Antioxidant properties of alkaline extracts from insoluble and soluble dietary fibre derived from selected whole-grain cereals

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ABSTRACT

The extraction yields of insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) from seven whole-grain cereals (WG) ranged from 11.73% to 23.71% and 2.28% to 5.15%, respectively. Eight monomeric phenolic acids and four diferulic acids were identified and quantified in cereal alkaline extracts by reversed phase high performance liquid chromatography coupled with quadrupole - time of flight mass spectrometry. IDF alkaline extracts had significantly ($p < 0.05$) higher levels of total phenolic content (TPC) and DPPH radical scavenging activity than WG and SDF extracts. Corn IDF (C-IDF) extracts exhibited the highest TPC and DPPH, followed by red rice. MTT cell viability assay indicated that 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) or xanthine-xanthine oxidase (X-XO) induced cell loss can be effectively reduced by pre-incubating Caco-2BBe or FHs 74 Int cells with certain levels of C-IDF extracts. This effect was speculated to be associated with the antioxidant activity of C-IDF linked phenolic compounds.

Key words: whole-grain cereals, insoluble dietary fibre, soluble dietary fibre, alkaline extracts, antioxidant activity

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LIST OF ABBREVIATIONS

AACC	American Association of Cereal Chemists
AAPH	2,2'-azobis (2-amidinopropane) dihydrochloride
AOA	Antioxidant activity
AXA	Arabinoxylans
ATCC	American Type Culture Collection
Corn IDF	C-IDF
DMEM	Dulbecco's Modified Eagle Medium
DF	Dietary fibre
DF-antioxidants	Dietary fibre-antioxidants
DF-PC	Dietary fibre-phenolic compounds
diFAs	Diferulic acids
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EGF	Epidermal growth factor
FA	Ferulic acid
FAE	Ferulic acid equivalent
FBS	Fetal bovine serum
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer
HPLC	Reversed phase high performance liquid chromatography
hTF	Human transferrin
IDF	Insoluble dietary fibre
L-gln	L-glutamine
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
Na/Pyr	Sodium pyruvate
$O_2^{-\bullet}$	Superoxide radical
•OH	Hydroxyl radical
PAs	Phenolic acids

PBS	Phosphate buffered saline
PDA	Photodiode array detection
P/S	Penicillin/streptomycin
Q-TOF	Quadrupole - time of flight
ROO•	Peroxyl
ROS	Reactive oxygen species
SDF	Soluble dietary fibre
SET	Single electron transfer
X	Xanthine
XO	Xanthine oxidase
X-XO	Xanthine-xanthine oxidase
TE	Trolox equivalents
TPA	Total phenolic acid content
TPC	Total phenolic content
WG	Whole-grain cereals

CHAPTER 1: Introduction

Cereal grains are major food sources worldwide. Whole-grain cereals, which contain the principal anatomical components – the germ, endosperm, and bran, are rich sources of energy, protein, dietary fibre, vitamins, and minerals for the human diet. The mainly consumed cereal grains include wheat, rice, maize, oats, rye, barley, triticale, millet, and sorghum (Fardet, 2010). Three cereals: wheat, maize and rice together comprise at least 75% of the world's grain production (Cordain, 1999).

Generally, an intact cereal kernel comprises three parts: germ, endosperm, and multiple bran layers (**Figure 1.1 (a)**). From the inner to the outer side, the bran layers are named as aleurone layer, testa, and pericarp (**Figure 1.1 (b)**). Aleurone layer is the outermost layer of endosperm but is removed with the testa and pericarp to form the bran (Hoseney, 1994). In spite of structural similarities, different species of cereal grains display variations in size, shape, and proportions of cereal components (Kent & Evers, 1994). As seen in **Table 1.1,** the endosperm is the largest tissue of the grain. Wheat grains have a relatively higher proportion of bran that ranges from 14.1% to 15.9%. The maize grain is the largest of the common cereal grains and has a relatively big germ. The aleurone layer is generally one cell thick in most cereals. However, barley has two to three cell thick aleurone layers and the aleurone cells are smaller than those in wheat (Kent & Evers, 1994).

The bioactive compounds in cereal grains are unevenly distributed (Fardet, 2010). In general, bran is a rich source of dietary fibre, proteins, vitamins, minerals, and cell wall bound phenolic compounds; endosperm is mainly composed of starch and proteins; germ is relatively high in protein, sugar, oil, vitamins, and enzymes (Hoseney, 1994; Fardet, 2010). The

bioactive compounds also display inconsistent composition among cereal species. Take dietary fibre as an example. Cereal dietary fibre is mainly composed of non-starchy polysaccharides and lignin and functions as cell wall structural components in cereal grains. Therefore, the size of cereal cells, the thickness of the cell walls, and the distribution of dietary fibre polysaccharides (cellulose in the pericarp and testa; arabinoxylan and β-glucan in the aleurone and starchy endosperm cell walls) decides dietary fibre levels in different types of cereals (Mälkki, 2001; Fastnaught, 2001; Căpriţă & Căpriţă, 2011). The multiple layers of aleurone cells result in barley having a relatively higher dietary fibre content. Barley, rye, and oats with high β-glucan content in their endosperm cell walls also decide their higher dietary fibre yield. Wheat endosperm walls are rich in arabinoxylans but poor in β-glucan, and thus display high amounts of insoluble dietary fibre in the bran fraction (**Table 2.2**). Maize has a thicker and more robust pericarp than the smaller grains so the dietary fibre content in its bran fraction is extremely high (**Table 2.2**). Moreover, different cereal cultivars and growing environment also influence the dietary fibre composition in cereals (Fastnaught, 2001).

Table 1.1 Proportions of parts of cereal grains (%).

Cereal	Bran (Pericarp + Testa + Aleurone)	Endosperm	Germ (Embryonic axis + Scutellum)
Wheat	14.1-15.9	81.4-84.1	2.5-3.6
Barley	8.8	87.6	3.4
Oats	12.0	84.0	3.7
Rice	5.0-7.0	90.7-91.7	2.3-3.3
Maize	5.1-6.5	79.7-82.0	11.7-15.2

Source: Kent & Evers (1994)

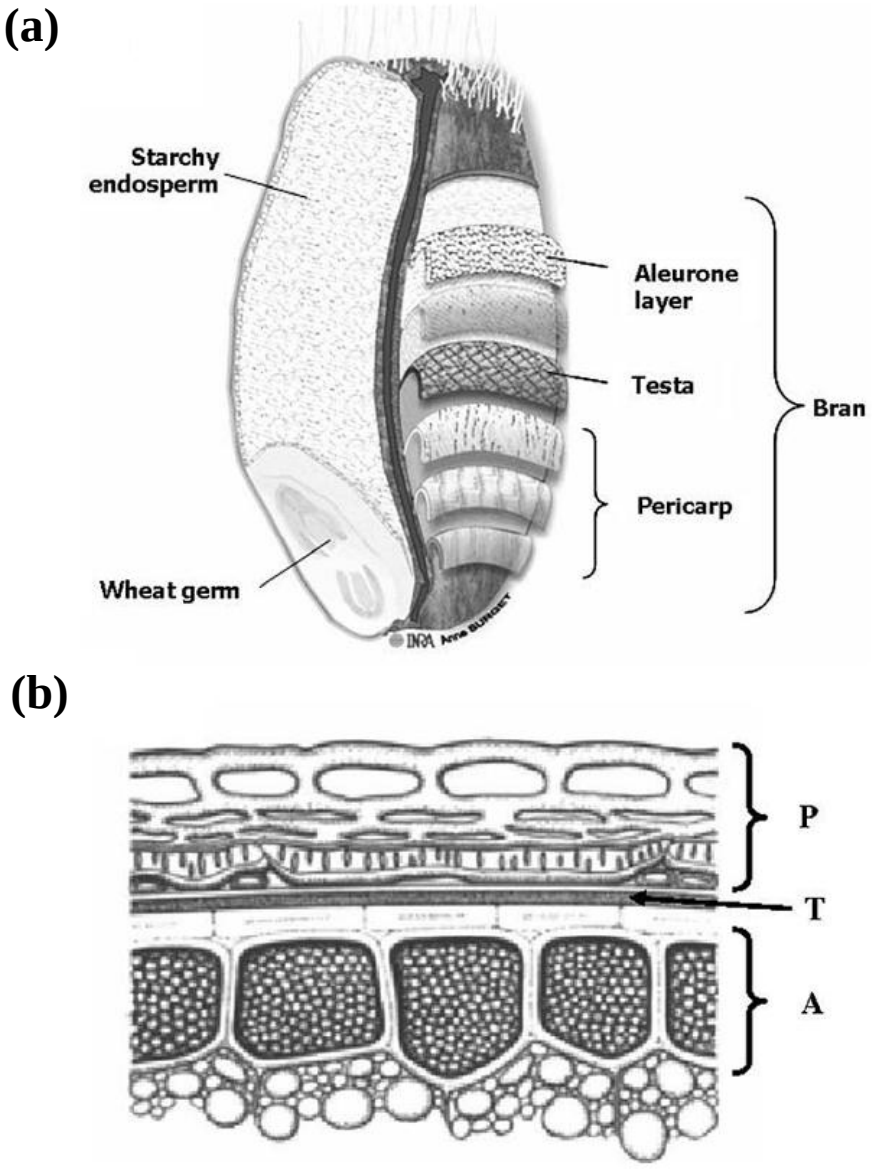


Figure 1.1 The structure of wheat whole grain kernel (a) and the constitutive layers of wheat bran (b). P: pericarp; T: testa; A: aleurone layer.

