin, luteolin, vitexin, isovitexin) and flavonols (quercetin). On the other hand flavan-3-ol monomers and procyandins were mainly detected in finger millet varieties apart from flavonols (quercetin, myricetin), and flavanonols (taxifolin) (*26*). Thus, bioactivities of extracts belonging to different millet types examined in the present study may partly be attributed to these compositional differences.

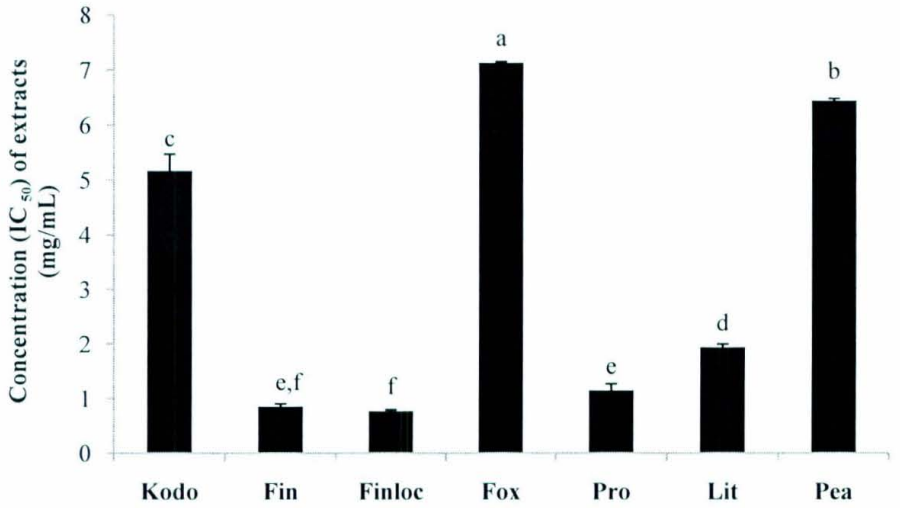


Figure 5.1 Concentrations of whole millet grain phenolic extracts required to chelate 50% of ferrous ion (IC_{50}) in the in vitro assay system. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters have significantly different ($p < 0.05$) mean values. The letter 'a' represents the highest value.

5.4.3 Ferrous ion chelating activity

Ferrous ion contributes to lipid peroxidation as well as DNA oxidation by generating hydroxyl radical through Fenton reaction. In addition, ferrous ion accelerates decomposition of lipid hydroperoxides into peroxyl and alkoxyl radicals (*28*). In this study, millet extracts showed chelating activity of ferrous ions in a dose-dependent manner. **Figure 5.1** shows the concentration of millet phenolics extracts needed to achieve 50% chelation of ferrous ion available in the assay medium. Among different varieties tested finger (Ravi), finger (local) and proso millets showed high ferrous ion chelating activities with low IC_{50} values of 0.85, 0.77 and 1.14 mg/mL, respectively. Foxtail millet showed the least ferrous ion chelating efficacy followed by pearl and

kodo millet extracts with high IC_{50} values. It is noteworthy that, kodo millet which displayed a relatively high antioxidant and antiradical activities in a number of assays (*20, 11*) demonstrated a low ferrous ion chelating activity. On the other hand, two finger millet varieties which showed high chelating activity were reported to contain flavan-3-ol monomers as well as dimers such as procyanidin B1 and B2 which could be attributed to their high ferrous ion chelating activity (*26*). Results of the present study showed that millet extracts act as chelating agents thus may help inhibiting lipid peroxidation.

5.4.4 Singlet oxygen inhibition

Singlet oxygen reacts with a number of biological molecules. Kanofsky et al. (*29*) showed that singlet oxygen was produced by human eosinophils. Eosinophils are known to exert a variety of toxic effects that destroy infectious agents during host-defense responses in the body, but can also damage normal tissues under inflammatory disease states. Furthermore, singlet oxygen is a highly reactive, long-lived and diffusible molecule, thus allowing more destructive effects on biomolecules in the cells (*29*). Furthermore, Bose et al. (*30*) showed that singlet oxygen is involved in the UVA induced lipid peroxidation of liposomal membranes.

Phenolic extracts of millets showed varying degrees of scavenging activity against singlet oxygen. Ferulic acid exhibited the highest activity of 42% and ranged from 9 to 25% at a concentration of 1 mg/mL (**Table 5.2**). Inhibition percentage of singlet oxygen by millet phenolic extracts was generally lower compared to that of other ROS such as hydrogen peroxide and hydroxyl radicals (*11*). Previously, Wang and Jiao (*31*) had also observed a similar pattern with the extract of berry crops. **Figure 5.2** shows the percent singlet oxygen inhibition activity of millet extracts at

different concentrations. The trend showed a dose dependent inhibition activity against singlet oxygen. It is noteworthy that except proso and little millets all other millets had decreased singlet oxygen inhibition activities with increasing concentration of the extracts, suggesting the involvement of constituent phenolic compounds in these extracts.

Table 5.2 Percentage inhibition of UVA induced liposome oxidation (Liposome), and Xanthine oxidase activity (XO) and scavenging of singlet oxygen ($^1O_2$) by phenolic extracts of whole millets

Millet type	Liposome	XO	$^1O_2$
Ferulic Acid	91 ± 4 a	75 ± 2 a	42 ± 1 a
Kodo	80 ± 5 c	69 ± 2 a	25 ± 1 b
Finger (Ravi)	85 ± 1 b,c	14 ± 3 d	14 ± 3 c
Finger (local)	87 ± 3 a	23 ± 1 b	15 ± 1 c
Foxtail	93 ± 1 a	13 ± 1 d	20 ± 1 c
Proso	88 ± 1 a	19 ± 1 c	9 ± 1 d
Little	87 ± 1 b	11 ± 1 d	18 ± 2 c
Pearl	80 ± 1 c	11 ± 1 d	25 ± 1 b

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

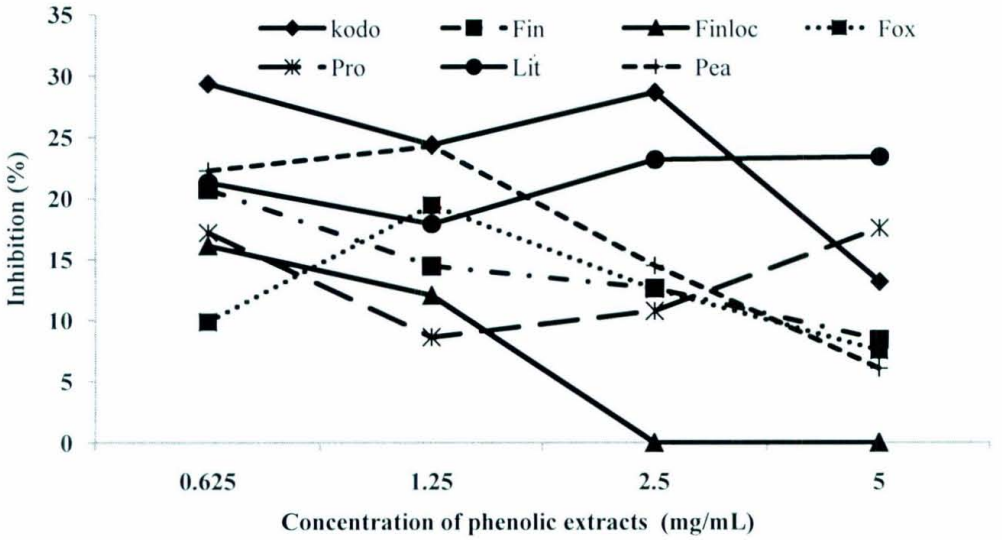


Figure 5.2 Percent inhibition of singlet oxygen at different concentrations of whole millet phenolic extracts. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets.

5.4.5 Inhibition of liposome peroxidation induced by UVA light

Ultraviolet radiation is known to damage lipid membranes directly or via a process mediated by ROS (*30, 32, 33*). UVA (365 nm) is the major component of UV solar spectrum that reaches the earth. Some evidences indicate a relationship between exposure to sunlight and incidence of skin cancers suggesting the involvement of free radicals in photocarcinogenesis (*34, 35*). Antioxidant activity of millet grain phenolics were determined on the basis of their ability to inhibit conjugated diene hydroperoxide formation in phosphatidylcholine (PC) liposome membranes which were oxidized under UVA light.

The results of the present study demonstrated that millet phenolics were effective against liposome peroxidation induced by UVA light at a concentration of 1 mg/mL of

the extracts (**Table 5.2**). A ferulic acid solution of 1 mg/mL, which was used as a standard, showed 91% inhibition whereas millet phenolics inhibited lipid oxidation ranging from 80 to 93%. It is noteworthy that the extract of finger (local), foxtail, and proso millet grains had a high inhibition percentage similar to that of pure ferulic acid. Ferulic acid has been reported to possess the highest antioxidant activity among phenolic compounds in a PC liposome system in which oxidation is induced by cupric acetate (*36*). The relatively high content of ferulic acid in millet extracts may contribute to its effective oxidation inhibitory activity in liposome in the present work. The antioxidant activity of phenolic compounds may be related to both their structural characteristics as well as ability to interact with the liposome. Furthermore, Verstraeten et al. (*37*) reported that the interaction of flavonols and procyanidins with phospholipid head groups especially with those containing hydroxyl groups is responsible for a reduced rate of membrane lipid oxidation. Thus, finger millet varieties with a high proportion of such compounds tested in this study may exert their antioxidant activity in a liposome system by limiting the access of oxidants to the bilayer and the propagation step of lipid oxidation in the hydrophobic membrane matrix. Erlejman et al. (*38*) found that hydrophilicity of the phenolic compounds, degree of flavonol oligomerization and the number of hydroxyl groups in the respective molecule was associated with the protection against membrane oxidation. The flavonol and quercetin may protect against UV radiation through absorbance of UV radiation, antioxidant activity and interaction with signal transduction pathways (*39-41*). Recently, Fahlaman and Krol (*42*) showed that quercetin inhibited UVA and UVB radiation induced lipid oxidation mainly by scavenging radical species. These evidences suggest that peroxidation inhibition demonstrated by millet phenolic

extracts in the present study could be due to their ability to scavenge free radicals generated during UV radiation.

5.4.6 Xanthine oxidase inhibition

Xanthine oxidase (XO) is one of the enzymes belonging to the group of molybdenum iron-sulphur flavin hydroxylases and regulates the metabolism of purine substrates (*43*). XO reduces dioxygen into hydrogen peroxide and superoxide anion radicals by two electrons and one electron reduction mechanism, respectively, in the process of converting hypoxanthine to xanthine and then uric acid. In addition, XO plays a major role as a source of ROS in ischemic reperfusion injuries of heart and small intestine (*44-46*). Furthermore, excessive level of uric acid in the body may result in disease conditions such as gout which is caused by the deposition of uric acid in the joints and is associated with inflammation. In addition, uric acid contributes to the formation of renal calculi and is also associated with a spectrum of renal disorders. Thus, foods with XO inhibitory activity serve as a clinically useful nutraceutical as well as a preventive functional food ingredient for such diseases. XO inhibitory activity of polyphenols, tannin, and flavonoids has previously been reported (*47-49*).

Table 5.2 presents the XO inhibition activity *in vitro* of millet phenolic extracts at a concentration of 1 mg/mL. Ferulic acid, which was employed as the standard compound inhibited XO activity by 75%. Among millet varieties tested in this study, kodo millet exhibited the highest activity (69 %) whereas other millet varieties had an activity ranging from 11 to 23%. The high activity of kodo millet extract against XO inhibition could partly be due to its high *trans*-ferulic acid content. Chandrasekara and Shahidi (*26*) showed that 53% of hydroxycinnamic acids and their derivatives in the soluble phenolic extracts of kodo millet were composed of *trans*-

ferulic acid. Two finger millet varieties showed high TPC and TFC compared to other varieties examined in this study, yet they demonstrated low XO inhibition activity. Characterization of phenolic composition of these two varieties showed that monomeric flavonol (catechin) contents were 82 and 77% for finger (Ravi) and finger (local) millets, respectively, of their total flavonoids in the soluble extracts (*25*). According to Nagao et al. (*50*) flavonols such as catechin and epicatechin do not inhibit XO effectively and this may partly explain the poor inhibitory activity of finger millet extracts against XO as noted in the study reported here. Furthermore, it has been shown that flavanones, flavanols and dihydroflavonols, which are potent ROS scavengers, are not effective XO inhibitors as they are devoid of a double bond between carbon-2 and carbon-3 in the C-ring of flavonoids (*51, 52*).

5.4.7 Supercoiled plasmid DNA strand scission inhibition

Permanent modification of DNA as a result of oxidative damage is the first step in several pathological conditions, including cancer and aging. The free radical mediated damage to DNA may vary and includes base modification, production of base-free sites, strand breaks, DNA-protein cross links and abnormal chromosomal arrangements, among others (*53*). Hydroxyl radical can abstract a hydrogen atom from the deoxyribose sugar moiety as well as pyrimidine and purine bases of DNA, thus producing single strands (*54, 55*). Double strand breaks, which occur near to each other on both strands, may be due to the multiple hydroxyl radical attacks and could lead to lethal damage of the cells (*56*). In this study, free radical induced DNA strand scission inhibition was tested for two concentrations of crude phenolic extracts, 0.25 and 0.5 mg/mL, and determined as the percentage of the retention of supercoiled

DNA (**Table 5.3**). The results showed that both peroxyl and hydroxyl radicals induced DNA strand scission.

Table 5.3 Retention percentage of supercoiled pBR 322 plasmid DNA in peroxyl radical-mediated and hydroxyl radical-mediated *in vitro* systems with phenolic extracts of whole millets

	Peroxyl radical		Hydroxyl radical	
	0.5 mg/mL[a]	0.25 mg/mL[a]	0.5 mg/mL[a]	0.25 mg/mL[a]
Control	17±2		27±3	
Ferulic acid	98 ± 2 a	99 ± 1 a	90 ± 6 a	41 ± 6 b
Kodo	100 ± 1a	97 ± 1 a	30 ± 9 d	68 ± 1 a
Finger (Ravi)	100 ± 1a	97 ± 2 a	54 ± 3 c	64 ± 4 a
Finger (local)	98 ± 2 a	97 ± 1 a	54 ± 3 c	45 ± 6 b
Foxtail	52 ± 1 d	16 ± 1 c	60 ± 8 b,c	54 ± 5 b
Proso	53 ± 3 d	25 ± 1 b	68 ± 3 b	68 ± 6 a
Little	63 ± 3 b	17 ± 1 c	88 ± 4 a	42 ± 9 b
Pearl	58 ± 2 c	23 ± 1 b	56 ± 3 c	42 ± 4 b

[a] Concentrations of extracts

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

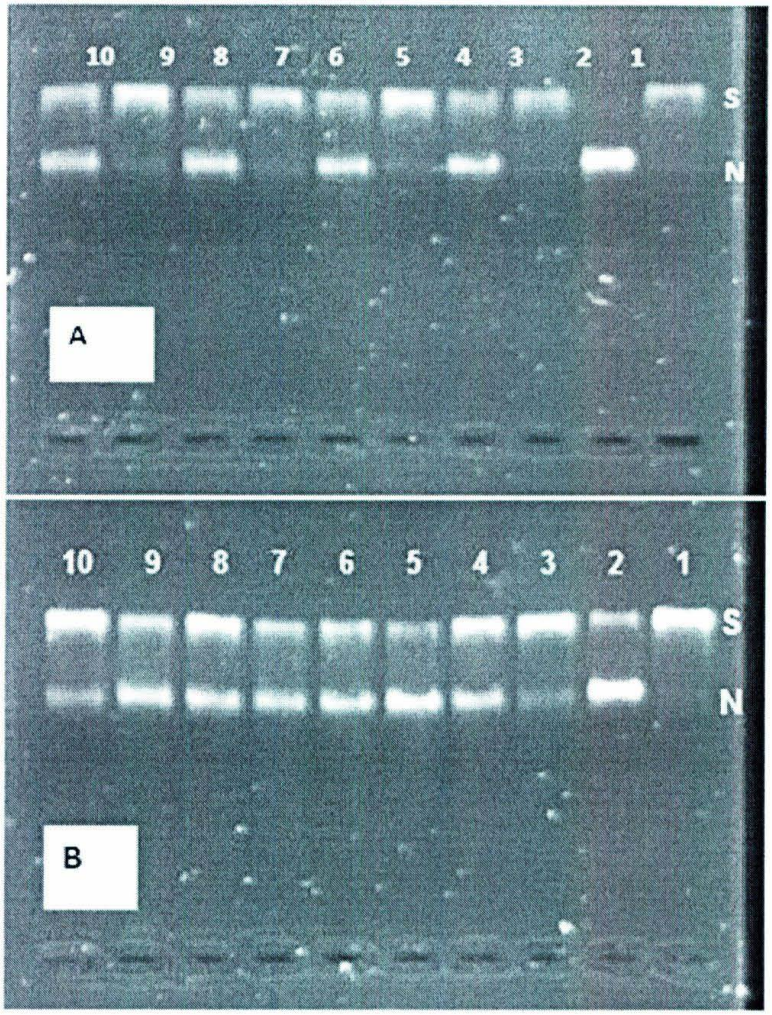


Plate 5.1 Agarose gel electrophoresis of DNA treated with peroxyl (A) and hydroxyl (B) radicals (R) in the presence of millet phenolic extracts at 37^0C. Lane 1= DNA, blank; Lane 2= DNA+R, control; Lane 3=DNA+R+trans ferulic acid; Lane 4=DNA+R+foxtail; Lane 5= DNA+R+kodo; Lane 6= DNA+R+pearl; Lane 7=DNA+R+finger (local); Lane 8= DNA+R+proso; Lane 9= DNA+R+finger (Ravi); Lane 10= DNA+R+little; S= Supercoiled DNA strands; N= Nicked DNA strands.

It has been shown that polyphenols may be chemopreventive through antioxidative as well as a number of other mechanisms (*57*). In general, phenolic extracts of millet grains demonstrated effective inhibitory activity against peroxyl radical induced DNA scission at both concentrations tested. Kodo, and two finger millet varieties achieved total DNA scission inhibition and in this respect were similar to that of ferulic acid at 0.5 mg/mL (**Table 5.3, Plate 5.1A**). It is interesting to note that kodo and two finger millet varieties had similar (97%) inhibition activity against peroxyl radical-mediated DNA scission whereas extracts of foxtail, proso, little and pearl millets showed 3.3, 2.1, 3.7, and 2.5 times less activities, respectively, after the extracts were diluted by a factor of two. The inhibition of DNA scission induced by peroxyl radicals has previously been reported by Madhujith and Shahidi (*6*) for phenolic extracts of barley grains. Inhibition of peroxyl radical induced supercoiled DNA scission of barley extracts ranged from 78 to 92% at a concentration of 4 mg/mL. In the present assay system the concentration of extracts used was 10 and 5 times higher than that of DNA for high and low concentrations of extracts, respectively. The observed scission inhibition could be due to the scavenging of peroxyl radicals by phenolic compounds present in the extract.

For hydroxyl radical induced DNA scission, however little millet extract showed the highest inhibitory activity which was similar to that of ferulic acid at a concentration of 0.5 mg/mL **(Table 5.3, Plate 5.1B**). The supercoiled DNA retention ranged from 30 to 90% against hydroxyl radical induced oxidation in the presence of millet grain extracts. It is noted that at a similar concentration of the extract (0.5 mg/mL), kodo and two finger millet varieties inhibited DNA scission induced by peroxyl radicals 2 to 3 times higher than that of hydroxyl radicals. In contrast, foxtail,

proso and little millets extracts inhibited DNA scission induced by hydroxyl radicals more favourably than that of peroxyl radicals. The same pattern was observed at a lower concentration (0.25 mg/mL) of the extracts in this study. Madhujith and Shahidi (*6*) also showed that phenolic extracts of different barley varieties were more effective against DNA scission inhibition induced by peroxyl radicals compared to hydroxyl radicals. These results further suggest that phenolic compounds present in the crude extracts of cereal grains could be more effective against the scavenging of peroxyl radicals compared to hydroxyl radicals.

In addition to the reaction with purine, and pyrimidine bases and deoxyribose backbone of the DNA molecule, hydroxyl radical may be added to DNA bases, thus producing a number of oxidative products. The inhibition of supercoiled DNA scission induced by hydroxyl radicals is possible via two mechanisms of chelating ferrous ions needed to initiate and catalyze the decomposition of H_2O_2 or scavenging of H_2O_2 itself, thus preventing the generation of hydroxyl radicals. The second mechanism is the capacity of phenolic extracts for scavenging of hydroxyl radicals produced in the system. The results of the present study showed that two finger millet varieties, proso and little millets were effective metal chelators (**Figure 5.1**). In a previous study we showed that millet phenolic extracts scavenged H_2O_2 as well as hydroxyl radicals effectively in *in vitro* systems (*11*). Thus, results obtained in this study could be due to a multifactorial effect of all possible inhibitory activities aforementioned, hence suggesting that millet phenolics are effective against DNA scission, mediated by hydroxyl radicals. This lends further support to the findings of Liyana-Pathirana et al. (*20*) who showed that wheat phenolic extracts were effective against hydroxyl radical-mediated inhibition of DNA scission. These results suggest

that quantity as well as concentration of different phenolic compounds present in the grains may be responsible for the observed effects. The TPC and TFC of these extracts showed that in general kodo and two finger millet varieties were rich in total phenolics and flavonoids than foxtail, proso, little and pearl millets. In a previous study Noroozi et al. (*58*) reported that flavonoids were effective against hydrogen peroxide initiated oxidative DNA damage to human lymphocytes. In the present study, kodo and two finger millet varieties had high inhibition ability against peroxyl radical mediated DNA oxidation and possibly due to their high content of flavonoids compared to other millet varieties employed. Notwithstanding, little millet, with less TPC and TFC, demonstrated significantly high inhibition activity against DNA scission which was similar to that of ferulic acid standard used in this study.

5.4.8 Human colon adenocarcinoma cell proliferation inhibition

In general, blocking agents hamper initiation of cancer by preventing interaction of cellular target molecules such as DNA. Meanwhile suppressing agents inhibit expression of initiated cells either in promotion or progression stages of cancer. Millet phenolic extracts employed in this study showed suppressing action demonstrating time and dose dependent antiproliferation of HT-29 human colon adenocarcinoma cells. **Figure 5.3A** shows the antiproliferative activities of millet extracts at a concentration of 0.5 mg/mL, from day 0 to day 4. At the beginning, none of the varieties showed any antiproliferative activity. After 24 h, kodo and proso millet extracts exhibited 58-64% antiproliferative activity whereas the activity of the rest of millet varieties tested ranged from 10 to 39%. At each time point examined kodo and proso millet extracts demonstrated superior antiproliferative activity compared to that of two finger millet varieties, foxtail, little and pearl millet. At the

end of day 4, all varieties except pearl millet, demonstrated their highest antiproliferative activity. The order of antiproliferative activity was in the decreasing order of kodo ≥ proso > finger (Ravi) ≥ little > finger (local)> foxtail > pearl millets. It is interesting to note that crude extract of proso millet had 5 and 1.2 times lesser TPC and TFC, respectively, than that of kodo millet, although both varieties displayed equal antiproliferative activity against HT-29 cells. Kampa et al. (*59*) reported that an antiproliferative activity of phenolic acids against 747 D human breast cancer cells with the following potency: caffeic > *trans*-ferulic = protocatechuic = 3,4-dihydroxyphenylacetic acid > sinapic = syringic. Furthermore, their results suggested that two hydroxyl groups on the phenolic ring and the three carbon side chain were essential for the observed antiproliferative activities. In addition, shortening of the side chain resulted in the loss of antiproliferative activity and this was more apparent with the methylation of one or both hydroxyl groups and *p*-hydroxy substitution as in syringic and sinapic acids. Caffeic and gallic acids are known for their bioactivity related to modulation of carcinogenesis (*60, 61*). Characterization of phenolic composition of soluble extracts of millet showed that the quantity of caffeic acid of kodo and proso soluble extracts was 7 and 14% of the total hydroxycinnamic acids present, respectively (*26*). In addition, it was noted that proso millet extracts were rich in gallic acid which accounted for 14% of the content of their hydroxybenzoic acids in the free phenolic fraction (*26*). Relatively high caffeic and gallic acids contents present predominantly as free phenolic acids, which are readily available for action, may contribute to the high antiproliferative activity of proso millet extracts observed in this study.

Figure 5.3B shows the inhibition of cell proliferation by extracts at the two concentrations employed. At a concentration of 0.5 mg/mL, extracts of both kodo and proso millets completely inhibited cell proliferation. Except for foxtail and pearl millets, all other varieties had 50% inhibition of cell proliferation at a concentration less than 0.5 mg/mL. However, antiproliferative activity ranged from 3 to 51% at the lower concentration of 0.1 mg/mL, suggesting the dose dependency of extracts on cell proliferation inhibition. Furthermore, at the lower concentration little and pearl millets showed a significantly ($p \leq 0.05$) higher inhibitory activity compared to other varieties tested. In agreement with the present results, Madhujith and Shahidi (*6*) also reported a dose dependent activity for barley phenolics. They demonstrated that barley extracts at 0.05 mg/mL concentration, had a less antiproliferative activity compared to a higher concentration of 0.5 mg/mL on Caco-2 colon cancer cells. However, antiproliferative activity demonstrated by different barley cultivars only ranged from 29 to 51 % at 0.5 mg/ml concentration at the end of day 4 and was at a lower range compared with the results obtained in this study at a similar concentration for millet extracts, thus suggesting their superior activity over barley as chemopreventive agents (**Figure 5.3B**). Parry et al. (*62*) also reported that grape, black raspberry and cranberry seed flours showed effective proliferative inhibition of HT-29 cells in a dose and time dependent manner. Similarly, pomegranate juice showed 30-100% inhibition of proliferation of a number of colon cancer cell lines including HT-29 (*63*).

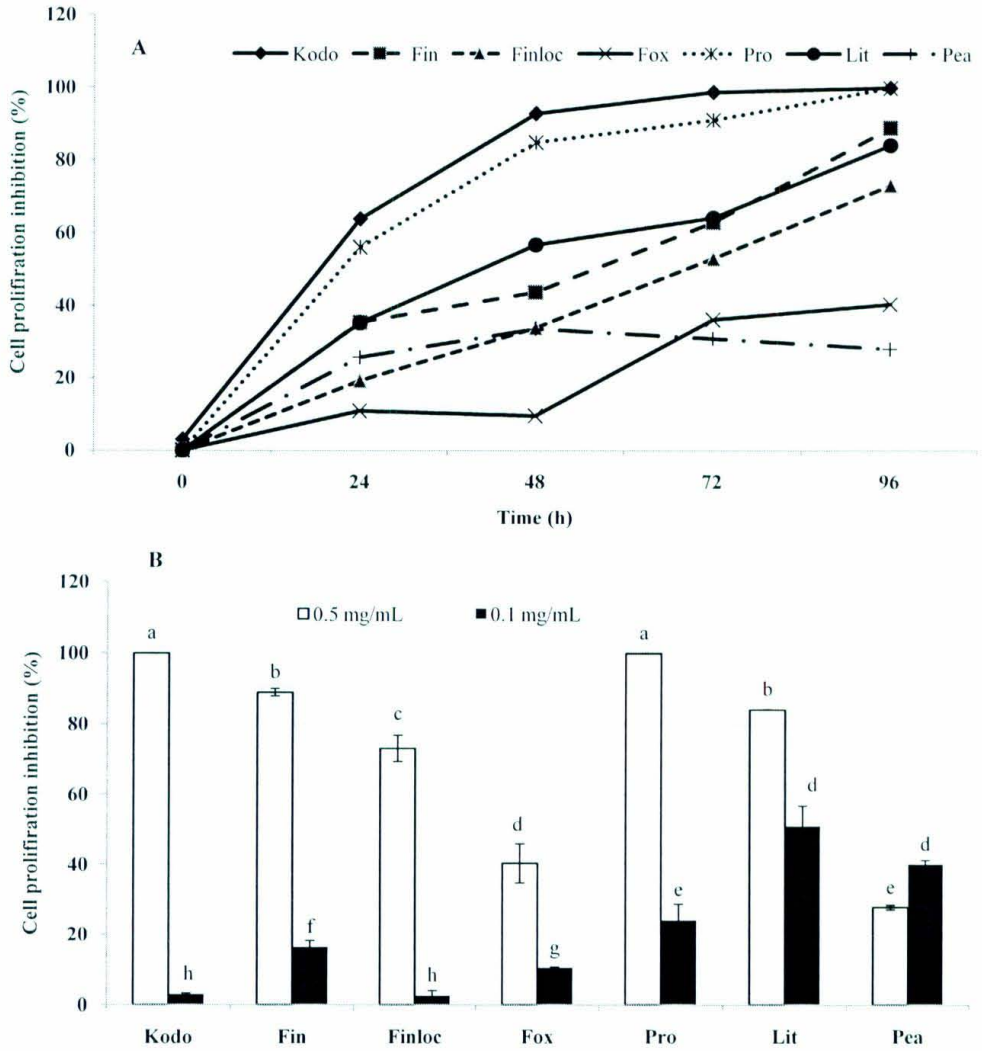


Figure 5.3 Percent inhibition of colon adenocarcinoma cell (HT-29) proliferation by whole millet extracts at a concentration of 0.5 mg/mL, from day 0 to day 4 of incubation (A) and at concentrations 0.5 and 0.1 mg/mL at the end of day 4 of incubation (B). Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters have significantly different ($p < 0.05$) mean values. The letter 'a' represents the highest value.

Flavones such as apigenin, and luteolin have shown strong antiproliferative activities against a number of human cancer cell lines, including colon carcinoma, namely SW 480, HT-29 and Caco-2 (*64, 65*). Flavonoids such as kaempherol, apigenin, luteolin, and quercetin were reported in millet extracts used in this study and may contribute to the observed antiproliferative activities *in vitro* (*26*). The results of this study clearly show that millet phenolics are effective against *in vitro* cancer cell proliferation. However, their bioavailability in the body to exert these effects has to be confirmed.

5.5 Conclusion

The results of the present study together with those we previously reported suggest that millet phenolics are effective antioxidants and possess antiproliferative and DNA scission inhibitory activities which would help regulating carcinogenesis at the initiation as well as progression stages. Phenolic extracts from millets showed high liposome peroxidation inhibition activity. However, they exhibited low singlet oxygen and XO inhibitory activities, suggesting the differential effects of constituent phenolics of the extract in diverse assay systems. The study of mechanisms involved in the DNA scission inhibition and antiproliferation of millet extracts is beyond the objectives of this work but the present findings may shed light on future *in vivo* studies on prevention and management of cancer. Thus, use of untapped millets as nutraceutical and functional food ingredients or inclusion in therapeutic diets for patients with diseases linked to oxidative stress may be recommended; further *in vivo* and clinical studies are warranted to expand the knowledge on bioavailability, bioaccessibility and bioactivity of millet grain phenolics.

5.6 References

1. Darley-Usmar, V.; Halliwell, B. Blood Radicals: Reactive nitrogen species, reactive oxygen species, transition metal ions and the vascular system. *Pharmaceutical Res.* **1996**, 13, 649-662.

2. Boyd, N.F.; Macguri, V. The possible role of lipid peroxidation in breast cancer risk. *Free Radical Biol.Med.* **1991**, 10, 185-190.

3. Marnett, L.J. Lipid peroxidation-DNA damage by malondialdehyde. *Mutat. Res. Fund. Mol. Mech. Mut.* **1999**, 424, 83-95.

4. Valko M.; Leibfritz D.; Moncola J.; Cronin M.D. Free radicals and antioxidants in normal physiological functions and human disease. Review. *Int. J. Biochem. Cell Biol.* **2007**, 39, 44-84.

5. Liyana-Pathirana, C.; Dexter, J.; Shahidi, F. Antioxidant properties as affected by pearling. *J. Agric. Food Chem.* **2006**, 54, 6177-6184.

6. Madhujith, T.; Shahidi, F. Antioxidative and antiproliferative properties of selected barley (*Hordeum vulgarae* L.) cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. *J. Agric. Food Chem.* **2007**, 55, 5018-5024.

7. Popkin, B.M. The nutrition transition and obesity in the developing world. *J. Nutr.* **2001**, 131, 8715-8735.

8. Jemal, A.; Murray, T.; Ward, E.; Samuels, A.; Tiwari, R.C.; Ghafoor, A.; Feuer, E.J.; Thun, M.J. Cancer Statistics. *CA: Cancer J. Clinicians.* **2005**, 55, 10-30.

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

10. Chandrasekara, A.; Shahidi, F. The content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *J. Agric. Food Chem.* **2010,** 58, 6706-6714.

11. Chandrasekara, A.; Shahidi, F. Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. *J. Agric. Food Chem.* **2011,** 59, 428-436.

12. Chethan, S.; Dharmesh, S.M.; Malleshi, N.G. Inhibition of aldose reductase from cataracted eye lenses by finger millet (*Eleusine coracana*) polyphenols. *Bioorganic Med. Chem.* **2008,** 16, 10085-10090.

13. Viswanath, V.; Urooj, A.; Malleshi, N.G. Evaluation of antioxidant and antimicrobial properties of finger millet (*Eleusine coracana*). *Food Chem.* **2009,** 114, 340-346.

14. Shobana, S.; Harsha, M.R.; Platel, K.; Srinivasan, K.; Malleshi, N.G. Amelioration of hyperglycaemia and its associated complications by finger millet (*Eleusine coracana* L.) seed coat matter in streptozotocin-induced diabetic rats. *Brit. J. Nutr.* **2010,** 104, 1787-1795.

15. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965,** 16, 144-158.

16. Kim, D.; Jeong, S.W.; Lee, C.Y. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem.* **2003**, 81, 321-326.

17. Dinis T.C.P.; Madeira, V.M.C.; Almeida, L.M. Action of phenolic derivates (acetaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophy.* **1994,** 315, 161-169.

18. Maldonado, P.D.; Rivero-Cruz, I.; Mata, R.; Pedraza-Chaverr, J. Antioxidant activity of A-type proanthocyanidins from *Geranium niveum* (Geraniaceae). *J. Agric. Food Chem.* **2005,** 53, 1996-2001.

19. Hiramoto, K.; Ojima, N.; Sako, K.; Kikugawa, K. Effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. *Biol. Pharm. Bull.* **1996,** 19, 558-563.

20. Liyana-Pathirana, C.; Shahidi, F. Importance of insoluble-bound phenolics to antioxidant properties of wheat. *J. Agric. Food Chem.* **2006,** 54, 1256-1264.

21. Wang, C.Y.; Wang, S.Y.; Yin, J-J.; Parry, J.; Yu, L.L. Enhancing antioxidant, antiproliferation, and free radical scavenging activities in strawberries with essential oils. *J. Agric. Food Chem.* **2007,** 55, 6527-6532.

22. Qiao, L.; Koutsos, M.; Tsai, L-L.; Kozoni, V.; Guzman, J.; Shiff, S. J.; Rigas, B. Staurosporine inhibits the proliferation, alters the cell cycle distribution and induces

apoptosis in HT-29 human colon adenocarcinoma cells. *Cancer Lett.* **1996,** 107, 83-89.

23. Yoshida, S.; Honda, A.; Matsuzaki, Y.; Fukushima, S.; Tanaka, N.; Takagiwa, A.; Fujimoto, Y.; Miyazaki, H.; Salen, G. Antiproliferative action of endogenous dehydroepiandrosterone metabolites on human cancer cell lines. *Steroids.* **2003**, 68, 73-83.

24. Huang, S-W.; Frankel, E.N. Antioxidant activity of tea catechins in different lipid systems. *J. Agric. Food Chem.* **1997**, 45, 3033-3038.

25. Noro, T.; Oda, Y.; Miyase, T.; Ueno, A.; Fukushima, S. Inhibitors of xanthine oxidase from the flowers and buds from *Daphne genkwa*. *Chem. Pharm. Bull.* **1983**, 31, 3984-3987.

26. Chandrasekara A.; Shahidi F. Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n. *J. Functional Foods.* **2011**, 3, 144-158.

27. Shahidi, F.; Naczk, M. Phenolics in Food and Nutraceuticals. CRC press, Boca Raton, FL, **2004**, pp 1-82.

28. Halliwell, B. Biochemistry, and role in human disease resistance. *Am. J. Med.* **1991**, 91, 14-19.

29. Kanofsky, J.R.; Hoogland, H.; Wever, R.; Weiss, S.J. Singlet oxygen production by human eosinophils. *J. Biol. Chem.* **1988,** 263, 9692-9696.

30. Bose, B.; Agarwal, S.; Chattergee, S.N. UV-A induced lipid peroxidation in liposomal membranes. *Radiat. Environ. Biophys.* **1989**, 28, 59-65.

31. Wang, S.Y.; Jiao, H. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. *J. Agric. Food Chem.* **2000**, 48, 5677-5684.

32. Krutmann, J. Ultraviolet A radiation-induced biological effects in human skin: relevance for photoaging and photodermatosis. *J. Dermatol. Sci.* **2000**, 23 (Suppl), S22-S26.

33. Einspahr, J.G.; Stratton, S.P.; Bowden, G.T.; Alberts, D.S. Chemoprevention of human skin cancer. *Crit. Rev.Oncol. Hematol.* **2002**, 41, 269-285.

34. Freeman, B.A.; Crapo, J.D. Biology of disease: free radicals and tissue injury. *Lab. Invest.* **1982**, 47, 412-426.

35. Black, H.S. Potential involvement of free radical reactions in ultraviolet light mediated cutaneous damage. *Photochem. Photobiol.* **1987**, 46, 213-221.

36. Heinonen, M.; Rein D.; Satu-gracia, T.M.; Huang, S-W.; German, J. B.; Frankel, E.N. Effect of protein on the antioxidant activity of phenolic compounds in a lecithin-liposome oxidation system. *J. Agric. Food Chem.* **1998,** 46, 917-922.

37. Verstraeten, S.V.; Keen, C.L.; Schmitz, H.H.; Fraga, C.G.; Oteiza, P.I. Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and disruption of the bilayer structure. *Free Radic. biol. Med.* **2003**, 34, 84-92.

38. Erlejman, A.G.; Verstraeten, S.V.; Fraga, C.G.; Oteiza, P.I.T. The interaction of flavonoids with membranes: potential determinant of flavonoid antioxidant effects. *Free Radic. Res.* **2004**, 38, 1311-1320.

39. Bachelor, M. A.; Bowden, G. T. UVA-mediated activation of signaling pathways involved in skin tumor promotion and progression. *Sem. Cancer Biol.* **2004**, 14, 131-138.

40. Casagrande, R.; Georgetti, S. R.; Verri, W. A. Jr.; Dorta, D. J.; dos Santos, A. C.; Fonseca, M.J. Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. *J. Photochem. Photobiol. B,* **2006**, 84, 21-27.

41. Skaper, S. D.; Fabris, M.; Ferrari, V.; Dalle, C. M.; Leon, A. Quercetin protects cutaneous tissue-associated cell types including sensory neurons from oxidative stress induced by glutathione depletion: cooperative effects of ascorbic acid. *Free Radic. Biol. Med.* **1997**, 22, 669-678.

42. Fahlman, B.M.; Krol, E.S. Inhibition of UVA and UVB radiation-induced lipid oxidation by quercetin. *J. Agric. Food Chem.* **2009**, 57, 5301-5305.

43. Borges, F.; Fernandes, E.; Roleira, F. Progress towards the discovery of xanthine oxidase inhibitors. *Cur. Med. Chem.* **2002**, 9, 195-217.

44. Zulueta, J.J.; Sawhney, R.; Yu, T.S.; Cote, C.C.; Hassoun, P.M. Intracellular generation of reactive oxygen species in endothelial cells exposed to anoxia-reoxygenation. *Am. J. Physiol.* **1997**, 272, L897-L902.

45. Zhang, Z.; Blake, D.R.; Stevens, C.R.; Kanczler, J.M.; Winyard, P.G.; Symons, M.C.; Benboubetra, M.; Harrison, R. A reappraisal of xanthine dehydrogenase and

oxidase in hypoxic reperfusion injury: the role of NADH as an electron donor. *Free Radic. Res.* **1998**, 28, 151-164.

46. Lin, J.K.; Chen, P.C.; Ho, C.T.; Lin-Shiau, S-Y. Inhibition of xanthine oxidase and suppression of intracellular reactive oxygen species in HL-60 cells by theaflavin-3,3'-digallate, (-)-epigallocatechin-3-gallate, and propyl gallate. *J. Agric. Food Chem.* **2000,** 48, 2736-2743.

47. Hatano, T.; Yasuhara, T.; Yoshihara, R.; Agata, I.; Noro, T.; Okuda, T.; Effects of interaction of tannins with co-existing substances. Inhibitory effects of tannins and related polyphenols on xanthine oxidase. *Chem. Pharm. Bull.* **1990**, 38,1224-1229

48. Costantino, L.; Albasini, A.; Rosetelli, G.; Bevenutt, S. Activity of polyphenolic crude extracts as scavengers of superoxide radicals and inhibitors of xanthine oxidase. **1992**, *Planta Med.* 58, 342-344.

49. Cakir, A.; Mavi, A.; Yildirim, A.; Duru, M.E.; Harmandar, M.; Kazaz, C. Isolation and characterization of antioxidant phenolic compounds from the aerial parts of *Hypericum hyssopifolium* L. by activity-guided fractionation. *J. Ethnopharmacol.* **2003**, 87, 73-83.

50. Nagao, A.; Seki, M.; Kobayashi, H. Inhibition of xanthine oxidase by flavonoids. *Biosc. Biotechnol. Biochem.* **1999**, 63, 1787-1790.

51. Cos, P.; Ying, L.; Calomme, M.; Hu, J.P.; Cimanga, K.; Poel, B.V.; Pieters, L.; Vlietinck, A.J.; Berghe, D.V. Structure-Activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J. Nat. Prod.* **1998,** 61, 71-76.

52. Lopez-Lazaro, M. Flavonoids as anticancer agents: structure-activity relationship study. *Curr. Med. Chem.* **2002**, 2, 691-714.

53. Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C.J.; Telser, J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol.Cell. Biochem.* **2004**, 266, 37-56.

54. Breen, A.P; Murphy, J.A. Reactions of oxyl radicals with DNA. *Free Radic. Biol. Med.* **1995**, 18, 1033-1077.

55. Stolc, S.; Valko, L.; Valko, M.; Lombardi, V. A technique for the fast sampling of biological tissues for electron paramagnetic resonance spectroscopy. *Free Radic. Biol. Med.* **1996**, 20, 89-91.

56. Ward, J.F. Biochemistry of DNA lesions. *Radiat. Res.* **1985**, 104, S103-S111.

57. Fresco, P.; Borges, F.; Diniz, C.; Marques, M.P.M. New insights on the anticancer properties of dietary polyphenols. *Med. Res. Rev.* **2006**, 26, 747-766.

58. Noroozi, M.; Angerson, W.J.; Lean, M.E.J. Effects of flavonoids and vitamin C on oxidative damage to human lymphocytes. *Am.J.Clin. Nutr.* **1998**, 67, 1210-1218.

59. Kampa, M.; Alexaki, V-I.; Notas, G.; Nifli, A-P.; Nistikaki, A.; Hatzoglou, A.; Bakogeorgou, E.; Kouimtzoglou,E.; Blekas, G.; Boskou, D.; Gravanis,A.; Castanas, E. Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Res.* **2004**, 6, R63-R74.

60. Gomes, C.A.; da Cruz, T.G; Andrade, J.L.; Milhazes, N.; Borges, F.; Marques, M.P.M. Anticancer activity of phenolic acids of natural or synthetic origin: A structure-activity study. *J. Med. Chem.* **2003**, 46, 5395-5401.

61. Fiuza, S.M.; Gomes, C.; Teixeira, L.J.; Girao da Cruz, M.T.; Cordeiro, M.N.; Milhazes, N.; Borges, F.; Marques, M.P.M. Phenolic acid derivatives with potential anticancer properties-a structure-activity relationship study. Part 1: Methyl, propyl and octyl esters of caffeic and gallic acids. *Bioorg. Med. Chem.* **2004**, 12, 3581-3589.

62. Parry, J.; Su, L.; Moore, J.; Cheng, Z.; Luther, M.; Rao, J. N.; Wang, J-Y.; Yu, L. L. Chemical compositions, antioxidant capacities, and antiproliferative activities of selected fruit seed flours. *J. Agric. Food Chem.* **2006**, 54, 3773-3778.

63. Seeram, N.P.; Adams, L.S.; Henning, S.M.; Niu, Y.; Zhang, Y.; Nair, M.G.; Heber, D. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J. Nutr. Biochem.* **2005**, 16, 360-367.

64. Wang, W.; Heideman, L.; Chung, C. S.; Pelling, J. C.; Koehler, K. J.; Birt, D. F. Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Mol. Carcinog.* **2000**, 28, 102-110.

65. Manthey, J.A.; Guthrie, N. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. *J. Agric. Food Chem.* **2002**, 50, 5837-5843.

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Antioxidant phenolics of millet control lipid peroxidation in human LDL cholesterol and food systems

6.1 Abstract

Phenolic extracts from seven millet varieties, namely kodo, finger (Ravi), finger (local), proso, foxtail, little and pearl were evaluated for their inhibitory effects on lipid peroxidation in *in vitro* copper-mediated human LDL cholesterol oxidation and several food model systems, namely cooked comminuted pork, stripped corn oil, and linoleic acid emulsion. Total phenolic content (TPC) and free radical scavenging activities were measured. The TPC ranged from 146 to 1156 μmol ferulic acid eq/g crude extract and the corresponding values based on defatted weight of grain ranged from 8.6 to 32.4 μmol FAE/ g. At a final concentration of 0.05 mg/mL, millet extracts inhibited LDL cholesterol oxidation by 1- 41 %. All seven varieties exhibited effective inhibition of lipid oxidation in food systems used in this study and kodo millet exhibited superior inhibition of lipid peroxidation, similar to butylated hydroxyanisole (BHA) at 200 ppm. Thus, millets may serve as a natural source of antioxidants in food application and as a nutraceutical and functional food ingredient in health promotion and disease risk reduction.

6.2 Introduction

Lipid oxidation is a major cause of food quality deterioration during processing and storage, leading to rapid development of rancid and stale odors and unfavorable tastes. Furthermore, lipid oxidation leads to loss of nutrients by destruction of fat soluble vitamins and essential fatty acids, apart from the health hazards arising from both the primary and secondary lipid oxidation products. Thus, antioxidants are used in foods to prevent lipid oxidation. Synthetic antioxidants currently used by the food industry include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ). However, their use has been limited during the past few years due to concerns associated with the potential carcinogenic effect which displayed at high concentrations in some animal models (*1*). Therefore, there has been a desire to focus on finding natural sources of antioxidants to substitute for synthetic antioxidants. A general feature of many antioxidants is their phenolic structure.

Phenolic compounds are natural antioxidants ubiquitously found in all plant organs. A number of studies have reported the antioxidant activity of phenolics extracted from different plant parts such as leaves, flowers, stems, roots and fruits. Several cereal grains have also been studied for their antioxidant activity in food models as well as *in vitro* low density lipoprotein (LDL) cholesterol (*2-4*).

In the body, oxidative modification of LDL, which is the major cholesterol carrier in the blood, is known to play a key role in atherogenic plaque formation leading to coronary heart disease (*5*). The changes of LDL are initiated by peroxidation of polyunsaturated fatty acids in the LDL cholesterol. The degradation products of LDL include peroxyl radicals, conjugated dienes (CD), aldehydes and

ketones, among others. Therefore, inhibition of LDL peroxidation by supplementation of antioxidants is a therapeutic strategy for prevention of atherosclerosis and coronary heart disease.

Depending on the test system used, the antioxidant activity of compounds depend on factors such as solubility and their activity in the lipid system, and the stability and possible synergistic and antagonistic interactions with other molecules present in the system. In addition, it has been shown that polar antioxidants are more effective in non-polar medium, whereas non-plolar antioxidants are more active in polar lipid emulsions that describe the polar paradox theory (*6*). Therefore, investigation of natural antioxidants that may have different polarities and multiple constituents in food systems is needed.

Cereals have played a major role as staple food from the beginning of human civilization. Millets are considered as the first cultivated cereals in the world. Furthermore, millets are unexploited as a food in the developed countries. In 2008, millet grain production in North America was 0.07% of total cereal production and a major part of it was used for feed and as bird seed (*7*). Hence, a very small proportion of millet grains is utilized for human consumption, as specialty foods in niche markets. Since millets are gluten-free, they are increasingly used to produce gluten-free cereals for individuals suffering from celiac disease. In addition, millets are becoming popular as organic cereals due to their ability to thrive on soils with the least nutrient requirements and under harsh environmental conditions. There are different millet species in the world and a majority (80%) of millet global production is utilized as food in the developing countries.

In previous studies we have shown that millet phenolics act as natural antioxidants by different mechanisms, including free radical scavenging, acting as reducing agents and as chelators of transition metal ions (*8, 9*). Furthermore, phenolic compounds belonging to phenolic acids and flavonoids were identified in millet grains tested and may contribute to these observed effects (*10*). However, the existing literature lacks information on the effect of millet phenolics against LDL cholesterol oxidation and lipid peroxidation in food systems. The antioxidant activity of millet phenolics demonstrated in our previous studies suggests that they could serve as potential natural antioxidants in foods and biological systems. Hence, the objectives of the present study were to: (a) determine the inhibition of copper-mediated LDL cholesterol peroxidation; and (b) determine the antioxidant activity of millet phenolics in food models, namely cooked comminuted pork, bulk oil, and linoleic acid emulsion.

6.3 Material and methods

Seven millet grain samples, namely foxtail (*Setaria italica)*, proso (*Panicum miliacium)*, two finger (*Elusine coracana*) varieties (Ravi and local), kodo (*Paspalum scrobiculatum*), little (*Panicum sumatrense*) and pearl (*Pennisetum glaucum*) millets harvested in the crop year 2007 were used in this study. Pearl millet (dark green cultivar), grown in India, was kindly provided by Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India. All other grain samples were obtained from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka.

Folin Ciocalteu's reagent, ferulic acid, trichloroacetic acid (TCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide, 5,5-dimethyl-1-1-pyrroline-*N*-

oxide (DMPO), ferrous chloride, ferrous sulphate, copper sulphate, ammonium thiocyanate, thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), mono- and dibasic potassium phosphates, Tween 40 (polyoxyethylene sorbitan monopalmitate), β-carotene, linoleic acid, butylated hydroxyanisole (BHA), human low density lipoprotein (LDL) cholesterol were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Hexane, acetone, methanol, ethanol, and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada). Stripped corn oil (SCO) was procured from Acros Organics (Morris Plains, NJ, USA)

6.3.1 Sample preparation

Cleaned whole millet grains were ground in a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc. Brockville, ON, Canada) to obtain a fine powder which passed through mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH, USA). All samples were defatted by blending with hexane (1:5, w/v, 5 min, 3 times) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA) at ambient temperature. Defatted samples were vacuum packed in polyethylene pouches and stored at -20°C until used within one week for the extraction of phenolics.

6.3.2 Extraction of soluble phenolic compounds

Ultrasonic-assisted extraction procedure was used for soluble phenolic compounds. Defatted meal (5 g) was mixed with 100 mL of 70% (v/v) acetone and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) and sonicated at the maximum power for 25 min under refluxing

conditions. After centrifugation of the resulting slurry for 5 min at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA), the supernatant was collected and extraction was repeated two more times. Combined supernatants were evaporated *in vacuo* at 40°C (Buchi, Flawil, Switzerland) and lyophilized for 72 h at -46°C and 34 x 10^{-3} mbar (Freezone, Model 77530, Labconco Co., Kansas City, MO, USA). Residues of whole grain samples were air dried for 12 h and stored at -20°C until used to extract bound phenolic compounds within a week. During all stages, extracts were protected from light by covering them with aluminium foil. Lyophilized crude phenolic extracts were stored at -20°C until used for further analysis.

6.3.3 Determination of total phenolic content (TPC)

The total phenolic content of each extract was determined using the method described by Singleton and Rossi (*11*) with slight modifications, as explained elsewhere (*8*). The content of total phenolics in each extract was determined using a standard curve prepared for ferulic acid and expressed as micromoles ferulic acid equivalents (FAE) per gram of crude extract.

6.3.4 DPPH radical scavenging capacity assay using electron paramagnetic resonance (EPR) spectroscopy

The effect of extracts on the scavenging of DPPH radicals was determined according to the method described by Madhujith and Shahidi (*12*) with slight modifications as previously explained (*9*). Phenolic extracts in methanol were used at different concentrations (0.062-2.5 mg/mL). Methanol was used as the control in place of

extract. The concentration of extracts required to inhibit 50% (IC_{50}) of DPPH radicals was then calculated.

6.3.5 Hydroxyl radical scavenging assay using EPR spectroscopy

The hydroxyl radical scavenging capacity was determined according to the method explained by Chandrasekara and Shahidi (*9*). Extracts of soluble phenolic compounds of millets were dissolved in deionized water to obtain concentrations from 0.0625 to 5 mg/mL. Extracts (100 µL) were mixed with 100 µL of H_2O_2, (10 mM), and 200 µL of DMPO (17.6 mM) and 100 µL of $FeSO_4$ (0.1 mM). After 1 min the mixtures were introduced into the sample cavity of EPR spectrometer and the spectrum was recorded. Deionized water was used as the control in place of the extract. The (IC_{50}) values of millet extracts for hydroxyl radicals were then calculated.

6.3.6 Inhibition of copper-mediated human LDL peroxidation

Inhibitory activities of millet whole grain extracts against human low density lipoprotein (LDL) cholesterol oxidation was determined by measuring conjugated dienes (CD) produced in the system using the method described by Andreasen et al. (*13*). Human LDL cholesterol (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 LDL cholesterol was subsequently diluted to obtain a standard protein concentration of 0.1 mg/mL in PBS. The diluted LDL cholesterol solution (0.8 mL) was mixed with 100 µL of soluble extract (0.5 mg/mL in PBS) in an Eppendorf tube. Oxidation of LDL cholesterol was initiated by adding 0.1 mL of 100 µM CuSO4 solution in distilled water. The mixture was incubated at 37°C for 28 h. The initial absorbance (t=0) was read at 234 nm immediately after mixing and CD

hydroperoxides formed were measured at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 15, 20, 24, and 28 h intervals. The concentration of CD formed was calculated using molar extinct coefficient of 29500 M^{-1} cm^{-1}. The corrected absorbance at 4 h against 0 h was employed to calculate the percentage inhibition of CD formation using the following equation. Percentage inhibition of CD formation = (Abs $_{oxidative}$ – Abs $_{sample}$)/Abs $_{oxidative}$ – Abs $_{native}$)*100, where, Abs $_{oxidative}$ = absorbance of LDL mixture and distilled water with CuSO4 only; Abs $_{sample}$ = absorbance of LDL with extract and CuSO4; Abs $_{native}$ = absorbance of LDL with distilled water.

6.3.7 Oxidation inhibition in bulk oil

The efficacy of millet extracts on delaying the oxidation of stripped corn oil was measured under accelerated oxidative conditions using a Rancimat apparatus (Model 743, Rancimat Metrohm, Herisau, Switzerland). The extracts (0.2, 0.5 and 1 mg of extracts /g of oil) were added into the reaction vessels of the Rancimat apparatus containing 3 g of stripped corn oil (SCO) followed by vortexing for 3 min. A constant stream (20 L/h) of dry air, obtained by passing laboratory air through molecular sieve (0.3 nm), was blown through the samples in the reaction vessel which was maintained at 100ºC 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 continuously and recorded. The inflection point (IP) was obtained (743 Rancimat® PC software version 1.0, 2000, Metrohm Ion Analysis Ltd., Herisau, Switzerland) and recorded. A blank containing only SCO was used. Ferulic acid and BHA, at a concentration of 0.2 mg/g of oil, were used as positive controls. Results were reported as protection factor (PF), which were calculated as

follows. PF = $IP_{additive}$ / $IP_{control}$ where, $IP_{additive}$ = inflection point of SCO mixture containing the additive and $IP_{control}$ = inflection point of pure SCO.

6.3.8 Inhibition of oxidation in comminuted pork model system

The effect of millet phenolic extracts in the inhibition of production of thiobarbituric acid reactive substances (TBARS) in cooked pork was determined according to Wijeratne et al. (*14*). Ground pork was mixed with 20% (w/w) deionized water in Mason jars. Phenolic extracts and BHA were added separately to meat (100 g) and thoroughly mixed with a glass rod. A control sample containing no extract was also prepared. Samples were cooked in a thermostated water bath at 80 ± 2°C for 40 min while stirring every 5 min. Meat samples were mixed thoroughly, after cooling to room temperature and transferred into plastic bags, followed by storing in a refrigerator at 4°C for 14 days. Samples for the analyses of TBARS were drawn on days 0, 3, 5, 7 and 14 and were analyzed for TBARS according to the method of Siu and Draper (*15*) as described by Shahidi and Hong (*16*). In this, 2 g of meat sample were weighed in a 50 mL centrifuge tube to which 5 mL of a 10% (w/v) solution of trichloroacetic acid (TCA) were added and vortexed (Fisher Vortex Genie 2; Fisher Scientific, Nepean, ON, Canada) at high speed for 2 min. Thiobarbituric acid (TBA) solution (0.02 M, 5 mL) was then added to each centrifuge tube, followed by vortexing for 30 s. The samples were subsequently centrifuged at 3000 x g for 10 min and the supernatants were filtered through a Whatman No. 3 filter paper. Filtrates were heated in a boiling water bath for 45 min, cooled to room temperature in an ice bath, and the absorbance of the resultant pink-coloured chromogen read at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane as a precursor of the malondialdehyde (MDA). The TBARS values were then calculated using the standard

curve and expressed as µmol MDA equivalents per kg of sample. The percentage inhibition of TBARS formation was calculated as follows. Inhibition percentage = $\{(TBARS_{control}-TBARS_{sample})/ TBARS_{control}\}*100$, where $TBARS_{control}$= TBARS formed in the control and $TBARS_{sample}$= TBARS formed in the sample.

6.3.9 Inhibition of oxidation in linoleic acid emulsion system

The peroxidation inhibition activity of millet phenolic extracts was determined in a linoleic acid emulsion system according to the method described by Osawa and Namiki (*17*). Linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid and 0.2804 g of Tween 40 with 50 mL of phosphate buffer solution (PBS, pH 7, 0.2 M). Phenolic extracts were dissolved in absolute ethanol (2 and 5 mg/mL). The sample (0.5 mL) was mixed with 2.5 mL of linoleic acid emulsion and 2 mL of PBS and incubated at 37°C, and the degree of oxidation was measured using the thiocyanate method (*18*). At different time intervals of 0, 24, 48, 72, and 96 h, samples (0.5 mL) were drawn from the reaction mixture. In the thiocyanate method, 4.7 mL of ethanol (75%), 0.1 mL of 30% ammonium thiocyanate, 0.1 mL of sample and 0.1 mL ferrous chloride (0.02 M in 3.5% HCl) were sequentially mixed and vortexed. After 3 min absorbance was measured at 500 nm. The control consisted of 0.5 mL of absolute ethanol and BHA and ferulic acid, at a concentration of 200 ppm in the final assay were used as positive controls. The percentage inhibition of linoleic acid peroxidation was calculated at the end of 48h as follows. Lipid peroxidation inhibition (%) = $\{1-(Abs_{500}$ of sample at 48h/ Abs_{500} of control at 48h)*100$\}$.

6.3.10 Statistical analysis

All experiments were carried out in triplicates and data were reported as mean ± standard deviation. The Student's t test at $p < 0.05$ was used to determine the significance of differences between two concentrations of the extracts of millets. The differences of mean values among millet varieties was determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p<0.05$ significance level. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL).

6.4 Results and discussion

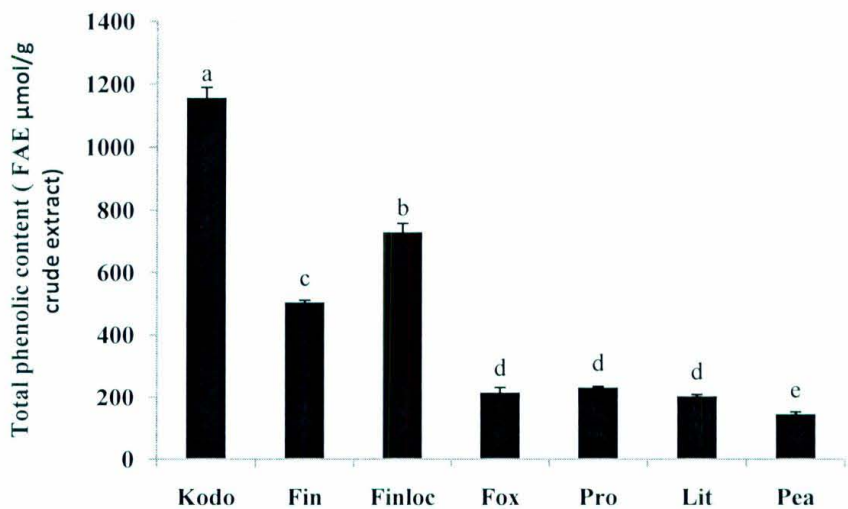

Figure 6.1 Total phenolic content of soluble phenolic extracts of whole millets. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters have significantly different ($p < 0.05$) mean values. The letter 'a' represents the highest value.

6.4.1 Total phenolic content (TPC)

The TPC of soluble extracts of whole grains ranged from 146 to 1156 µmol FAE/ g extract (**Figure 6.1**). Kodo millet had the highest TPC followed by finger (local), finger (Ravi), proso, foxtail, little and pearl millets. The corresponding values based on defatted weight of grain ranged from 8.6 to 32.4 µmol FAE/ g. In general, millet varieties employed in the present study contained more TPC than that in other cereals such as wheat, barley, and rice (*2, 4, 19*).

Table 6.1 IC_{50} values of millet whole grain soluble extracts for hydroxyl and DPPH radicals in the final assay as measured by electron paramagnetic resonance spectroscopy

Millet types	Hydroxyl radical	DPPH radical
Kodo	$0.22\pm0.01d^{1}$	$0.07\pm0.01e^{2}$
Finger	$0.53\pm0.02c^{1}$	$0.10\pm0.01d^{2}$
Finger (local)	$0.31\pm0.02d^{1}$	$0.05\pm0.01e^{2}$
Foxtail	$0.98\pm0.06b^{1}$	$0.97\pm0.07a^{1}$
Proso	$0.75\pm0.01b^{1}$	$0.61\pm0.04b^{1}$
Little	$0.54\pm0.07c^{1}$	$0.16\pm0.01d^{2}$
Pearl	$1.95\pm0.10a^{1}$	$0.35\pm0.01c^{2}$

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

6.4.2 Hydroxyl and DPPH radical scavenging activities

Table 6.1 presents the IC_{50} values of millet extracts for hydroxyl and DPPH radicals extrapolated from dose dependent curves. IC_{50} value shows the concentration of the extract required for scavenging 50% of radicals present in the test medium and hence a lower value represents a higher activity. IC_{50} values for hydroxyl radical scavenging activity ranged from 0.22 to 1.95 mg/mL in the final assay medium. Kodo millet showed the highest activity whereas pearl millet had the lowest. On the other hand, finger (local) millet displayed the highest DPPH radical scavenging activity whereas foxtail millet showed the lowest IC_{50} values ranging from 0.05 to 0.97 mg/mL for soluble whole grain extracts tested in the present study. The IC_{50} values of millet grain extracts for DPPH radicals were lower generally than that of hydroxyl radicals suggesting a higher effectiveness of millet phenolic extracts in scavenging DPPH radicals. In agreement with the present results, Madhujith and Shahidi (*12*) showed that IC_{50} values obtained for six barley cultivars for DPPH radical were lower than those of hydroxyl radicals and ranged from 1.51 to 3.33 and 2.2 to 9.65 mg/mL, for DPPH and hydroxyl radicals, respectively. The different scavenging activities of millet extracts of the two radical species could be due to the content and composition of phenolics of the extract. It has been shown that depending on the variety, millet phenolic extracts contain variable amounts of hydroxycinnamic and hydroxybenzoic acids and their derivatives as well as flavonoids (*10*).

6.4.3 Inhibition human LDL oxidation

Natural antioxidants from dietary sources that may inhibit LDL cholesterol oxidation are of great importance in the prevention of atherosclerosis and associated

cardiovascular diseases. It has been shown that uptake of oxidized LDL by macrophages and smooth muscle cells leads to the formation of fatty streaks or vascular lesions which accumulate lipids further (*5,20,21*). Several studies have shown that phenolics from cereal grains such as wheat and barley are potential sources of antioxidants that inhibit *in vitro* LDL cholesterol oxidation (*2,4*). Inhibitory effects of phenolic compounds against LDL cholesterol may arise from their free radical scavenging activity or by metal ion chelation properties. In addition, phenolic compounds can protect endogenous antioxidants such as tocopherols, β-carotene, lycopene and ubiquinol in LDL cholesterol molecule, or inhibit enzymes such as xanthine oxidase involved in the initiation of oxidation or cell mediated LDL cholesterol oxidation. In this study, the protective activity of extracts of millet grain phenolics for chelating cupric ions and thus reducing metal catalyzed oxidation of LDL cholesterol was demonstrated. It is noteworthy that at the beginning the rate of CD formation was slow as LDL cholesterol molecules contain antioxidant compounds such as tocopherols, β-carotene and lycopene. The rapid oxidation has started after the depletion of endogenous antioxidants of LDL cholesterol molecule (**Figure 6.2A**). **Figure 6.2B** shows the inhibitory activities of millet extracts at a concentration of 0.5 mg/mL against human LDL cholesterol oxidation induced by cupric ion. Ferulic acid, the major hydroxycinnamic acid present in millets was used as a standard compound at a concentration of 0.5 mg/mL and exhibited a 55% inhibition at the end of 4 h incubation period and showed 71% inhibition at the end of 20 h incubation. Millet varieties examined in the present work showed 1.3 to 41% inhibition after 4 h incubation and decreased with continuous incubation. The ability of phenolic compounds to inhibit copper ion-mediated LDL cholesterol oxidation may be

attributed to their capacity to remove cupric ions from the LDL cholesterol molecule (*22*). It has been shown that copper mediated oxidation of typtophan residues in apolipoprotein B could be a leading cause for initiating cholesterol oxidation in the LDL molecule (*23*).

It was noted that finger (Ravi) millet had a low LDL cholesterol oxidation inhibition, accounting for 1.3% and finger (local) millet did not show any protective effect at a concentration of 0.05 mg/mL in the final assay whereas other millet extracts had inhibitory activities against LDL cholesterol oxidation at a similar concentration. The CD content formed in the presence of extract of finger (local) millet was higher than that of the control suggesting a pro-oxidant effect. The TPC of finger (local) millet extract, at the concentration used was 0.037 μmol FAE in 1 mL of assay. The results showed that at 5 times lower TPC (0.007 μmol FAE in 1 mL of assay), extracts of pearl millet had 9% inhibition of LDL cholesterol oxidation. Therefore, the present results suggest that irrespective of the TPC, differences in phenolic composition of grain extracts play a major role in the observed effects.

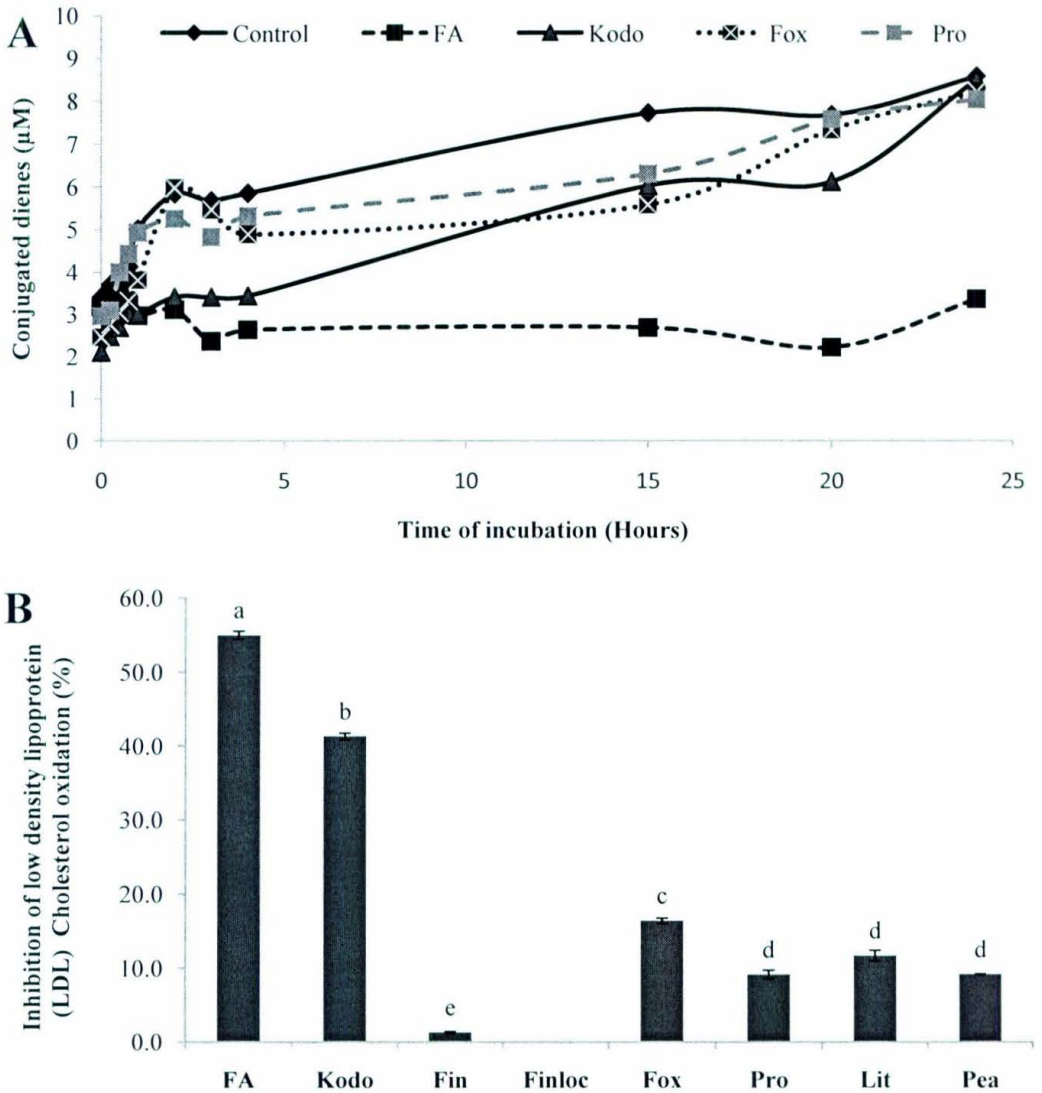

Figure 6.2 Representative graph showing the trend of conjugate dienes formation of LDL cholesterol in presence of whole millet extracts at a concentration of 0.5 mg/mL (A) and percentage inhibition of LDL cholesterol oxidation at a concentration of 0.5 mg/mL at the end of 4 h incubation (B). FA, ferulic acid; Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters have significantly different ($p < 0.05$) mean values. The letter 'a' represents the highest value.

Two finger millet varieties, Ravi and local, reported to contain flavan-3-ol monomers such as catechin, epicatechin, gallocatechin, and epigallocatechin and dimers such as procyanidin B1 and B2 in the phenolic extracts in the free and esterified forms (*10*). Cirico and Omaye (*24*) have shown that catechin, hesperidin, ferulic acid and quercetin exerted both antioxidant and pro-oxidant effects on LDL cholesterol oxidation depending on the concentration when used individually, whereas a mixture of these compounds had a significant antioxidant activity. Nevertheless, in the present study ferulic acid, used as a positive control exhibited a high inhibitory activity against LDL cholesterol oxidation (**Figure 6.2A**). It has been shown that procyanidins can complex with proteins (*25*). Hence, their contribution to the inhibition of oxidation of cholesterol at the concentration of finger millet extracts used in the present work could be low or ineffective. However, previous studies have shown that procyanidins are effective antioxidants and inhibit LDL cholesterol oxidation *in vitro* (*26-30*).

On the other hand, kodo millet which also has a high TPC showed the highest inhibition of LDL cholesterol oxidation of 41% after 4 h of incubation. The flavonoids present in the soluble extracts of kodo millet were mainly vitexin, luteolin, isovitexin, and apigenin (*10*). These compounds may effectively inhibit LDL cholesterol oxidation, thus producing low levels of CD in the presence of the respective extracts leading to a higher oxidation inhibition.