Source: Hemery et al. (2009)

The consumption of whole-grain cereals has been revealed to be associated with the reduced risk of developing chronic diseases, such as cardiovascular disease, type II diabetes, obesity, and cancer (Okarter & Liu, 2010). These health benefits are in part attributed to the antioxidant capacity of phenolic compounds in cereals (Liu, 2007). The predominant group of phenolic antioxidants in cereal grains is phenolic acids, which can be further classified into hydroxybenzoic acids and hydroxycinnamic acids. Hydroxycinnamic acid derivatives, particularly ferulic acid, are the most abundant type of phenolic acids in cereal grains and found esterified or etherified to cereal cell wall components. As the major constituent of plant cell walls, dietary fibre is regarded as an abundant source of phenolic acids. The phenolic acids linked to dietary fibre polysaccharides can be released by bacterial enzymes in the large intestine (Buchanan et al., 1996; Kroon et al., 1997; Vitaglione et al. 2008). The free phenolic components can function as antioxidants and provide an important health protection in the human body. Therefore, to reveal the antioxidant potential of dietary fibre-linked phenolics in commonly consumed cereal grains, the objectives of the present study were:

(1) To prepare dietary fibre fractions form barley, wheat, corn, rice and oats whole-grain cereals and to release the bound phenolic acids from whole-grain cereals and its dietary fibre fractions;

(2) To confirm the phenolic acid composition in alkaline extracts obtained from whole-grain cereals and its dietary fibre fractions;

(3) To evaluate the antioxidant capacity of dietary fibre alkaline extracts based on chemical assays; and

(4) To investigate the antioxidant potential of dietary fibre alkaline extracts in cell culture models.

CHAPTER 2: Literature review

2.1 Dietary fibre (DF)

2.1.1 Definition, composition, and classification of DF

The first physiological-related definition of dietary fibre (DF) was described as plant cell wall remnants that are resistant to hydrolysis by human alimentary enzymes (Trowell, 1974). Over the following 30 years, such a definition has been gradually modified and improved. AACC (American Association of Cereal Chemists, 2001) gave a more detailed definition of DF based on its chemical nature and physiological properties: *"DF is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. DF includes polysaccharides, oligosaccharides, lignin, and associated plant substances. DF promotes beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation".* More recently, Health Canada developed a revised DF definition as *carbohydrates with a DP (degree of polymerization or number of saccharide units) of 3 or more that naturally occur in foods of plant origin and that are not digested and absorbed by the small intestine.* In addition, *novel fibres*, which are ingredients manufactured to be sources of DF, were also included in the above definition. Once a novel fibre source has been accepted by Health Canada, it is labelled as DF (Health Canada, 2012). Such a definition allows for the flexibility to incorporate new fibre sources developed in the future. Although DF has been defined in a variety of ways, some essential components of proposed definitions of DF are common, such as the chemical structure, the site of digestion,

the acceptable sources of fibre, and a reference to measurable beneficial effects (Health Canada, 2010).

Most of our DF intakes come from the cell walls of the plant food sources. The principal components of DF in plant cell walls are non-starchy polysaccharides (mainly cellulose, hemicellulose, pectin), lignin, and some proteins. However, their compositions vary with the types of plant materials (**Table 2.1**). The primary difference between dicotyledons and cereal grains is their pectic substance content. The cell wall polysaccharides of fruits, vegetables, and legumes are mainly pectic substance, cellulose, and some hemicellulose components, whereas those of cereals are predominantly arabinoxylans (AXA) and β-glucans, the two of which comprise the hemicellulose part of cereal DF. Phenolic acid esters are mainly found in cereal cell walls. Their cross-linkages with cereal DF components, such as AXA and lignins, have been revealed (Renger & Steinhart, 2000; Mathew & Abraham, 2004).

Table 2.1 Components of dietary fibre (DF).

DF sources	Tissue types	Main DF polymers
Cereals	Endosperm Seed coats	Hemicellulose (AXA and β-glucans) Cellulose Lignin
Fruits and vegetables	Parenchymatous flesh Vascular tissue Epidermal tissue	Cellulose Hemicellulose (xyloglucans, glucuronoxylans) Pectic substances Lignin Cutin Waxes
Seeds other than cereals	Cotyledons Endosperm walls	Cellulose Hemicellulose (xyloglucans, galactomannans) Pectic substances
Food additives		Gums

Source: Selvendran (1984); Pilch (1987)

DF is conventionally divided into two categories according to their water solubility, namely insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). Each category displays different DF composition. IDF mainly consists of water insoluble cell wall components such as cellulose, part of hemicellulose (water-insoluble AXA), and lignin, while SDF is composed of noncellulosic polysaccharides (including pectin, gums, and mucilages) and some hemicelluloses (water-soluble AXA and β-glucan) (Charalampopoulos et al., 2002). About 75% of the dietary fibre in foods is found in the insoluble fraction (Dreher, 2001). Whole-grain cereals and their bran fractions are rich in IDF. In addition to this, other sources of IDF include dried beans, peas, vegetables, and nuts. Good sources of SDF include whole-grain oats and barley, some fruits, dried beans, and legumes.

2.1.2 Cereal DF

Cereal grains are important plant food sources of DF. In western countries, wheat, barley, oat, rye, rice and maize contribute about 50% of DF consumption (Vitaglione et al., 2008). Both types of insoluble and soluble dietary fibre exist in cereals and serve as structural constituents in maintaining cell wall strength (Izydorczyk & Biliaderis, 1995).

The presence of DF in cereal cell walls is complex. **Figure 2.1** gives a simplified model of DF in cereal endosperm primary cell walls. Cellulose microfibrils are the predominant components of cereal primary cell walls. They are embedded in a network of matrix of hemicelluloses, which consist mainly of AXA and/or β-glucans. Some of hemicellulose components are cross-linked by phenolic esters and/or proteins. The lignin present in secondary cell wall may be covalently bound to hemicelluloses and proteins (Stone, 2006).

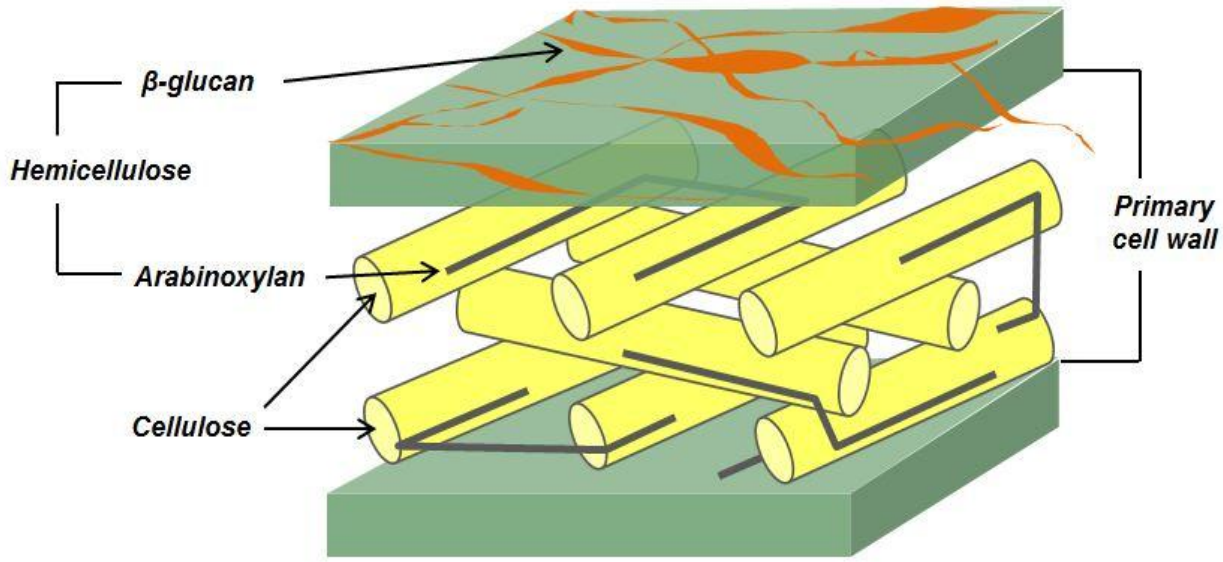


Figure 2.1 A simplified model of cereal endosperm primary cell wall.

Source: Stone (2006)

The distribution of DF is uneven in cereal structures. Generally, an intact cereal kernel consists of three parts: germ, endosperm, and multiple bran layers. The majority of DF occurs in decreasing amounts from the outer bran layers to the endosperm (Charalampopoulos et al., 2002). For example, in wheat bran fraction, the proportion of DF is up to 45%, while in endosperm fraction, the amount of DF is usually less than 10% (Fardet, 2010). It is reported that cereal bran concentrates 30-80% of DF in whole grains (Vitaglione et al., 2008). Therefore,

the refining processes that remove the bran and germ fractions from whole-grain cereals may result in the loss of DF up to 50% (Selvendran, 1984; Fardet, 2010).

The content and the proportion of IDF and SDF are various among different types of cereals (**Table 2.2**). This is mainly due to their differences in seed morphology. Most cereal grains, such as wheat, maize, and barley have much higher content of IDF than SDF. In oat, the interest is due to its relatively high content of SDF, which is mainly composed of β-glucans (about 40% in flour) (Renger & Steinhart, 2000).

Table 2.2 Amount of total DF (TDF), insoluble DF (IDF), and soluble DF (SDF) of the whole grain and the bran fraction of five main western diet cereals (g/100 g).