Table 6.2 Effect of whole millet grain extracts at different concentrations on prevention of autoxidation of stripped corn oil (SCO) as measured by Rancimat

Millet types	0.2 mg/g of oil	0.5 mg/g of oil	1 mg/g of oil
Kodo	1.00±0.12c[1]	1.18±0.00b[2]	1.43±0.00d[3]
Finger	0.95±0.01d[1]	1.10±0.02d[2]	1.53±0.02b[3]
Finger (local)	1.01±0.02c[1]	1.17±0.00b[2]	1.47±0.06c[3]
Foxtail	0.93±0.01d[1]	1.09±0.05d[2]	1.31±0.07e[3]
Proso	0.93±0.00d[1]	1.17±0.00b[2]	1.57±0.01a[3]
Little	1.00±0.03c[1]	1.13±0.02c[2]	1.43±0.02d[3]
Pearl	0.93±0.01d[1]	1.45±0.03a[2]	1.39±0.02d[3]
BHA*	2.48±0.13a	-	-
Ferulic acid	1.15±0.01b	-	-

*Butylated hydroxyanisole

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

6.4.5 Oxidation inhibition in bulk oil

The antioxidant activities of millet phenolic extracts in stripped corn oil were measured by the Rancimat method. The Rancimat method is an accelerated method that uses elevated temperatures in the presence of excess oxygen, thus results could be obtained within a relatively short period. The protection factors calculated are shown in **Table 6.2** and a higher protection factor (PF) suggests a stronger antioxidant

activity of the additive employed. In general, PF higher than 1.0 indicates the protection from autoxidation. The results obtained in the present study showed that millet phenolics increased the oxidative stability of SCO, which was devoid of any endogenous antioxidative compounds. The PF of whole millet extracts varied with the concentration used in the bulk SCO system. At a higher concentration of 1 mg/ g of oil, all extracts exerted a better ($p \leq 0.05$) PF that ranged from 1.31 to 1.57 than those at a low concentration of 0.2 mg /g of oil. Ferulic acid and BHA, showed PF of 1.15 and 2.48, respectively, at a concentration of 0.2 mg/g of oil and were higher than PFs obtained for millet phenolic extracts at a similar concentration. However, at a 2.5-fold higher concentration of the extracts, all millets examined in this study, except foxtail millet, demonstrated a significantly ($p \leq 0.05$) higher protection against oxidation of bulk oil than pure ferulic acid. These results further suggest that millet phenolics may prevent the oxidation propagation by scavenging free radicals formed in unsaturated fatty acids of SCO. The main unsaturated fatty acid in SCO is linoleic acid that accounts for about 59% of total fatty acid content.

Liyana-Pathirana and Shahidi (*2*) showed that at a concentration of 25 mg/g of oil, extract of two wheat cultivars had PFs of 1.47 and 1.66 in SCO as determined by the Rancimat method at 120°C. Furthermore, at a concentration of 20 mg/g of oil, whole barley extracts exhibited PFs between 1.31 and 1.59 at 120°C (*4*). The PFs obtained for whole millet grain phenolic extract in the present work were in the range of those obtained in earlier studies despite a lower concentration of extract used, thus suggesting the efficacy of millet phenolics as a source of natural antioxidant in this food system. In a previous study we have shown that different varieties of millet grains contain hydroxycinnamic and hydroxybenzoic acids and flavonoids in different

amounts that may contribute to the protection against accelerated lipid oxidation observed in this study (*10*).

6.4.6 Inhibition of oxidation in comminuted pork model system

In general, the presence of millet grain extracts at a concentration of 0.2 % (w/w) in comminuted pork significantly ($p \leq 0.05$) reduced the lipid oxidation except for pearl millet, in the storage period compared to the control with no additives (**Figure 6.3A**). In the present work the production of thiobarbituric acid reactive substances (TBARS), the secondary products of lipid oxidation in cooked comminuted pork, was measured and expressed as μmol MDA equivalents per kg of sample. The 2-thiobarbituric acid method is widely used for measuring lipid peroxidation in muscle foods due to its simplicity. The oxidation inhibition trend resulted by additives varied slightly during storage period of 14 days; thus results as percentage inhibition of TBARS formation at the end of day 3 and day 14 were presented to demonstrate the activity differences of millet extracts (**Figure 6.3B**). BHA was used as a positive control at a level of 0.02% (w/w). The results showed that the percentage inhibition of TBARS formation in the BHA treated sample was 27-99 and 0-96% for day 3 and day 14 of storage, respectively. BHA exhibited the highest inhibition throughout the entire 14 days of storage (**Figure 6.3A**). It is worth noting that kodo, finger (Ravi) and finger (local) millet varieties demonstrated a similar effect as BHA, inhibiting TBARS formation during the refrigerated storage suggesting their high potential as natural sources of antioxidants in meat systems. The observed inhibition of lipid oxidation kodo and finger millet extracts may be attributed to their high TPC (**Figure 6.1**). At the end of day 14, pearl millet extracts showed no protective effect against lipid oxidation (**Figure 6.3**). However, in the bulk SCO system, millet varieties with

high TPC showed the same antioxidant activity as varieties with low TPC (**Table 6.2**). Therefore it appears that the antioxidant activity of millet phenolic extract is system dependent. In comminuted pork model system, iron released from haem moieties act as a catalyst of lipid oxidation in cooked meat. It has been shown that the rate of iron release from porphyrin ring depends on time, intensity and temperature of cooking (*31*). Free non-haem iron catalyzes the oxidation of polyunsaturated fatty acyl components. It appears that millet phenolics may act as effective chelators of free iron ions as well as scavengers of peroxyl radicals formed during initiation and propagation steps in lipid oxidation. Finger millet phenolics demonstrated effective ferrous ion chelating activities whereas kodo millet showed a weak activity (*8*).

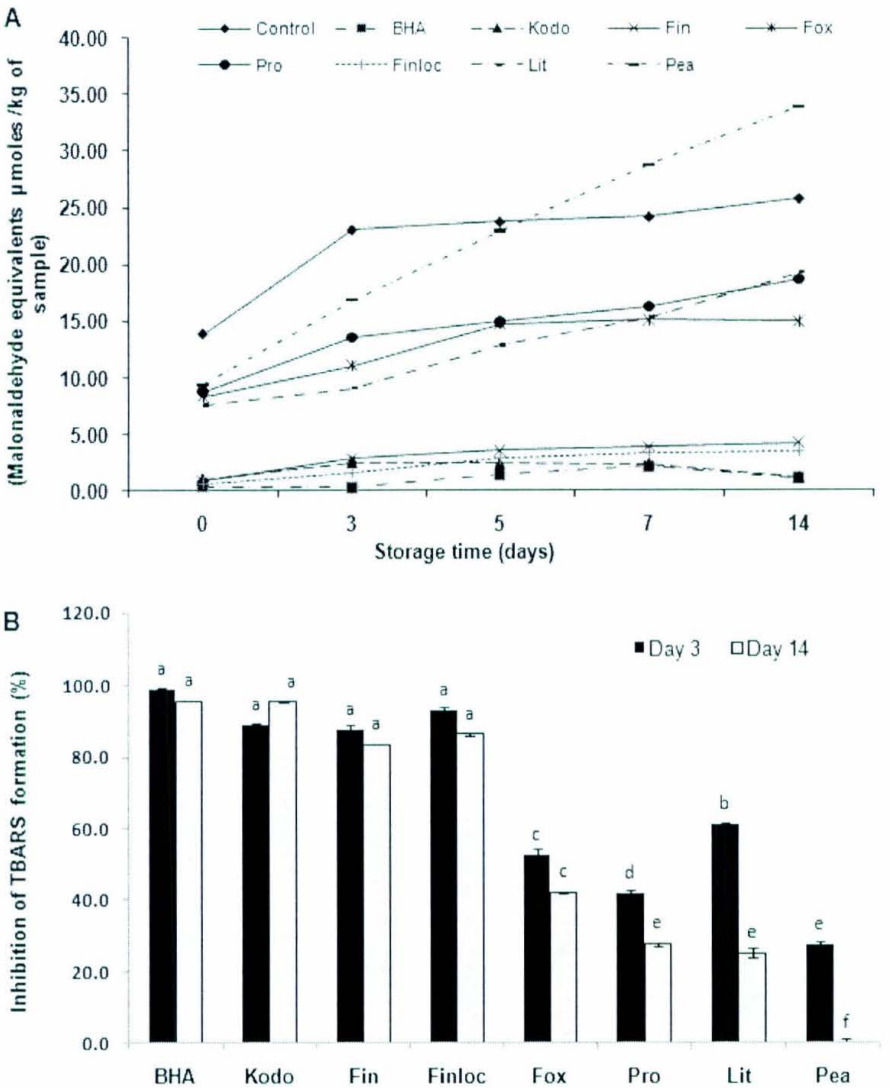

Figure 6.3 Effects of millet grain phenolic extracts on the formation of thiobarbituric acid reactive substances (TBARS) (A) and inhibition percentage of TBARS at the end of day 3 and day 14 of incubation (B). BHA, butylated hydroxyanisole; Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters have significantly different ($p < 0.05$) mean values. The letter 'a' represents the highest value.

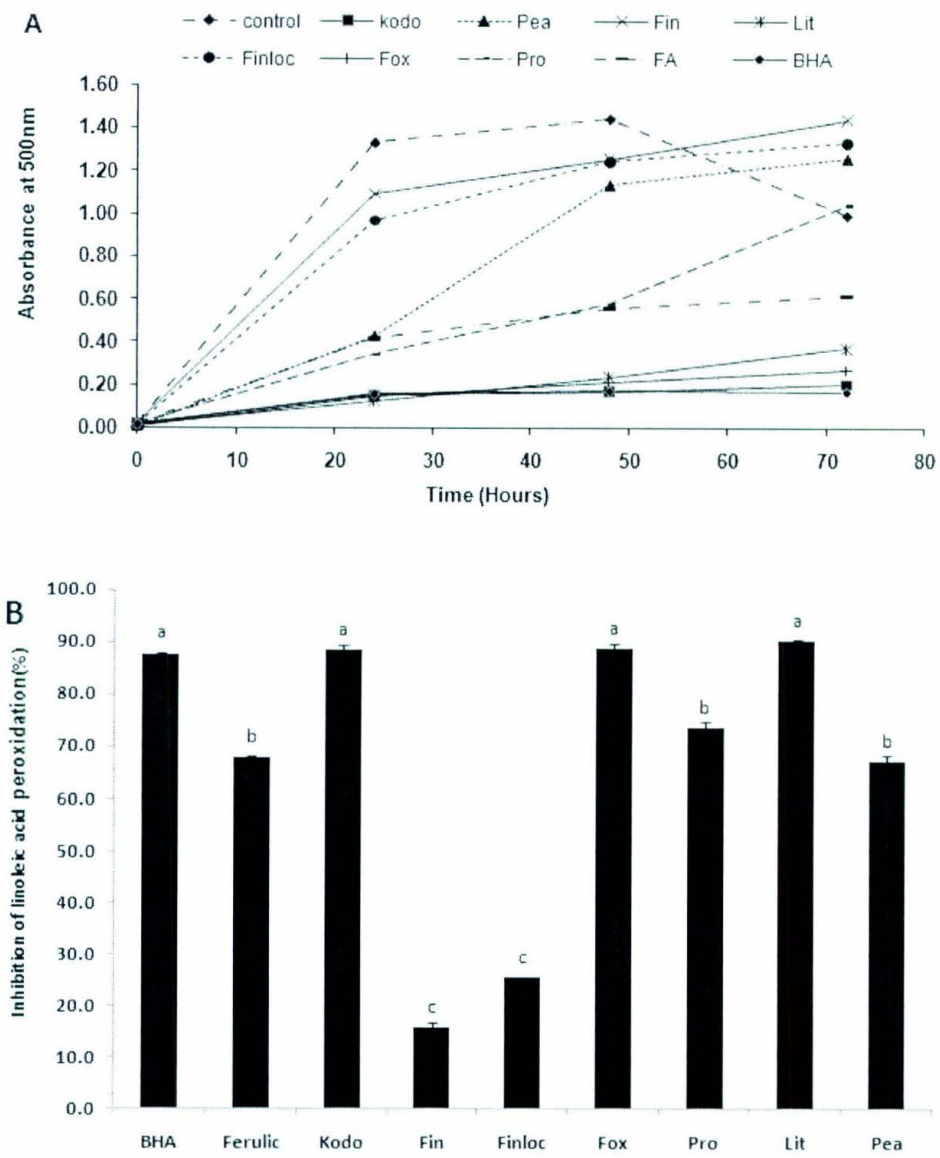

Figure 6.4 Effect of millet phenolic extracts on inhibition of linoleic acid oxidation (A) and percentage inhibition of linoleic acid peroxidation at the end of 48 h incubation. FA, ferulic acid; BHA, butylated hydroxyanisole; Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets. Bars with different letters have significantly different ($p < 0.05$) mean values. The letter 'a' represents the highest value.

6.4.7 Inhibition of oxidation in linoleic acid emulsion system

Figure 6.4 presents the antioxidant activity of millet phenolic extracts in linoleic acid system, an oil-in-water emulsion. In the course of incubation, the control which was devoid of any added extract showed a decrease in absorbance at 500 nm after 48 h, possibly due to the breakdown of primary oxidation products of linoleic acid. Thus, percentage inhibition of linoleic acid peroxidation at 48 h was selected to compare the efficacy of different extracts. At a concentration of 5 mg/mL all millet extracts, except two finger millet varieties, exhibited more than 67% inhibition of peroxidation similar to that of BHA, thus suggesting high effectiveness of millet phenolics in such systems. However, pure ferulic acid tested in the same system exhibited a significantly ($p \leq 0.05$) lower inhibition compared to that of BHA as well as kodo, foxtail and little millets. It should be noted that the two finger millet varieties which contained a higher TPC of 504-730 μmol FAE /g crude extract exhibited only 16-26% inhibition. Catechin which is a hydrophilic phenolic compound is the predominant flavonoid in two finger millet varieties (*10*). Catechin may be less effective in the oil-in-water emulsion system to protect the oil at the oil-water interface at the concentration used in the present study.

These findings further confirmed the fact that TPC and constituent phenolic compounds in a mixture of plant extract is important to their antioxidant activity. Kodo millet extracts which contained a high amount of phenolic acids and flavonoids such as vitexin, isovitexin, luteolin, quercetin, apigenin and kaempherol showed high antioxidant activity in the linoleic acid emulsion system. The formation of hydroperoxides in emulsion systems is dependent on the effective concentration of antioxidants in the oil and water phases and the interface (*32*). BHA exhibited a high

antioxidant activity in pork, bulk oil and oil-in-water emulsion systems employed in the present work. However, Chen and Ho (*32*) showed that BHT had high inhibitory activity in oil-in-water emulsion whereas a weak antioxidant was noted in bulk oil using the Rancimat method.

6.5 Conclusion

To the best of our knowledge this study is the first to report the use of millet grain phenolics against oxidation of human LDL cholesterol and oxidation in several food systems such as bulk oil, muscle food and linoleic acid emulsion. The results of the present work clearly demonstrated that millet phenolics may serve as potential sources of natural antioxidants for inhibition of lipid peroxidation in LDL cholesterol and food systems. The antioxidant activity of extracts which is system and dose dependent may be exerted via metal ion chelation and free radical scavenging effects. The content as well as the chemical nature of the phenolic constituents in the extract of millet grains may be responsible for the observed antioxidant effects. Thus, the foregoing results suggest the potential use of millet grains as sources of natural antioxidants in food systems and for disease risk reduction.

6.6 References

1. Shahidi, F.; Zhong, Y. Lipid oxidation and the improving the oxidative stability. *Chem. Soc. Rev.* **2010**, 39, 4067-4079.

2. Liyana-Pathirana, C.; Shahidi, F. Antioxidant properties of wheat as affected by pearling. *J. Agric. Food Chem.* **2006**, 54, 1256-1264.

3. Liyana-Pathirana, C.; Shahidi, F. The antioxidant potential of milling fractions from breadwheat and durum. *J. Cereal Sci.* **2007,** 45, 238-247.

4. Madhujith, T.; Shahidi, F. Antioxidative and antiproliferative properties of selected barley (*Hordeum vulgarae* L.) cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. *J. Agric. Food Chem.* **2007,** 55, 5018-5024.

5. Steinberg, D. Low density lipoprotein oxidation and its pathological significance. *J. Biol. Chem.* **1997**, 272, 20963-20966.

6. Frankel, W.N.; Waterfouse, A.L.; Teissedre, P.L. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *J. Agric. Food Chem.* **1995**, 43,890-894.

7. FAOSTAT, 2011 http:// www. faostat.fao.org. (Accessed on 12 Feburary, 2011).

8. Chandrasekara, A.; Shahidi, F. The content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *J. Agric. Food Chem.* **2010**, 58, 6706-6714.

9. Chandrasekara, A.; Shahidi, F. Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. *J. Agric. Food Chem.* **2011**, 59, 428-436.

10. Chandrasekara, A.; Shahidi, F. Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n. *J. Functional Foods.* **2011**, 3, 144-158.

11. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticul.* **1965**, 16, 144-158.

12. Madhujith, T.; Shahidi, F. Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *J. Agric. Food Chem.* **2006**, 54, 8048-8057.

13. Andreasen, M.F.; Landbo, A-K.; Christensen, L.P.; Hansen, A.; Meyer, A.S. Antioxidant effects of phenolic rye (*Secale cereale* L.) extracts monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low density lipoprotein. *J. Agric. Food Chem.* **2001**, 49, 4090-4096.

14. Wijeratne, S.S.K.; Amaraowicz, R.; Shahidi, F. Antioxidant activity of almonds and their by-products in food model systems. *JAOCS*, **2006**, 83, 223-230.

15. Siu, G.M.; Draper, H.H. A survey of the malonaldehyde content of retail meats and fish. *J. Food Sci.* **1978**, 43, 1147-1149.

16. Shahidi, F.; Hong, C. Evaluation of malonaldehyde as a marker of oxidative rancidity in meat products. *J. Food Biochem.* **1991**, 15, 97-105.

17. Osawa, T.; Namiki, M. A novel type of antioxidant isolated from leah wax of eucalyptus leaves. *Agric. Biol. Chem.* **1981**, 45, 735-739.

18. Mitsuda, H.; Yasumoto, K.; Iwami, K. Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyo to Shokuryo*. **1966,** 19, 210-214.

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

20. Kume, N.; Cybulsky, M.I; Gimbrone, M.A. Jr. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J. Clin. Invest.* **1992**, 90, 1138-1144.

21. Kume, N.; Gimbrone, M.A. Jr. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. *J. Clin. Invest.* **1994**, 93, 907-911.

22. Decker, E.A.; Ivanov, V.; Zhu, B.Z.; Frei, B. Inhibition of low-density lipoprotein-oxidation by carnosine and histidine. *J. Agric. Food Chem.* **2001**, 49, 511-516.

23. Giessauf, A.; Steiner, E.; Esterbauer, H. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. *Biochim. Biophys. Acta*. **1992**, 1256, 221-232.

24. Cirico, T.L.; Omaye, S.T. Additive or synergetic effects of phenolic compounds on human low density lipoprotein oxidation. *Food Chem. Toxicol*. **2006**, 44, 510-516.

25. Riedl, K. M.; Hagerman, A. E. Tannin-protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* **2001**, 49, 4917-4923.

26. Ariga, T.; Hamano, M. Radical scavenging action and its mode in procyanidins B-1 and B-3 from Azuki beans to peroxyl radicals. *Agric. Biol. Chem*. **1990**, 54, 2499-2504.

27. Ricardo da Silva, J. M.; Darmon, N.; Fernandez, Y.; Mitjavila,S. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. *J. Agric. Food Chem.***1991**, 39, 1549-1552.

28. Mangiapane, H.; Thomson, J.; Salter, A.; Brown, S.; Bell, G.D.; White, D.A. The inhibition of the oxidation of low density lipoprotein by (1)-catechin, a naturally occurring flavonoid. *Biochem. Pharmacol.* **1992**, 43,445-450.

29. Teissedre, P.L.; Frankel, E.N.; Waterhouse, A.L.; Peleg, H.; German, J.B. Inhibition of *in vitro* human LDL oxidation by phenolic antioxidants from grapes and wines. *J. Sci. Food. Agric.* **1996**, 70, 50-61.

30. Pikul, J. The oxidation of lipids and the development of warmed over flavor in heated and stored meat, part 1. *Gospodarka Mie Sna*. **1992**, 7, 20-23.

31. Frankel, E.N.; Meyer, A.S. The problems of using one dimensional method to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric*. **2000**, 80, 1925-1941.

32. Chen, J.H.; Ho, C-T. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *J. Agric. Food Chem.* **1997**, 45, 2374-2378.

CHAPTER 7

Effect of processing on the antioxidant activity of millet grains

7.1 Abstract

Millets are generally dehulled and subjected to a hydrothermal treatment before consumption, thus hulls can be used as a potential source of antioxidants. Several millet grains, namely kodo, finger (Ravi), finger (local), proso, foxtail, little and pearl millet were studied. Antioxidant activities of phenolic extracts obtained from whole grains and their corresponding dehulled and cooked grains and hulls were studied for their total phenolic content (TPC), radical scavenging capacity, and antioxidant activity in a β-carotene/linoleate emulsion. Phenolics present in whole grains were identified and quantified using HPLC and HPLC/MS and results were expressed as total for each of phenolic groups. The TPC ranged from 2 to 112 µmol ferulic acid equivalents / g defatted meal. All varieties exhibited effective inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, peroxyl and superoxide radicals. Dehulling and cooking affected the TPC and radical scavenging and antioxidant activities of grains depending on the variety. In general antioxidant activity of phenolic extracts was in order of hull > whole grain > dehulled grain > cooked dehulled grain. Except for two finger millet varieties hulls of other millet grains had high TPC thus, demonstrating their superior antioxidant activity. Hydroxybenzoic acids, hydroxycinnamic acids and flavonoids in whole grains were identified and these contributed to the observed effects. Therefore, dehulling of grain and hydrothermal treatments affect the phenolic content and antioxidant potential of millet grains.

7.2 Introduction

Cereals play an important role in the human diet. Thus, daily consumption of 3.5 ounces of whole grain products has been recommended by the US Department of Health and Human services and the US Department of Agriculture (*1*). Cereals belong to the grass family *Poaceae* that includes wheat, rice, barley, oats, rye, maize, sorghum and millets as major grain crops in the world market. Millets are small seeded cereals that are cultivated as subsistence crops mainly in semiarid and tropical regions in Asia and Africa. Pearl millet is the most widely grown millet type followed by foxtail, proso, and finger millets. There are other minor cultivated millet species, namely kodo, little millet, Japanese barnyard millet and teff millet which are economically important in ecological sub-regions where they grow. As a group, millets are used for both forage and grain. A wide variety of traditional foods and beverages is produced in countries where millets are grown for consumption. Millet foods produced from meal or flour include flat breads (fermented or unfermented) couscous and porridges in addition to snack foods such as 'Halepe' in Sri Lanka prepared with finger millet flour.

The principal anatomical components of the grain are pericarp, germ and endosperm. In utricle type kernels such as finger millets the pericarp is loosely attached to the endosperm at only one point. In caryopses type kernels, such as sorghum and pearl millets the pericarp is completely fused to the endosperm, thus making the separation from each other hard. Generally, in Asia and Africa, before preparing the flour for food use, millets are dehulled mechanically or traditionally using wooden mortar and pestle. These waste fractions may serve as a potential source of natural antioxidants. In cereal grains, polyphenols and phytates are mainly

concentrated in the pericarp, seed coat and aleurone layer (*2-4*). Dehulling and decortication are reported to decrease polyphenolic and phytic acid contents of pearl millet (*5*). On the other hand, dehulling increases the starch content and *in vitro* protein digestibility of pearl and finger millets (*6-8*). Pericarp of finger millet is membranous and thin layered. Therefore it detaches during harvesting or by simple abrasion (*9*). Seed coat (testa) of finger millets is usually pigmented and is fused with aleurone layer which is rigidly attached to the endosperm (*2*). In the process of dehulling the outermost cover of the grain, pericarp is separated from the seed. The successive steps may detach outer layers of the grain, depending on the time and degree of abrasion used which is commonly referred to as decortication.

Millets are prepared for consumption after submission to a wide range of thermal and hydrothermal treatments. Several studies on cereals have reported that thermal treatments may reduce or increase the phenolic content and their antioxidant activities, depending on severity of heat treatment, time of exposure and type of cereal tested (*10-12*). However, the available information is limited to only a few millet species (*11*). Effects of different processing conditions such as malting, fermentation, and germination on phenolic content and their antioxidant activities of millet have been examined and reported (*13, 14*). Malting of finger millet changed the composition of free and bound phenolic acids contents (*14*). According to Siripriya et al. (*13*) fermentation and germination of finger millet decreased their DPPH radical quenching ability compared to the raw finger millet. Shobana and Malleshi (*9*) showed that hydrothermal treatment and decortication of finger millets reduced polyphenolic content by 14 and 74%, respectively. Furthermore, Towo et al. (*10*) reported that hydrothermal treatment of finger millet reduced the total phenolic

content (TPC) by 1.7 times compared to that of the raw grains. Processing may increase the bioavailability of bioactive compounds in grains; however processing may decrease their levels (*15*). There are different methods in grain processing such as milling, baking, malting, extrusion, puffing and cooking. Depending on the severity and time of exposure of process variables, the activity and availability of bioactive compounds may vary. The available literature on the effects of millet processing on levels of phenolic compounds is scarce.

Liukkonen et al. (*16*) showed that several classes of bioactive compounds, namely sterols, folates, tocopherols, tocotrienols, alkylresorcinols, lignans and phenolic acids were concentrated in the bran layers of the rye grain. They further showed that germination and sourdough fermentation increased the levels of folate and extractable phenolic compounds whereas the content of some compounds such as tocopherols and tocotrienols was reduced (*16*).

Our previous studies have demonstrated that millet whole grains are rich sources of phenolics and polyphenolics (*17, 18*). The presence of phenolic acids as well as flavonoids has been reported in millets. Depending on the species of millet and the form of phenolic compounds present in the grain, the contents of hydroxycinnamic acids, hydroxybenzoic acids and flavonoids differ (*19*).

In general, phenolics are not distributed evenly in the grain. Thus, processing of cereals may exert a noted effect on their antioxidant activity (*20*). Several studies have exhibited that the outmost layers of the grains posses a high phenolic content and antioxidant activity (*20-22*). However, the concentration of antioxidants present in the grains and their antioxidant activities may vary depending on the species, cultivar, and growing location and environmental conditions, among others (*23-25*).

Phenolics are notable among bioactive phytochemicals in foods and in supplements for their role in disease risk reduction and improving health and wellness. Cereal-based foods are viable vehicles for bioactive compounds due to their widespread consumption as staple foods by much of the world's population.

It is important to know how the content of phenolics and their antioxidant activities are affected by the dehulling and thermal treatments. There is limited information on the phenolic content and antioxidant activities of different species of millet hulls and grains. Therefore, the aims of this study were to determine the TPC and their antioxidant activities in whole grains, dehulled grains and cooked dehulled grains and to evaluate hulls as a source of natural antioxidants.

7.3 Materials and methods

Seven samples of millet grains, namely foxtail (*Setaria italica)*, proso (*Panicum miliacium)*, two finger millet (*Elusine coracana*) varieties (Ravi and local), kodo (*Paspalum scrobiculatum*), little millet (*Panicum sumatrense*), and pearl millet (*Pennisetum glaucum*) were used in this study. All grain samples, with the exception of pearl millet, were obtained from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka. Pearl millet (dark green cultivar), grown in India, was obtained through Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India. The grains from both sources were harvested in 2007 and subsequently cleaned to remove foreign particulates before further use.

Folin Ciocalteu's reagent, ferulic acid, 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), H_2O_2, $FeSO_4$, 5,5-dimethyl-1-1-pyrroline-*N*-oxide (DMPO), nitroblue tetrazolium, mono-

and dibasic sodium phosphates, mono- and dibasic potassium phosphates, Tween 40 (polyoxyethylene sorbitan monopalmitate), β-carotene, linoleic acid, xanthine, xanthine oxidase were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Hexane, acetone, methanol, chloroform, acetonitrile, hydrochloric acid, formic acid, sodium hydroxide and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON).

7.3.1 Sample preparation

Whole millet grains cleaned from debris and soil particles were used in the experiments. Whole grains were directly ground using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc. Brockville, ON) to obtain a fine powder which passed through mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH, USA). Another portion of whole millet grains was dehulled using Seedburo hand grinder (Seedburo Equipment Company, Chicago, IL, USA). The hulls were separated by air classification on a 757 South Dakota seed blower equipped with a column which utilizes an air flow generated by a blower motor combination to separate seed fractions by size and density (Seedburo Equipment Company, Chicago, IL, USA). In this process, hulls and dehulled grains were procured along with one or more intermediatery fractions in which fragments such were not separated from hulls. A portion of dehulled grains and separated hulls were ground separately to obtain fine powders in a similar manner. Dehulled millet grains (30 g) were boiled in 300 mL of distilled water on a hot plate for 15 min (total cooking time was 30 min) with intermittent stirring using a glass rod to a consistency of a cooked rice-like product. After cooling to room temperature, cooked grains were transferred to –20°C freezer followed by freeze drying at -46°C and 34 x 10^{-3} mbar (Freezone, Model 77530,

Labconco Co., Kansas City, MO, USA). Freeze dried grain samples were ground using a coffee bean grinder to obtain a fine powder which passed through mesh 16. All samples, namely whole, dehulled, cooked grains and hulls were defatted by blending with hexane (1:5, w/v; 5 min, 3 times) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA) at ambient temperature. Defatted samples were vacuum packed in polyethylene pouches and stored at –20°C until used within one week for extraction of phenolics.

7.3.2 Extraction of soluble phenolic compounds

Ultrasound-assisted extraction procedure was used to extract phenolic compounds from defatted whole, dehulled and cooked grains and hulls. Defatted meal (5 g) was mixed with 100 mL of 70% (v/v) acetone and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) and sonicated at the maximum power for 25 min under reflux conditions. After centrifugation of the resulting slurry at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA) for 5 min, the supernatant was collected and extraction was repeated two more times. Combined supernatants were evaporated *in vacuo* at 40°C (Buchi, Flawil, Switzerland) and lyophilized for 72 h at -46°C and 34 x 10^{-3} mbar. Lyophilized crude phenolic extracts were stored at -20°C until used for further analysis. Residues of whole grain samples were air dried for 12 h and stored at -20°C until used to extract bound phenolics within a week.

7.3.3 Extraction of bound phenolics

The residue of the whole grain sample obtained after extraction of soluble phenolics was used to extract insoluble bound phenolics by subjecting the solids to alkaline

hydrolysis at room temperature and under a nitrogen atmosphere as explained by Chandrasekara and Shahidi (*17*). Phenolic compounds so obtained were reconstituted in 5 mL of HPLC grade methanol and stored at -20°C until used for HPLC analysis.

7.3.4 Determination of total phenolic content (TPC)

The total phenolic content of each extract was determined using the method described by Singleton and Rossi (*26*) with slight modifications, as explained previously (*17*). The content of total phenolics in each extract was determined using a standard curve prepared for ferulic acid and expressed as micromoles of ferulic acid equivalents (FAE) per gram of defatted meal.

7.3.5 HPLC analysis of phenolic compounds

Hydroxybenzoic and hydroxycinnamic acids and their derivatives as well as flavonoids of soluble and insoluble-bound phenolic fractions of whole millet grains were determined and quantified by HPLC and HPLC/MS analysis as explained previously (*17*). Results were expressed as total of each group of phenolic compounds as micrograms per gram of defatted meal.

7.3.6 DPPH radical scavenging capacity assay using electron paramagnetic resonance (EPR) spectroscopy

The effect of extracts on the scavenging of DPPH radicals was determined according to the method explained by Chandrasekara and Shahidi (*18*). The phenolic extracts (0.5 mL) were used at different concentrations of 0.25-1.0 mg/mL in methanol. Methanol was used as the control in place of extract; different concentrations (0.025-0.3 mg/mL) of ferulic acid in methanol were used for constructing the standard curve.

A Bruker E-scan EPR spectrometer (Bruker E-scan, Bruker Biospin Co. Billercia, MA, USA) was used to record the spectrum. The DPPH radical scavenging activity was expressed as micromoles of ferulic acid equivalents (FAE) per gram of defatted meal.

7.3.7 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was determined according to the method previously explained by Chandrasekara and Shahidi (*18*). In brief, phenolic extracts (100 μL) in deionized water were mixed with 100 μL of H_2O_2, (10 mM), 200 μL of DMPO (17.6 mM) and 100 μL of $FeSO_4$ (0.1 mM). After 1 min, each mixture was introduced into the sample cavity of EPR spectrometer and its spectrum recorded. Ferulic acid dissolved in deionized water was used to prepare the standard curve (0.05-2.0 mg/mL). Hydroxyl radical scavenging capacities of the extracts were calculated using the following equation. Hydroxyl radical scavenging capacity (%) = {(EPR signal intensity for the control – EPR signal intensity for the sample)/ EPR signal intensity for the control} x 100. The hydroxyl radical scavenging activity of the extracts was expressed as micromoles of FAE per gram of defatted meal.

7.3.8 Hydrogen peroxide (H_2O_2) scavenging activity

The efficacy of phenolic extracts in scavenging H_2O_2 was based on the method explained by Wettasinghe and Shahidi (*27*) and as reported previously (*18*). In brief, the extracts (0.4 mL) dissolved in distilled water at a concentration of 1 mg/mL were added to 0.6 mL of H_2O_2 (40 mM). Total reaction volume was made to 2 mL with 45 mM sodium phosphate buffer. The absorbance was measured at 230 nm after 40 min incubation at 30^0 C. Blanks were run for each sample replacing H_2O_2 with buffer for

background correction. A standard curve was prepared with ferulic acid. Percentage of H_2O_2 scavenging activity of millet phenolic extracts was calculated using the following equation. H_2O_2 scavenging activity (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. The results were expressed as micromoles of FAE per gram of defatted meal.

7.3.9 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed as previously explained by Chandrasekara and Shahidi (*18*). The extracts were diluted in 75 mM phosphate buffer (pH 7.0). Determination of ORAC was carried out using a plate reader equipped with a built-in incubator (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany). ORAC values of extracts were expressed as micromoles of trolox equivalents (TE) per gram of defatted meal.

7.3.10 Superoxide radical scavenging activity

Superoxide radical scavenging activity of millet extracts were determined according to the method explained by Chun et al. (*28*) with some modifications. Tetrazolium blue solution was prepared by dissolving xanthine (1 mM) and NBT (1 mM) in 50 mM potassium phosphate buffer (PBE) (pH 7.4) with 0.05 mM EDTA. Samples were dissolved and diluted in 50% (v/v) methanol to obtain a concentration of 1 mg/mL. Tetrazolium blue solution (0.45 mL) was added to 0.05 mL of sample and reaction was initiated by mixing 0.5 mL of xanthine oxidase prepared in PBE (0.25 units/mL of PBE). The mixture was then incubated at 37^{0} C for 20 min and to which 1 mL of 2 M HCl was added to terminate the reaction. The blue colour formed was measured at 560 nm using a spectrophotometer (Model HP 8452A diode array spectrophotometer,

Agilent Technologies, Palo Alto, CA, USA). A blank was prepared for each sample in which 2 M HCl was mixed prior to the addition of xanthine oxidase solution. The percentage of superoxide radical scavenging activity was calculated using the following equation. Superoxide radical scavenging activity (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. The results were expressed as millimoles of FAE per g defatted meal.

7.3.11 Antioxidant activity in *β*-carotene/linoleate model system

The antioxidant activity of extracts was evaluated in a *β*-carotene /linoleate model system as explained by Chandrasekara and Shahidi (*17*). Absorbance measurement was carried out using a microplate reader equipped with a built-in incubator (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany). Antioxidant activity coefficient (AAC) after 120 min of incubation was calculated using the following equation; $AAC = (A_{a(120)} - A_{c(120)}) / (A_{c(0)} - A_{c(120)})$ where $A_{a(120)}$ and $A_{c(120)}$ are the absorbance values measured at 120 min for the sample and the control, respectively, and $A_{c(0)}$ is the absorbance value of the control, at 0 min. The results were expressed as AAC per gram of defatted meal.