		TDF	IDF	SDF
Wheat	Whole	11.6-17.0	10.2-14.7	1.4-2.3
	Bran	36.5-52.4	35.0-48.4	1.5-4.0
Oat	Whole	11.5-37.7	8.6-33.9	2.9-3.8
	Bran	18.1-25.2	14.5-20.2	3.6-5.0
Barley	Whole	14.6-27.1	12.0-22.1	2.6-5.0
	Bran	70.0	67.0	3.0
Corn	Whole	13.1-19.6	11.6-16.0	1.5-3.6
	Bran	86.7	86.5	0.2
Rye	Whole	15.2-20.9	11.1-16.0	3.7-4.5
	Bran	35.8	30.5	5.3

Source: Vitaglione et al. (2008); Fastnaught (2001)

2.1.3 Physiological effect of DF

According to the definition, DF is not digested by mammalian enzymes. Therefore, it passes into the intestine and results in physiological effects along the gastrointestinal tract. The

beneficial physiological effects of DF have been widely reported, such as laxation, regulation of blood sugar, lowering of blood cholesterol, and prevention of bowel cancer (Ferguson & Harris, 1999; Slavin, 2003; Wood, 2007; Topping, 2007; Anderson et al., 2009). Such physiological benefits are mostly due to different actions of IDF and SDF that possess different compositions.

The SDF components (water-soluble AXA and β-glucans) have high viscosity in water, so they can form viscous solutions, thereby slowing intestinal transit and delaying gastric emptying (Charalampopoulos et al., 2002). SDF can also lower serum cholesterol (Braaten et al., 1994) and reduce the post-prandial blood glucose (Casiraghi et al., 2006). Once fermenting in the colon, SDF degrades into short-chain fatty acids, which may inhibit hepatic cholesterol synthesis (Dreher, 2001). The cholesterol-lowering effect of oat bran that is rich in soluble β-glucans has been demonstrated in rats and humans (Atuio, 2006).

IDF shows different bio-functions from SDF due to its unique chemical composition. IDF, mainly composed of cellulose and water-insoluble AXA, do not have the ability to produce viscous solutions, so it cannot be fermented in the intestine. In the colon, the undegraded DF tends to increase faecal bulking, shorten bowel transit time, and thus decrease the contact between carcinogens and the colon epithelial cells (Fardet et al., 2010; Charalampopoulos et al., 2002). This may be important in the prevention of colon cancer (Negri et al., 1998).

AACC International reported three physiological effects of DF that have been scientifically recognized: a positive effect on laxation, attenuation of blood cholesterol levels, and/or attenuation of blood glucose levels (AACC report, 2003). In the newest report by Health Canada (2012), the beneficial effects of DF are summarized as:

- *improves laxation or regularity by increasing stool bulk;*
- *reduces blood total and/or low-density lipoprotein cholesterol levels;*
- *reduces post-prandial blood glucose and/or insulin levels;and*
- *provides energy-yielding metabolites through colonic fermentation.*

Additionally, DF can also be used as a source of prebiotic. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health (Gibson & Roberfroid, 1995). The water soluble DF components comply with the prebiotic criteria. SDF in colon can be degraded into short-chain fatty acids, which can increase the population of beneficial colonic bacteria, such as lactobacilli and bifidobacteria, and thereby act as prebiotics (Charalampopoulos et al., 2002).

Epidemiological studies have revealed an inverse relationship between DF consumption and the incidence of various diseases, such as obesity (Slavin, 2005), diabetes (Anderson, 2000; Chandalia et al., 2000), heart disease (Pereira et al., 2004), and colon and breast cancer (Gerber, 1998; Cohen, 1999; Aune et al., 2011). However, due to the complexity of human diet and the various mechanisms involved, the role of DF in human nutrition is still being studied.

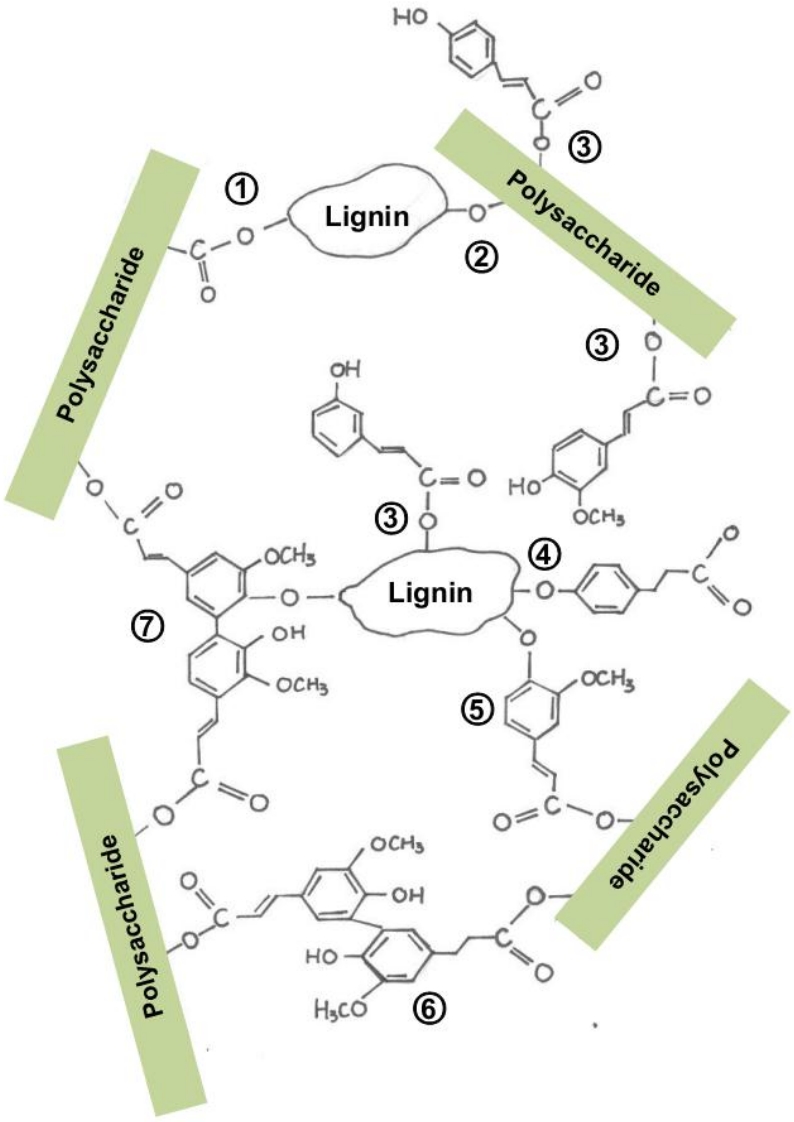
2.2 Dietary fibre-antioxidants (DF-antioxidants)

2.2.1 Concept of DF-antioxidants

In a study by Saura-Calixto (1998), the idea of antioxidant DF was first introduced. It mainly refers to the fruit or vegetable derived DF, which contains high levels of polyphenolic compounds and SDF/IDF ratio. It is also known that whole grains, particularly their bran

fractions, are an abundant source of DF. Unlike fruits or vegetables, however, the dominant forms of DF in cereals are insoluble and their associated phenolic compounds are mainly phenolic acids. With the increasing focus on the potential role of whole grain cereals and cereal bran enriched food products in human health, the concept of DF-antioxidants or DF-PA was proposed (Vitaglione et al., 2008) and their *in vitro* and *in vivo* release, bioavailability, and consequent bioprotection have been hypothesised and studied (Kroon et al., 1997; Andreasen et al., 2001; Kern et al. 2003; Pellegrini et al., 2006).

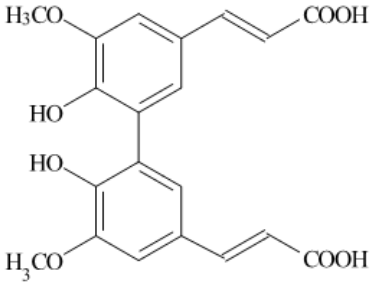
Ferulic acid (FA) and its dehydrodimers are important as the predominant phenolic compounds bound to the cereal DF matrix. As seen in **Figure 2.2**, FA attaches DF polysaccharides (mainly AXA) and serves as a part of lignin by ether or ester linkages. Dehydrodimeric FAs (diFAs) cross-link DF components and play an important role in maintaining plant cell wall integrity (Mathew & Abraham, 2004). The major FA dehydrodimers in cereals include 5-5'-coupled, 8-5'-(linear and benzofuran form)-coupled, 8-8'-(linear and aryltetralin form)-coupled, and 8-*O*-4'-coupled forms (**Figure 2.3**). In addition to these, 5-5'-methylated, 8-5'-decarboxylated, and 8-8'-tetrahydrofuran diFA were also detected in corn stover and corn IDF fraction (Dobberstein & Bunzel, 2010). FA and diFA are chiefly found in bran layers of the grain. For example, the aleurone layer and the pericarp account for 98% of the total FA in wheat grain (Dreher, 2001). Other than FA monomer and dehydrodimers, *p*-coumaric acids are also detected in cereal fibres and mainly link with lignin at the benzyl position (Lam et al., 2001; Ralph et al., 2004).



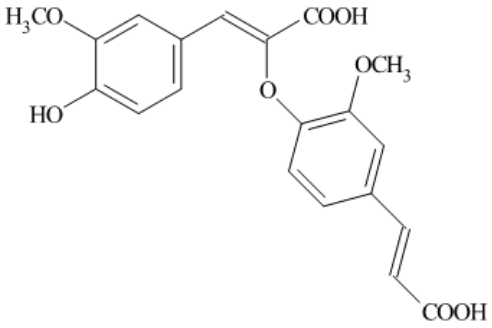
① **Direct ester linkage**
② **Direct ether linkage**
③ **Hydroxycinnamic acid ester**
④ **Hydroxycinnamic acid ether**
⑤ **Ferulic acid bridge**
⑥ **Dehydrodiferulic acid diester bridge**
⑦ **Dehydrodiferulic acid diester-ether bridge**

Figure 2.2 Possible linkages between dietary fibre components and hydroxycinnamic acids.

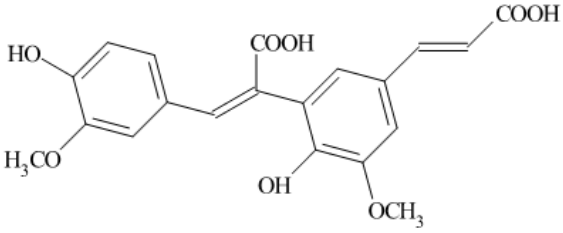
Source: Scheller (2007)



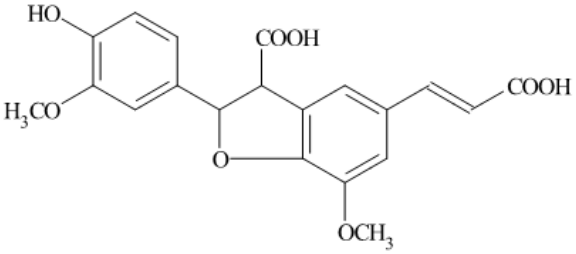
5-5’-coupled



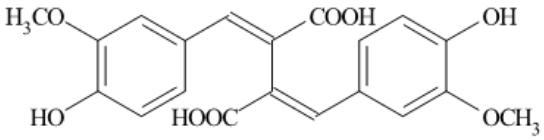
8-*O*-4’-coupled



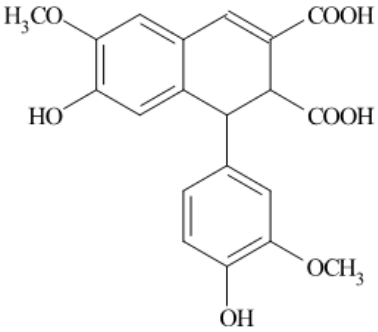
8-5’-linear form-coupled



8-5’- benzofuran form-coupled



8-8’-linear form-coupled



8-8’- aryltetralin form-coupled

Figure 2.3 Major ferulic acid dehydrodimers in cereals.

Source: Waldron et al. (1996)

2.2.2 *In vitro* antioxidant potential of cereal DF-antioxidants

The antioxidant properties of DF associated antioxidants have been measured by chemical assays in DF rich sources, such as whole-grain flour, cereal bran, and DF enriched food products (Adom & Liu, 2002; Pérez-Jiménez & Saura-Calixto, 2005; Pellegrini et al., 2006). From the result of Adom & Liu (2002), corn insoluble fibre fractions showed the highest total phenolic content (13.43 μmol gallic acid equivalent/g of grain), followed by wheat, oats, and rice. A similar pattern was also found in their total antioxidant activities. In a study by Pellegrini et al. (2006), the total phenolic content of the insoluble residue ranged from 2510 gallic acid equivalents mg/kg in wheat flour to 16430 gallic acid equivalents mg/kg in wheat bran. The higher levels of antioxidant capacity were found in cereal bran fraction (30.59 μmol Trolox/g of oat bran and 108.30 μmol Trolox/g of wheat bran). These results suggested that DF from different cereal sources with different compositions and PA linkages may decide its antioxidant capacity.

The antioxidant activities of free and insoluble bound PAs were also compared. Pellegrini et al. (2006) applied methanol and alkaline to extract both free and bound phenolics from cereal kernels, flours, and products. They found that the antioxidant capacity in cereal bound fractions was generally higher than that of free extracts. Pérez-Jiménez & Saura-Calixto (2005) also reported a much higher total phenolic content and antioxidant capacity of hydrolysable phenolic extracts compared with aqueous-organic extracts from a list of cereal products. Similarly, Qiu et al. (2010) observed a higher antioxidant activity of insoluble fractions than that of soluble fractions from eleven wild rice varieties. It is also reported that bound phenolics contribute 90% of the total phenolics in wheat, 87% in corn, 71% in rice, and 58% in oats (Adom & Liu, 2002). Therefore, due to the larger proportion, higher phenolic

content, and better antioxidant capacity of DF bound antioxidants, a complete release of these is essential to fully reveal the antioxidant properties of whole-grain cereals and their fibre concentrated fractions. Nevertheless, unlike free phenolic compounds, DF-bound phenolics display low solubility in water and organic solvent (Vitaglione et al., 2008), so their methanol or ethanol extracts usually reflect a small proportion of the real antioxidant capacity in cereals. To solve this problem, appropriate treatments are needed, such as alkali saponification, acidic hydrolysis, or enzymatic processes (Mathew & Abraham, 2004).

In vitro physiological evidence suggested that cereal antioxidants are available using small intestine digestion models that mimic the gastro-intestinal system (Pérez-Jiménez & Saura-Calixto, 2005; Serrano et al., 2007). The following colonic fermentation of the indigestible fraction in rat model indicated the bioaccessibility of insoluble antioxidants in the large intestine (Serrano et al., 2007). Less antioxidant capacity released from the fermentation process compared with enzymic digestion implied that some of the antioxidants released from the food matrix during colonic fermentation may be utilized for bacterial metabolism (Serrano et al., 2007). The presence of microbial cinnamyol esterase, xylanase, and ferulic acid esterase may be responsible for the liberation and degradation of insoluble antioxidants from the fibre fraction (Kroon et al., 1997; Andreasen et al., 2001).

2.2.3 *In vivo* antioxidant potential of cereal DF-antioxidants

2.2.3.1 Release of DF-Phenolic compounds (DF-PC) in human gastrointestinal tract

Vitaglione et al. (2008) hypothesised that the slow and continuous release of DF bound antioxidants in the gut determines the health benefits of cereal bran and whole grain consumption. On the basis of the proposed hypothesis, the mechanism of the phenolics

released from DF in the gastrointestinal tract is simply illustrated in **Figure 2.4**. Briefly, DF bound phenolic compounds survive digestion in the stomach and small intestine and arrive in the colon. In the colon, phenolics can be released by colonic bacterial enzymes, cross the intestinal epithelium into the blood plasma, and exert the antioxidant properties there.

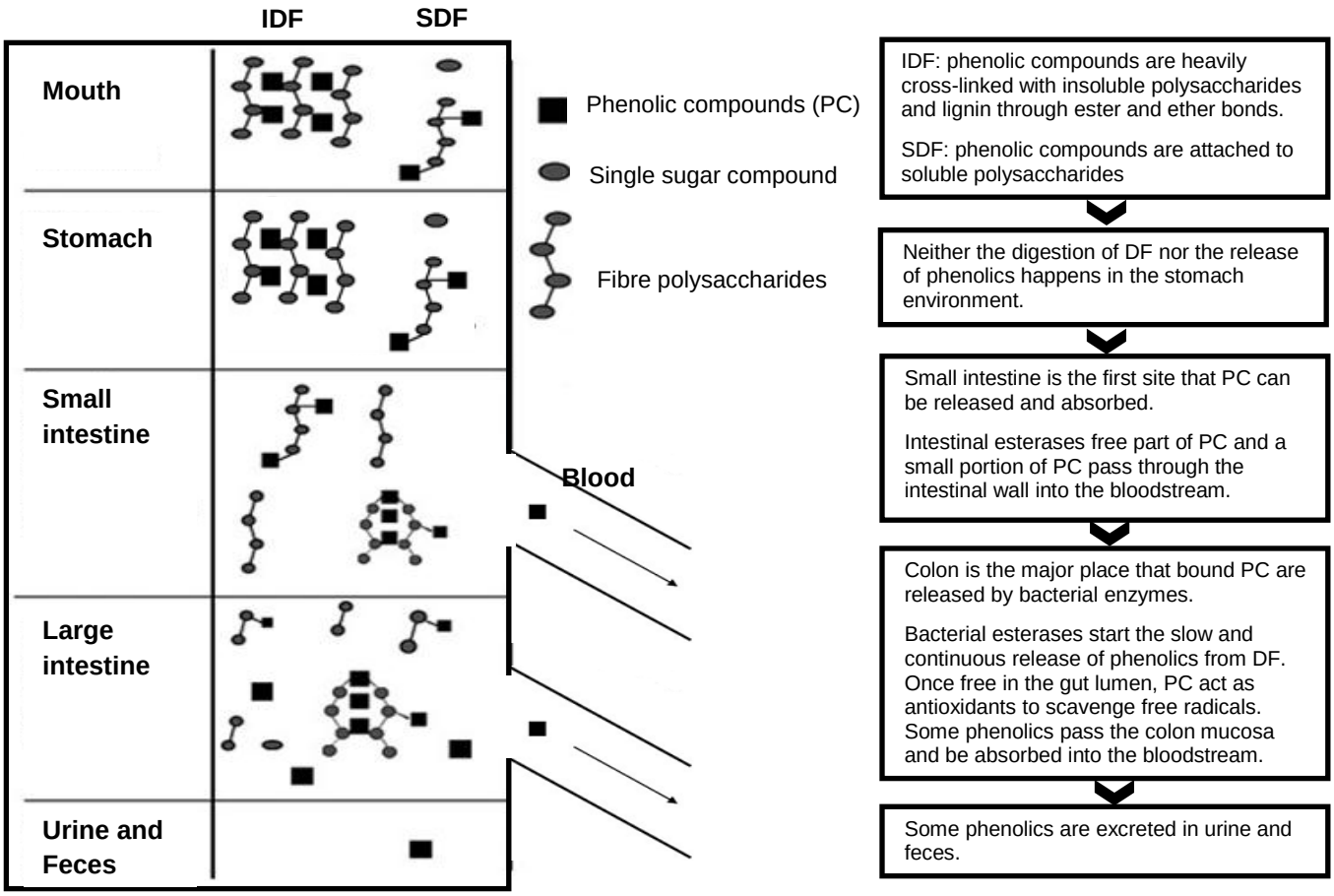


Figure 2.4 The simplified illustration of phenolic antioxidants released from DF in the gut.