7. 3.12 Statistical analysis

All experiments were carried out in triplicates unless otherwise stated and data were reported as mean ± standard deviation. The differences of mean values among millet varieties was determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p \leq 0.05$ significance level. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

7.4 Results and Discussion

The present work was aimed at assessing the phenolic contents and antioxidant properties of millet grains submitted to dehulling and consequent hydrothermal treatment. Hulls, a byproduct of millet grain processing, were evaluated as a natural source of antioxidants.

Table 7.1 Total phenolic content (µmol ferulic acid equiv/g defatted meal) of whole (WG), dehulled (DG), cooked (CG) grains and hulls of millets

Millet types	WG	DG	CG	Hull
kodo	32.4±0.93b[1]	6.86±0.19c[4]	6.06±0.11c[5]	112±1.37a[1]
Finger (Ravi)	21.2±0.31a[2]	16.8±0.02c[2]	15.0±0.26d[2]	19.3±0.29b[5]
Finger (local)	31.4±1.22a[1]	27.6±0.47b[1]	17.7±0.18d[1]	20.2±0.99c[4,5]
Foxtail	10.8±0.82b[3]	3.80±0.22c[5]	3.67±0.04c[6]	22.8±0.88a[4]
Proso	7.19±0.12b[4]	2.04±0.10c[6]	1.95±0.01c[7]	15.9±0.50a[6]
Little	12.7±0.33b[3]	8.77±0.28c[3]	8.58±0.13 c[3]	26.5±1.51a[3]
Pearl	8.63±0.38b[4]	8.50±0.03b[3]	8.13±0.06 b[4]	34.3±1.69a[2]

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

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

7.4 1Total phenolic content (TPC)

The total phenolic content (TPC) expressed as µmol FAE /g of defatted meal of whole grains (WG), dehulled grains (DG), cooked grains (CG), and hulls of millets are presented in **Table 7.1**. The results showed that depending on the type of millet, TPC

varied among different grains obtained after processing of millet whole grains. The TPC of WG, DG, CG and hulls ranged from 5.45 to 64.7, 2.97 to 63.7, 2.61 to 44.9 and 11.9 to 127 μmol FAE /g of defatted meal, respectively. In general, dehulling decreased the TPC of WG millets and this change was essentially due to the removal of the outer layers of the grain. The loss of TPC in DG compared to the corresponding WG was 78, 21, 12, 65, 72, 35 and 2% for kodo, finger (Ravi), finger (local), foxtail, proso, little and pearl millets, respectively. In accordance with the present results, Hag et al. (*29*) also showed that dehulling decreased the TPC of pearl millets. Thus, the results of this study lend further support to the fact that phenolic compounds of cereal grains are mainly concentrated in the outer layers of the grain (*4, 23*). The present results also showed that effect of dehulling on the TPC varies with the type of millet and could be due to the variable distribution of polyphenolics in different grain layers, namely testa, aleurone layer and pericarp.

Furthermore, hulls had the highest TPC in the tested millets, except for two finger millet varieties. In agreement with present results, layers of the outermost part of the grain are reported to contain high amounts of polyphenolic compounds in cereals such as wheat, barley, oat, buckwheat and sorghum (*4, 23, 30-32*). Peterson et al. (*33*) also reported that phenolic compounds are mainly concentrated in the bran of oats. In wheats, bran had the highest phenolic content and antioxidant activities while the endosperm possessed the lowest amount (*22, 31, 32*). In addition, the outermost fraction of barley possessed the highest phenolic content as well as antioxidant activity (*30*). According to Zielinski and Kozlowska (*23*), TPC and antioxidant activities of hulls of barley, oat and buckwheat were higher than those of their corresponding dehulled grains. These researchers reported that dehulled grains

of barley, oat and buckwheat had TPC of 27.96, 16.33 and 9.72 mg catechin equivalents /g of lyophilized extract, respectively, whereas corresponding hulls had the TPC of 31.33, 45.0 and 381.86 mg catechin equivalents /g of lyophilized extract, respectively.

Hydrothermal treatment of foods may affect their content of phytochemicals (*10, 12*). Cereals are generally subjected to a hydrothermal treatment prior to their consumption. Thus, in this study dehulled grains were cooked in boiling water for 15 minutes as a representative hydrothermal treatment. Cooked grains of finger (local) millet had the highest TPC whereas that of proso millet showed the least. It was further noted that TPC of DG and CG of millet varieties did not differ significantly except for two finger millet varieties. However, few studies have shown an increase in TPC after the hydrothermal treatment (*12, 34-36*). According to Zielinski et al. (*34*) phenolic acid content of wheat, barley, rye and oat increased under extrusion cooking except for sinapic and caffeic acids. Dewanto et al. (*35*) showed a significant increase of total free phenolics and antioxidant activity in sweet corn following thermal treatment with increased heating times and temperatures. In addition they have showed that hydrothermal treatment released phenolic compounds from esterified and insoluble bound forms of the grain suggesting the breakdown of cellular constituents by thermal application (*35*). Bryngelsson et al. (*36*) also showed that various hydrothermal processes such as steaming and autoclaving of oats were effective in increasing the assayable phenolic contents, particularly ferulic and *p*-coumaric acids which could be released from their bound forms. According to Zielinski et al. (*12*) extrusion of buckwheat seeds increased the phenolic acids content primarily due to the increased release of phenolics from their matrix. However,

Bryngelsson et al. (*36*) also demonstrated that drum drying of oats decreased the phenolic compounds such as those of ferulic acid, *p*-coumaric acid and vanillin, possibly due to their oxidation under the processing conditions employed. Thus, insignificant differences of TPC between DG and CG of kodo, foxtail, proso, little and pearl millets observed in this study could be due to the release of bound phenolics which compensated the losses experienced during thermal treatment.

The present results showed that thermal treatment reduced the TPC of dehulled finger (Ravi) and finger (local) millets by 11 and 36%, respectively. In agreement with these results, Towo et al. (*10*) reported that boiling of finger millet and red sorghum for 15 min reduced their total extractable phenolics by 40 and 80%, respectively. The reduced TPC in finger millet grains could be due to the degradation of phenolics upon heat treatment or leaching into the endosperm to form complexes with proteins and other macromolecules, thus making phenolics less extractable. Kodo millet showed the highest TPC for WG and hulls among the different types of millets examined in this study. However, finger (local) millet had the highest TPC for DG and CG, among others, suggesting the importance of screening different millet types for their use as a functional food ingredient or a nutraceutical.

7.4.2 HPLC analysis of phenolic contents

The total contents of phenolic acids and flavonoids in millet varieties are given in **Figure 7.1**. Both hydroxybenzoic and hydroxycinnamic acids in whole grains were identified and quantified. The total content of hydroxybenzoic acid derivatives in the soluble and insoluble bound extracts of millets ranged from 103 to 269 and from 32 to 215 µg/g defatted meal, respectively. The major hydroxybenzoic acids identified were gallic, vanillic, protocatechuic, *p*-hydroxybenzoic, syringic and gentisic acids.

In general, hydroxybenzoic acid derivatives were mainly concentrated in the soluble fraction. On the other hand hydroxycinnamic acids and their derivatives were mainly found in the insoluble bound fraction of whole millet grains and their contents ranged from 45 to 682 and 468 to 3687 μg/g defatted meal, for soluble and insoluble bound fractions, respectively. Sinapic, cholorogenic, caffeic, *p*-coumaric, cinnamic, and ferulic acids were among the major hydroxycinnamic acids identified (*19*). Flavonoids were mainly present in the soluble fraction of whole grains and their content ranged from 54 to 2100 μg/g defatted meal. It is noteworthy that soluble fractions of the two finger millet varieties had 30-39 % higher contents of flavonoids compared to those of proso millet which had the least amount of 54 μg/g defatted meal of grains examined in this study. In a previous study, Hernanz et al. (*38*) reported that the outer fraction of barley grain consisting mainly of the husk and outer layers had the highest concentration of ferulic acid, *p*-coumaric acid and dehydrodimers of ferulic acid with contents ranging from 77 to 82, 78 to 86 and 79 to 87% of their total amounts in the whole grain, respectively. However, in this study no attempts were made to quantify individual phenolic compounds in the dehulled and cooked grains and hulls.

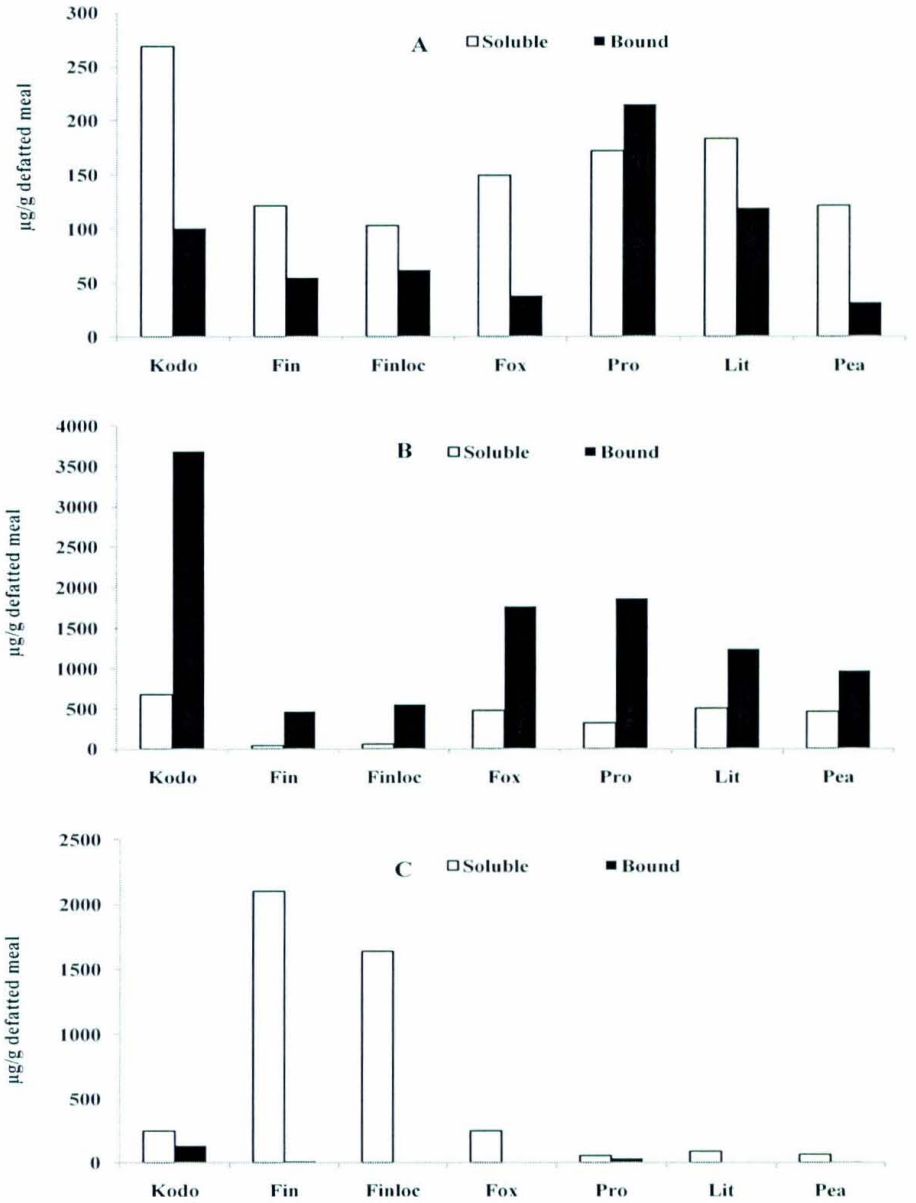

Figure 7.1 Total contents of hydroxybenzoic acids and derivatives (A) hydroxycinnamic acids and derivatives (B) and flavonoids (C) in soluble and insoluble bound fractions of whole millet grains. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets.

Table 7.2 DPPH radical scavenging activity (µmol ferulic acid equiv /g defatted meal) of whole (WG), dehulled (DG), cooked (CG) grains and hulls of millets

Millet types	WG	DG	CG	Hull
kodo	33.3±0.75b[2]	10.6±0.39c[4]	10.2±0.59c[3]	127±1.62a[1]
Finger (Ravi)	35.7±6.92a[2]	31.6±0.41a[2]	30.9±1.02a[2]	17.9±0.40b[3]
Finger (local)	64.7±3.81a[1]	63.7±1.33a[1]	44. 9±1.41b[1]	17.6±0.95c[3]
Foxtail	5.86±0.15c[3]	6.77±0.24b[5]	6.55±0.35b[4]	11.9±0.16a[5]
Proso	5.45±0.06b[3]	2.97±0.10c[6]	2.61±0.27c[5]	14.9±0.62a[4]
Little	10.3±0.33b[3]	10.1±0.52b[4]	8.07±0.33c[4]	17.9±1.09a[3]
Pearl	13.8±1.23b[3]	13.8±0.06b[3]	11.2±0.61c[3]	38.8±1.07a[2]

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

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

7.4.3 DPPH radical scavenging capacity

The DPPH radical scavenging activity (DRSA) of WG, DG, CG and hulls were measured using EPR spectroscopy and results are presented in the **Table 7.2**. The WG of two finger millet varieties showed a high DRSA whereas hulls had high activities in kodo, foxtail, proso, little and pearl millets tested in this study.

Cooked finger (Ravi) and (local) millets showed 11 and 36% lesser DRSA than that of the dehulled grains. However, CG of all other varieties examined in this study did not show significant differences when compared to their dehulled counterparts; this is in agreement with the TPC values. DRSA of cooked grains ranged from 2.61 to 44.9 FAE µmol /g of defatted meal; finger (local) millet had the

highest DRSA which was 17 times higher than that of proso millet with the least value. However, some researchers (*39, 40*) showed that thermal processing decreased the DPPH radical scavenging activity of buckwheat. This could be due to the binding of phenolics to other molecules such as proteins or some of phenolics may not be readily extractable after the thermal processing.

The DRSA of hulls ranged from 11.9 to 127 FAE μmol /g of defatted meal and kodo millet hulls had the highest DRSA which was 3-10 times higher than that of other varieties examined in this study. High TPC present in hulls may contribute to the high DRSA of hulls observed in this study suggesting the potential use of hulls as nutraceuticals for improving health and reducing disease risk.

7.4.4 Hydroxyl radical scavenging activity

Trapping of hydroxyl radicals by phenolic compounds led to a decline in the formation of DMPO-OH adduct. Hydroxyl radical is highly reactive and short living in biological systems and can damage DNA, proteins and other biomolecules. Thus, hydroxyl radical scavenging activity of millet phenolic compounds was determined using EPR spectroscopy. The decrease of the signal intensity of DMPO-OH adduct was measured with reference to the control and expressed as FAE μmol/g defatted meal. **Table 7.3** presents the hydroxyl radical scavenging activity (HRSA) of WG and corresponding processed grains of millets. The HRSA of WG, DG, CG and hulls ranged from 26 to 66.8, 2.13 to 28.4, 1.49 to 14, and 18.1 to 196 FAE μmol/g defatted meal, respectively. In general, hulls of millet showed the highest HRSA, consistent with the results obtained for total phenolic contents in this study. It is noteworthy that hulls of two finger millet varieties which showed relatively low DRSA in this study (**Table 7.2**) showed a 2-7 times higher HRSA than that of their WG counterparts.

These results further demonstrate that antiradical activity of phenolic extracts depends on the nature of free radicals employed in the assay in addition to their concentration and structural characteristics as well as synergistic interactions with other compounds present. The hydroxyl radical quenching ability of phenolic compounds has directly been correlated with the number of hydroxyl groups substituting at aromatic rings of phenolics and to the nature of substitution at the *para* position.

Table 7.3 Hydroxyl radical scavenging activity (µmol ferulic acid equiv /g defatted meal) of whole (WG), dehulled (DG), cooked (CG) grains and hulls of millets

Millet types	WG	DG	CG	Hull
kodo	56.4±6.76b[1]	16.4±1.13c[2]	14.0±1.95c[1]	133±23.0a[2]
Finger (Ravi)	26.8±2.29b[3,4]	9.69±0.11c[3]	3.01±0.32c[4,5]	183±29.6a[1]
Finger (local)	66.8±3.70b[1]	27.2±3.64c[2]	9.12±2.16c[2]	128±13.8a[2,3]
Foxtail	38.0±4.28a[2,3]	28.4±4.44a[1]	9.00±1.07b[2]	39.1±6.57a[4]
Proso	26.0±1.37a[4,5]	7.76±1.05c[3,4]	5.08±0.22d[3,4]	18.1±0.66b[4]
Little	44.8±3.35b [2]	23.5±1.04c[1]	7.96±0.70d[2,3]	85.9±4.47a[3]
Pearl	56.0±4.77b[1,2]	2.13±0.89c[4]	1.49±0.36c[5]	196±8.26a[1]

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

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

Table 7.4 Hydrogen peroxide scavenging capacity (µmol ferulic acid equiv /g defatted meal) of whole (WG), dehulled (DG), cooked (CG) grains and hulls of millets

Millet types	WG	DG	CG	Hull
kodo	$28.4\pm0.51b^{4}$	$21.7\pm0.73c^{6}$	$21.8\pm0.11c^{4}$	$84.0\pm5.02a^{3}$
Finger (Ravi)	$38.9\pm1.23b^{3}$	$37.2\pm0.53b^{5}$	$31.1\pm0.18b^{3}$	$131\pm5.86a^{1}$
Finger (local)	$40.3\pm0.70c^{3}$	$49.4\pm2.09b^{4}$	$49.0\pm0.94b^{1}$	$93.4\pm0.66a^{2}$
Foxtail	$44.5\pm0.66b^{2}$	$61.6\pm1.88a^{2}$	$23.0\pm0.73c^{4}$	$42.1\pm1.05b^{4}$
Proso	$28.8\pm0.16b^{4}$	$24.4\pm0.30c^{6}$	$19.9\pm0.12d^{4,5}$	$50.0\pm0.13a^{4}$
Little	$53.6\pm1.64c^{1}$	$69.6\pm0.42b^{1}$	$30.9\pm0.88d^{3}$	$77.4\pm1.68a^{3}$
Pearl	$52.9\pm0.45c^{1}$	$57.2\pm0.17b^{3}$	$46.2\pm1.09d^{2}$	$130\pm0.83a^{1}$

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

Bors et al. (*41*) reported that methoxylation of hydroxyl group at the ortho position, as in ferulic acid, results in a decrease in the rate constant of phenolic antioxidants upon reduction with hydroxyl radicals. It has previously been shown that two finger millet varieties are rich sources of flavonoids, particularly catechin, which may contribute to the observed HRSA in the present work.

It is also noted that CG of two finger millet varieties, proso and little millets, had significantly ($p<0.05$) lesser HRSA than their dehulled counterparts. These results further suggest that dehulling and cooking of millet grains invariably affect the antiradical activity owing to the compositional changes occurring in mechanical and hydrothermal processes involved.

7.4.5 Hydrogen peroxide (H_2O_2) scavenging activity

The scavenging capacity of phenolic extracts procured from processed millet grains and hulls against hydrogen peroxide was examined (**Table 7.4**). In general hulls showed the highest activity and the magnitude which varied depending on the variety employed. In spite of the low TPC, finger (Ravi) millet hulls had a higher value compared to other varieties tested. The present results showed that cooked grains of foxtail, proso, little and pearl millets had a lower hydrogen peroxide scavenging activity than those of their dehulled grain counterparts, suggesting the negative effect of thermal processing on the antioxidant activity of polyphenolics of grains examined. In addition, dehulled grains of some varieties such as finger (local), foxtail, little and pearl millets showed significantly higher hydrogen peroxide scavenging activity compared to the corresponding whole grains.

7.4.6 Oxygen radical absorbance capacity (ORAC)

In the ORAC assay, the ability of WG and their processed counterparts to scavenge peroxyl radical was demonstrated. Peroxyl radicals which are less active than hydroxyl radicals are a common radical species found in food and biological systems. Generally hulls showed high ORAC values with the exception of finger (local) and little millets (**Table 7.5**), similar to the pattern observed for radical species such as DPPH and hydroxyl radicals. The ORAC values of millet hull extracts ranged from 104 to 234 TE μmol/g of defatted sample. In general, cooked millet grains of millet had lower ORAC values compared to their corresponding WG and DG and ranged from 19 to 133 TE μmol/g of defatted sample. The present results showed that hydrothermal treatment unfavourably affected the peroxyl radical scavenging activity

of cooked millet grains. Zielinski et al. (*42*) also showed that hydrophilic ORAC values obtained for roasted buckwheat seeds were less than those of raw whole seeds possibly due to the thermal deterioration of hydrophilic antioxidative compounds such as flavonoids, namely rutin and isovitexin during roasting.

Table 7.5 Oxygen radical absorption capacity (μmol trolox equiv /g defatted meal) of whole (WG), dehulled (DG), cooked (CG) grains and hulls of millets

Millet types	WG	DG	CG	Hull
Kodo	95.7±2.37b^{2}	41.1±1.39c^{4}	24.3±0.23d^{6}	226±1.54a^{1,2}
Finger (Ravi)	101±5.54c^{2}	129±1.82ba^{2}	116±3.24b^{2}	132±8.39a^{3}
Finger (local)	143±4.51b^{1}	165±3.26a^{1}	133±0.41c^{1}	126 ±5.16c^{3}
Foxtail	59.8±9.19c^{3}	111±10.1b^{3}	39.1±0.89d^{5}	234±0.34a^{1}
Proso	35.0±3.49b^{4}	33.2±4.38b^{4}	19.7±1.89c^{6}	104±4.46a^{4}
Little	87.3±0.74c^{2}	148±6.12a^{1}	71.4±1.75d^{3}	106±2.33b^{4}
Pearl	60.3±8.39c^{3}	95.9±10.1b^{3}	65.5±3.10c^{4}	219±7.35a^{2}

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

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

7.4.7 Antioxidant activity in *β*-carotene/linoleate model system

Antioxidant activities of whole, dehulled, cooked grains and hulls which were evaluated in the *β*-carotene/linoleate emulsion system and antioxidant activity coefficients (AAC) of grains and hulls are presented in **Figure 7.2**. The *β*-carotene/linoleate emulsion system has widely been used for measuring the antioxidant activity of cereal phenolic extracts (*17, 30, 43*). In this assay phenolic

compounds scavenge free radicals formed due to the oxidation of linoleic acid and minimize the oxidation of β-carotene, thus preserving the colour which is measured at 450 nm. In the present work, extracts obtained from all grain samples and hulls inhibited β-carotene oxidation. Similar to the results obtained in other *in vitro* assays, hulls displayed the highest antioxidant activity and values ranged from 1711 to 7524 AAC / g defatted meal. Hulls of pearl millets had the highest AAC followed by kodo, little, finger (Ravi), finger (local), foxtail and proso millets. Generally, cooked grains of millets had a lower AAC compared to those of their uncooked counterparts, thus suggesting that thermal processing may lower the antioxidant activity of phenolics present in the grains.

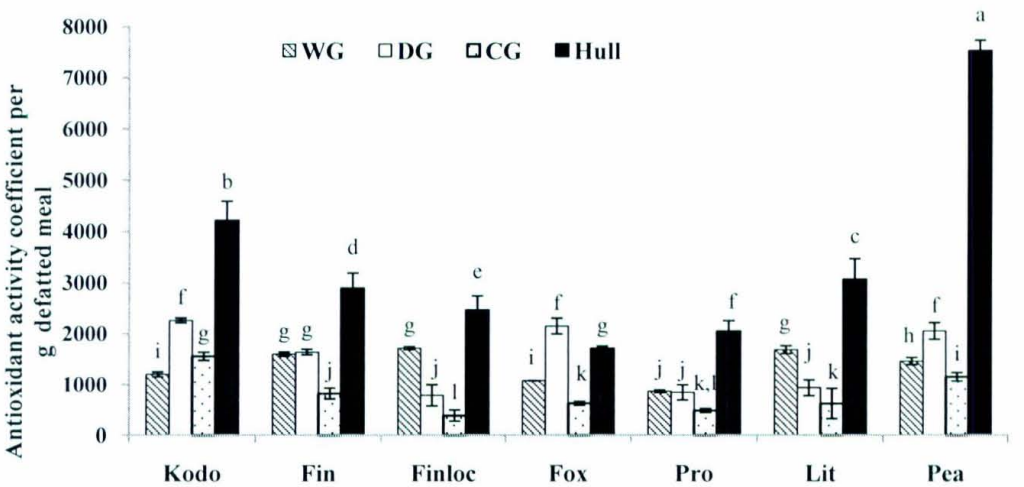

Figure 7.2 Antioxidant activity of whole (WG), dehulled (DG), cooked (CG) grains and hulls of millets in β-carotene/linoleate emulsion system. Bars with different letters have significantly different ($p < 0.05$) mean values. The letter 'a' represents the highest value. Kodo, kodo; Fin, finger (Ravi); Finloc, finger (local); Fox, foxtail; Pro, proso; Lit, little; Pea, pearl millets.

Table 7.6 Superoxide radical scavenging capacity (mmol ferulic acid equiv /g defatted meal) of whole (WG), dehulled (DG), cooked (CG) grains and hulls of millets

Millet types	WG	DG	CG	Hull
Kodo	$0.50\pm0.05b^{5}$	$0.49\pm0.01b^{4}$	$0.47\pm0.04b^{4}$	$0.90\pm0.10a^{2}$
Finger (Ravi)	$1.00\pm0.02a^{3}$	$0.72\pm0.01b^{3}$	$0.57\pm0.03c^{3}$	$0.44\pm0.02d^{3}$
Finger (local)	$0.87\pm0.01a^{4}$	$0.75\pm0.11ab^{3}$	$0.48\pm0.02c^{4}$	$0.69\pm0.05b^{2,3}$
Foxtail	$1.31\pm0.05b^{2}$	$1.57\pm0.05a^{1}$	$0.65\pm0.03d^{2}$	$0.81\pm0.02c^{2}$
Proso	$0.81\pm0.02a^{4}$	$0.67\pm0.01b^{3}$	$0.50\pm0.01c^{4}$	$0.80\pm0.03a^{2,3}$
Little	$1.30\pm0.01b^{2}$	$1.50\pm0.01a^{1,2}$	$0.52\pm0.01c^{3,4}$	$0.55\pm0.08c^{3}$
Pearl	$1.41\pm0.01b^{1}$	$1.42\pm0.04b^{2}$	$1.09\pm0.01c^{1}$	$3.20\pm0.20a^{1}$

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

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

7.4.8 Superoxide radical scavenging activity

Table 7.6 presents the superoxide anion radical scavenging activity (SORSA) of whole grains and processed counterparts of different millet varieties. Superoxide anion radicals are generated *in vivo* when xanthine oxidase reduces molecular oxygen instead of nicotinamideadeninedinucleotide (NAD) under stress conditions (*44*). The SORSA of millet phenolic extracts was measured in the hypoxanthine-xanthine oxidase system as inhibition of superoxide anion radical generation compared to a control in the absence of antioxidative compound. The superoxide anion radical reduces yellow nitro blue tetrazolium solution into ink blue formazan at pH 7.4 and at

37° C. Antioxidative compounds reacting with superoxide anion radical inhibit production of formazan and reduce absorbance at 560 nm. The present results showed that millet phenolics are effective scavengers of superoxide anion radicals. The SORSA of whole, dehulled, cooked grains and hulls ranged from 0.50 to 1.41, 0.49 to 1.57, 0.47 to 1.09 and 0.44 to 3.20 FAE mmol/g defatted sample, respectively. In general, cooked grains of millet varieties had low SORSA and values ranged from 0.47 to 1.09 FAE mmol/g defatted meal. This is in agreement with the findings of Hedge and Chandra (*11*) that showed that the roasting and boiling of kodo and finger millets reduced their free radical quenching activity. Among all grain samples examined in this study, hulls of pearl millets had the highest SORSA which accounted for 3.2 FAE mmol /g defatted meal and it was 7 times higher than that of finger (Ravi) millet that showed the least activity. Superoxide anion radical plays an important role in several pathophysiological conditions owing to its ability to transform into more reactive hydroxyl radicals. Furthermore, superoxide radical itself also initiates lipid peroxidation (*45*). In a previous study it was shown that, except kodo millet, millet varieties examined were not effective xanthine oxidase inhibitors (*46*). Unno et al. (*47*) suggested that certain antioxidants may directly scavenge superoxide anion radical without inhibiting the xanthine oxidase activity. Previously Yen and Duh (*48*) also reported that antioxidant activity of flavonoids could be due to their scavenging of superoxide anion radicals. HPLC profiles of millet whole grain phenolics showed that, in addition to hydroxybenzoic and hydroxycinnamic acids, flavonoids as well were important constituents present in the extracts depending on the millet type (**Figure 7.1**; *19*) and may contribute to the observed effects. It should also be noted that in the present study, kodo whole grains displayed a superoxide radical scavenging

activity of 0.5 mmol FAE /g defatted meal which was 1.8 to 2.8 times less ($p \leq 0.05$) than those of other millet varieties employed in the present work. The HRSA observed for all samples examined was less than that of SORSA of the grain and hull extracts in this study. In agreement with the present results Nam et al. (*49*) showed that rice bran extracts had less HRSA than SORSA.

Madhujith et al. (*30*) reported that SORSA of barley extracts, Falcon and Metcalfe, at a concentration of 0.5 mg/mL, ranged from 82 to 94 and 73- 87%, respectively. The outermost fraction of the barley grain exhibited the highest SORSA (*30*). Furthermore, according to Liyana-Pathirana and Shahidi (*32*), bran of Amber durum and hard red spring wheat varieties had a higher SORSA compared to the corresponding flour fractions.

7.5 Conclusion

Health benefits of cereal phenolics and polyphenolics are promising for prevention of chronic diseases related to oxidative stress. The results presented in this study clearly demonstrated that dehulling of millet grains had a profound effect on the phenolic content and their antioxidant and antiradical properties. The magnitude of the antioxidant activity with dehulling depended on the millet type. In general hulls, undervalued waste product of millets had a high total phenolic content and antioxidant activities and may serve as a potential natural source of antioxidants. The findings of this study further showed that hydrothermal treatment employed, boiling for 15 min, does not affect the total phenolic content. However, the antioxidant activity differed depending on the type of millet and the assay employed. The final cooked millet grain product had a considerable phenolic content and antioxidant activity and thus

may play an important role in the prevention of chronic diseases and in promotion of health.

7.6 References

1. USDA, The 2005 dietary guidelines for Americans. **2005**, www.dietaryguidelines.gov
2. McDonough, C.M.; Rooney, L.W.; Earp, C.F. Structural characteristics of *Elusine coracana* (finger millet) using scanning electron and fluorescence microscopy. *Food Microstruc.* **1986**, 5, 247-256.
3. Ravindren, G. Studies on millets: Proximate composition, mineral composition and phytate and oxalate contents. *Food Chem.* **1991**, 39, 99-107.
4. Awika, J.M.; McDonough, C.M.; Rooney, L.W. Decorticating sorghum to concentrate healthy phytochemicals. . *J. Agric. Food Chem.* **2005**, 53, 6230-6234.
5. Monawar, L.Y. Food value of Sudanese indigenous cereal grains. PhD thesis, **1983**, University of Khartoum, Sudan
6. Ramachandra, G.; Virupaksha, T.K.; Shadaksharaswamy, M. Relationship between tannin levels and in vitro protein digestibility in finger millet (*Eleusine coracana* Gaertn). *J. Agric. Food Chem.* **1977**, 25, 1101-1104.
7. Dhankher, N.; Chauhan, B.M. Effect of temperature and period of fermentation on protein and starch digestibility (in vitro) of rabadi-pearl millet fermented food. *J. Food Sci.* **1987**, 52, 828-829.
8. Almeida-Dominguez, H.D.; Serna-Saldivar, S.O.; Gomezma, M.H.; Rooney, L.W. Production and nutritional value of weaning foods from mixtures of pearl millet and cowpeas. *Cereal Chem.* **1993**, 70, 14-18.
9. Shobana, S.; Malleshi, N.G. Preparation and functional properties of decorticated finger millet (*Eleusine coracana*). *J. Food Eng.* **2004**, 79, 529-538
10. Towo, E.E.; Svanberg, U.; Ndossi, G.D. Effect of grain pre-treatment on different extractable phenolic groups in cereals and legumes commonly consumed in Tanzania. *J. Sci. Food Agric.* **2003**, 83, 980-986.

11. Hegde, P.S.; Chandra, T.S. ESR spectroscopic study reveals higher free radical quenching potential in kodo millet (*Paspalum scrobiculatum*) compared to other millets. *Food Chem.* **2005**, 92, 177-182.

12. Zielinski, H.; Michalska, A.; Piskula, M.K.; Kozlowska, H. Antioxidants in thermally treated buckwheat groats. *Mol. Nutr. Food Res.* **2006,** 50, 824-832.

13. Sripriya G.; Chandrasekaran, K.; Murty, V.S.; Chandra, T.S. ESR spectroscopic studies on free radical quenching action of finger millet (*Elusine coracana*). *Food Chem.* **1996**, 57, 537-540.

14. Rao, M.V.S.S.T.S.; Muralikrishna, G. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Eleusine coracana* Indaf-15). *J. Agric. Food Chem.* **2002**, 50, 889-892.

15. Slavin, J.L.; Jacobs, D.; Marquart, L.; Wiemer, K. Grain processing and nutrition. *Crit. Rev. Biotechnol.* **2001**, 21, 49-66.

16. Liukkonen, K.; Katina, K.; Wilhelmsson, A.; Myllymaki, O.; Lampi, A.; Kariluoto, S.; Piironen, V.; Heinonen, S.; Nurmi, T.; Adlercreutz, H.; Peltoketo, A.; Pihlava, J.; Hietaniemi, V.; Poutanen, K. Process-induced changes on bioactive compounds in whole grain rye. *Proc. Nutr. Soc.* **2003**, 62, 117-122.

17. Chandrasekara, A.; Shahidi, F. The content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *J. Agric. Food Chem.* **2010**, 58, 6706-6714.

18. Chandrasekara, A.; Shahidi, F. Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. *J. Agric. Food Chem.* **2011**, 59, 428-436.

19. Chandrasekara, A.; Shahidi, F. Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n. *J. Functional Foods* **2011**, 3, 144-158.

20. Shahidi, F. Nutraceutical and functional foods: whole versus processed foods. *Trends Food Sci. Technol.* **2009**, 20, 376-387.

21. Garcia-Conesa, M.T.; Puumb, G.W.; Kroon, P.A.; Walace, G.; Williamson, G. Antioxidant properties of ferulic acid dimmers. *Redox Rep.* **1997**, 3, 239-244.

22. Zhou, K.; Laux, J.; Yu, L. Comparison of Swiss red wheat grain and fractions for their antioxidant properties in wheat bran. *J. Agric. Food Chem.* **2004**, 52, 1118-1123.

23. Zielinski, H.; Kozlowska, H. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *J. Agric. Food Chem.* **2000**, 48, 2008-2016.

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

25. Bonoli, M.; Verardo, V.; Marconi, E.; Caboni, M.F. Antioxidant phenols in barley (*Hordeum vulgare* l.) flour: comparative spectrophotometric study among extraction methods of free and bound phenolic compounds. *J. Agric. Food Chem.* **2004**, 52, 5195-5200.

26. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, 16, 144-158.

27. Wettasinghe, M.; Shahidi, F. Scavenging of reactive oxygen species and DPPH free radicals by extracts of borage and evening primrose. *Food Chem.* **2000**, 70, 17-26.

28. Chun, O.K.; Kim, D.; Lee, C.Y. Superoxide radical scavenging activity of the major polyphenols in fresh plums. *J. Agric. Food Chem.* **2003**, 51, 8067-8072.

29. Hag, M.E.E.; Tinay, A.H.E.; Yousif, N.E. Effect of fermentation and dehulling on starch, total polyphenols, phytic acid content and in vitro protein digestibility of pearl millet. *Food Chem.* **2002**, 77, 193-196.

30. Madhujith, T.; Izydorczyk, M.; Shahidi, F. Antioxidant properties of pearled barley fractions. *J. Agric. Food Chem.* **2006**, 54, 3283-3289.

31. Liyana-Pathirana, C.; Shahidi, F. The antioxidant potential of milling fractions from breadwheat and durum. *J. Cereal Sci.* **2007**, 45, 238-247.

32. Liyana-Pathirana, C.; Shahidi, F. Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chem.* **2007**, 101, 1151-1157.

33. Peterson, D.M.; Emmons, C.L.; Hibbs, A.H. Phenolic antioxidants and antioxidant activity in pearling fractions of oat groats. *J. Cereal Sci.* **2001**, 33, 97-103.

34. Zielinski, H.; Kozlowska, H.; Lewczuk, B. Bioactive compounds in cereal grains before and after hydrothermal processing. *Inn. Food Sci. Emerg. Technol.* **2001**, 2, 159-169.

35. Dewanto, V.; Wu, X.; Liu, R.H. Processed sweet corn has higher antioxidant activity. *J. Agric. Food Chem.* **2002**, 50, 4959-4964.

36. Bryngelsson, S.; Dimberg, L.H.; Kamal-Eldin, A. Effects of commercial processing on levels of antioxidants in oats (*Avena sativa* L) *J. Agric. Food Chem.* **2002**, 50, 1890-1896.

37. Hernanz, D.; Nunez, V.; Sancho, A.I.; Faulds, C.B.; Williamson, G.; Bartolome, B.; Gomez-Cordoves, C. Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley. *J. Agric. Food Chem.* **2001**, 49, 4884-4888.

38. Vogrincic, M.; Timoracka, M.; Melichacova, S.; Vollmannova, A.; Kreft, I Degradation of rutin and polyphenols during the preparation of tartary buckwheat bread. *J. Agric. Food Chem.* **2010**, 58, 4883-4887.

39. Zielinska, D.; Szawara-Nowak, D.; Zielinski, H. Comparison of spectrophotometric and electrochemical methods for the evaluation of the antioxidant capacity of buckwheat products after hydrothermal treatment. *J. Agric. Food Chem.* **2007**, 55, 6124-6131.

40. Bors, W.; Michel, C.; Saran, M Inhibition of the bleaching of the carotenoid crocin, a rapid test for quantifying antioxidant activity. *Biochim. Biophys. Acta.* **1984**, 796, 312-319.

41. Zielinski, H.; Michalska, A.; Amigo-Benavent, M.; Castillo, M.D.D.; Piskula, M.K. Changes in protein quality and antioxidant properties of buckwheat seeds and groats induced by roasting. *J. Agric. Food Chem.* **2009**, 57, 4771-4776.

42. Liyana-Pathirana, C.; Shahidi, F. Antioxidant activity of comercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions. *J. Agric. Food Chem.* **2005**, *53*, 2433-2440.

43. Halliwell, B.; Gutteridge, J. M. C. Role of free radicals and catalytic metal ions in human diseases: An overview. *Methods Enzymol.* **1990**, 186, 1-86.

44. Wickens, A.P. Aging and the free radical theory. *Resp. Physiol.* **2001**, 128, 379-391.

45. Chandrasekara, A.; Shahidi, F. Antiprolifirative potential and DNA scission inhibitory activity of phenolics from whole millet grains. *J. Functional Foods.* **2011**, 3, 159-170.

46. Unno T.; Sugimoto A.; Kakuda T. Scavenging effect of tea catechins and their epimers on superoxide anion radicals generated by a hypoxanthine and xanthine oxidase system. *J. Sci. Food Agric.* **2000**, 80, 601-606.

47. Yen, G.C.; Duh, P.D. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. *J. Agric. Food Chem.* **1994**, *42*, 629-632.

48. Nam S.H.; Choi, S.P.; Kang, M.Y.; Koh H.J.; Kozukue N.; Friedman M. Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. *Food Chem.* **2006**, 94, 613-620.

CHAPTER 8

Bioactivities of millet grains and hulls

8.1 Abstract

Antioxidant activities of phenolic extracts of kodo and pearl millet whole grains, dehulled grains, and hulls were examined by monitoring inhibition of radical-induced DNA scission, human low density lipoprotein (LDL) cholesterol and phospholipid liposome oxidation. Total phenolic content (TPC), hydroxyl and peroxyl radical inhibition and antiproliferative activities against HT-29 cells were also determined. Major hydroxycinnamic acids in dehulled grains and hulls were identified and quantified using HPLC. Phenolic extract of kodo millet exhibited higher inhibition activities against oxidation of LDL cholesterol and liposome than those of pearl millet. All phenolic extracts exhibited a dose-dependent inhibition of DNA scission. The TPC of hulls of kodo and pearl millets were three times higher than those of their corresponding whole grains. At the end of 96 h incubation kodo millet extracts inhibited cell proliferation in the range of 75-100%. Antioxidant activities of phenolic extracts was in order of hull > whole grain > dehulled grain. Dehulling reduced the antioxidant potential of whole millet grains. Ferulic and *p*-coumaric acids were the major hydroxycinnamic acids and their contents ranged from 17.8 to 1685 and 3.5 to 680 μg/ g defatted meal, respectively. Dehulled grains as well as the hull fraction, may serve as potential sources of nutraceutical and functional food ingredients in health promotion.

8.2 Introduction

The consumption of whole grain has been encouraged due to numerous health benefits arising from their bioactive constituents. Several epidemiological studies have shown a positive relationship between whole grain consumption and decrease of chronic diseases such as cardiovascular disorders, type 2 diabetes, several types of cancer, obesity and neurodegenerative diseases *(1)*. The protective effect of whole grains could be due to the presence of a number of phytochemicals, including phenolic compounds that may play a major role in disease risk reduction. Several *in vitro* studies have shown that polyphenolic compounds from a number of cereal grains and their products are effective antioxidants (*2-12*). Free radical mechanisms are implicated in the etiology of several degenerative diseases and phenolic compounds found in fruits, vegetable, spices, beverages and cereals have shown to be effective as antioxidants in a number of *in vitro* and *in vivo* studies (*13, 14*).

Cereals serve as a major staple food for many populations around the globe and millets are placed sixth among other cereals, accounting for about 1% of total cereal production in 2009 (*15*). Pearl millet is the major millet type cultivated mainly in Asia, and Africa and constitutes about 50% of global millet production. Kodo millet, which is considered as a minor millet, is predominantly grown in the Indian subcontinent. Previous studies have shown that kodo millet grain extracts possess a high *in vitro* antioxidant capacity, among others (*11, 12, 16*). In addition, antidiabetic effect of kodo whole millet grain in alloxan-induced diabetic rats has been reported (*17*).

Phenolic acids are the major phenolics identified in kodo and pearl millet grains with flavonoids being present in small amounts (*18*). Phenolics in cereal grains

exist as free, soluble conjuagated and insoluble bound compounds and it has been shown that the latter fraction in cereal grains is predominant (*5, 11, 19, 20*). However, enzymatic hydrolysis under acidic and alkaline conditions in the gastrointestinal tract may release phenolic compounds and hence could affect the antioxidant activity in the intestine, locally as well as systemically upon absorption (*19*). In addition, insoluble bound phenolics in cereal grains are released during fermentation in the colon (*21*). Thus, determination of antioxidant and other bioactivities of bound phenolics of grains is important.

Free radical attacks on biological molecules such as lipids, protein and DNA may be considered as an initiating stage for several chronic diseases. Free radical induced or metal ion dependent oxidation of low density lipoprotein (LDL) cholesterol is an important step in developing atherosclerotic lesions that leads to coronary heart diseases (*22*). It is well established that elevated levels of LDL cholesterol are associated with increased risk of atherosclerosis. Thus, dietary antioxidants that inhibit LDL oxidation may help reducing the occurrence of coronary heart disease. A number of early studies have indicated that phenolics in cereal grain are effective inhibitors of human LDL cholesterol oxidation (*23, 24*).

In addition, several studies have shown that phenolic compounds are effective against liposome oxidation, but no such information is available for millet grains. Cellular membrane damage and consequent peroxidation of phospholipids leads to interruption of the membrane assembly, and leads to changes in fluidity and permeability as well as alterations of ion transport and inhibition of metabolic processes and several pathological conditions (*25*). In a previous study, we showed that soluble millet phenolic extracts from whole grains were effective against DNA

scission and HT-29 cell proliferation (*26*). However, as millets are dehulled before consumption, it is necessary to examine the potential bioactivities of the edible part of the grain as well as hulls, as a processing byproduct, which can be used in pharmaceuticals and nutraceuticals applications. Therefore, the objectives of this study were (a) to evaluate the antioxidant activity of soluble phenolic extracts of whole grains, dehulled grains and hulls and bound fraction extracts of kodo and pearl millets (b) to determine the bioactivities, namely liposome oxidation inhibition, DNA oxidation inhibition, antiproliferation against adenocarcinoma cells and human LDL cholesterol oxidation inhibition of whole grains, dehulled grains and hulls and bound fraction extracts of kodo and pearl millets.

8.3 Materials and methods

Kodo (*Paspalum scrobiculatum*), and pearl millets (*Pennisetum glaucum*) harvested in 2007, were used in this study. Kodo millet samples were obtained from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka. Pearl millet (dark green cultivar), grown in India, was kindly supplied by Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India.

Folin Ciocalteu's reagent, ferulic acid, *p*-coumaric acid, 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), trolox, ferrous sulphate, ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), mono- and dibasic potassium phosphates, hydrogen peroxide, L-α-phosphatidylcholine type XVI-E, pBR 322 plasmid DNA, agarose, tris acetate, bromophenol blue, xylene cyanol, and glycerol, 5,5-dimethyl-1-1-pyrroline-*N*-oxide (DMPO), human LDL cholesterol were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). SYBR safe gel

stain was purchased from Probes Invitrogen (Eugene, OR, USA). Biological grade dimethyl sulphoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). McCoy's 5A medium was purchased from Invitrogen Co. (Carlsbad, CA, USA) and HT-29 cells were purchased from American Type Culture Collection (Rockville, MD, USA). Diethyl ether, ethyl acetate, hexane, acetone, methanol, chloroform, acetonitrile, formic acid, hydrochloric acid, sodium hydroxide and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada).

8.3.1 Sample preparation

Whole millet grains, cleaned from debris and soil particles, were used in the experiments. Whole grains were directly ground using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc., Brockville, ON, Canada) to obtain a fine powder which passed through mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH, USA). Another portion of whole millet grains was dehulled using Seedburo hand grinder (Seedburo Equipment Company, Chicago, IL, USA). The hulls were separated by air classification on a 757 South Dakota seed blower equipped with a column which utilizes an air flow generated by a blower motor combination to separate seed fractions by size and density (Seedburo Equipment Company, Chicago, IL, USA). A portion of dehulled grains and procured hulls were ground separately to obtain fine powders in a similar manner. All samples were defatted by blending with hexane (1:5, w/v, 5 min, 3 times) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA) at ambient temperature. Defatted samples were vacuum packed in polyethylene pouches and stored at –20°C until used within one week for extraction of phenolics.

8.3.2 Extraction of soluble phenolic compounds

Ultrasound-assisted extraction procedure was used for soluble phenolic compounds. Defatted meal (5 g) was mixed with 100 mL of 70% (v/v) acetone and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) and sonicated at the maximum power for 25 min under reflux conditions. After centrifugation of the resulting slurry for 5 min at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA), the supernatant was collected and extraction was repeated two more times. Combined supernatants were evaporated *in vacuo* at 40°C (Buchi, Flawil, Switzerland) and lyophilized for 72 h at -46°C and 34 x 10^{-3} mbar (Freezone, Model 77530, Labconco Co., Kansas City, MO, USA). The residues of whole grain samples were air dried for 12 h and stored at -20°C until used for extraction of bound phenolic compounds within a week. During all stages, extracts were protected from light by covering them with aluminium foil. Lyophilized crude phenolic extracts were stored at -20°C until used for further analysis.

8.3.3 Extraction of free and esterified phenolic compounds

Free phenolic acids and those liberated from soluble esters of whole grains, dehulled grains and hulls were extracted from the lyophilized crude phenolic extract based on a previously explained method (*11*). In brief, the pH of aqueous suspension of extract (250 mg in 10 mL) was adjusted to 2 with 6 M HCl; free phenolics were then extracted 5 times into a mixture (1:1, v/v) of diethyl ether and ethyl acetate. The free phenolic extract was evaporated to dryness under vacuum at room temperature. The water phase was neutralized to pH 7 with 2 M NaOH and then lyophilized. The

resulting residue was dissolved in 10 mL of 2 M NaOH and hydrolyzed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 with 6 M HCl, phenolic acids released from soluble esters were extracted from the hydrolyzates 5 times with a mixture (1:1, v/v) of diethyl ether and ethyl acetate and evaporated to dryness under vaccum.

8.3.4 Extraction of bound phenolic compounds

The residue of the grain samples obtained after extraction of soluble phenolics was hydrolyzed with 2 M NaOH at room temperature for 4 h with stirring under nitrogen. The resulting slurry was acidified to pH 2 with 6 M HCl and extracted 5 times with hexane to remove fatty acids released during alkaline hydrolysis. Bound phenolic compounds were extracted five times with diethyl ether and ethyl acetate (1:1, v/v) and subsequently desolventized to dryness at room temperature in a rotary evaporator. Phenolic compounds were reconstituted in 5 mL of HPLC grade methanol and stored at -20°C until used.

8.3.5 Determination of total phenolic content (TPC)

The total phenolic content of each extract was determined using the method described by Singleton and Rossi (*27*) with slight modifications. Briefly the crude extracts of soluble phenolic compounds were dissolved in methanol to obtain a concentration of 1 mg/mL. Folin Ciocalteu's reagent (0.5 mL) was added to centrifuge tubes containing 0.5 mL of extract and the contents mixed thoroughly by vortexing. The reaction was neutralized by adding 1 mL of saturated sodium carbonate to each tube, followed by the addition of distilled water (8 mL) and thorough mixing. Tubes were allowed to stand at room temperature in the dark for 35 min followed by centrifugation at 4000 x

g for 10 min. The absorbance of the resulting blue colour supernatant was measured at 725 nm (Model HP 8452A diode array spectrophotometer, Agilent Technologies, Palo Alto, CA, USA) using appropriate blanks for background subtraction. The content of total phenolics in each extract was determined using a standard curve prepared for ferulic acid and expressed as micromoles ferulic acid equivalents (FAE) per gram of defatted meal.

8.3.6 Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity measures antioxidant scavenging activity of test compounds against peroxyl radical generated by AAPH. The ORAC assay was based on the method explained by Madhujith and Shahidi (*23*) as reported elsewhere (*12*). All reactions mixtures were prepared in duplicate and three independent runs were performed for each sample. ORAC values of extracts were expressed as micromoles of trolox equivalents (TE) per gram of defatted meal using the standard curve calculated for each experiment.

8.3.7 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was determined according to the method explained by Madhujith and Shahidi (6) with slight modifications and as described by Chandrasekara and Shahidi (12), using electron paramagnetic resonance (EPR) spectrometry. Hydroxyl radical scavenging capacity of the extracts was calculated using the following equation. Hydroxyl radical scavenging capacity (%) = {(EPR signal intensity for the control – EPR signal intensity for the sample)/ EPR signal intensity for the control} x 100. The hydroxyl radical scavenging activity of the

extracts was expressed as micromoles of ferulic acid equivalents (FAE) per gram of defatted meal.

8.3.8 Determination of ferulic and *p*-coumaric acids contents by HPLC and HPLC/MS

Identification and quantification of phenolic compounds in whole millet grains showed that ferulic and *p*-coumaric acids were predominant phenolic acids, among others, in soluble and bound phenolic fractions of the grains (*18*). Therefore, ferulic and *p*-coumaric acids content of free, esterifed and bound phenolic extracts of dehulled grains and hulls were determined by HPLC analysis (*11*). The phenolic acid contents of free and esterified phenolic fractions are given as the total phenolics in the soluble fraction. Ferulic and *p*-coumaric acids were identified by comparing their relative retention times, and UV and ESI-MS spectra with authentic standards. An external standard method with ferulic and *p*-coumaric acids was used for quantification purposes.

8.3.9 Supercoiled plasmid DNA strand scission inhibition

Inhibition activity of millet phenolics against supercoiled strand DNA scission induced by peroxyl and hydroxyl radicals was evaluated according to Hiramoto et al. (*28*) and Liyanapathirana and Shahidi, (*24*) with slight modifications. Supercoiled plasmid DNA (pBR 322 from *Escherichia coli* RRI) was dissolved at a concentration of 50 μg/mL in 0.5 M, pH 7.4 phosphate buffer solution (PBS). Different concentrations of soluble millet phenolic extracts (0.0625 - 0.5 mg/mL) were prepared in PBS. In an Eeppendorf tube (500 μL), 2 μL of a solution of supercoiled plasmid DNA, PBS , phenolic extract, H_2O_2 (1 mM) and $FeSO_4$ (0.5 mM) were added in the

order stated to determine the inhibitory activity of millet extracts against hydroxyl radical induced DNA strand scission. The mixture was incubated at 37°C for 1 h in the dark (*28*). The loading dye (2 µL), consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol in distilled water, was added to the reaction mixture at the end of the incubation period.

In another experiment inhibitory effect of millet extracts against peroxyl radical induced DNA scission was investigated. In this, AAPH was dissolved in PBS in order to attain a final concentration of 9 mM which was then mixed with DNA and the extracts to a final volume of 10 µL. The incubation was done for 1 h at 37°C. A control with DNA alone and a blank devoid of phenolic extracts were prepared with each set of phenolic extracts tested.

The samples were electrophoresed using a 0.7% (w/v) agarose gel prepared in Tris-acetic acid-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5). SYBR safe was added at a concentration of 100 µL/L of TAE buffer as a gel stain. Submarine gel electrophoresis was run at 60 V for 5 h using a model B1A horizontal mini gel electrophoresis system (Owl Separation systems Inc., Portsmonth, NH, USA) and a model 300V power supply (WMR International Inc., West Chester, PA, USA) at room temperature in TAE buffer. The bands were visualized under transillumination of UV light using AlphaImager™ gel documentation system (Cell Biosciences, Santa Clara, CA, USA). The images were analyzed using Chemilmager 4400 software (Cell Biosciences, Santa Clara, CA, USA) to quantify DNA scission. The protective effect of millet phenolic extracts was calculated using retention percentage of the normalized supercoiled DNA as given below. DNA retention % = (Intensity of supercoiled DNA with the oxidative radical and extract / Intensity of

supercoiled DNA in control) X 100. The concentration of extracts that retain 50% (EC_{50}) of supercoiled DNA was then calculated.

8.3. 10 Inhibition of cupric ion-induced human LDL peroxidation

The method described by Andreasen et al. (29) was adopted to determine the human low density lipoprotein (LDL) cholesterol oxidation inhibitory activities of grain extracts. Human LDL cholesterol (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 LDL was subsequently diluted to obtain a standard protein concentration of 0.1 mg/mL with PBS. The diluted LDL cholesterol solution (0.8 mL) was mixed with 100 µL of extract (0.125 and 0.5 mg/mL) in an Eppendorf tube. Oxidation of LDL cholesterol was initiated by adding 0.1 mL of 100 µM CuSO4 solution in distilled water. The mixture was incubated at 37°C for 20 h. The initial absorbance (t=0) was read at 234 nm immediately after mixing and conjugated diene (CD) hydroperoxides formed at the end of 20 h were measured. The corrected absorbance at 20 h against 0 h was employed to calculate the percentage inhibition of CD formation using the following equation. Percentage inhibition of CD formation = ($Abs_{oxidative} - Abs_{sample}$)/ ($Abs_{oxidative} - Abs_{native}$)*100, where, $Abs_{oxidative}$ = absorbance of LDL mixture and distilled water with CuSO4 only; Abs_{sample} = absorbance of LDL with extract and CuSO4; Abs_{native} = absorbance of LDL with distilled water.

8.3. 11 Inhibition of liposome oxidation

The liposome suspension was prepared according to a previously described method (*30*). Briefly, α-phosphatidylcholine (PC, 20 mg) was dissolved in chloroform in a round bottom flask and the solvent was evaporated in a rotary evaporator *in vacuo* at

room temperature. After removing traces of solvent by nitrogen flush, 10 mL of 10 mM phosphate buffered saline (PBS) (pH 7.4, 150 mM NaCl) were added. The mixture was vortexed and sonicated for 1 and 3 min, respectively, before making the PC mixture to a final concentration of 0.5 mg/mL in PBS. To evaluate the inhibition of liposome oxidation induced by peroxyl radicals by the extracts, 0.8 mL of PC and 0.1 mL of extracts (0.5 mg/mL for whole grain, dehulled grain and hull extract: 50µL/mL for bound extract) were mixed in an Eppendorf tube (1.5 mL) and the mixtures were incubated for 5 min at 37° C before adding 0.1 mL of AAPH in PBS (10 mM). Conjugated diene hydroperoxides formed were measured on a 100 µL of sample diluted to 1 mL with methanol at 234 nm. The samples were drawn at regular intervals at 0, 6, 9 and 12 h. The control was prepared with PC mixture and PBS, and blanks were prepared with samples and PBS.

In another experiment, inhibitory activity of grain extracts against liposome peroxidation induced by hydroxyl radicals was determined. In this 0.8 mL of PC and 0.1 mL of extract were pre- incubated for 5 min at 37° C and 50 µL of each hydrogen peroxide (1 mM) and $FeSO_4$ (0.5 mM) were added followed by incubation; CD was measured as explained above. The results were expressed as percentage inhibition of liposome oxidation.

8.3. 12 Human colon adenocarcinoma cell proliferation inhibition

Cell proliferation inhibition was studied using HT-29 colorectal cancer (CRC) cell line, according to Wang *et al.* (*31*). The HT-29 cells were propagated in T-150T flasks in McCoy's 5A medium supplemented with 10% fetal bovine serum albumin (FBS) and 1% antibiotic/antimycotic. Cells were grown in a humidified atmosphere

containing 5% CO_2 at 37°C (*32,33*). The cells were plated at 2500 cells per well in a 96-well microplate and incubated for 24 h at 37°C. Soluble millet phenolic extracts dissolved in DMSO were introduced into the wells containing cell culture media to obtain final concentrations of 0.1 and 0.5 mg/mL. The concentration of bound extracts dissolved in ethanol was 0.015 and 0.003 µL/mL for high and low doses, respectively. The control consisted of cell culture media and DMSO. Both treatment levels and the control contained a final concentration of 0.99 % DMSO. The live cells on each of the wells were studied using an ATP-Lite 1 step kit (Perkin Elmer, Shelton, CT, USA) which produces luminescence proportionately to the amount of ATP present in viable cells. Luminescence readings were taken using a Victor multi-well plate reader (PerkinElmer, Shelton, CT, USA) immediately prior to the treatment and 4, 24, 48, 72 and 96 h afterwards. The treatment and control media were replaced every 24 h up to 96 h during the incubation period. The antiproliferative effects of millet phenolics against HT-29 cells were expressed as percent inhibition calculated against the control.

8.3. 13 Statistical analysis

All experiments were carried out in triplicates and data were reported as mean ± standard deviation. The differences of mean values among millet grain fractions was determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p<0.05$ significance level. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

8.4 Results and discussion

This study reported for the first time *in vitro* human LDL cholesterol oxidation inhibition, antiproliferation of adenocarcinoma cells, DNA scission inhibition and liposome oxidation inhibitory activities of phenolics extracted from dehulled grains of kodo and pearl millets and their corresponding hulls. These two millets were selected in this study based on the importance of pearl millet as the major type in global production and reported high antioxidant activity of kodo millet. The information generated in this study demonstrated the potential of using dehulled millet grains as therapeutic dietary agents and hulls, the processing byproducts, as potential nutraceuticals for reduction of diseases in which oxidative stress plays a role in their initiation and progression.

Table 8.1 Phenolic contents and antioxidant activities of whole millet grains, dehulled grains and hulls

	Whole grain (Soluble)	Dehulled grain (Soluble)	Hulls (Soluble)	Whole grain (Bound)
Total phenolic content (ferulic acid equiv µmol/g defatted meal)				
Kodo	32.4±0.93*c[1]	6.86±0.19d[1]	112±1.37a[1]	81.6±0.15*b[1]
Pearl	8.63±0.38*b[2]	8.50±0.03b[2]	34.3±1.69a[2]	9.14±0.17*b[2]
Oxygen Radical absorbance capacity (trolox equiv µmol/g defatted meal)				
Kodo	95.7±2.37c[1]	41.0±1.39d[1]	216±1.54b[1]	793±7.76a[1]
Pearl	60.3±8.38d[2]	95.9±10.1c[2]	214±0.35a[1]	176±18.2b[2]
Hydroxyl radical scavenging activity (ferulic acid equiv µmol/g defatted meal)				
Kodo	56.4±6.76c[1]	16.4±1.13d[1]	133±23.0b[1]	499±34.0a[1]
Pearl	59.6±1.01c[1]	2.13±0.89d[2]	196±8.26a[2]	80.9±0.49b[2]

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

For individual assays, values in each column having the same superscript values ([1] or [2]) are not significantly different ($p > 0.05$).

* Values are adapted from reference *(11)*.

8.4.1 Total phenolic content (TPC) and antioxidant activities

Table 8.1 presents the TPC and free radical scavenging activities of whole grain, dehulled grain, and hull soluble phenolic extracts and whole grain insoluble bound phenolic extracts from kodo and pearl millet grains. The TPC ranged from 6.9 to 112 and from 8.5 to 34.3 µmol FAE/g defatted meal for kodo and pearl millets, respectively. As expected hulls of both millet types had 4-16 times higher ($p \leq 0.05$)

TPC compared to their dehulled grain counterparts. Our work lends further support to the earlier studies reporting that dehulling significantly reduced the polyphenolic content of the two pearl millet cultivars, Standard and Ugandi (*34*). In addition, hulls of barley, oat and buckwheat grains showed higher TPC than that of the corresponding dehulled grains (*35*). Soluble extracts of kodo whole grain millet showed 3.8 times higher TPC than that of pearl millet.

It is noteworthy that upon dehulling pearl millet grains showed more TPC in the edible part than that of kodo millet indicating that the distribution of phenolic compounds in different grain fractions is dependent upon the variety tested. On the other hand, kodo millet hulls had 3 times more TPC than that of pearl millet suggesting its potential use as a source of natural antioxidant.

Oxygen radical absorbance capacity (ORAC) showed that millet grain phenolics were effective peroxyl radical scavengers. In biological systems peroxyl radical are formed through autooxidation of fats. In addition, compared to other oxygen centred radical species peroxyl radicals are stable, thus have ability to diffuse to distant cellular locations (*36*). Bound extracts of kodo millet grains showed the highest ORAC value, expressed as μmol trolox equivalents/ g defatted meal followed by soluble extracts of hulls, whole grains and dehulled grains (**Table 8.1**). It is noteworthy that despite the high TPC observed in the hull extracts of kodo millet, bound extracts showed the highest ORAC value suggesting that the content as well as composition of phenolics contribute to the antioxidant activity. In addition, the ORAC value of kodo hulls was 5 fold higher than that of the corresponding dehulled grains. Similarly, it was noted that pearl millet grain hulls had the highest ORAC value which was 2.2 times higher than that of its dehulled grain counterpart. The

edible fraction, pearl dehulled grains, had a 2 fold higher ORAC value compared to that of kodo.

Hydroxyl radicals can be generated in the body and may attack all biological molecules such as DNA, proteins, polyunsaturated fatty acids (PUFA) in membranes, among others. In the present study hydroxyl radicals were generated through the iron catalyzed Harber-Weiss reaction and EPR spectrometry was used to measure the presence of stable spin adduct formed between hydroxyl radical and DMPO. The hydroxyl radical scavenging activity (HRSA) of kodo and pearl millet grain fractions were 16.4-499 and 2.1-196 μmol FAE/g defatted meal, respectively. In addition, dehulled grains of both millet types had the least HRSA in accordance with the ORAC values and TPC. It is worthwhile to note that though dehulled pearl millet grains showed 2.3 fold higher ORAC value than that of kodo, a reverse trend existed for hydroxyl radical scavenging activity which was 8 times lower than that of kodo millet. Thus, the use of multiple test systems in the assessment of antioxidant activities of the extracts is warranted as antioxidant activity may differ based on the type of millet grain as well as the radical species used in the evaluation system employed. The present results demonstrated that millet hulls have a high potential as an attractive source of natural antioxidants. In general, the outer layers of plants such as peels, shells and hulls contain a high amount of phenolic compounds that protect the inner material from pest attacks, microbial invasion, and protection from environmental stress.

Table 8.2 Ferulic and *p*-coumaric acid contents (µg/g defatted meal) of whole millet grains, dehulled grains and hulls

	Ferulic acid		*p*-Coumaric acid	
	Kodo	Pearl	Kodo	Pearl
Whole grain (Soluble)	202±5.70*c[1]	82.5±1.40*c[2]	19.2±0.46*c[1]	20.8±0.18*b[1]
Dehulled grain (Soluble)	17.8±0.50d[1]	50.0±0.39d[2]	3.46±0.16d[1]	9.90±0.62c[2]
Hulls (Soluble)	617±0.14b[1]	222±0.89b[2]	25.7±1.69b[1]	73.6±0.49a[2]
Whole grain (Bound)	1685±1.00*a[1]	639±2.86*a[2]	680±6.33*a[1]	20.7±0.04*b[2]

Values in each column having the same letter are not significantly different ($p > 0.05$). For individual phenolic acid, values in each row having the same superscript values ([1] or [2]) are not significantly different ($p > 0.05$).

*Values are adapted from reference (*11*).

8.4.2 Determination of ferulic and *p*-coumaric acids contents

Table 8.2 summarizes the contents of ferulic and *p*-coumaric acids contents of different extracts of kodo and pearl millets as analyzed by HPLC and HPLC /MS. Ferulic and *p*-coumaric acids were mainly found in the insoluble bound extracts of the grain for both kodo and pearl millet grains and their contents ranged from 17.8 to 1685 and from 3.5 to 680 µg /g defatted meal. In addition, the results of the present study further established the fact that phenolic acids were mainly concentrated in the hulls of the grains. Kodo and pearl millets contained 34 and 4 fold, more ferulic acid,

respectively, compared to their dehulled counterparts. In general, *p*-coumaric acid content in hulls of kodo and pearl millet grains was 7 times higher when compared to their corresponding dehulled grains. In a previous study, Hernanz et al. (*37*) showed that the outermost fraction which mainly consists of husk and outer layers of barley grains had the highest concentrations of ferulic and *p*-coumaric acids as well as ferulic acid dehydrodimers that could contribute to their high antioxidant activity.

8.4.3 Supercoiled plasmid DNA strand scission inhibition

DNA molecules are easily attacked by free radicals and induced base modification as well as DNA strand scission that leads to mutagenesis could possibly be progressed to cancer. **Table 8.3** presents the effective concentrations of millet extracts needed to retain 50% (EC_{50}) of the supercoiled DNA (10 mM in the assay) in the presence of peroxyl and hydroxyl radicals. Thus, the extract with the least concentration required for 50% inhibition shows superior protection against oxidative damage to DNA. Kodo millet grain extracts showed higher ($p \leq 0.05$) protection against peroxyl radical induced DNA damage compared to corresponding pearl millet extract. **Plate 8.1** shows that decreased concentration of the whole grain extracts of kodo and pearl millets rendered a lesser protection against peroxyl radical induced DNA scission, thus demonstrating a dose dependent protection of extracts against oxidative stress on DNA molecules. The EC_{50} values of kodo extracts ranged from 0.02 to 0.17 mg/mL (4-34 mM in the assay). The results of the present work showed that millet antioxidants protected against both hydroxyl and peroxyl radicals induced DNA scission. In agreement with this finding, Madhujith and Shahidi (*7*) demonstrated that barley extracts at a concentration of 4 mg/mL inhibited oxidation of DNA by peroxyl and hydroxyl radicals by 83-92% and 53-65%, respectively.

Inhibition percentages of DNA strand scission induced by peroxyl radicals and hydroxyl radicals for different grain extracts of kodo and pearl millets at a concentration of 0.5 mg/mL (100 mM in the assay) is shown in **Figure 8.1**. All extracts tested in this study effectively protected DNA for scission and this may be partly attributed to the free radical scavenging activity of the extracts as demonstrated by ORAC values and HRSA in this study.

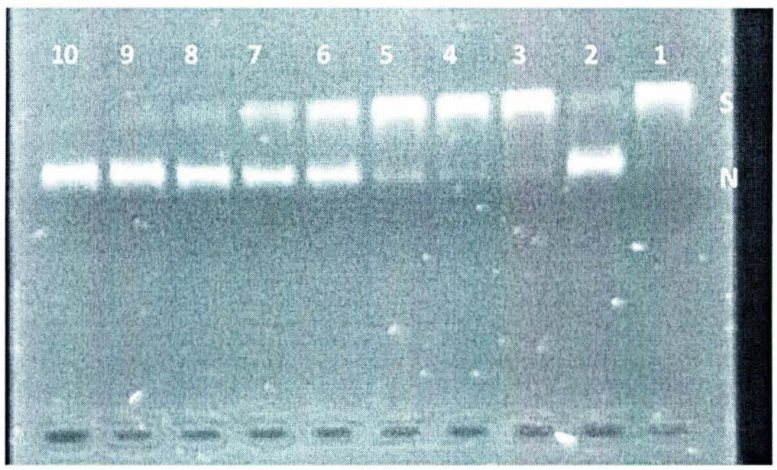

Plate 8. 1 Agarose gel electrophoresis of DNA treated with peroxyl radical (R) in the presence of millet whole grain (WG) soluble (S) phenolic extracts at 37° C. Concentrations of extracts were given in µg/mL of final assay. Lane 1= DNA, blank; Lane 2= DNA+R, control; Lane 3=DNA+R+kodo WG (S)100; Lane 4=DNA+R+ kodo WG (S)50 ; Lane 5= DNA+R+ kodo WG (S)25; Lane 6= DNA+R+ kodo WG(S) 12.5; Lane 7=DNA+R+ pearl WG(S) 100; Lane 8= DNA+R+ pearl WG (S) 50; Lane 9= DNA+R+ pearl WG(S) 25; Lane 10= DNA+R+ pearl WG (S) 12.5; S= Supercoiled DNA strands; and N= Nicked DNA strands.

The percentage inhibition of supercoiled DNA scission induced by peroxyl radicals of kodo and pearl millet extracts was 85-95 and 31-88 %, respectively (**Figure 8.1 A**). Despite the low content of TPC (**Table 8.1**) and phenolic acids (**Table 8.2**), dehulled grains of kodo millet had nearly 3 fold higher protection against peroxyl radical induced supercoiled DNA scission than that of pearl millet. This could be due to the presence of other phenolic compounds belonging to hydroxybenzoic acids and flavonoids that may possess effective antiradical activity (*18*). Percentage DNA strand scission inhibition induced by hydroxyl radicals by kodo and pearl millet extracts was 46-96 and 67-89%, respectively (**Figure 8.1B**). Hydroxyl radical is extremely reactive although short lived and has been implicated as a highly damaging free radical in cells. On the other hand, peroxyl radicals have a long half-life, thus greater affinity to diffuse into cells that leads to more macromolecular damage (*38*). This lends further support to previous findings that wheat phenolics extracts protected supercoiled plasmid pBR 322 DNA from scission by hydroxyl radicals (*24*). The outermost fractions obtained as byproducts of pearling were found to be more effective in the inhibition of DNA strand scission. The present work indicates that natural antioxidants in kodo and pearl millets dose dependently protected against DNA scission induced by both peroxyl and hydroxyl radicals, thus suggesting their potential use as a functional food ingredient to prevent carcinogenesis.