Source: Vitaglione et al. (2008); Palafox-Carlos et al. (2011)

The released free FA is the major form of FA that is available in the colon and the blood after passaging across the colonic barrier (Poquet et al., 2008), so the production of free FA from DF decides its health benefits. Compared with IDF, the phenolic compounds linked with SDF, which is less structured, are more readily freed by bacterial esterases (Kroon et al., 1997). Therefore, the higher SDF/IDF ratio of the cereal products are favoured to exhibit antioxidant protective benefits. Furthermore, using enzymes to convert IDF into SDF, maintaining the phenolic compounds linked to DF, is a promising strategy to enhance the possible health effects of cereal products (Vitaglione et al., 2008).

2.2.3.2 Bioavailability of DF-Ferulic acid (DF-FA) in animal and human studies

The *in vivo* physiologic importance of phenolic antioxidants depends upon its availability for absorption and interaction with target tissues (Adam et al., 2002). As the predominant phenolic acid in cereals, the bioavailability of FA in the gut environment decides the antioxidant potential of cereals.

In cereal grains, FA exists as free, soluble conjugated, and insoluble bound forms and the ratio of these is 0.1: 1: 100 (Adom and Liu, 2002). Free form FA has a good absorbability (Adam et al., 2002; Zhao et al., 2004; Poquet et al., 2008), but it is quickly metabolized in liver to form FA sulphate and/or glucuronides (Zhao et al., 2004). Due to this reason, the bioavailability of free type FA is low (Zhao & Moghadasian, 2008). In contrast, the cell wall polysaccharides bound FA remains for a long time in gastrointestinal digestion, so it is hypothesised that slow and continuous release of DF-PC may exert an antioxidant protection in gastrointestinal tract (Vitaglione et al., 2008). However, the evidence of the bioavailability of bound FA is inconsistent. Zhao et al. (2005) found that FA concentrated corn bran was less

bioavailable than free FA. In contrast, in the study by Rondini et al. (2004), wheat bran rich in bound FA showed higher metabolic fate than free FA. Such a difference may be attributed to the linkage form of FA and the physical structure of cereal fractions. As described in **2.2.1**, in cereal IDF fraction, FA monomeric and dimeric groups are complicatedly cross linked with water-insoluble AXA and lignin, while in cereal SDF fraction, mainly FA monomers are attached to the water-soluble AXA. Therefore, SDF-FA has the advantage of being released from the water soluble DF component and being utilized in the intestine. It is reported that wheat bran contains higher amount of SDF than corn bran (Kahlon & Chow, 2000), so more available FA existing in wheat bran SDF fraction determines its relatively higher bioavailability. Similarly, higher FA bioavailability from SDF than IDF was also demonstrated in cereal endosperm and bran fraction (Adam et al., 2002). This was due to the fact that a large proportion of SDF consisting of wheat endosperm cell wall facilities the release of bound FA by enzymatic and microbial degradation. Furthermore, the high concentration of diFA in corn bran reinforces the physical structure of fibre and consequently decreases the bioaccessibility of bound FA (Vitaglione et al., 2008). To sum up, cereal cell wall-linked FA may have advantages than free FA in producing protective effects in biological systems. However, the nature of linkages between FA and DF components determines the bioavailability of DF-FA.

In human studies, the research on the metabolic fate of DF-FA is limited. Kern et al. (2003) investigated the bioavailability of hydroxycinnamates from high-bran wheat breakfast in humans. According to their findings, it is concluded that the de-esterification and absorption of FA take place mainly in the human small intestine and from the soluble part of the cereal diet. Although from this work, the availability of bound type PA in the large intestine is undecided, Kern and coworkers hypothesized that the bound form of FA may be further

metabolized by colonic microflora. However, to date, the site and extent of bound FA absorption in human gastrointestinal tract is still unclear.

2.2.3.3 *In vivo* antioxidant activity of cereal DF-antioxidants

The *in vivo* antioxidant properties of whole-grain cereals and their bran fractions have been reported in animal and human studies (Rezar et al., 2003; Chen et al. 2004; Ardiansyah et al., 2006; Fardet et al., 2007; Price et al., 2008; Wang et al., 2011). In animal studies, wheat bran, oat bran, and rice bran displayed positive effects on some antioxidant biomarkers, including the level of malondialdehyde (MDA) excretion, the degree of DNA damage, and the regulation of glucose metabolism in rats and pigs (Rezar et al., 2003; Ardiansyah et al., 2006). In the work by Fardet et al. (2007), however, no changes in MDA and other antioxidant status were observed between whole wheat flour and refined wheat flour treated rats after 2 weeks feeding. Recently, a study carried out by Wang and coworkers (2011) suggested that the feruloyl oligosaccharides (FOs) derived from wheat bran IDF decreased the oxidative stress level in rats. They inferred that the enhanced antioxidant capacity of rat plasma after ingestion of FOs might be related to the increased phenolic levels caused by the metabolites of FOs in rats. Significantly higher total phenolic content and antioxidant potential were also observed in human plasma after consumption of wheat bran compared with ground rice (Price et al., 2008). In living organisms, numerous factors may influence the antioxidant action of phenolics, such as digestibility, bioavailability and metabolism, so more *in vivo* studies are needed to explore the real antioxidant potential of cereals.

2.3 Evaluation of antioxidant activity

2.3.1 Mechanism of phenolic acids as antioxidants

In biological systems, reactive oxygen species (ROS) that exist as free radicals and non-radical derivatives of oxygen are continuously produced during cellular metabolism. They function importantly in manipulating cell signaling, apoptosis, gene expression and ion transportation (Lü et al., 2010). However, excess accumulation of ROS causes oxidative stress to cell constituents (lipids, proteins, DNA), results in cell injury, and eventually develops into a number of degenerative diseases, such as inflammatory disease, cardiovascular disease (CVD), diabetes, Alzheimer's disease, cancer, and age-related function decline (Maxwell, 1995; Dhalla et al., 2000; Singh et al., 2004; Singh & Singh, 2008). Antioxidants are substances that can prevent or reduce ROS-induced oxidative damage through the following mechanisms: 1) scavenging free radicals; 2) suppressing free radical production by inhibiting the activity of some enzymes or chelating trace metals involved in free radical generating; 3) enhance the activity or expression of intracellular antioxidant enzymes (Cotelle, 2001; Lü et al., 2010). The formation of ROS and action of antioxidants are summarized in **Figure 2.5**.

Free radical scavenging activity is considered to be the notable mechanism of phenolics in their function as antioxidants in protecting cell constituents against oxidative damage (Nenadis & Tsimidou, 2010). The antioxidant activity of phenolic acids depends on the hydroxyl groups on the aromatic ring (Rice-Evan et al., 1996). Two principal reactions are involved in phenolic antioxidant properties: hydrogen atom transfer (HAT) and single electron transfer (SET) (Huang et al., 2005).

$$AOH + ROO^{\bullet} \rightarrow A^{\bullet} + ROOH \qquad \text{(HAT reaction)}$$

$$AOH + ROO^{\bullet} \rightarrow ROO^{-}\text{-}AH^{\bullet+} \rightarrow ROOH + A^{\bullet} \qquad \text{(SET reaction)}$$

Phenolic antioxidants quench free radicals by donating a hydrogen atom or an electron to a free radical in HAT reaction and SET reaction, respectively (Aruoma, 1998).

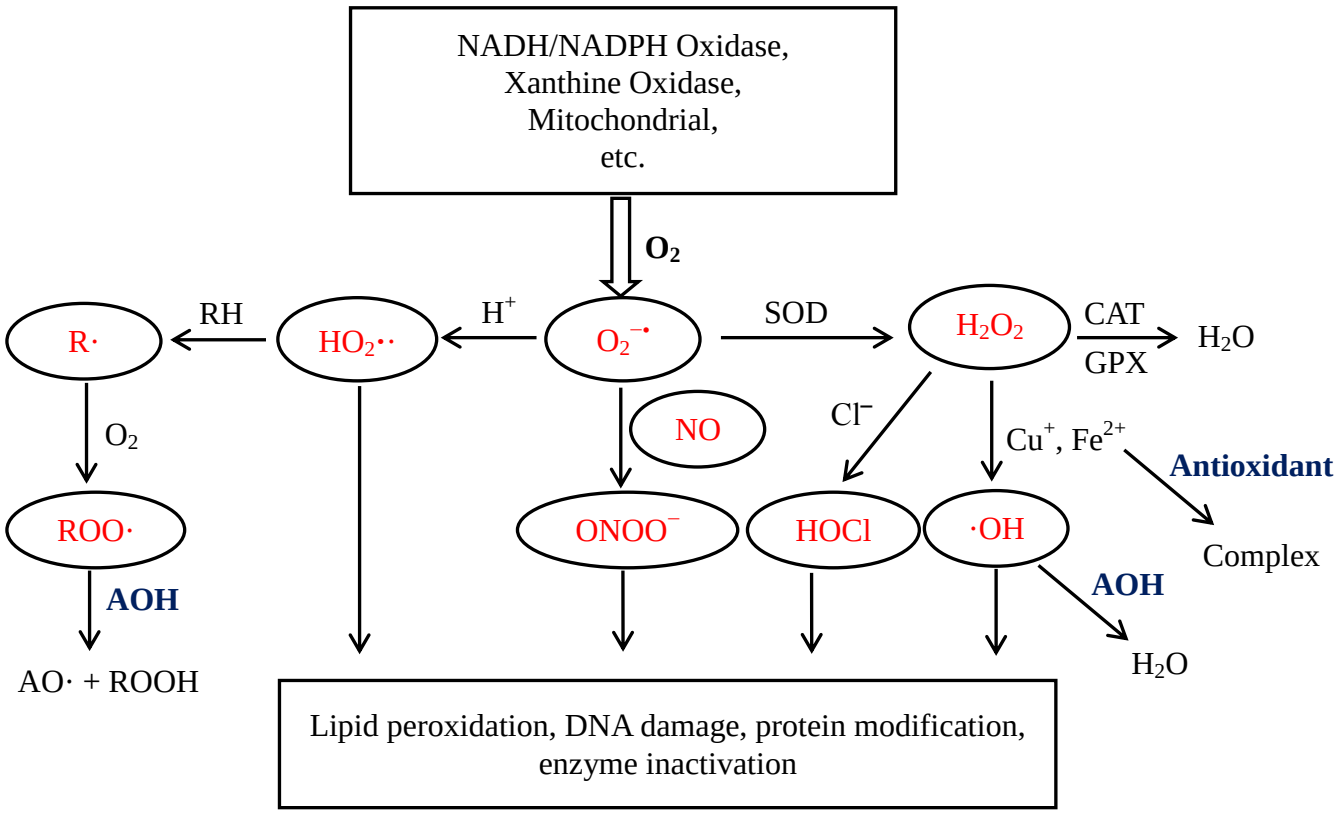


Figure 2.5 Summary of ROS formation and the action of antioxidants. AOH, antioxidant; $O_2^{-\bullet}$, superoxide anion; $HO_2\cdot$, perhydroxyl radical; ·OH, hydroxyl radical; H_2O_2, hydrogen peroxide; NO, nitric oxide; HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; R·, lipid alkyl radical; RH, lipid; ROO·, lipid peroxyl radical; ROOH, lipid hydroperoxide; SOD, superoxide dismutase; CAT, catalase; and GPX, glutathione peroxidase.

Source: Lü et al. (2010)

2.3.2 Chemical-based antioxidant activity assays

In vitro chemical-based antioxidant assays have the advantages of being relatively simple, inexpensive, and reproducible to carry out, so they are widely used to give a first insight of the antioxidant properties of plant-derived phenolic compounds. Based on the reaction mechanisms involved, major antioxidant capacity evaluation methods have been generally divided into two categories: HAT reaction-based assay and SET reaction-based assay (Huang et al., 2005). HAT-based assays measure the free radical scavenging activity of the antioxidant by donating hydrogen (Prior et al., 2005). ORAC (oxygen radical absorbance capacity), TRAP (total radical trapping antioxidant parameter), β-carotene or crocin bleaching assay, and inhibition of LDL oxidation are under this category. The representative SET-based assays include DPPH (diphenyl-1-picrylhydrazyl) radical scavenging activity, FRAP (ferric ion reducing antioxidant potential), TEAC (Trolox equivalent antioxidant capacity), and copper (II) reduction capacity assay. Those methods evaluate the ability of antioxidants to transfer electron in reducing free radicals (Karadag et al., 2009). Due to the undefined reaction conditions, the antioxidant evaluation mechanisms of some assays are disputed. According to Huang et al. (2005), Folin-Ciocalteu method measures the reducing capacity of antioxidants and is usually classified into a SET-based assay. Good correlations were also demonstrated between Folin-Ciocalteu assay and other well-known SET-based assays, such as FRAP (Dudonné et al., 2009). However, Prior et al. (2005) pointed out that Folin-Ciocalteu assay as well as DPPH and TEAC antioxidant activity assays involve both HAT and SET reaction mechanisms with electron transfer action predominantly. Actually, HAT and SET mechanisms almost always occur together in antioxidant reactions and their difference is hard to distinguish (Karadag et al., 2009). In addition, some chemical assays adopt biologically relevant radical

sources, such as peroxyl radicals generated by AAPH (2,2'-azobis-2-amidinopropane dihydrochloride) in ORAC, crocin bleaching, and lipid autoxidation assays. Such antioxidant activity assays better reveal the radical scavenging activity of antioxidants in biological system (Nenadis & Tsimidou, 2010). It is therefore suggested that a combination of two to three antioxidant assays in consideration of their different reaction mechanisms and biological relevance may provide a more reliable view of phenolic antioxidant activity.

2.3.3 Cell-based antioxidant activity assays

Although the antioxidant properties of plant-derived phenolic compounds have been extensively studied by using chemical systems, such *in vitro* assays do not consider the physiological conditions, bioavailability, and metabolism issues in living beings (Liu & Finley, 2005; Honzel et al., 2008). Thus, their relevance to *in vivo* health-protective activities is uncertain. On the other hand, animal models or human studies are complex, costly, and not suitable for the initial antioxidant screening of foods and dietary supplements (Liu & Finley, 2005). It is therefore advisable to use *ex vivo* models, such as cell culture models, prior to animal studies and human clinical trials to simulate the *in vivo* condition and reveal the effects of treatments in living organisms.

2.3.3.1 Oxidative stress on cellular components

Oxidative stress caused by excess formation of ROS leads to the damage of cell components, including DNA, lipids, and proteins. Free radicals, particularly hydroxyl radical, attack the sugar moiety of DNA and cause the formation of various sugar products, DNA strand breaks, base-free sites, and DNA-protein cross-links (Dizdaroglu et al., 2002). Such free

radical-derived DNA modifications are linked to DNA mutations in carcinogenesis (Farinati et al., 1998; Valko et al., 2004). Lipid peroxidation is a free radical-mediated process (Slater, 1984). A wide range of products are formed by the peroxidation of polyunsaturated fatty acids, such as reactive aldehydes, alkanes, lipid epoxides, and alcohols (Elisia, 2005). The lipid oxidation products may affect cell survival by modifying cell membrane, interfering with cell signaling pathways, and inducing cellular cytotoxicity (Leonarduzzi et al., 2000; Girotti, 2001). Proteins attacked by free radicals can generate amino-acid radicals, which may crosslink or react with oxygen to give peroxyl radicals. Further reactions of peroxyl radicals and proteins produce protein peroxides, which can generate even more radicals (Halliwell & Whiteman, 2004). Oxidative damage to proteins may be affecting the function of receptors, enzymes, transport proteins, generating new antigens, and contributing to secondary damage of other biomolecules, such as DNA (Halliwell & Whiteman, 2004).

2.3.3.2 Potential cell culture models for assessing phenolic antioxidants

To properly assay the antioxidant properties of phenolic compounds on cell basis, relevant cell models are needed to be adopted. Plant derived phenolics have been applied in different cells from human organisms that include macrophage cells, red blood cells, monocytic cells, and a list of cancer related cell lines, such as hepatocarcinoma cells (HepG2 cells, H4IIE cells), intestinal cells (Caco-2 cells, HT-29 cells), breast cancer cells, and prostate cancer cells (Nardini et al., 1995; Kanski et al., 2002; Meyers et al., 2003; Hu et al., 2003; Ferguson et al., 2005; Yuan et al., 2005; Chi et al., 2007; Wolfe & Liu, 2007; Yang & Liu, 2009). The antioxidant biomarkers in cell models include the level of intercellular antioxidant activity, the effect on cell apoptosis, the action in cell inflammatory suppressing, and the

activity on cancer cell proliferation inhibition. Among those biomarkers, the antioxidant activity of phenolic acids in protecting against free radical induced cellular oxidation is widely used. Yuan et al. (2005) evaluated the inhibitory activity of feruloyl oligosaccharides on rat erythrocyte hemolysis induced by AAPH. Wolfe & Liu (2007) evaluated the effect of fruit extracts in preventing the formation of fluorescent dichlorofluorescein (DCF) by AAPH-generated peroxyl radicals in human liver cancer cells. Nardini et al. (1998) evaluated the ability of caffeic acid to affect cellular response in human monocytic cells to t-butyl hydroperoxide-induced oxidative stress. Kanski et al. (2002) reported that the presence of ferulic acid in reducing free radical damage in hippocampal neuronal cells.

It is known that the release and absorption of DF linked phenolic compounds mostly occur in human intestinal system. The small intestine is the first site of phenolic compound absorption after their ester bond cleavage by intestinal esterases (Vitaglione et al. 2008). After passing through small intestine, DF with its unreleased phenolic compounds arrives in human colon and further continuous release of phenolic compounds takes place here by colon bacterial esterases (Vitaglione et al. 2008). Based on the metabolic pathway of DF and its associated antioxidant compounds in the human body, the cell models adopted for DF-antioxidant investigation are preferable from human gastrointestinal tract. Caco-2BBe and FHs 74 Int are two widely used cell models resemble human intestinal tract environment. Caco-2BBe is a human colon adenocarcinoma cell line cloned from Caco-2 cells. Like the parental Caco-2 cells, differentiated Caco-2BBe cells are structurally and functionally similar to the small intestinal epithelium and regarded as a simple *in vitro* model for the study of drug transportation and absorption in human intestinal mucosa (Hilger et al., 1990). FHs 74 Int cell line is a primary human fetal intestinal cell culture. Although genotypically similar to neonatal

enterocytes, it has been reported to show mature epithelial-like characteristics (Wagner et al., 1998).

Previous studies indicated that the phenolic compounds occurring in dietary fibre, such as ferulic acid, *p*-coumaric acid, and diferulic acids, function well in reducing oxidative stress in various cell and non-cell based systems (Garcia-Conesa et al., 1997; Kanski et al., 2002; Yuan et al., 2005; Ferguson et al., 2005). However, the cereal dietary fibre insoluble fraction, which is abundantly linked with ferulic acids and diferulic acids, has rarely been reported as bioactive components in cellular models. Little information is available about the protective effect of IDF extracts against free radical induced oxidation and its associated cytotoxicity in cultured cells.

CHAPTER 3: Antioxidant properties of alkaline extracts from insoluble and soluble dietary fibre derived from selected whole-grain cereals

3.1 Samples and chemicals

3.1.1 Samples

Seven different species of whole-grain cereals (WG) include barley (Lot no. 100526A), purple barley (Lot no. 9999001117), wheat (MSU D8006 soft white wheat), purple wheat, yellow corn (US P1395 XR), red rice (Jatiluwih red rice, Bali, Indonesia), and oats (Lot no. 10702tN11010AA) were used in the present study. Except red rice, all the cereal samples were provided from Kellogg (Battle Creek, Michigan, USA).