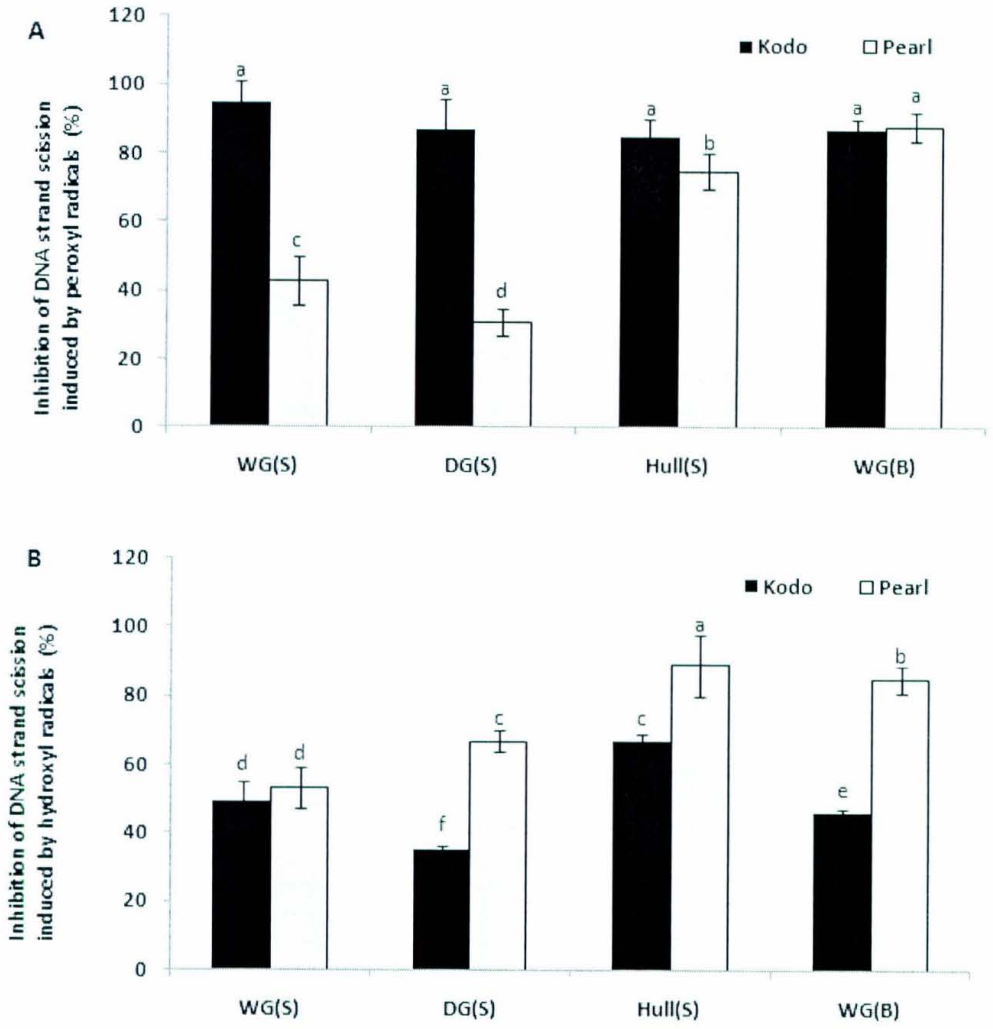

Figure 8.1 Retention percentage of supercoiled pBR 322 plasmid DNA in peroxyl radical-mediated (A) and hydroxyl radical-mediated (B) in vitro systems with phenolic extracts. WG(S),whole grain soluble; DG(S), dehulled grain soluble; hull(S), hulls soluble; and WG(B), whole grain bound at a concentration of 100 µg/mL and 20 µL/mL in final assay. Bars with different letters are significantly ($p < 0.05$) different from one another. The letter 'a' represents the highest value.

Table 8.3 EC_{50} values of grain extracts in retention of supercoiled DNA in the presence of peroxyl and hydroxyl radicals

	Peroxyl radical		Hydroxyl radical	
	Kodo	Pearl	Kodo	Pearl
Whole grain (Soluble)[a]	0.06±0.00b[1]	0.53±0.01a[2]	0.07±0.00c[1]	0.11±0.02b[2]
Dehulled grain (Soluble)[a]	0.17±0.04a[1]	0.61±0.01a[2]	0.23±0.04a[1]	0.22±0.01a[2]
Hulls (Soluble)[a]	0.02±0.00c[1]	0.20±0.01b[2]	0.15±0.04b[1]	0.10±0.01b[1]
Whole grain (Bound)[b]	3.00±0.00[1]	7.30±0.40[2]	3.80±0.40[1]	5.40±1.20[2]

Values in each column having the same letter are not significantly different ($p > 0.05$). For individual radical, values in each row having the same superscript values are not significantly different ($p > 0.05$).

[a] Concentration of extract is in mg/mL; [b] Concentration of extract is in µL/mL, 1 µL contains bound phenolics extracted from 1 µg of defatted meal.

8.4.4 Human colon adenocarcinoma cell proliferation inhibition

Colon cancer is the second most important cancer type in North America and its prevention and therapy is important. Consistent epidemiological evidences have indicated that a diet rich with fruits, vegetables and whole grains reduces the risk of many types of cancer suggesting that the dietary antioxidants could be effective in the reduction of cancer incidence (*39*). **Figure 8.2** and **Table 8.4** show that millet extracts effectively inhibited proliferation of HT-29 adenocarcinoma cells *in vitro*. These results further showed that cell proliferation inhibition by millet grain phenolic extracts was time and dose dependent. As shown in **Figure 8.2**, hull extracts at a

concentration of 0.5 mg/mL inhibited cell proliferation nearly 100% after a 24 h incubation and throughout the entire 96 h incubation period. **Table 8.4** presents the percentage of antiproliferative activity of millet extracts against HT-29 cells at the end of a 96 h incubation period. At a concentration of 0.5 mg/mL kodo and pearl hull extracts displayed 100 and 68% inhibition activity, respectively. On the other hand, even at a five-fold lower concentration (0.1 mg/mL), kodo hull extracts demonstrated 100% inhibition of cell proliferation . It was noted that hull extracts of both kodo and pearl millets exhibited superior antiproliferative activity than that of the corresponding dehulled grain extracts suggesting their potential use as natural agents in cancer therapy.

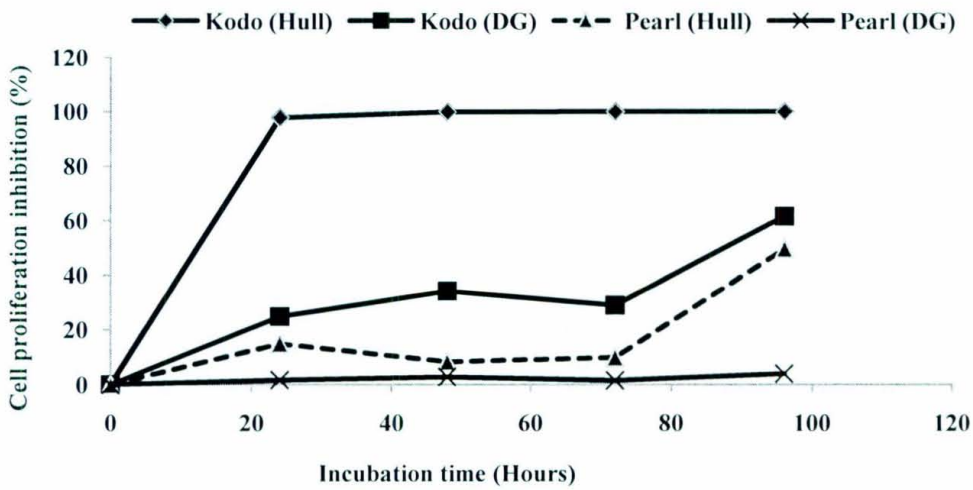

Figure 8.2 Percent inhibition of colon adenocarcinoma cell (HT-29) proliferation by millet soluble extracts. Dehulled grain (DG) and hulls at a concentration of 0.5 mg/mL, from day 0 to day 4 of incubation.

Table 8.4 Percentage inhibition HT-29 cell proliferation in the presence of millet extracts

Millets	Kodo		Pearl	
	0.5 mg/mL	0.1 mg/mL	0.5 mg/mL	0.1 mg/mL
Whole grain (Soluble)	100±0.01a[1]	35.5±2.27b[2]	53.6±2.83b[1]	52.7±4.80a[1]
Dehulled grain (Soluble)	75.5±7.40b[1]	24.8±4.01c[2]	33.3±0.54c[1]	43.8±3.40b[2]
Hulls (Soluble)	100±0.01a[1]	99.6±0.01a[1]	67.8±3.71a[1]	37.9±0.51b[2]
Whole grain (Bound) [a]	34.7±3.29c[1]	18.4±4.44d[2]	18.1±2.06d[1]	12.9±0.99c[2]

Values in each column having the same letter are not significantly different ($p > 0.05$). For individual millet type, values in each row having the same superscript values are not significantly different ($p > 0.05$).

[a]Concentration of extract are 0.015 and 0.003 µL/mL for high and low doses, respectively and 1 µL contains bound phenolics extracted from 1 µg of defatted meal.

The present results show that at a five fold lower concentration of the extracts pearl millet dehulled grain had 1.3 times higher antiproliferative activity than that at high concentration. It should be noted that at the lower concentration whole pearl millet grain and dehulled grain had higher inhibition of 52.7 and 43.8%, respectively, against HT-29 cells compared to their kodo millet counterparts. These results suggest that varying phenolic profiles of these two millet varieties may explain those observed

differences. In a previous study, we reported that soluble extracts of kodo millet grains also contain flavonoids such as vitexin, isovitexin, quercetin, luteolin, and apigenin at a higher concentration than that found in pearl millet grains (*18*). The potential of flavonoids as chemopreventive agents in carcinogenesis has been considered (*40*).

Table 8.5 Percentage inhibition of LDL cholesterol oxidation in the presence of millet extracts

	Kodo		Pearl	
	0.5 mg/mL	0.125 mg/mL	0.5 mg/mL	0.125 mg/mL
Whole grain (Soluble)	35.7±0.67c[1]	20.5±0.12c[2]	5.35±1.80c[1]	0.20±0.09d[2]
Dehulled grain (Soluble)	27.7±1.43d[1]	16.2±0.32d[2]	1.42±0.29d[1]	5.50±0.35c[2]
Hulls (Soluble)	58.2±0.16b[1]	24.5±0.96b[2]	9.34±0.82b[1]	14.5±3.02b[2]
Whole grain (Bound) [a]	70.8±0.58a[1]	34.1±1.54a[2]	32.9±1.67a[1]	18.6±1.10a[2]

Values in each column having the same letter are not significantly different ($p > 0.05$). For individual millet type, values in each row having the same superscript values are not significantly different ($p > 0.05$).

[a] Concentration of extract is 50 and 25 μL/mL for high and low doses, respectively, and 1 μL contains bound phenolics extracted from 1 μg of defatted meal.

8.4.5 Inhibition of human LDL cholesterol peroxidation

It has been shown that oxidation of LDL cholesterol plays a key role in the pathogenesis of atherescleorosis (*41*). Thus, prevention of LDL cholesterol oxidation reduces the diseases risk and complications. Antioxidant activity of millet grain phenolic extracts were determined by measuring the concentration of conjugated dienes formed during copper-catalyzed human LDL cholesterol oxidation *in vitro* and the results were expressed as percentage inhibition of oxidation based on the CD value after 20 h of incubation **(Table 8.5).** Bound phenolic extracts of kodo and pearl whole grain millets showed 71 and 31 % inhibition, respectively, at a concentration of 50 μL/mL. At each concentration of millet grain extract of 0.5 and 0.125 mg/mL, hulls displayed a higher inhibition against LDL cholesterol than that of dehulled grains. This may be attributable to the higher phenolic content of hulls as shown in the present work (**Tables 8.1 and 8.2**). Copper catalyzes the oxidation of unsaturated fatty acid moieties in the cholesterol molecule. The inhibition of LDL cholesterol oxidation by phenolics could account for chelation of cupric ions as well as scavenging of peroxyl radicals formed (*42*). Kodo millet extracts at 0.5 mg/mL had a higher inhibition percentage compared to that of the extracts at a lower concentration. On the other hand, pearl hulls and dehulled grain extracts at a low concentration (0.125 mg/mL) showed 1.6 and 3.9 times higher inhibition, respectively, than that at high concentration. Although the exact reason for this observation is not clear there is a possibility that phenolic compounds at high concentrations may complex with protein moieties of the LDL cholesterol molecules which makes them unavailable to inhibit oxidation of cholesterol. Several earlier studies have shown that phenolic compounds can inhibit protein oxidation by virtue of binding to the proteins, and

forming complexes with protein molecules (*43-45*). In addition, pearl millet contained proanthocyanidins at 5 μmol catechin equivalents/g defatted meal which may contribute to these observed effects (*11*).

Pure phenolic acids and flavonoids are known to inhibit copper induced LDL cholesterol oxidation (*46, 47*). Among different hydroxycinnamic acids, ferulic and *p*-coumaric acids showed weak inhibition of LDL cholesterol whereas caffeic acid displayed a high activity (*48*). The caffeic acid content of kodo and pearl millet grains was 48 and 30 μg/g defatted whole grain, respectively, suggesting their contribution to the observed effects (*18*). Furthermore, it has also been shown that mixtures of phenolic compounds are more effective in inhibition of LDL cholesterol oxidation (*48*). Synergistic activity of different phenolic compounds in a mixture could result in high antioxidant activity as demonstrated in the present work. In a previous study, Madhujith and Shahidi (*23*) showed that inhibition of LDL cholesterol oxidation of whole barley extracts ranged from 19 to 34% after 100 min of incubation. Furthermore, Liyana-Pathirana and Shahidi (*24*) showed that bound phenolic extracts of hard and soft wheats had a higher inhibition capacity of LDL oxidation than that of soluble extract as observed in the present work.

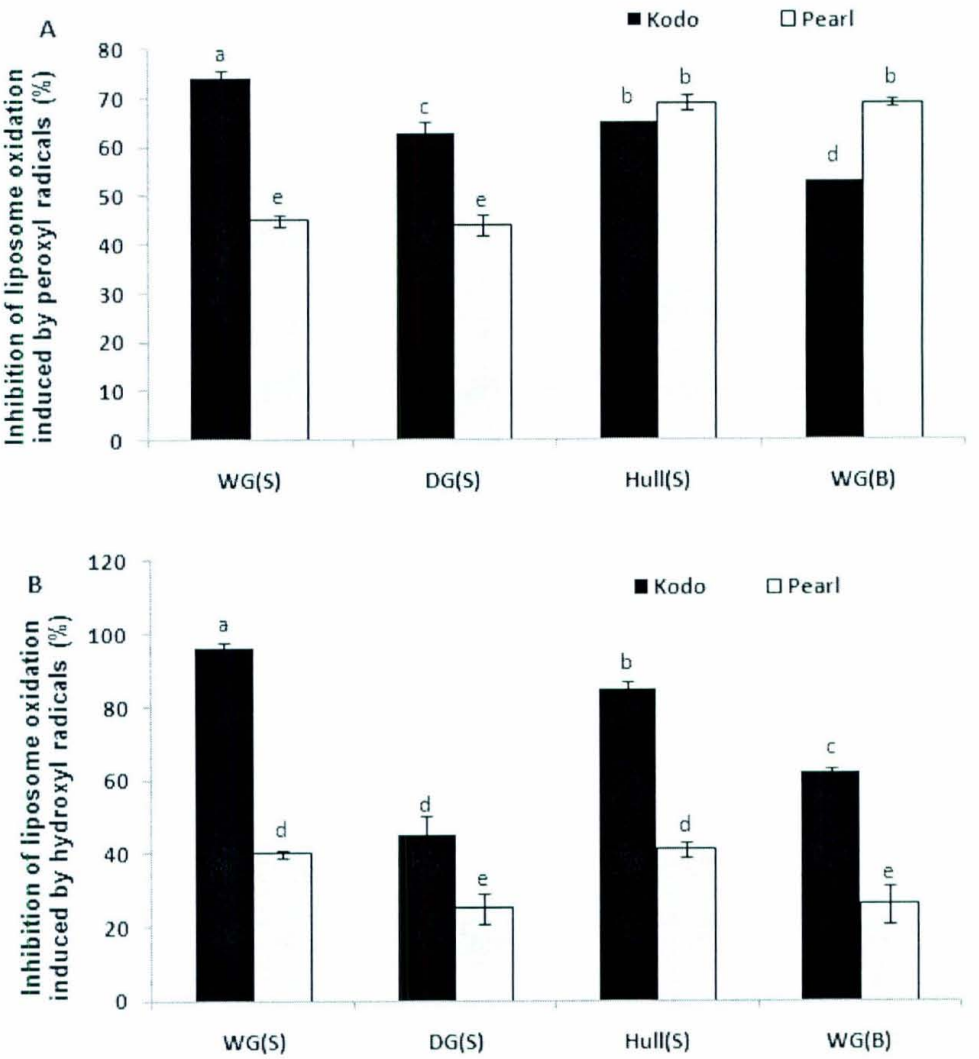

Figure 8.3 Inhibition of liposome oxidation induced by peroxyl radicals (A) and hydroxyl radicals (B) in the presence of phenolic extracts. WG(S), whole grain soluble; DG(S), dehulled grain soluble; hull(S), hulls soluble; WG (B), whole grain bound. Bars with different letters are significantly ($p < 0.05$) different from one another. The letter 'a' represents the highest value.

8.4.6 Inhibition of liposome oxidation

Figure 8.4 shows that millet grain phenolics are effective against inhibition of liposome peroxidation induced by peroxyl (**Figure 8.3A**) and hydroxyl (**Figure 8.3B**) radicals. The presence of kodo millet grain extracts caused a 53-74% inhibition of liposome oxidation induced by peroxyl radical, generated by AAPH after incubation for 6 hours at 37° C. Pearl millet extracts rendered 44-69% inhibition. Similarly, hull extracts of both millet types demonstrated a significantly ($p \leq 0.05$) higher liposome inhibition compared to that of dehulled grains, essentially due to their high phenolic content. Zielinski and Kozlowska (*35*) earlier demonstrated that hull extracts of buckwheat, oat and barley exhibited antioxidant activity in AAPH-induced lipid peroxidation in a PC-liposome system which was higher than that of dehulled grains. The protection of kodo and pearl millet extracts against hydroxyl radical induced liposome oxidation was 45-96% and 25-41%, respectively (**Figure 8.3B**).

Furthermore, hulls of kodo and pearl millet extracts had 1.9 and 1.6 fold higher inhibition percentage, respectively, compared to that of the dehulled grains. In addition, kodo millet grain extracts demonstrated higher protection against hydroxyl radical than that of pearl millet. Heinonen et al. (*49*) showed that ferulic acids had the highest antioxidant activity in a lecithin-liposome system and suppressed hydroperoxide formation. Furthermore, ferulic acid esters also showed potent inhibitory activity in PC liposome oxidation (*50*). The present results clearly show that millet grain phenolics are capable of protecting membrane lipids from oxidation caused by free radical reactions, thus saving cell membrane integrity and function. This protective effect could be due to the free radical scavenging and metal ion chelation activities of millet phenolics. Furthermore, phenolic compounds may

interact with membrane phospholipids by hydrogen bonding to the polar head groups of phospholipids and may accumulate at the surface of the membrane, thus preventing access of radicals to the lipid region. Verstraeten et al. (*51*) demonstrated that flavan-3-ols and procyanidins can potentially reduce liposome oxidation by limiting the access of oxidants to the bilayer.

8.5 Conclusion

The results of the present work demonstrated that dehulled grains of millet and hulls inhibited DNA scission, LDL cholesterol and liposome oxidation and proliferation of HT-29 adenocarcinoma cells. Bound phenolic extracts showed considerable bioactivity and release of these compounds in the colon upon microbial fermentation, hence may impart health benefits locally. Hydroxycinnamic acids, mainly ferulic and *p*-coumaric acids may contribute to the observed action of millet phenolics in addition to hydroxybenzoic acids and flavonoids. The results of the present study suggest that millet grains could serve as a promising cereal grain for incorporation in therapeutic diets. However, bioavailability and bioefficacy of millet phenolics upon absorption is yet to be unraveled.

8.6 References

1. McKeown, N.M. Whole grain intake is favorably associated with metabolic risk factors for type 2 diabetes and cardiovascular disease in the Framingham Offspring Study. *Am. J. Clin. Nutr.* **2002**, 76, 390-398.

2. Sun, T.; Ho, C-T. Antioxidant activities of buckwheat extracts. *Food Chem.* **2005**, 90, 743-749.

3. Awika, J.M.; McDonough, C.M.; Rooney, L.W. Decorticating sorghum to concentrate healthy phytochemicals. *J. Agric. Food Chem.* **2005**, 53, 6230-6234.

4. Yu, L.; Haley, S.; Perret, J.; Harris, M.; Wilson, J.; Qian, M. Free radical scavenging properties of wheat extracts. *J. Agric. Food Chem.* **2002**, 50, 1619-1624.

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

6. Wende, L.; Shan, F.; Sun, S.; Corke, H.; Beta, T. Free radical scavenging properties and phenolic content of Chinese black-grained wheat. *J. Agric. Food Chem.* **2005**, 53, 8533-8536.

7. Madhujith, T.; Shahidi, F. Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *J. Agric. Food Chem.* **2006**, 54, 8048-8057.

8. Liyana-Pathirana, C.; Shahidi, F. Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chem.* **2007**, 101, 1151-1157.

9. Viswanath V.; Urooj, A.; Malleshi, N.G. Evaluation of antioxidant and antimicrobial properties of finger millet (*Elusine coracana*). *Food Chem.* **2009**, 114, 340-346.

10. Qiu, Y.; Liu, Q.; Beta, T. Antioxidant activity of commercial wild rice and identification of flavonoid compounds in active fractions. *J. Agric. Food Chem.* **2009**, 57, 7543-7551.

11. Chandrasekara, A.; Shahidi, F. The content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *J. Agric. Food Chem.* **2010**, 58, 6706-6714.

12. Chandrasekara, A.; Shahidi, F. Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. *J. Agric. Food Chem.* **2011**, 59, 428-436.

13. Spencer, J.P.E. Flavonoids: modulators of brain function? *Brit. J. Nutr.* **2008**, 99, E-Suppl.1, ES60-ES77.

14. Manach, C.: Mazur, A.: Scalbert, A. Polyphenols and prevention of cardiovascular diseases. *Curr. Opin. Lipidol.* **2005**, 16, 77-84.

15. FAOSTAT, 2011 http:// www. faostat.fao.org. (Accessed on 12 Feburary, 2011).

16. Hegde P.S.; Chandra, T.S. ESR spectroscopic study reveals higher free radical quenching potential in kodo millet (*Paspalum scrobiculatum*) compared to other millets, *Food Chem.* **2005**, 92, 177-182.

17. Hegde P.S.; Rajasekaran N.S.; Chandra T.S. Effects of the antioxidant properties of millet species on the oxidative stress and glycemic status in alloxan-induced rats. *Nutr. Res.* **2005**, 25, 1109-1120.

18. Chandrasekara, A.; Shahidi, F. Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n. *J. Functional Foods.* **2011,** 3, 144-158.

19. Liyana-Pathirana, C.; Shahidi, F. Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions. *J. Agric. Food Chem.* **2005**, 53, 2433-2440.

20. Madhujith, T.; Shahidi, F. Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. *Food Chem.* **2009**, 117, 615-620.

21. Kroon, P.A.; Faulds, C.B.; Ryden, P.; Robertson, J.A; Williamson, G. Release of covalently bound ferulic acid from fiber in the human colon. *J. Agric. Food Chem.* **1997**, 45, 661-667.

22. Halliwell, B. Antioxidants and human diseases: A general introduction. *Nutr. Rev.* **1997**, 55, S44-S51.

23. Madhujith, T.; Shahidi, F. Antioxidative and antiproliferative properties of selected barley (*Hordeum vulgarae* L.) Cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. *J. Agric. Food Chem.* **2007**, 55, 5018-5024.

24. Liyana-Pathirana, C.; Shahidi, F. Antioxidant properties of wheat as affected by pearling. *J. Agric. Food Chem.* **2006**, 54, 1256-1264.

25. Nigam, S.; Schewe, T. Phospholipase A2s and lipid peroxidation, *Biochim. Biophys. Acta.* **2000**, 1488**,** 167–181.

26. Chandrasekara, A.; Shahidi, F. Antiprolifirative potential and DNA scission inhibitory activity of phenolics from whole millet grains. *J. Functional Foods.* **2011** 3,159-170.

27. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticul.* **1965**, 16, 144-158.

28. Hiramoto, K.; Ojima, N.; Sako, K.; Kikugawa, K. Effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. *Biol. Pharm. Bull.* **1996**, 19, 558-563.

29. Andreasen, M.F.; Landbo, A-K.; Christensen, L.P.; Hansen, A.; Meyer, A.S.; Antioxidant effects of phenolic rye (*Secale cereale* L.) extracts monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low density lipoprotein. *J. Agric. Food Chem.* **2001**, 49, 4090-4096.

30. Huang, S-W.; Frankel, E.N. Antioxidant activity of tea catechins in different lipid systems. *J. Agric. Food Chem.* **1997**, 45, 3033-3038.

31. Wang, C.Y.; Wang, S.Y.; Yin, J-J.; Parry, J.; Yu, L.L. Enhancing antioxidant, antiproliferation, and free radical scavenging activities in strawberries with essential oils. *J. Agric. Food Chem.* **2007,** 55, 6527-6532.

32. Qiao, L.; Koutsos, M.; Tsai, L-L.; Kozoni, V.; Guzman, J.; Shiff, S. J.; Rigas, B. Staurosporine inhibits the proliferation, alters the cell cycle distribution and induces apoptosis in HT-29 human colon adenocarcinoma cells. *Cancer Letters,* **1996**, 107, 83-89.

33. Yoshida, S.; Honda, A.; Matsuzaki, Y.; Fukushima, S.; Tanaka, N.; Takagiwa, A., Fujimoto, Y.; Miyazaki, H.; Salen, G. Antiproliferative action of endogenous dehydroepiandrosterone metabolites on human cancer cell lines. *Steroids.* **2003**, 68, 73-83.

34. Hag, M.E.E.; Tinay, A.H.E.; Yousif, N.E. Effect of fermentation and dehulling on starch, total polyphenols, phytic acid content and in vitro protein digestibility of pearl millet. *Food Chem.* **2002**, 77, 193-196.

35. Zielinski, H.; Kozlowska, H. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions *J. Agric. Food Chem.* **2000**, 48, 2008-2016.

36. Marnett, L.J. Peroxyl radical: potential mediators of tumor initiation and promotion. *Carcinogenesis.* **1987**, 8, 1365-1373.

37. Hernanz, D.; Nunez, V.; Sancho, A.I.; Faulds, C.B.; Williamson, G.; Bartolome, B.; Gomez-Cordoves, C. Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley. *J. Agric. Food Chem.* **2001,** 49, 4884-4888.

38. Hu, C.; Kitts, D.D. Evaluation of antioxidant activity of epigallocatechin gallate in biphasic model systems *in vitro*. *Mol. Cell. Biochem.* **2001**, 218, 147-155.

39. Egeberg, R.; Olsen, A.; Loft,S.; Christensen, J.; Johnsen, N.F.; Overvad, K.; Tjønneland, A. Intake of wholegrain products and risk of colorectal cancers in the Diet, Cancer and Health cohort study. *Brit. J. Cancer.* **2010**, 103, 730-734.

40. Pierini, R.; Gee, J.M.; Belshaw, N.J.; Johnson, T. Flavonoids and intestinal cancers. *Brit. J. Nutr.* **2008**, 99, ES53-ES59.

41. Esterbauer, H. Cytotoxicity and genotoxicity of lipid oxidation products. *Am. J. Clin. Nutr.* **1993**, 57, 7795-7855.

42. Decker, E.A.; Ivanov, V.; Zhu, B.Z.; Frei, B. Inhibition of low-density lipoprotein-oxidation by carnosine and histidine. *J. Agric. Food Chem.* **2001**, 49, 511-516.

43. Hagerman, A.E.; Butler, L.G. The specificity of proanthocyanidin-protein interaction. *J. Biol.Chem.* **1981**, 256, 4494-4497.

44. Siebert, K.J.; Troukhanova, N.V.; Lynn, P. Y. Nature of polyphenol-protein interactions. *J. Agric. Food Chem.* **1996**, 44 80-85.

45. Riedl, K. M.; Hagerman, A. E. Tannin-protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* **2001**, 49, 491-4923.

46. Meyer, A.S.; Heinonen, M.; Frankel, E.N. Antioxidant interactions of catechin, cyanidine, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chem.* **1998**, 61, 71-75.

47. Cirico, T.L.; Omaye, S.T. Additive or synergistic effects of phenolic compounds on human low density lipoprotein oxidation. *Food Chem. Toxicol.* **2006**, 44, 510-516.

48. Nardini, M.; D'Aquino, M.; Tomassi, G.; Gentili, V.; Felice, M.D.; Scaccini, C. Inhibition of human low density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Rad. Biol. Med.* **1995**, 19, 541-552.

49. Heinonen, M.; Rein, D.; Satu-Gracia, M.T.; Huang, S.; German, J.B.; Frankel, E.N. Effect of protein on the antioxidant activity of phenolic compounds in a lecithin-liposome oxidation system. **1998**, 46, 917-922.

50. Kikuzaki, H.; Hisamoto, M.; Hirose, K.; Akiyama, K.; Taniguchi, H. Antioxidant properties of ferulic acid and its related compounds. *J. Agric. Food Chem.* **2002**, 50, 2161-2168.

51. Verstraeten, S.V.; Keen, C.L.; Schmitz., H.H.; Fraga, C.G.; Oteiza, P.I. Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and disruption of the lipid bilayer structure. *Free Radic. Biol. Med.* **2003**, 34, 84-92.

CHAPTER 9

Bioaccessibility and antioxidant potential of millet grain phenolics as affected by simulated *in vitro* digestion and microbial fermentation

9.1 Abstract

Dehulled and cooked grains of five millet varieties (kodo, finger, proso, foxtail and pearl) were subjected to *in vitro* enzymatic digestion and microbial fermentation under physiological conditions in order to determine the bioaccessibility of their phenolic compounds. Extracts recovered as supernatants from enzymatic digestion and microbial fermentation were employed for the determination of their total phenolic content (TPC) and total flavonoid content (TFC), as well as inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH), peroxyl and hydroxyl radicals. Furthermore, trolox equivalent antioxidant capacity (TEAC), reducing power and ferrous ion chelating activity of the extracts so obtained were evaluated. The DPPH and hydroxyl radical inhibitions were determined using electron paramagnetic resonance (EPR) spectroscopy. The peroxyl radical activity was measured using oxygen radical absorbance capacity (ORAC) assay. The TPC ranged from 12.7 to 35.4 and 21.2 to 47.4 μmol ferulic acid equivalents per gram of grain, on a dry weight (dw) basis at the end of intestinal digestion and colonic fermentation, respectively. All five millet varieties exhibited effective antioxidant activity and the order of efficacy differed according to the assay employed. The present study showed that phenolic compounds of processed millets were bioaccessible and colonic fermentation released phenolics bound to the insoluble fibre in the grain.

9.2 Introduction

Evidences from epidemiological studies have shown that diets rich in plant foods are protective against several degenerative diseases such as cancer, cardiovascular ailments, diabetes, metabolic syndrome and Parkinson disease, among others (*1,2*). There are several phytochemicals which are responsible for those observed effects, polyphenols being the predominant ones (*3*). Phenolics and polyphenolics represent several groups of compounds that include hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, stilbenes and lignans (*4*).

There is a wide variability in the bioavailability of different phenolic and polyphenolic compounds (*2*). In recent years, a number of studies have reported the antioxidant activity of phenolic extracts from different plant sources. Many *in vitro* studies have shown that phenolic extracts exert antioxidant effects and their level of activity depends on the constituent phenolic compounds present and their synergistic interactions (*4*).

Millets are considered as one of the first cereal grains cultivated from the early human civilization and a recent archeobotanical study has shown that common millet, also known as proso millet (*Panicum miliacium*), was domesticated as a staple food 10,000 years ago in Northern China (*5*). At present millets are common staple foods for populations in Asian and African countries, particularly those belonging to lower income levels. In the western world, millets are primarily used as feed and forage for livestock. However, their popular use for multigrain products and applicability in niche markets for gluten free products and organic cereals is growing.

A limited number of *in vivo* studies on millet grains published so far has shown that millets are hypoglycaemic, hypocholesterolaemic, nephroprotective and anti-

cataractogenic (*6-9*). It should be noted that much of the early work has been concentrated on finger millet (*7,8*). Previously reported studies have shown that different millet types are rich sources of phenolic compounds with *in vitro* antioxidant and antiproliferative activities (*10-13*). In those studies, soluble and insoluble bound phenolic compounds present in whole grain millets were extracted using 70% acetone (v/v) and alkaline hydrolysis, respectively. These extracts that exhibited a wide range of antioxidant activity depended on the type of millet and assay employed.

Phenolics in the soluble extracts of grains are present as free or as conjugates bound to sugars, sugar alcohols, or amines. It has been shown that depending on the type of millet, a major proportion of phenolic compounds is linked to cell wall polysaccharides and as such constitutes the insoluble bound phenolics in the grain (*13*). This is similar to other cereals such as barley, wheat, corn, oats and rice (*14-17*). Hydroxycinnamates such as ferulic acids, the most abundant phenolics in the cereal grains, are ester bound to arabinoxylans in the cell walls by acetylating the hydroxyl group at the C5 position of α-L-arabinofuranosyl residue (*18*). They may also be etherified to lignin by cross linking with polysaccharides such as arabinoxylan. On the other hand, ferulates are oxidatively coupled via cross-linking with polysaccharide chains in the cell wall catalyzed by the action of peroxidase, to form di- and triferulates (*19*). The potential antioxidant activity of dehydrodimers of ferulates in the insoluble dietary fibre of cereals has been reported by Garcia-Conesa et al. (*20*).

Bioavailability of a compound can be defined as the quantity that passes through the cell membranes in the intestine and is available for action within the cells. Several studies have shown that phenolics from different plant extracts are bioavailable in *in vivo* studies using both animal models and human volunteers (*21-27*). On the other

hand, bioaccessibility is referred to the amount of compound that is released from the solid food matrix into the gut. Phenolic compounds released via enzymic digestion in the gastro-intestinal phase of the digestion and microbial fermentation in the colon are available in the gut and may potentially be bioavailable. The quantity of bioaccessible phenolics may vary depending on the food material and such information serves as a valuable guide to design *in vivo* bioavailability studies and *in vitro* studies to assess their bioactivities.

In vitro digestion and colonic fermentation models have previously been used to mimic the physiological release of phenolic compounds in some cereals and cereal-based products, among others (*28-31*). The determination of antioxidant activity before and after digestion and colonic fermentation processes also allows the evaluation of the stability of antioxidative phenolic compounds of foods in this respective environment, after released under physiological conditions. However, the release of phenolics from millet grain matrix under physiological conditions and their antioxidant activities has not yet been reported. Therefore, the objectives of the present study were (a) to determine the antioxidant activity of millet grain phenolics after being subjected to *in vitro* physiological pH variations and enzymic digestion and (b) to evaluate the availability and the antioxidant activity of insoluble bound phenolics of millet after *in vitro* microbial fermentation.

9.3 Materials and methods

Five millet grain samples, namely foxtail (*Setaria italica)*, proso (*Panicum miliacium)*, finger (*Elusine coracana*) (local), kodo (*Paspalum scrobiculatum*), and pearl (*Pennisetum glaucum*) millets harvested in the crop year 2007 were used in this

study. Pearl millet (dark green cultivar), grown in India and harvested in 2007 was obtained through Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India. All other grain samples were procured from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka.

Folin Ciocalteu's reagent, ferulic acid, 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium chloride, sodium bicarbonate, potassium chloride, calcium chloride, magnesium chloride, urea, trolox, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, ferrous chloride, cobalt chloride, sodium sulphate, ferrous sulphate, manganese sulphate, zinc sulphate, copper sulphate, ammonium molybdate, ascorbic acid, ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), mono- and dibasic sodium phosphates, 3-(2-pyridyl)-5-6-diphenyl-1,2,4,-triazine-4,4-disulphonic acid sodium salt (ferrozine), L-cysteine, methylene blue, acetic, propionic and butyric acids and porcine α-amylase, pepsin, bile salt, pancreatin and mucin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Methanol, hydrochloric acid, sodium hydroxide and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON).

9.3.1 Preparation of millet grain samples

Whole millet grains, cleaned to remove any debris and soil particles, were used in this study. Whole grains were dehulled using a Seedburo hand grinder (Seedburo Equipment Company, Chicago, IL) and the hulls were separated by air classification using a 757 South Dakota seed blower (Seedburo Equipment Company, Chicago, IL). The dehulled grains (30 g) so obtained were boiled with distilled water (300 mL) on a hot plate for 15 min with intermittent stirring, using a glass rod. After cooling to room

temperature, cooked grains were transferred to a –20°C freezer and lyophilized at -46°C and 34 x 10^{-3} mbar (Freezone, Model 77530, Labconco Co., Kansas City, MO). The lyophilized samples were ground to pass through a 16 mesh size sieve (sieve opening 1 mm, Tylor test sieve, Mentor, OH) and used for *in vitro* enzymic digestion and subsequent microbial fermentation.

9.3.2 Extraction of phenolics with water

Phenolics from millet grains were extracted into distilled water by incubating at 37°C in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific, Edison, NJ) at 200 rev/min for 5 h and 15 min (equal to the total period of enzymic digestion simulated). At the end of incubation, the resulting slurries were centrifuged for 10 min at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA). The supernatant was subsequently collected and stored at -20°C, followed by freeze drying for 72 h at -46°C and 34 x 10^{-3} mbar (Freezone, Model 77530, Labconco Co., Kansas City, MO).

9.3.3 Simulated *in vitro* enzymic digestion of millet grains

In vitro digestion of cooked dehulled grains was performed according to the method of Aura et al. (*32*) with slight modifications. The ground grain sample was weighed (1000 mg) into a 50 mL screw capped conical flask. Distilled water (15 mL) and 10 mL of 0.85% (w/v) sodium chloride solution were added to each sample followed by incubation at 37°C for 10 min in a Gyrotory water bath shaker at 200 rev/min. Four glass marbles were added to each flask for uniform mixing during incubation. After samples were brought to 37°C, 1 mL of porcine α-amylase (50 units/mL, EC 232-565-6) diluted in 20 mM sodium phosphate buffer (pH 6.9) containing 1 mM calcium

chloride were added. At the end of 5 min incubation period, 4.5 mL of 0.15 M hydrochloric acid were added and the pH was maintained below 2.5. Porcine pepsin (1 mL of 20 mg/mL, EC 232-629-3) dissolved in 20 mM hydrochloric acid was mixed with the sample and incubated for 2 h under the same conditions. Subsequently, 4 mL of bile salt (150 mg/mL), dissolved in 0.15 M sodium bicarbonate, 4 mL of porcine pancreatin (18.75 mg/mL, EC 232-468-9) diluted in 0.15 M sodium bicarbonate and 1 mL of porcine mucin (75 mg/mL, EC 282-010-7) dissolved in distilled water were added and the incubation was continued for another 3 h. The pH was maintained at 6.9. The resulting slurries were centrifuged at 4000 x g for 10 min and the supernatant was decanted. The centrifugation was repeated two times after adding 25 mL of distilled water and mixing well at each time to collect soluble compounds from the samples. The supernatants so obtained were combined and stored immediately at -20°C, before subsequent freeze drying for 72 h. The residue obtained after enzymatic digestion was lyophilized and used for the *in vitro* microbial fermentation.

To evaluate the effect of digestion during gastric phase, on the release of phenolics and their antioxidant activities, grain samples were prepared separately and incubated with relevant buffers and enzymes in a similar manner up to the end of gastric phase (2 h) followed by centrifugation, as explained above. The supernatants collected were combined, stored at -20°C, and subsequently lyophilized. Three replicates were used for each grain sample employed for the gastric and gastrointestinal digestion phases.

A blank (without added grain sample) was incubated under the same conditions and used in the determinations of phenolic content and antioxidant activity

for the correction of interference from the digestive enzymes and buffers. Under the same enzymatic and incubation conditions sugars and aromatic amino acids released may interfere with the analysis of phenolics and antioxidant activities. Therefore, preliminary studies were conducted using corn starch, glucose, egg albumin, and tyrosine; the results indicated that such interference were negligible.

9.3.4 Extraction under simulated gastric pH conditions

To determine the effect of changing gastrointestinal pH conditions throughout the digestion process phenolics from millet grains were extracted into buffers. In this, samples were treated similarly as in the simulated enzymic digestion process explained above but only distilled water, acid, alkali or buffer solutions were added and incubated at 37°C in a water bath shaker for 5 h and 15 min.

9.3.5 *In vitro* microbial fermentation

9.3.5.1 Culture medium

The culture medium was comprised of the following constituents in distilled water (g/L) $NaHCO_3$, 9.240; $Na_2HPO_4.2H_2O$ 3.542; NaCl 0.470; KCl 0.450; $Na_2SO_4.10H_2O$ 0.227; anhydrous $CaCl_2$; 0.055; $MgCl_2.6H_2O$ 0.100; and urea, 0.400. The trace elements mixture was prepared with 3680, 1159, 440, 120, 98, 17.4 mg/L of $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, $Mo_7(NH_4)_6O_{24}.4H_2O$, respectively, and added 10 mL per litre of culture medium (*33*). Subsequently, the culture medium was boiled for 15 min and after cooling to room temperature, its pH was adjusted to 7 with 5 M HCl. Then filter sterilized L-cysteine was added at 250 mg/L as a reducing agent and stored at 4° C until used after purging

with N_2 gas. A separate control culture medium, in which methylene blue was added as a redox indicator, was prepared and maintained throughout the experiment to assure the maintenance of reduced conditions in the medium.

9.3.5.2 Preparation of inocula

Fresh feces were collected from three male and three female Yucatan miniature pigs from the Vivarium pig breeding station, Animal care services, Memorial University of Newfoundland. The age and weight of animals were 9-18 months and 50-60 kg, respectively. Animals were fed with pig pusher, a standard diet for growing pigs, and had not received antibiotics at any time. Fresh faeces were immediately collected in a BD GasPak ^{TM} EZ pouch systems (BD Diagnostics, Sparks, MD) which generate an anaerobic environment. Under anaerobic conditions, faeces were quickly pooled and homogenized with sterile, anaerobic culture medium in a Waring blender (Model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT) for 2 min to prepare 10% (w/v) faecal slurry. The slurry was filtered using a sterile cheesecloth to remove large particles and used immediately as the inoculum for the fermentation.

9.3.5.3 *In vitro* colonic fermentation

The batch microbial fermentation was carried out as explained by Karppinen et al. (*33*) with minor modifications. Under anaerobic conditions, 5 mL of sterile culture medium were added to the lyophilized residue obtained after enzymic digestion in autoclaved 50 mL glass flasks for 16 hours before the start of the fermentation experiment in order to adequately hydrate the insoluble residue. Capped and sealed flasks were stored at 4^{o} C to minimize microbial growth. Two hours before the

inoculation, flasks were transferred to an anaerobic chamber at room temperature. Immediately after the addition of 20 mL of inoculum, flasks were flushed with nitrogen, sealed and incubated at 37° C for 24 h in a shaking water bath at 100 rev/min. A similar volume of faecal inoculum and culture medium without any added substrates were incubated simultaneously as blanks. All fermentations were carried out in triplicate. At the end of incubation, 1 mL from each sample was drawn and stored at -20°C for the analysis of short chain fatty acids (SCFA) which are known to be indicative of occurrence of active fermentation by colonic microflora. Fermented slurries were centrifuged for 15 min at 4000 x g (4°C) and the collected supernatants were immediately stored at -80°C and subsequently lyophilized.

9.3.5.4 Analysis of short chain-fatty acids

The thawed fermented sample (1 mL) in eppendorf tube was centrifuged at 10,000 x g for 5 min (Model 5415, Centrifuge eppendorf, Brinkmann Instruments Inc., Westbury, NY) and the supernatant filtered using 0.45 µm polytetrafluoroethylene (PTFE) membrane syringe filter (Whatman Inc., Florham Park, NJ). Aqueous solutions of fermented samples so obtained were directly analyzed by HPLC using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) according to the method explained by Anderson and Hedlund (*34*) with slight modifications. Separations were conducted with a SUPELCOGEL^{TM} C-610H column (30 cm × 7.8 mm; SUPELCO, Bellefonte, PA). The mobile phase consisted of 0.1% phosphoric acid and isocratic elution was used at a flow rate of 0.7 mL/min. The column temperature was maintained at 30° C. The detection of compounds was performed at 210 nm. Acetic,

propionic and butyric acids were identified by comparing their relative retention times with authentic compounds and external standard method was used for quantification.

9.3.6 Total phenolic content

The total phenolic content of each extract was determined using the method described by Singleton and Rossi (*35*) with slight modifications as explained elsewhere (*10*). Lyophilized samples were dissolved in methanol to obtain a concentration of 2 to 5 mg/mL. The content of total phenolics in each sample was determined using a standard curve prepared for ferulic acid and expressed as micromoles ferulic acid equivalents (FAE) per gram of cooked grain (dw).

9.3.7 Total flavonoid content

Total flavonoid content was determined using a colourimetric method as explained by Kim et al. (*36*) and Chandrasekara and Shahidi (*10*). Methanolic solutions of lyophilized samples were used for the analysis. Total flavonoid content calculated from a standard curve for catechin, was expressed as micromoles catechin equivalents (CE) per gram of cooked grain (dw).

9.3.8 DPPH radical scavenging activity

The determination of the effect of lyophilized samples on the scavenging of DPPH radicals was based on a procedure previously described by Chandrasekara and Shahidi (*11*). A Bruker E-scan EPR spectrometer (Bruker E-scan, Bruker Biospin Co. Billercia, MA) was used to record the spectrum. DPPH radical scavenging capacities of the extracts were calculated using the following equation. DPPH radical scavenging capacity (%) = {(EPR signal intensity for the control – EPR signal

intensity for the sample)/ EPR signal intensity for the control} x 100. The standard curve was prepared using ferulic acid and the DPPH radical scavenging activity was expressed as micromoles FAE per gram of cooked grain (dw).

9.3.9 Trolox equivalent antioxidant capacity (TEAC)

The total antioxidant capacity of the millet extracts was determined according to the method of van den Berg et al. (*37*) with slight modifications as explained by Chandrasekara and Shahidi (*10*). Digested and fermented millet samples were dissolved in PBS at a concentration of 5 mg/mL and further diluted to fit them within the range of values in the standard curve (6.25-50 μM prepared using trolox). TEAC values were expressed as micromoles trolox equivalents (TE) per gram of cooked grain (dw).

9.3.10 Reducing power

The reducing power of digested and fermented millet lyophilized samples was determined according to the method of Oyaizu (*38*) as summarized by Chandrasekara and Shahidi (*10*). The standard curve was prepared using ascorbic acid. Increased absorbance of the reaction mixture indicated increased reducing power. The results were expressed as micromoles ascorbic acid equivalents (AAE) per gram of cooked grain (dw).

9.3.11 Ferrous ion chelating activity

The ability of millet phenolic extracts to chelate ferrous ions was measured according to the method described by Dinis et al. (*39*) as explained elsewhere (*10*). Lyophilized samples obtained from enzymic digestions and microbial fermentation dissolved in

distilled water at different concentrations (2-5 mg/mL) were used to measure their chelating activity for ferrous ions. The standard curve was prepared using Na_3EDTA and the inhibition percentage of ferrozine-ferrous ion complex formation was calculated by the following equation. Metal chelating activity (%) = {1-(Absorbance of the sample / Absorbance of the control)} x 100. The results were expressed as micromoles EDTA equivalents per gram of cooked grain (dw).

9.3.12 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was based on that previously explained by Chandrasekara and Shahidi (*11*) with minor modifications. The samples, standards and other reactants were prepared in 75 mM phosphate buffer (pH 7.0) and the final reaction mixture (295 µL) contained 200 µL (0.11 µM) of fluorescein as oxidizable substrate, 20 µL of extract or trolox and 75 µL of AAPH (63.4 mM). The reaction was carried out in a Costar® 3695 flat bottom 96 well black microplates (Corning Incoperated, Corning, NY). Determination of ORAC was carried out using a plate reader equipped with a built-in incubator (Bio Tek®, BioTek Instruments, Inc., Winooski, VT). Fluorescein (200 µL) was manually pipeted into the wells containing the extract or standards (20 µL), followed by incubation for 15 min at 37°C. At the end of incubation, APPH (75 µL) was manually pipette into wells. The plate was automatically shaken for 4 s after addition of AAPH and the microplate reader was programmed to perform additional shaking of the contents in wells before each reading was taken. A gain adjustment was performed before the beginning of the measurements to optimize the maximum sensitivity. Fluorescence recorded every minute for 25 cycles and each cycle was 210 s. A control (phosphate buffer, fluorescein and AAPH) and different concentrations

of Trolox (6.25 -50 μM), as the standard, were used in each assay. All reaction mixtures were prepared in duplicate and three independent runs were performed for each sample. ORAC values of extracts were expressed as micromoles TE per gram of cooked grain (dw) using the standard curve calculated for each experiment.

9.3.13 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was determined according to the method explained by Chandrasekara and Shahidi (*11*). Lyophilized digested and fermented samples of millets were dissolved in deionized water and diluted appropriately. Ferulic acid dissolved in deionized water was used to prepare the standard curve (0.05-2.0 mg/mL). Hydroxyl radical scavenging capacities of the extracts were calculated using the following equation. Hydroxyl radical scavenging capacity (%) = {(EPR signal intensity for the control – EPR signal intensity for the sample)/ EPR signal intensity for the control} x 100. The hydroxyl radical scavenging activity of the extracts was expressed as micromoles FAE per gram of cooked grain (dw).

9.3.14 HPLC analysis of enzyme digested and fermented phenolic compounds

Phenolic compounds were extracted into ethyl acetate and diethyl ether after acidifying to pH 2 with 2 M HCl of aqueous solutions of enzymic digested and fermented millet grain samples. Phenolic compounds present were determined by HPLC and HPLC/MS analysis as explained previously (*10*).

9.3.15 Statistical analysis

All experiments were carried out in triplicates unless otherwise stated and data were reported as mean ± standard deviation. The differences of mean values among millet

varieties and differently treated samples within a single millet variety was determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple rank test at $p \leq 0.05$ significance level. Correlation analysis was performed between phenolic contents and antioxidant activities of extracts using Pearson correlation analysis. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

9.4 Results and discussion

The present study investigated the bioaccessibility of millet grain phenolics as affected by *in vitro* enzymic digestion and colonic fermentation. The information generated in the present study is important since the phenolic compounds released from food matrices are potentially bioavailable and may exert antioxidant activity in the gastrointestinal tract. Plant extracts may exert their antioxidative function by virtue of donating hydrogen or electrons to free radicals, chelating transition metals such as ferrous ion, or quenching singlet oxygen, among others. Thus, the use of different methods to measure antioxidant activity of such extracts serves to understand their mode of action. To the best of our knowledge, this study reports for the first time the bioaccessibility of soluble as well as insoluble bound phenolic compounds of different millet grains released under simulated physiological conditions and their antioxidant activities.

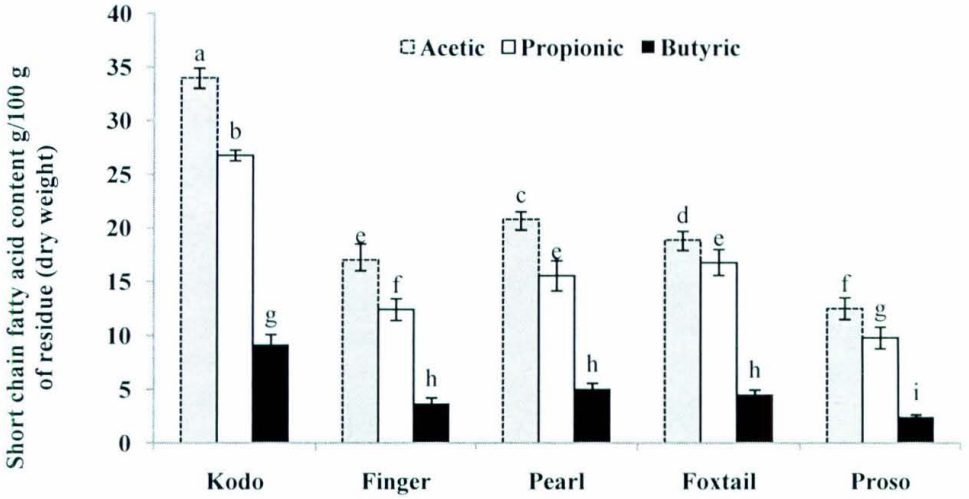

Figure 9.1 The contents of acetic, propionic and butyric acids produced in the *in vitro* microbial fermentation of millet grains

9.4.1 Short chain fatty acid content

Indigestible carbohydrates were fermented by the colonic microflora, and short chain fatty acids (SCFA), comprised mainly of acetic, propionic and butyric acids, were produced during colonic fermentation (*40*). In the present study, *in vitro* fermentation of insoluble fraction of millet grains by microbes was followed by measuring the production of SCFA at the end of 24 h fermentation period (**Figures A.1-2**). The contents of insoluble residue (mainly consisted of insoluble fibre) obtained after enzymic digestion of kodo, finger, pearl, foxtail and proso millets were 81, 179, 132, 157, and 210 mg /g of cooked grain (dw), respectively. **Figure 9.1** shows the contents (corrected values for fermentation due to inoculum) of acetic, propionic and butyric acids produced during the fermentation of millet grains. The total content of SCFA, which included acetic, propionic and butyric acids produced ranged from 25 to 70 g/100 g of insoluble residue (dw) of millet grains. In general acetic acid (50%) was the

main SCFA produced upon fermentation of millet grains, followed by propionic (37-41%) and butyric (9-13%) acids. In a previous study, Karppinen et al. *(33)* showed production of 58-60% acetic acid in total SCFA at the end of a 24 h *in vitro* microbial fermentation of rye, wheat and oat bran by human faecal inoculum.

Table 9.1 Phenolic contents of millet grain aqueous extracts (AQE), and extracts obtained subjecting to simulated *in vitro* gastrointestinal pH conditions (EPH), gastric digestion (EGD), gastrointestinal digestion (EGID) and colonic fermentation (EFM)

Millet	AQE	EPH	EGD	EGID	EFM
Total phenolic content (µmol FAE/g cooked grain, dry weight)					
Kodo	4.33±0.08d^{2}	2.98±0.36d^{3}	10.3±0.12b^{4}	12.7±1.12a^{4}	9.19±0.53b^{2}
Finger	12.0±0.22c^{1}	5.89±0.16d^{1}	26.9±0.21b^{1}	31.2±0.60a^{2}	4.47±0.30e^{2}
Pearl	4.24±0.36d^{2}	4.56±0.36d^{2}	18.5±0.28b^{2}	35.4±0.96a^{1}	6.73±0.02c^{2}
Foxtail	2.26±0.23d^{3}	1.46±0.07d^{4}	10.2±0.46c^{4}	30.8±0.98a^{2}	16.6±0.81b^{1}
Proso	2.01±0.17d^{3}	1.54±0.20d^{4}	11.2±0.17c^{4}	28.6±1.86a^{3}	17.1±0.15b^{1}
Total flavonoid content (µmol CE/g cooked grain, dry weight)					
Kodo	1.28±0.01c^{2}	0.82±0.01d^{2}	2.14±0.19b^{2}	4.09±0.22a^{3}	1.13±0.12c,d^{3}
Finger	2.92±0.08b^{1}	0.71±0.08c^{2}	2.74±0.09b^{1}	6.48±0.56a^{2}	0.53±0.08c^{4}
Pearl	0.79±0.02c^{3}	2.23±0.06b^{1}	2.18±0.14b^{2}	9.84±0.46a^{1}	0.75±0.02c^{4}
Foxtail	0.37±0.07d^{4}	0.37±0.01d^{3}	0.82±0.04c^{3}	4.18±0.11a^{3}	1.56±0.14b^{2}
Proso	0.65±0.00c^{3}	0.78±0.09c^{2}	0.64±0.03c^{3}	3.61±0.16a^{3}	2.21±0.07b^{1}

Values in each row having the same letters are not significantly ($p > 0.05$) different. In individual assays, values in each column having the same superscript values are not significantly ($p > 0.05$) different.

9.4.2 Total phenolic and total flavonoid contents

Table 9.1 presents the total phenolic (TPC) and total flavonoid (TFC) contents of aqueous extracts (AQE) of millet grains, and extracts after simulated gastrointestinal pH conditions (EPH), gastric digestion (EGD), gastrointestinal digestion (EGID) and colonic fermentation (EFM). The TPC in aqueous extracts ranged from 2 to 12 μmol FAE / g cooked grain (dw) whereas that of EPH ranged from 1.5 to 5.9 μmol FAE / g cooked grain (dw). Except for finger millet, no significant difference was observed in AQE and EPH of other millet grains suggesting the lesser effect of pH changes alone under physiological conditions on the release of phenolics from food matrix. Nevertheless, high TPC in AQE and EPH of finger millet grains noted in the present study could be due to the higher content of flavonoids in the free phenolic extract of grains (*13*).

In contrast to the results obtained in the present work, Liyana-Pathirana and Shahidi (*16*) demonstrated that simulated gastrointestinal pH conditions significantly increased the TPC and antioxidant potential of phenolic extracts obtained from soft and hard wheat whole grains and their flour, germ and bran fractions. However, in the present work cooked millet grains were employed whereas in the earlier work (*16*) raw wheat grains and their fractions were used for the analysis of TPC and antioxidant activities. Thus, the possible effects of cereal type as well as pretreatment may be responsible for the differences observed between two studies.

The TPC and TFC of millet extracts obtained following enzymic digestion in the gastric phase (EGD) ranged from 10.2 to 26.9 μmol FAE / g cooked grain (dw) and from 0.64 to 2.74 μmol CE / g cooked grain (dw), respectively. The present results showed that at the end of gastric phase, 2-5 times more total phenolics were

released than those released from corresponding aqueous extracts of samples. Furthermore, TFC of kodo, pearl and foxtail millets in EGD were also 2-3 times higher than those of their corresponding aqueous extracts. In the process of digestion TPC and TFC increased for all millet grains employed in this study. This is in agreement with the results obtained by Gawlik-Dziki et al. (*30*) for TPC of wheat bread under simulated digestion. However, in contrast to present results, their study showed a decrease in TFC after the small intestine digestion. The difference observed in the present work and the study by Gawlik-Dziki et al. (*30*) could be attributed to different cereal samples used in the two studies. In the gastric phase, primarily proteins present in grains were digested and some of phenolics bound with proteins may be released at this point. It is noteworthy that phenolics released in the gastric phase may be absorbed and they may have some local antioxidant effect in the small intestine as well due to their solubility in the digesta. However, some authors have previously shown that phenolic acids such as gallic, caffeic, ferulic, coumaric and chlorogenic acids are absorbed from the stomach (*22,24, 27,41*).

Table 9.2 Antioxidant activities of millet grain aqueous extracts (AQE), and extracts obtained subjecting to simulated *in vitro* gastrointestinal pH conditions (EPH), gastric digestion (EGD), gastrointestinal digestion (EGID) and colonic fermentation (EFM)

Millet	AQE	EPH	EGD	EGID	EFM
Trolox equivalent antioxidant activity (µmol TE /g cooked grain, dry weight)					
Kodo	2.55±0.62c[1]	23.2±3.29b[2]	14.7±1.48b,c[3]	55.2±9.41a[3]	56.1±5.52a[2]
Finger	18.3±1.03d[2]	57.7±9.57b[1]	32.3±3.32c,d[2]	96.4±11.2a[2]	5.54±0.13e[4]
Pearl	2.54±0.46c[1]	8.02±0.61c[3]	49.6±0.01b[1]	142±15.5a[1]	50.6±5.42b[2]
Foxtail	1.51±0.24c[3]	21.5±2.57b[2]	20.0±4.20b,c[3]	155±19.4a[1]	33.0±3.64b[3]
Proso	1.06±0.10e[3]	18.5±1.67d[2]	42.3±4.45c[1]	62.2±1.56b [2,3]	81.3±6.49a[1]
Reducing power (µmol AAE/g cooked grain, dry weight)					
Kodo	3.61±0.04c[2]	2.31±0.03d[3]	2.84±0.05c,d[3]	11.9±0.65a[3]	5.39±0.32b[3]
Finger	12.6±0.24b[1]	7.09±0.00c[1]	12.0±0.38b[1]	23.5±0.59a[1]	3.56±0.07d[4]
Pearl	2.87±0.06d[3]	2.64±0.07d[2]	5.62±0.17b[2]	15.4±0.22a[2]	4.61±0.40c[3]
Foxtail	1.06±0.04b[3]	1.20±0.10b[4]	1.76±0.02b[4]	11.8±0.88a[3]	12.2±0.43a[2]
Proso	1.05±0.05c,d[3]	0.87±0.04d[5]	1.59±0.01c[4]	10.6±0.31b[3]	16.6±0.36a[1]
Ferrous ion chelating activity (µmol EDTA equivalents/g of cooked , dry weight)					
Kodo	76.0±0.17c[3]	161±0.43b[3]	5.09±0.90d[3]	529±5.28a[4]	72.4±7.61c[3]
Finger	124±5.46c[1]	174±8.59b[3]	5.87±0.00e[3]	725±28.9a[1]	72.8±0.45d[3]
Pearl	81.3±2.45c[2]	217±5.10b[2]	11.7±0.66d[1]	601±2.26a[3]	78.5±7.69c[3]
Foxtail	36.3±0.00d[4]	145±10.2c[4]	1.49±0.42e[4]	591±2.28a[3]	194±2.63b[2]
Proso	34.9±0.11d[4]	407±7.89b[1]	8.63±0.05d[2]	684±48.0a[2]	264±3.89c[1]

Values in each row having the same letter are not significantly ($p > 0.05$) different.
In individual assays, values in each column having the same superscript values are not significantly ($p > 0.05$) different.

In the present work both TPC and TFC were significantly ($p \leq 0.05$) increased after completion of the gastrointestinal digestion (GID). The TPC and TFC of different millet grain EGID ranged from 12.7 to 35.4 µmol FAE and from 3.6 to 9.8 µmol CE/g of cooked grain (dw), respectively. Extracts of pearl millet showed the highest TPC and TFC, among others. In a previous study we showed that the soluble TPC of different cooked millet grains extracted into aqueous acetone (70%, v/v) ranged from 1.95 to 17.7 µmol FAE/ g defatted meal (*42*). It is worthwhile to note that in the present work, digestion in the GI tract released 2 -15 % more phenolic compounds than those of the same millet grain samples extracted with 70% acetone. In agreement with the present results, Perez-Jimenez and Saura-Calixto (*28*) showed that boiled rice extracted under simulated GI conditions had 15 imes more TPC than that extracted in an aqueous organic solvent. This could be due to the partial release of phenolics bound to the cell wall material of endosperm fraction of the grain.

In vitro batch fermentation method used in the present study provided quantitative data on the fermentation of non-starch polysaccharides by colonic microflora in humans. In addition, *in vitro* fermentation for 24 h was sufficient to mimic the polysaccharide degradation *in vivo* (*43*). In the present work the TPC determined in the extracts following *in vitro* colonic fermentation for 24 h ranged from 4.5 to 17.1 FAE/g of cooked grain (dw). Proso and foxtail millet residues had 3.8 times higher TPC than that of finger millet which showed the lowest. In a previous study, the TPC of finger millet insoluble residue extracted under alkaline hydrolysis condition and a nitrogen atmosphere at room temperature exhibited the least amount of phenolics among other millets employed, thus confirming the low content of bound phenolics of finger millet grains, as also observed in the present

work (*11*). Therefore, these results lend further support to the existing knowledge that insoluble bound phenolics in cereals are attached to cell wall polysaccharides and are released in the colon by fermentation. Couteau et al. (*44*) isolated cinnamoyl esterase producing organisms that released ferulic acid from its ethyl esters in human faecal inoculum and identified them as *Escherichia coli*, *Bifidobacterium lactis* and *Lactobacillus gasseri*. Furthermore, Andreasen et al. (*45*) showed that cinnamoyl esterases which can hydrolyze dietary hydroxycinnamate esters are distributed throughout the intestinal tract of humans. They further showed that mucosal cells in the small intestine were responsible for esterase activity whereas microbial originated esterases were predominantly responsible in the colon (*45*). Thus, mere presence of liberated phenolics in the colon may exert a local effect and their further absorption via epithelium cells may render unique systemic health benefits in different tissues and organs in the body.

9.4.3 Trolox equivalent antioxidant capacity (TEAC)

The TEAC of millet grains subjected to *in vitro* enzymic digestions and colonic fermentation were measured using ABTS radical anion and the results are presented in **Table 9.2**. The TEAC values of the millet grain AQE and EPH ranged from 1.1 to 18.3 and from 8.0 to 57.7 µmol TE /g cooked grain (dw), respectively. In general, EPH of millet varieties in the present work showed 3-18 times higher ($p \leq 0.05$) TEAC values compared to their water extracted counterparts. In accordance with the present results, Liyana-Pathirana and Shahidi (*16*) showed that simulated gastrointestinal pH conditions increased TEAC of soft and hard wheat whole grains, and their fractions. It should be noted that in the present work TPC of AQE and EPH did not differ significantly. However, the high antioxidant activity obtained for EPH of millets

could be due to the compositional changes of phenolics released under changing pH conditions.

Furthermore, in the present study TEAC values of EGD and EGID showed significantly ($p \leq 0.05$) higher values compared to their corresponding AQE, and EPH. The TEAC values of EGID showed 22, 5, 57, 104 and 62 fold higher antioxidant capacity than their corresponding water extracts of kodo, finger, pearl, foxtail and proso millets, respectively. In addition, the TEAC values of millet EFM ranged from 5.5 to 81.3 µmol TE /g cooked grain (dw). It is interesting to note that finger millet EFM showed the least TEAC value and this may associate with its lower phenolic content present in their insoluble fibre. Proso millet EFM had the highest TEAC value and this was 1.4-15 times higher than those of other millets studied in this study.

9.4.4 Reducing power (RP)

The reducing power of a compound determines its ability to donate an electron to oxidized compounds by acting as an antioxidant. Extracts with high reducing power values, as denoted by ascorbic acid equivalents, exhibit a better reducing activity. The results of the present study demonstrated that millet EGID had a higher RP than their corresponding AQE (**Table 9.2**). The reducing power of millet AQE and EGID ranged from 1.05 to 12.6 and 10.6 to 23.5 µmol AAE/g cooked grain (dw), respectively. Finger millet had the highest RP whereas proso millet showed the least for both AQE and EGID. Except for kodo and finger millets no significant difference was observed in their RP of millet AQE and EPH employed in the present work. Hence, the present results suggest that changing gastrointestinal pH levels alone may not release phenolic compounds with potential reducing power from cooked millet grains. However, enzymatic treatments that hydrolyze starch and proteins may favour

the release of phenolics and could be attributed to higher RP in EGD and EGID. Nonetheless, Gawlik-Dziki et al. (*30*) have shown a decrease of RP upon progression of digestion of wheat bread.

Reducing power of millet EFM ranged from 3.56 to 16.6 AAE/g cooked grain (dw). In accordance with the results obtained for other assays in the present study, finger millet extract showed the lowest RP and accounted for 4.6 fold less value than that of proso millet which showed the highest RP among the EFM. The low reducing power of finger millet EFM may essentially be attributed to its low phenolic content compared to that of proso millet.

9.4.5 Ferrous ion chelating activity (FCA)

Ferrous ions are key catalysts in Fenton reaction and generate highly reactive and destructive hydroxyl radicals in biological systems. Chelating agents of transition metal ions are capable of acting as secondary antioxidants in such systems. The results of the present study showed that extracts of millet phenolics have effective FCA depending on the variety and the treatment received by millet grains (**Table 9.2**). Notwithstanding the other assays in the present work, millet EGD exhibited the least FCA which ranged from 1.49 to 11.6 µmol EDTA equivalents/g of cooked grain (dw). Interestingly, millet EPH showed 1.4 to 12 times higher FCA ($p \leq 0.05$) compared to their corresponding water extracts. In addition, millet EGID demonstrated 7, 6, 7.4, 16, and 20 fold higher FCA than that for kodo, finger, pearl, foxtail and proso millet water extracts, respectively. It was also noted that millet EGID had higher FCA than that of EGD. This is in agreement with the results obtained for FCA, after simulated in vitro digestion of wheat bread added with Tartary buckwheat flavones (*30*). Millet EFM exhibited a considerable FCA, which ranged from 72.4 to 264 µmol EDTA

equivalents/g of cooked grain (dw) and the order of activity among different millet varieties were: kodo < finger < pearl < foxtail < proso (**Table 9.2**). The results of the present work further showed that FCA of millet phenolics released under gastric and intestinal digestion depend on the pH conditions in the medium.

9.4.6 DPPH radical scavenging activity (DRSA)

The DPPH radical scavenging activity (DRSA) of different millet extracts was 1.9-16.2, 4.9-33.5, 59.3-84.6, 12.3-114 and 17-32.7 μmol FAE /g of cooked grain (dw) for AQE, EPH, EGD, EGID and EFM, respectively (**Table 9.3**). DPPH radical is artificial, stable, organic nitrogen radical, commonly used in the assessment of antioxidant activity of phenolic extracts. The present results showed that millet aqueous extracts had the lowest DRSA compared to the other samples that received different treatments for the extraction of phenolics. It is noteworthy that EPH demonstrated a higher DRSA compared to their corresponding AQE though phenolic contents showed a reverse trend in the present work. These results suggest that phenolic content alone may not explain the activity exerted by these extracts but their composition of phenolic compounds which can be affected by the extraction conditions may also contribute to the observed differences. Furthermore, it was noted that millet EGD had significantly higher DRSA compared to those of their corresponding EGID in the present work except for finger millet. This trend contradicts those obtained from other antioxidant activity assays such as TEAC, reducing power and ferrous chelating activity employed in this work. The DPPH assay employed in the present study used 10 min for completion of the reaction between DPPH radicals and phenolic compounds, thus exhibiting intermediate rate kinetic behaviour in the reaction (*46*).

DPPH assay used in this work may involve electron transfer reactions and acids and alkali present in extracts may influence the ionization equilibrium of phenolic compounds resulting in a reduction or enhancement of the reaction rate, respectively (*47*). The difference in pH conditions of millet EGD and EGID used in the present study may also affect the results obtained for DRSA assay in this work and exhibited a different trend observed in other antioxidant activity measurements such as hydroxyl radical scavenging activity (HRSA) and oxygen radical absorbance capacity (ORAC).

9.4.7 Hydroxyl radical scavenging activity (HRSA)

In the present study hydroxyl radicals were generated via Fenton reaction and millet grain extracts dissolved in distilled water. The HRSA of millet AQE ranged from 40.9 to 161.9 μmol FAE /g of cooked grain (dw) and foxtail millet had the highest activity whereas finger millet showed the least (**Table 9.3**). In general, millet EPH demonstrated a significantly ($p \leq 0.05$) higher HRSA than that of EAQ, similar to the results obtained for DRSA in the present study. On the other hand millet EGID showed 11 to 46 fold higher HRSA which ranged from 1120 to 1873 μmol FAE /g of cooked grain (dw) compared to their corresponding EGD and this contradicted the results obtained for DRSA. The observed trend could be due to the existing differences of two types of radicals used in two assays and their varying reaction rates and mechanisms with antioxidant compounds in the extracts. In addition, phenolic compounds present in millet extracts were reported to be effective ferrous ion chelating agents as shown by the results in the present work. Therefore, HRSA observed in the present analysis could be due to direct scavenging of hydroxyl radicals and /or chelating of ferrous ions which are needed to generate hydroxyl radicals in the

assay system. These results further implicated that intestinal epithelium cells may be more efficiently protected from free radical damage by millet grain phenolics that exhibited a higher scavenging ability compared to those in the gastric phase.

Millet EFM showed a considerable HRSA which ranged from 155.7 to 1179 µmol FAE /g of cooked grain (dw) and activity differed with the millet type. Proso millet EFM exhibited the highest activity whereas finger millet had the least in the present study. Thus, present results could be speculated to have a protective effect of bound phenolics which are released during fermentation process in the colon, against oxidative stress caused by hydroxyl radicals.