3.1.2 Chemicals

(1) Sample preparation

Heat-stable *α*-amylase Termamyl 120 L, protease Alcalase 2.4 L, and amyloglucosidase AMG 300 L, used in DF preparation, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ethanol, acetone, and ethyl acetate (HPLC grade) were also from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

(2) HPLC and HPLC-MS/MS analysis

Phenolic acid standards (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringe, *p*-coumaric, ferulic, sinapic, *iso*-ferulic, *o*-coumaric, *trans*-cinnamic acid) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Deionized distilled water, HPLC grade methanol and acetic acid were used in HPLC and HPLC-MS/MS analysis.

(3) Chemical model assay

Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

(4) Cell culture assay

All cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin/streptomycin (P/S), L-glutamine (L-gln), sodium pyruvate (Na/Pyr), human transferrin (hTF), and epidermal growth factor (EGF) were from Sigma-Aldrich (Oakville, ON, Canada). Phosphate buffered saline (PBS) used for sample preparation was also purchased from Sigma-Aldrich (Oakville, ON, Canada). Trypsin-EDTA used for cell dissociation was obtained from Invitrogen (Burlington, ON, Canada). MTT cell proliferation kit (ATCC® 30-1010k) used for cell viability assay was from ATCC (American Type Culture Collection; Manassas, VA, USA). Xanthine (X), xanthine oxidase (XO, Grade I from bovine milk), and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) used as cell oxidation generators were obtained from Sigma-Aldrich (Oakville, ON, Canada).

3.2 Analytical methods

3.2.1 Sample preparation

WG were ground into powder by using a coffee grinder, then a mortar and pestle was used to get an even particle size. After that, the fine powder was passed through a 0.5 mm sieve screen and stored at -20 ℃ before analysis.

3.2.2 Extraction

3.2.2.1 Insoluble and soluble dietary fibre (DF) preparation

The extraction method of insoluble and soluble DF from WG was determined based on AOAC Official Method 991.43 and Bunzel et al. (2001) with modifications. Extraction procedures are summarized in **Figure 3.1**. Briefly, 5 g of milled sample in duplicate was subjected to sequential enzymatic digestion by heat-stable α-amylase (250 µL, boiling water bath for 30 min), protease (50 mg/mL, 500 µL, pH 7.5, 60 ℃ water bath for 30 min), and amyloglucosidase (1500 µL, pH 4.5, 60 ℃ water bath for 30 min) to remove starch and protein. After centrifugation (10,000 rpm, SS-34 Rotors, RC5C Sorvall Instruments, Thermo Scientific, Asheville, NC, USA), the residue that had been washed with hot distilled water, ethanol (95%), and acetone (95%) was vacuum filtered and referred to as IDF. The supernatant combined with the washings of the residue was precipitated in ethanol (80%, preheated to 60 ℃, 4 volumes) overnight and referred to as SDF. Both the IDF and SDF were placed in a fume hood to remove organic solvent and then dried at 35-40 ℃ overnight in a vacuum oven (Model 5831, Napco Scientific Company, Tualatin, OR, USA). The dried fibre fractions were ground into fine powder by using a mortar and pestle and then passed through a 0.5 mm sieve. The procedures described were repeated several times to get sufficient IDF and SDF for further analysis.

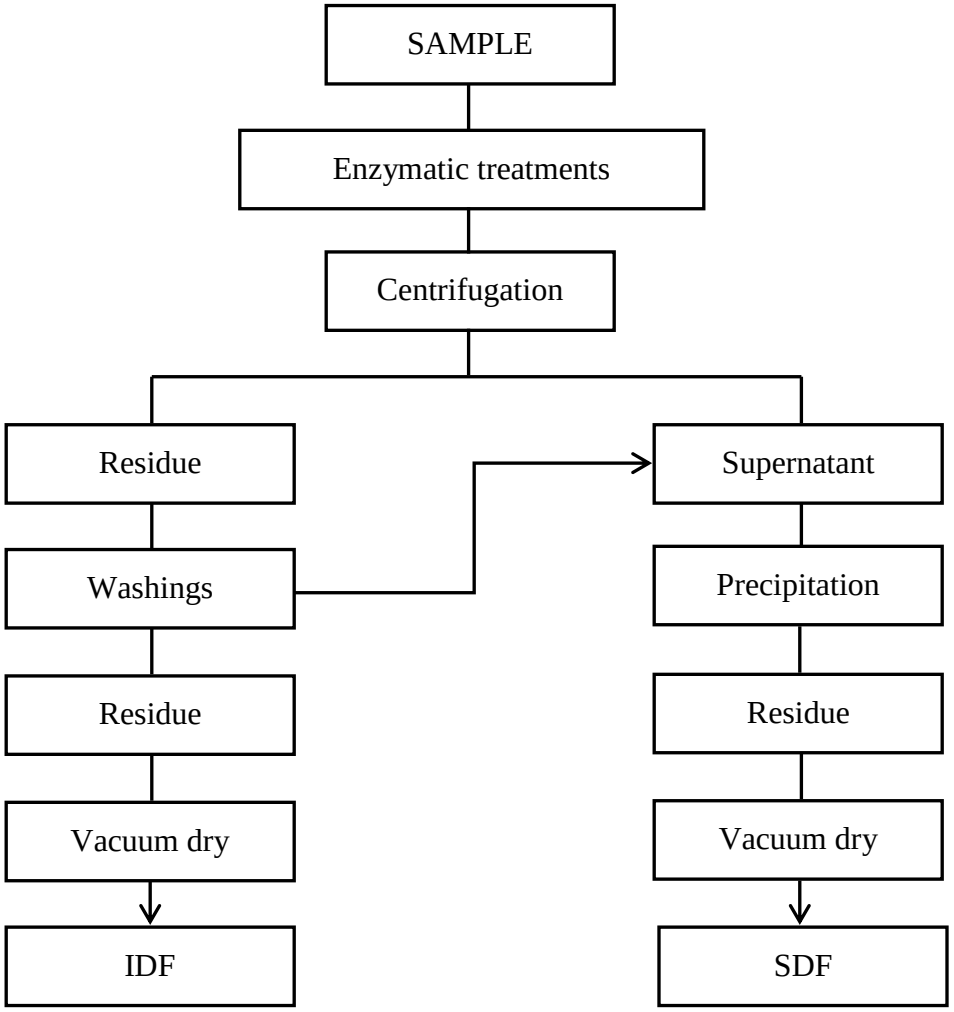


Figure 3.1 Analytical scheme for insoluble and soluble dietary fibre preparation.

3.2.2.2 Phenolic acid extraction

In the present study, alkaline hydrolysis and liquid-liquid partitioning steps were applied to extract ester linked phenolics from WG and their fibre fractions (Grabber et al., 1995; Bunzel et al., 2001; Bunzel et al., 2003). Specifically, WG (100 mg), IDF (100 mg), and SDF (50 mg) were placed in dark-colored screw-cap bottles with degassed NaOH (4 M, 5 ml). The head-space of the bottles was flushed with a stream of N_2 to remove the air. The bottles were shaken slowly on a rotary shaker (Fermentation Design Inc., Allentown, PA) for 4 hrs at room temperature and in the absence of light. Samples were then acidified (pH 1.5-2) with HCl (6 M) and extracted into ethyl acetate (twice the volume, three times). After centrifuging (Thermo Scientific IEC CL31 Multispeed Centrifuge, Thermo Fisher Scientific Inc., USA) at 4000 rpm for 20 min, the supernatants obtained from each time were combined and concentrated to dryness by using a rotary evaporator (IKA RV10, IKA® Works Inc., North Carolina, USA). The dried alkaline extracts were re-dissolved in 1 mL of MeOH/H_2O (50/50, v/v) for the determination of total phenolic content and DPPH radical scavenging activity. Extracts were filtered with a 0.45 μm PTFE filter before HPLC-MS analysis. The phenolic acid extracts in cell culture assay were also prepared as described above, but with different dissolving solvent. The dried alkaline extracts obtained from rotary evaporator were re-dissolved in 1 mL of PBS by using a sonicator to prepare a stock solution of 100 mg/mL. The working solutions at concentrations of 0.2, 0.5, 1, 2, 3, 5, 10, 20 mg/mL were diluted from the stock solution with PBS. Samples were filtered with 0.2 μm sterile filters (Corning Life Science, Pittston, PA, US) before applying to cells.

3.2.3 HPLC and HPLC-MS/MS analyses

3.2.3.1 Identification of phenolic acids by HPLC and HPLC-MS/MS analyses

The identification of major phenolic acids in WG and DF alkaline extracts was carried out on an HPLC (Waters 2695) equipped with a photodiode array detector (PDA) (Waters 996) and an autosampler (Waters 717 plus) (Waters, Milford, MA). The analytical column was a 25 cm × 4.6 mm, 5 µm RP C18 column (Shim-pack HRC-ODS, SHIMADZU Corp., Tokyo, Japan). The mobile phase consisted of A (0.1% acetic acid in water) and B (0.1% acetic acid in methanol). A 70 min-linear gradient established in our laboratory is summarized in **Table 3.1**. The injection volume of sample was 10 µL and the flow rate was 0.9 mL/min. The monitor wavelength was set at 280 nm and 320 nm for hydroxybenzoic acids and hydroxycinnamic acids, respectively. The preliminary identification of detected monomeric phenolic acids was achieved by comparison of the retention time of the alkaline extracts with external phenolic acid standards.

Table 3.1 Solvent gradient of HPLC for phenolic acids.