Table 9.3 Free radical scavenging activities of millet grain aqueous extracts (AQE), and extracts obtained subjecting to simulated in vitro gastrointestinal pH conditions (EPH), gastric digestion (EGD), gastrointestinal digestion (EGID) and colonic fermentation (EFM)

Millet	AQE	EPH	EGD	EGID	EFM
DPPH radical scavenging activity (µmol FAE /g cooked grain, dry weight)					
Kodo	5.14±0.29e^{2}	17.8±0.61c^{2}	67.2±2.91a^{2}	12.3±1.54d^{5}	32.7±0.21b^{1}
Finger	16.2±0.18d^{1}	33.5±1.19c^{1}	65.3±0.88b^{2}	114±1.31a^{1}	17.0±3.29d^{2}
Pearl	5.27±0.28d^{2}	17.7±2.31c^{2}	83.3±2.73a^{1}	43.2±2.42b^{2}	17.4±0.40c^{2}
Foxtail	1.94±0.05d^{3}	7.47±0.16d^{3}	59.3±0.13a^{3}	33.2±4.42b^{3}	19.2±2.16c^{2}
Proso	2.03±0.08d^{3}	4.85±0.54d^{3}	84.6±0.90a^{1}	20.8±2.01c^{4}	26.9±3.20b^{1}
Hydroxyl radical scavenging activity (µmol FAE /g cooked grain, dry weight)					
Kodo	60.1±8.1e^{3}	330±0.6b^{4}	106±12.6d^{2}	1120±9.0a^{3}	231±3.5c^{5}
Finger	40.9±4.1d^{4}	387±2.8b^{3}	33.9±1.73d^{4}	1518±17a^{2}	156±8.9c^{4}
Pearl	89.0±3.7d^{2}	453±2.5b^{1}	72.2±0.00d^{3}	1862±34a^{1}	334±9.8c^{3}
Foxtail	161±1.3d^{1}	332±3.2c^{4}	46.6±5.27e^{4}	1873±23a^{1}	943±5.4b^{2}
Proso	159±1.4e^{1}	405±2.8d^{2}	120±3.22c^{1}	1857±18a^{1}	1179±11b^{1}
Oxygen Radical absorbance capacity (µmol TE /g cooked grain, dry weight)					
Kodo	0.17±0.00b,c^{2}	0.07±0.01c^{3}	0.33±0.03b^{5}	0.67±0.15a^{3}	0.30±0.03b^{3}
Finger	0.53±0.04b^{1}	0.31±0.04c^{1}	0.60±0.05b^{1}	1.14±0.03a^{3}	0.21±0.01c^{4}
Pearl	0.16±0.01c^{2}	0.19±0.00c^{2}	0.54±0.02b^{2}	1.40±0.25a^{2,3}	0.36±0.03b,c^{3}
Foxtail	0.07±0.01c^{3}	0.03±0.01c^{3}	0.42±0.05b,c^{4,5}	1.94±0.46a^{1}	0.81±0.07b^{2}
Proso	0.07±0.01d^{3}	0.04±0.02d^{3}	0.55±0.14b,c^{3}	0.82±0.30a^{3}	1.01±0.01a^{1}

Values in each row having the same letter are not significantly ($p > 0.05$) different.
In individual assays, values in each column having the same superscript values are not significantly ($p > 0.05$) different.

9.4.8 Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity measures the peroxyl radical scavenging capacity of the millet extracts and results were expressed as μmol trolox equivalents (TE) /g of cooked grain (dw) (**Table 9.3**). In general, a significant difference was not observed between millet AQE and EPH in the present study, similar to the results obtained for TPC. The ORAC values of AQE and EPH ranged from 0.07 to 0.53 and 0.03 to 0.31 μmol TE/g of cooked grain (dw), respectively. Furthermore, millet EGID had significantly ($p \leq 0.05$) higher ORAC values that ranged from 0.6 to 1.9 μmol TE/g of cooked grain (dw) compared to that of EGD. The ability of millet EFM to scavenge peroxyl radicals was in the order of proso > foxtail > pearl ≥ kodo > finger millets.

9.4.9 Correlation analysis between phenolic content and antioxidant activities

Table 9.4 presents the coefficient values of correlations of TPC with the antioxidant activity determinants used in the present study for millet extracts obtained under different treatment conditions. Generally, the TPC of AQE demonstrated strong and positive correlation with antioxidant activities determined except for HRSA, suggesting their contribution to the observed effects in different systems. On the other hand, HRSA had a negative significant association with TPC and this could be due to other contributory factors in the extracts which may be responsible for the observed effects. Reducing power and ORAC positively correlated with the TPC in the EGD whereas HRSA was negatively associated with that of millet grains. In addition, TPC of the EGID positively associated with TEAC, ORAC, HRSA and FCA in the present study. There was a significantly positive and strong correlation between TPC and RP,

ORAC, HRSA and FCA of millet EFM suggesting contribution of released phenolics from insoluble fibre under microbial fermentation in the colon. The present results showed an inconsistency of association between TPC and antioxidant activities as determined by different methods of the millet grains subjected to a range of treatments suggesting contribution of other compounds such as carotenoids and tocopherols, among others, in the antioxidant activity of millet extracts.

Table 9.4 Correlation coefficients of antioxidant activities with total phenolic contents (TPC) of millet grain aqueous extracts (AQE), and extracts obtained subjecting to simulated in vitro gastrointestinal pH conditions (EPH), gastric digestion (EGD), gastrointestinal digestion (EGID) and colonic fermentation (EFM)

Treatment	TEAC[a]	RP[b]	ORAC[c]	DRSA[d]	HRSA[e]	FCA[f]
AQE	0.960**	0.992**	0.990**	0.994**	0.628*	0.872**
EPH	0.303*	0.813**	0.900**	0.866**	0.164	0.137
EGD	0.146	0.958**	0.386*	0.014	0.386	0.088
EGID	0.516**	0.144	0.407*	0.235	0.679**	0.265
EFM	0.312*	0.906**	0.895**	0.076	0.903**	0.848**

** Significance $p \leq 0.0001$; * $p \leq 0.05$

TEAC[a], trolox equivalent antioxidant activity; RP[b], reducing power; ORAC[c], oxygen radical absoption capacity; DRSA[d], DPPH radical scavenging activity; HRSA[e], Hydroxyl radical scavenging activity; FCA[f], ferrous chelating activity.

9.4.10 HPLC analysis of phenolic compounds

In a previous study we showed that soluble and insoluble bound phenolic extracts of millet grains contained a number of compounds belonging to hydroxycinnamic and hydroxybenzoic acids as well as flavonoids (*13*). In the present work, the HPLC and tandem mass spectrometric analyses of the phenolic extracts of simulated digested (**Figure A.3**) and fermented (**Figures A.4**) grain samples revealed that the phenolic profile were not similar to those previously observed in raw millet grain soluble and bound fractions, respectively. This could be due to the transformation of compounds present in the raw grain in the processes of heat treatment and subsequent *in vitro* simulated enzymic digestion and microbial fermentation. Thermal processing may alter endogenous phenolics in cereal grains by degradation or polymerization of simple phenolics. Furthermore, during digestion and colonic fermentation enzymatic reactions produce compounds which might be different from those originally present in the grains. However, *in vitro* antioxidant activities demonstrated by stable compounds that were present in the extracts in the present work further proved that transformed phenolics could serve as active antioxidative compounds *in vivo* and protect gastrointestinal tract from oxidative damage. Therefore, identification of these newly formed compounds is of much interst and need to be further studied in future.

It has been reported that hydroxycinnamic acids such as ferulic acid from cereals are mainly absorbed in the small intestine and these are from soluble fraction of the grain (*48*). Furthermore, they have shown that a minor quantity of ferulic acid was absorbed after releasing from insoluble fibre in the large intestine. Nevertheless, these phenolics may act locally to protect other dietary antioxidants from degradation, at the intestinal level. Thus, they may enhance the total antioxidant status of the body,

exerting a protective effect against diseases associated with oxidative stress. This study also indicated that millet grain phenolic compounds may undergo structural changes during digestion as well as microbial fermentation in the body. Therefore, information generated in the present study lays the foundation for future studies for identifying the compounds formed upon digestion and fermentation that exhibited *in vitro* antioxidant activities.

9.5 Conclusion

The results of the present study clearly showed that millet grain phenolics are released during gastrointestinal digestion and colonic fermentation. The compounds so produced were soluble in the medium, thus are considered accessible for absorption into the body and may exhibit bioactivity. Several antioxidant activity assays employed in the present work demonstrated that bioaccessible phenolic compounds had a considerable antioxidant activity and exerted their activities through different mechanisms such as free radical scavenging, ferrous ion chelating, and reducing activity. The findings of this study further demonstrated that different millet types may possess varying antioxidant activities under physiological conditions. Furthermore, it was shown that phenolic compounds bound to the insoluble fibre released during colonic fermentation may exert their health benefits locally as well as systemically upon absorption. Nevertheless, identification of phenolics in the digesta as well as fermented end products is warranted in order to determine the fate of phenolics in millet grains *in vivo*.

9.6 References

1. Scalbert, A.; Manach C.; Morand C.; Remesy C.; Jimenez, L. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, 45, 287-306.

2. Manach, C.; Mazur A.; Scalbert, A. Polyphenols and prevention of cardiovascular diseases. *Curr. Opin. Lipidol.* **2005**, 16, 77-84.

3. Shahidi, F.; Chandrasekara, A.; Zhong, Y. Bioactive phytochemicals in vegetables. *In Handbook of vegetables and vegetable processing*. (*ed by N.K.Singha*), John Wiley & Sons Publishing Ltd., Ames, IA, **2011**, pp 125-158.

4. Shahidi, F.; Naczk, M. Phenolics in Food and Nutraceuticals. CRC press, Boca Raton, FL, **2004**, pp 1-82.

5. Lu, H.; Zhang, J.; Liub, K.; Wu, N.; Li, Y.; Zhou, K.; Ye, M.; Zhang, T.; Zhang, H.; Yang, X.; Shen,L.; Xu,D.; Li, Q. Earliest domestication of common millet (Panicum miliaceum) in East Asia extended to 10,000 years ago. *PNAS*, **2009**, 106, 7367-7372.

6. Hegde, P.S.; Rajasekaran, N.S.; Chandra, T.S. Effects of the antioxidant properties of millet species on the oxidative stress and glycemic status in alloxan-induced rats. *Nutr. Res.* **2005**, *25*, 1109-1120.

7. Lakshmi Kumari, P.; Sumathi, S. Effect of consumption of finger millet on hyperglycemia in non-insulin dependent diabetes mellitus (NIDDM) subjects. *Plant Foods Human Nutr.* **2002**, 57, 205-213.

8. Shobana, S.; Harsha, M.R.; Platel, K.; Srinivasan, K.; Malleshi, N.G. Amelioration of hyperglycaemia and its associated complications by finger millet (*Eleusine coracana* L.) seed coat matter in streptozotocin-induced diabetic rats. *Brit. J. Nutr.* **2010**, 104, 1787-1795.

9. Lee, S.H.; Chung, I.M.; Cha, Y.S.; Park, Y. Millet consumption decreased serum concentration of triglyceride and C-reactive protein but not oxidative status in hyperlipidemic rats. *Nutr. Res.* **2010**, 30, 290-296.

10. Chandrasekara, A.; Shahidi, F. The content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *J. Agric. Food Chem.* **2010**, 58, 6706-6714.

11. Chandrasekara, A.; Shahidi, F. Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. *J. Agric. Food Chem.* **2011**, 59, 428-436.

12. Chandrasekara, A.; Shahidi, F. Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n. *J.Functional Foods.* **2011**, 3, 144-158.

13. Chandrasekara, A.; Shahidi, F. Antiproliferative potential and DNA scission inhibitory activity of phenolics from whole millet grains. *J. Functional Foods.* **2011**, 3, 159-170.

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

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

16. Liyana-Pathirana, C.; Shahidi, F. Antioxidant activity of comercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions. *J. Agric. Food Chem.* **2005**, 54, 1256-1264.

17. Madhujith, T.; Shahidi, F. Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. *Food Chem.* **2009**, 117, 615-620.

18. Ralph, J.; Bunzel, M.; Marita, J.M.; Hatfield, R.D.; Lu, F.; Kim, H.; Schatz, P.F.; Grabber, J.H.; Steinhart, H. Peroxidase-dependent cross-linking reactions of *p*-hydroxycinnamates in plant cell walls. *Phytochem. Rev.* **2004**, 3, 79-96.

19. Saulnier, L.; Thibault, J.F. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *J. Sci. Food Agric.* **1999**, 79, 396-402.

20. Garcia-Conesa, M.T.; Puumb, G.W.; Kroon, P.A.; Walace, G.; Williamson, G. Antioxidant properties of ferulic acid dimers. *Redox Rep.* **1997**, 3, 239-244.

21. Bourne, L.C.; Rice-Evans, C. Bioavailability of ferulic acid. *Biochem. Biophy. Res. Comm.* **1998**, 253, 222-227.

22. Adam, A.; Crespy, V.; Levrat-Verny, M.A.; Leenhardt, F.; Leuillet, M.; Demigne, C.; Remesy, C. The bioavilability of ferulic acid is governed primarily by the food matrix rather than its metabolism in intestine and liver in rats. *J. Nutr.* **2002,** 132, 1962-1968.

23. Olthof, M.R.; Hollman, P.C.H.; Buijsman, M.N.C.P.; van Amelsvoort, J.M.M.; Katan, M.B. Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. *J. Nutr.* **2003**, 133, 1806-1814.

24. Zhao, Z.; Egashira, Y.; Sanada, H. Ferulic acid is quickly absorbed from rat stomach as the free form and then conjugated mainly in the liver. *J. Nutr.* **2004**, 134, 3083-3088.

25. Rondini, L.; Peyrat-Maillard, M.N.; Marsset-Bagliweri, A.; Fromentin, G.; Durand, P.; Tome, D.; Prosy, M.; Berset, C. Bound ferulic acid from bran is more bioavailable than the free compound in rat. *J. Agric. Food Chem.* **2004**, 52, 4338-4343.

26. Chen, C.-Y.; Milbury, P.E.; Kwak, H.-K.; Collins, F.W.; Samuel, P.; Blumberg, J.B. Avenanthramides and phenolic acids from oats are bioavailable and act synergistically with vitamin C to enhance hamster and human LDL resistance to oxidation. *J. Nutr.* **2004**, 134, 1459-1466.

27. Lafay, S.; Gil-Izquierdo, A.; Manach, C.; Morand, C.; Besson, C.; Scalbert, A. Chlorogenic acid is absorbed in its intact form in the stomach of rats. *J. Nutr.* **2006**, 136, 1192-1197.

28. Perez-Jimenez, J.; Saura-Calixto, F. Literature data may underestimate the actual antioxidant capacity of cereals. *J. Agric. Food Chem.* **2005**, 53, 5036-5040.

29. Nagah, A.M.; Seal, C. In vitro procedure to predict apparent antioxidant release from wholegrain foods measured using three different analytical methods. *J. Sci. Food Agric.* **2005**, 85, 1177-1185.

30. Gawlik-Dziki, U.; Dziki, D.; Baraniak, B.; Lin, R. The effect of simulated digestion in vitro on bioactivity of wheat bread with Tartary buckwheat flavones addition. *LWT- Food Sci. Technol.* **2009**, 42, 137-143.

31. Saura-Calixto, F.; Serrano, J.; Goni, I. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chem.* **2007**, 101, 492-501.

32. Aura, A.-M.; Harkonen, H.; Fabritius, M.; Poutanen, K. Development of an *in vitro* enzymic digestion method for removal of starch and protein and assessment of its performance using rye and wheat breads. *J. Cereal Sci.* **1999**, 29, 139-152.

33. Karppinen, S.; Liukkonen, K.; Aura, A.-M.; Forssell, P.; Poutanen, K. *In vitro* fermentation of polysaccharide of rye, wheat and oat brans and inulin by human faecal bacteria. *J. Sci. Food Agric.* **2000**, 80, 1469-1476.

34. Anderson, R.; Hedlund, B. HPLC analysis of organic acids in lactic acid fermented vegetables. *Z Lebensm Unters Forsch.* **1983**, 176, 440-443.

35. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, 16, 144-158.

36. Kim, D.; Jeong, S.W.; Lee, C.Y. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem.* **2003**, 81, 321-326.

37. van den Berg, R.; Haenen, G.R.M.M.; van den Berg, H.; Bast, A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurement of mixtures. *Food Chem.* **1999**, 66, 511-517.

38. Oyaizu, M. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* **1986**, 44, 307-315.

39. Dinis T.C.P.; Madeira, V.M.C.; Almeida, L.M. Action of phenolic derivates (acetoaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* **1994**, 315, 161-169.

40. Cummings, J.H. Short chain fatty acids. In '*Human Colonic Bacteria. Role in Nutrition, Physiology, and Pathology*', Gibson, G.R. & Macfarlane, G.T. (eds), CRC Press, Boca Raton, FL, USA. **1995**, pp 101–130.

41. Konishi, Y.; Zhao, Z.; Shimizu, M. Phenolic acids are absorbed from the rat stomach with different absorption rates. *J. Agric. Food Chem.* **2006**, 54, 7539-7543.

42. Chandrasekara, A.; Naczk, M.; Shahidi, F. Effect of processing on the antioxidant of millet grains. *Food Chem.* **2011**, (submitted)

43. Wisker, E.; Daniel, M.; Rave, G.; Feldheim W. Fermentation of non starch polysaccharides in mixed diets and single fibre sources: comparative studies in human subjects and in vitro. *Brit. J. Nutr.* **1998**, 80, 253-261.

44. Couteau, D.; McCartney, A.L.; Gibson, G.R.; Williamson, G., Faulds, C.B. Isolation and characterization of human colonic bacteria able to hydrolise chlorogenic acid. *J. App. Micriobiol.* **2001**, 90, 873-881.

45. Andreasen, M.F.; Kroon, P.A.; Williamson, G.; Garcia-Conesa, M. Esterase activity able to hydrolyze dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals. *J. Agric. Food Chem.* **2001**, 49, 5679-5684.

46. Sánchez-Moreno, C. Methods used to evaluate the free radical scavenging activity in foods and biological systems, *Food Sci. Technol. Intern.* **200**2, 8, 121-137.

47. Foti, M.; Ruberto, G. Kinetic solvent effects on phenolic antioxidant determined by spectrophotometric measurements. *J. Agric. Food Chem.* **2001**, 49, 342–348.

48. Kern, S.M.; Bennett, R.N.; Mellon, F.A. Absorption of hydroxycinnamates in humans after high-bran cereal consumption. *J. Agric. Food Chem.* **2003**, 51, 6050-6055.

CHAPTER 10

Summary and future directions

Millets are underutilized cereals in the developed countries while they serve as staple foods for millions of people in parts of Africa and Asia. In addition to serving as a source of macro- and micro-nutrients, similar to other principal cereals in the world, millets are rich in bioactive phytochemicals, particularly phenolics. Moreover, millets are staple food substitutes for celiac patients as they are gluten free. This study investigated the antioxidant and several other bioactivities of millet grain phenolics in a number of *in vitro* chemical, biological and food systems. Seven millet grain varieties, namely proso, kodo, little, foxtail, pearl and two finger millet varieties were used as intact whole grains, or as dehulled grains. Hulls, as process byproducts, were tested as a natural source of antioxidants. Phenolic contents and antioxidant activity in soluble and insoluble bound phenolic fractions were studied and their phenolic profiles elucidated.

A wide variation existed in the phenolic content and antioxidant capacity of millet grains as determined by Folin-Ciocalteu's assay, oxygen radical absorbance capacity (ORAC), hydroxyl radical scavenging and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging as well as reducing activities and metal ion chelation abilities. Both soluble and insoluble bound phenolics contributed to these effects. Over 50 phenolic compounds in millet grains were positively and/or tentatively identified and quantified using HPLC coupled with UV-DAD and ESI-tandem mass spectrometry. The main classes of phenolic compounds identified were hydroxycinnamic and hydroxybenzoic acids as well as flavonoids. The contents and distribution of them in the free, soluble conjugated and insoluble bound fractions

varied and these were also varietal dependent. Millet phenolics showed effective inhibition of DNA scission and antiproliferation of HT-29 adenocarcinoma cell line, thus suggesting their potency in regulating carcinogenesis at the initiation as well as progression stages. Furthermore, millet grain extracts showed high inhibition of liposome peroxidation while exhibiting low singlet oxygen and xanthine oxidase inhibitory activities. These may suggest varying effects of constituent phenolics in different assay systems which may be influenced by different mechanisms of actions. In food systems such as bulk oil, cooked muscle system and linoleic emulsion millet grain extracts exhibited noticeable antioxidant activity which was comparable to that of butylated hydroxyanisole (BHA), depending on the millet type, concentration of the extract and the test system employed. The results suggest the potential use of millet extracts as a natural source of antioxidants in foods. Furthermore, extracts of millet grains exhibited effective inhibition of oxidation of low density lipoprotein (LDL) cholesterol, suggesting a protective effect against cardiovascular disease.

Dehulling of millets demonstrated a significant effect on the phenolic content and antioxidant activities and depended on the millet variety. Hulls were noted for their high phenolic content and antioxidant activities, thus may serve as a potential source of natural antioxidants. Hydrothermal treatment, ie cooking for 30 min, employed in the present study, showed no significant effect on the total phenolic content. Therefore, use of millet grains in therapeutic diets for diseases associated with oxidative stress is suggested. However, detailed *in vivo* studies are still needed in order to demonstrate the efficacy of millet grains in risk reduction for such diseases.

It has been shown that millet grain phenolics are released under physiological conditions using a simulated *in vitro* digestion model and colonic fermentation. The

bioaccessible millet grain phenolics demonstrated effective antioxidant activity through different mechanisms such as free radical scavenging, ferrous ion chelating and reducing activity. Furthermore, bound phenolics present in the insoluble fibre released during colonic fermentation provide protective effects and may reduce the risk of colon cancer. Most of the research work on cereal grains has ignored this important fact and hence results that do not consider their contribution often do not correlate with benefits of cereal grains. Thus, the importance of bound phenolic fraction in the assessment of antioxidant activity of whole grains was further established in this present study. Dehulled grains and hulls of millets inhibited DNA scission, LDL cholesterol and liposome oxidation and proliferation of HT-29 adenocarcinoma cells effectively. These observations are similar to those noted for the intact whole grain of millets, thus suggesting their potency as nutraceuticals and functional food ingredients.

Overall, the results of this study suggest that millet grain phenolics, are bioaccessible, possess bioactivities against several pathophysiological conditions and may serve as potential natural sources of antioxidants in food and biological systems. Therefore, findings of this research fill some of the existing gap in the available literature on phenolic compounds and antioxidant properties of different millet varieties. Thus, use of millets, as nutraceuticals and specialty foods in disease risk reduction and overall health and wellness is suggested. Further, clinical studies are needed to examine the use of millet grains in specific disease conditions such as those of cancer and cardiovascular ailments.

In the present study millet samples used were grown under similar environmental conditions and harvested in the same crop year that minimized the

environmental impact on the phenolic content. Furthermore, major millet types currently consumed as foods in respective countries were evaluated for their antioxidant and bioactivities. Different tests with proper analytical techniques were employed which explained the different mechanisms underlined. However, except for finger millet, only one cultivar from each variety was used in this study that may abate the generalization of the findings of this study. Thus, further studies need to be carried out using different cultivars of different millet species. In addition, to understand bioavailability and bioefficacy of millet grain phenolics further investigations *ex vivo* and *in vivo* using cell lines and animal models as well as human clinical studies are warranted.

PUBLICATIONS ORIGINATED FROM THE STUDY

Articles published or submitted for publication considerations that are referred to in the text by their chapter numbers are as follows.

Chapter 2 - **Chandrasekara A,** Shahidi F (2010) The content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *Journal of Agricultural and Food Chemistry* 58: 6706-6714.

Chapter 3- **Chandrasekara A**, Shahidi F (2011) Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. *Journal of Agricultural and Food Chemistry 59:*428-436.

Chapter 4- **Chandrasekara A**, Shahidi F (2011) Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n . *Journal of Functional Foods, 3, 144-158.*

Chapter 5- **Chandrasekara A**, Shahidi F (2011) Antiproliferative potential and DNA scission inhibitory activity of phenolics from whole millet grains. *Journal of Functional Foods, 3, 159-170.*

Chapter 6- **Chandrasekara A**, Shahidi F (2011) Antioxidant phenolics of millet control lipid peroxidation in human LDL cholesterol and food systems. *Journal of American oil Chemists' Society, (DOI 10.1007/s11746-011-1918-5)*

Chapter 7- **Chandrasekara A**, Naczk M, Shahidi F (2011) Effect of processing on the antioxidant activity of millet grains. *Food Chemistry* (*accepted*)

Chapter 8- **Chandrasekara A**, Shahidi F (2011) Bioactivities of millet grains and ullhs. *Journal of Agricultural and Food Chemistry, (dx.doi.org/10.1021/jf201849d*)

Chapter 9- **Chandrasekara A**, Shahidi F (2011) Bioaccessibility and antioxidant potential of millet grain phenolics as affected by simulated *in vitro* digestion and microbial fermentation. *Journal of Functional Foods (submitted)*

APPENDIX

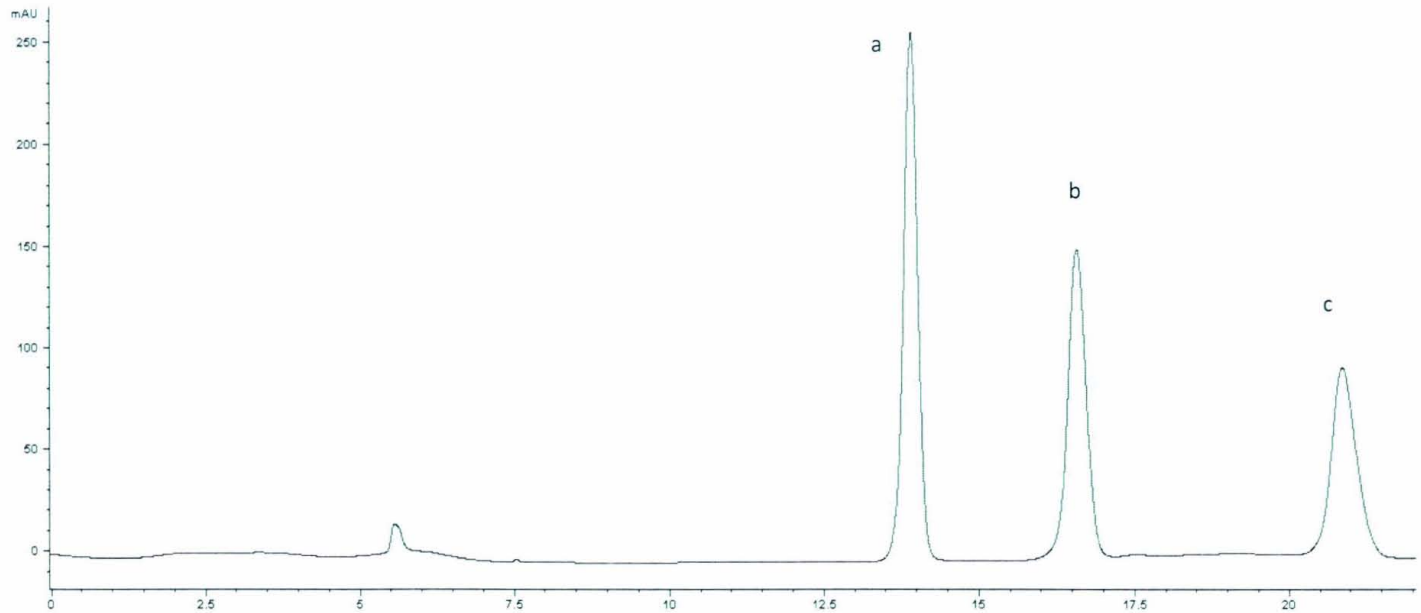

Figure A.1 Representative HPLC chromatogram of short chain fatty acid standards: a, acetic acid; b, propionic acid; and c, butyric acid

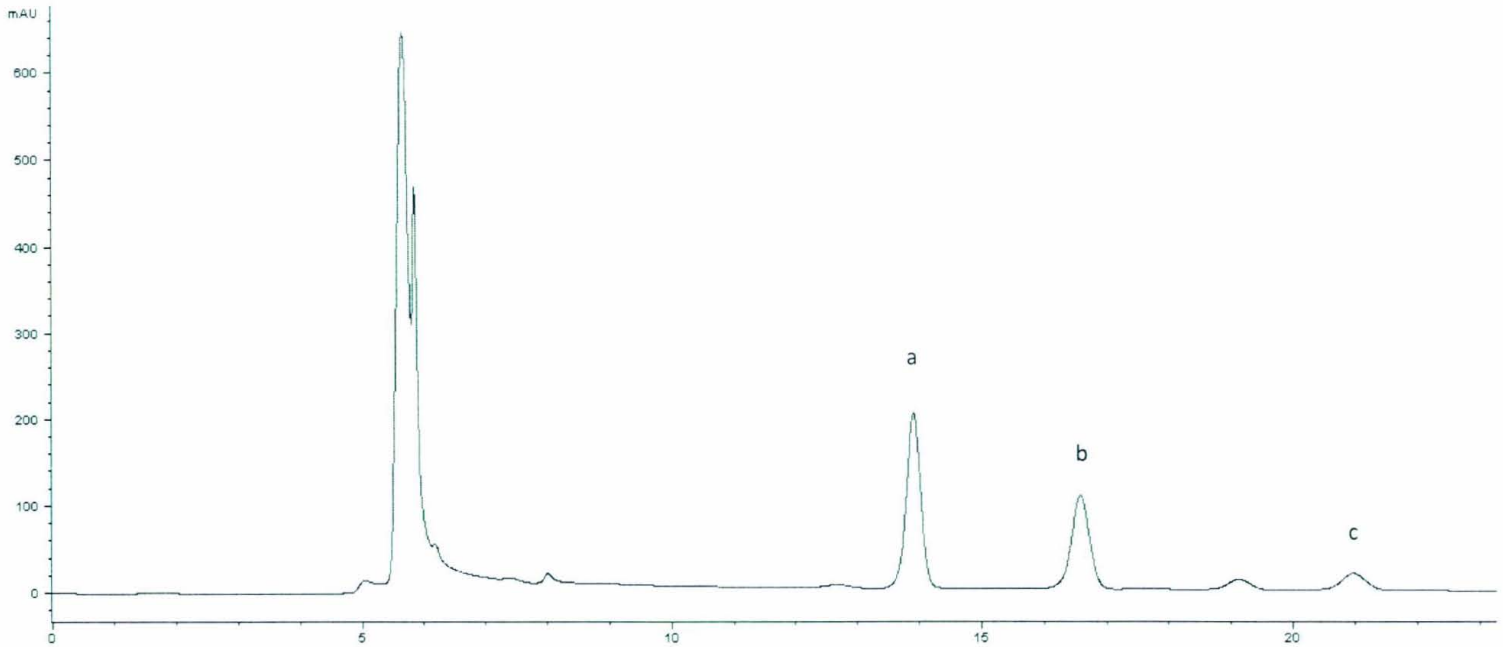

Figure A.2 Representative HPLC chromatogram of a, acetic acid; b, propionic acid; and c, butyric acid produced in the *in vitro* microbial fermentation of foxtail millet grains

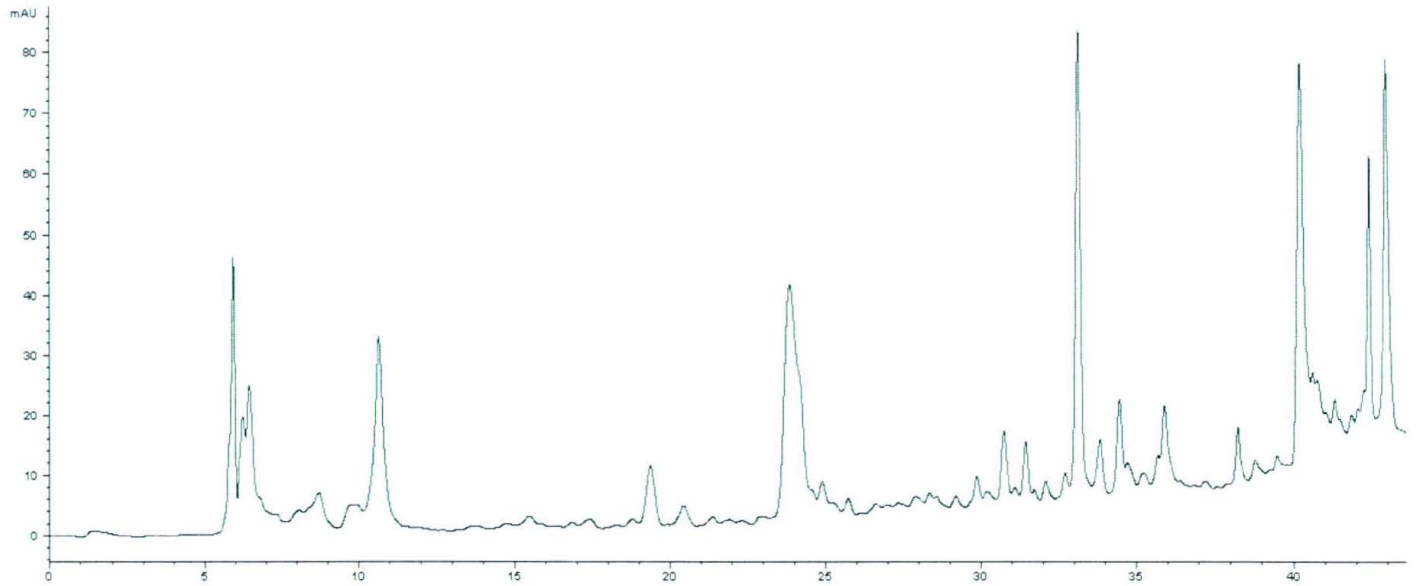

Figure A.3 Representative HPLC chromatogram of phenolic compounds released at the end of intestinal phase in the *in vitro* enzymic digestion of cooked dehulled foxtail millets

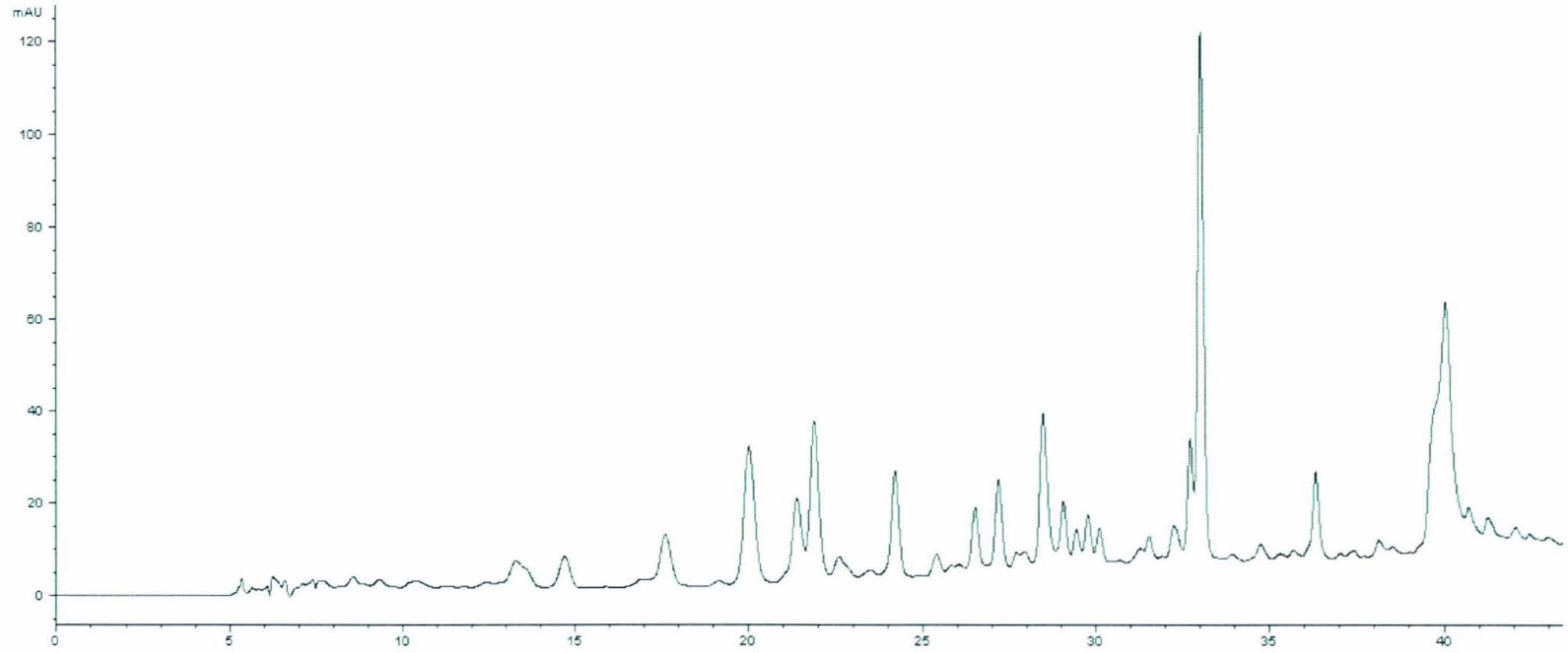

Figure A. 4 Representative HPLC chromatogram of phenolic compounds released in the *in vitro* microbial fermentation of cooked dehulled foxtail millets