Time (min)	Solvent A (%)	Solvent B (%)
0 – 11	91 – 86	9 – 14
11 – 14	86 – 85	14 – 15
14 – 17	85 – 85	15 – 15
17 – 24	85 – 83.5	15 – 16.5
24 – 28	83.5 – 81	16.5 – 19
28 – 30	81 – 75	19 – 25
30 – 36	75 – 74	25 – 26
36 – 38	74 – 72	26 – 28
38 – 41	72 – 65	28 – 35
41 – 46	65 – 60	35 – 40
46 – 48	60 – 52	40 – 48
48 – 53	52 – 47	48 – 53
53 – 70	47 – 30	53 – 70

The structural composition of HPLC-detected monomeric phenolic acids and dimeric ferulic acids was further confirmed by using the same HPLC analysis system mentioned above coupled with a quadrupole - time of flight mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA) linked to Mass Lynx software version 4.1 (Waters) for data processing. The full mass spectra (MS) were recorded in negative ion mode by using the following conditions: capillary voltage 1500 V, cone voltage 30 V, desolvation gas (N_2) flow rate 900 L/hr, cone gas (He) flow rate 50 L/hr, desolvation gas temperature 300 ℃, ion source temperature 150 ℃, mass range 100-1000 amu. The MS/MS spectra (MS/MS) were acquired by using the collision energy of 10 V for monomeric phenolic acids and a set of collision energies at 10, 20, and 30 V for dimeric ferulic acids. Phenolic acid monomers and ferulic acid dehydrodimers were identified by comparison of mass fragmentation patterns with their unique chemical structures and the available literature.

3.2.3.2 Quantification of phenolic acids by HPLC analysis

The quantification of phenolic acids in alkaline extracts was accomplished by using the same HPLC equipment and elution program as described above. Each phenolic acid standard was properly diluted from 1 mg/mL to generate a standard curve for phenolic acid content determination. Due to the lack of the standards for diferulic acids, ferulic acid dehydrodimers were quantified by using *trans*-cinnamic acid as a standard.

3.2.4 Chemical model assays

3.2.4.1 Determination of total phenolic content (Folin-Ciocalteu method)

Total phenolic content (TPC) of WG and DF alkaline extracts was determined by Folin-Ciocalteu method (Singleton & Rossi, 1965) as modified by Gao et al. (2002). Briefly, a 0.2 mL of appropriately diluted crude extract was added with 1.5 mL of freshly made 10-fold diluted Folin-Ciocalteu reagent. After 15 min equilibration, the mixture was neutralized with 1.5 mL of sodium carbonate (60 g/L), mixed well by a vortex (Fisherbrand, Bohemia, NY, USA), and the test tube sealed with paper film. After 90 min reaction in the dark, the absorbance of the mixture was measured at 725 nm with a UV-Vis spectrophotometer (Ultrospec 1100 pro, Biochrom, England) against 50% MeOH as a blank. Ferulic acid (0 to 200 µg/mL) was used as a standard and the results were expressed as milligrams of ferulic acid equivalent (FAE) per gram of sample (mg FAE/g sample).

3.2.4.2 Evaluation of antioxidant activity (DPPH radical scavenging capacity assay)

Antioxidant activity (AOA) of WG and DF alkaline extracts was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity assay (Brand-William et al., 1995) as modified by Li et al. (2007). Briefly, a 0.1 mL of appropriately diluted crude extract was combined with 3.9 mL of freshly made DPPH radical solution (60 µmol/L, prepared in 95% methanol). The absorbance of the extract with DPPH was measured at t=60 min (60 minutes' incubation with DPPH at room temperature and in the absence of light) and described as A_{sample}. The absorbance of the blank (95% methanol) with DPPH was measured at t=0 min (immediately after the addition of DPPH) and described as $A_{control}$. The DPPH radical scavenging capacity (%) of both alkaline extracts and trolox standard was calculated according to the equation: $(1 - A_{sample}/A_{control}) \times 100\%$. A standard curve was generated based on trolox concentration (25 to 500 µmol/L) and its DPPH radical scavenging activity (%). According to

the equation of the standard curve and the DPPH radical scavenging activity (%) of extracts, the AOA of extracts was further expressed as micromole of trolox equivalents (TE) per gram of sample (μmol TE/g sample).

3.2.5 Cell culture assays

3.2.5.1 Cell lines and cell culture

To reveal the potential effect of corn IDF (C-IDF) released phenolic acids on human gut health, Caco-2BBe and FHs 74 Int cell lines were employed in the present study. Caco-2BBe cell line is an epidermoid carcinoma cell line derived from human colon. It resembles large intestinal cells and is widely used as a model to simulate the gastrointestinal tract. Similarly, FHs 74 Int cell line is also used as a model of intestinal response, but it is a normal epithelical cell line obtained from the small intestine of a healthy 3 to 4 months-old female. The cells used in the present study were between passages 9 to 15 for Caco-2BBe cells and 7 to 14 for FHs 74 Int cells.

The complete growth medium for maintaining both cell lines was prepared as described in **Table 3.2**. Both intestinal epithelial cell lines are grown in DMEM with essential supplements, including FBS, P/S, L-gln, Na/Pyr, and hTF. For FHs 74 Int cells, additional EGF is essential for increasing the rate of cell proliferation. Cells cultured with complete growth medium in T-25 flasks were incubated at 37 ℃ in a 5% CO_2 humidified incubator. The growth medium was changed twice a week to maintain a normal cell growth. At 80-90% confluency, the cells were passaged at a sub-cultivation ratio of 1:4 for Caco-2BBe and 1:2 for FHs 74 Int.

Table 3.2 Complete growth medium for cell culture.

Name	Functionality
Dulbecco's Modified Eagle Medium (DMEM with high glucose content)	Cell culture medium
Fetal bovine serum (FBS), 10%	A serum-supplement provides growth promoting and survival enhancing factors to cells in culture
Penicillin/ streptomycin (P/S), 10 kU/mL pen, 10 mg/mL strep	Antibiotics prevent bacterial contamination
L-glutamine (L-gln), 200 mM	A source of cellular energy, next to glucose
Sodium pyruvate (Na/Pyr), 100 mM	An additional source of energy for cells
Human transferrin (hTF), 1 mg/mL	An agent binds and delivers ions in cell culture
Epidermal growth factor (EGF), 10 µM	An extra growth factor increases the rate of cell proliferation

3.2.5.2 Cytotoxicity assay

The cytotoxicity of various concentrations of corn IDF extracts, AAPH, and xanthine-xanthine oxidase was measured by MTT assay as described by Hirawan et al. (2011) with minor modifications. The flow chart of cytotoxicity assay is displayed in **Figure 3.2** and the arrangements of treatments in wells are summarized in **Table 3.3**. Specifically, detached intestine cell suspension from T-25 flask at a density of 2×10^4 was seeded in a 96-well plate (100 µL/well) and cultured at 37 ℃ with 5% CO_2 until confluent. After that, the culture medium in all wells was carefully pipetted out. Treatments were loaded to appropriate sample wells and PBS was added to control and blank wells. After 4 h of incubation, 10 µL of MTT reagent was added to all except the blank wells. The plate was then incubated for 3 h until the intracellular punctate purple precipitate was visible under the microscope. 100 µL of detergent

reagent was then added to all except the blank wells. The plate was swirled gently and then left in the dark overnight at room temperature. The absorbance at 560 nm was measured by using Opsys MR 96-well plate reader (DYNEX Technologies, Chantilly, VA, USA). The cytotoxicity of treatments was expressed as cell viability (%) and determined by comparing the absorbance of treated cells to that of control cells. Cell viability (%) = (absorbance of treatments) / (absorbance of controls) × 100%. Results were from six replicates run in three separate experiments.

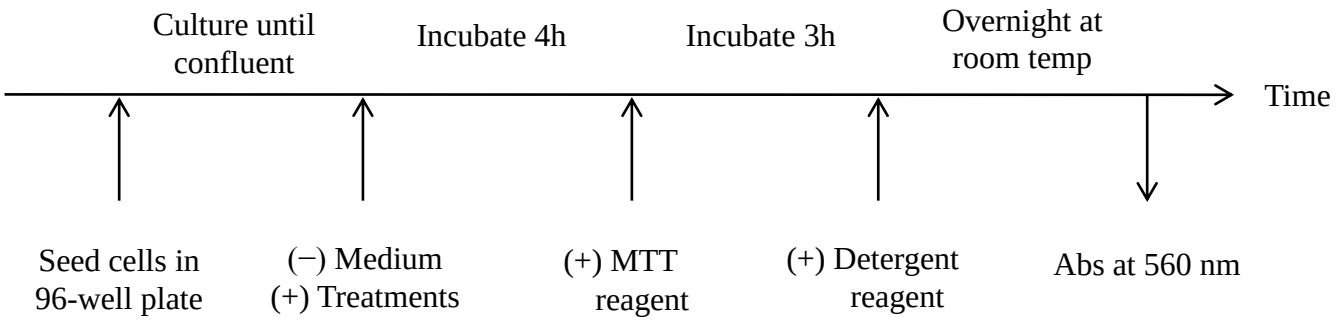


Figure 3.2 Flow chart of cytotoxicity assay.

Table 3.3 Arrangement of treatments in cytotoxicity assay.

Sample wells	Control wells	Blank wells
Cells	Cells	Cells
Treatment	PBS	PBS
MTT	MTT	-
Detergent reagent	Detergent reagent	-

3.2.5.3 Cytoprotective activity against AAPH induced oxidation

The cytoprotection of corn IDF (C-IDF) extracts against AAPH induced oxidation was determined by MTT assay according to Gliwa et al. (2011) with some modifications. The flow chart of cytoprotective activity assay is displayed in **Figure 3.3** and the arrangements of treatments are summarized in **Table 3.4**. Specifically, Caco-2BBe and FHs 74 Int cells were grown to confluence in 96-well plates. The culture medium in all wells was then discarded and different concentrations of C-IDF extracts (100 µL) were added to appropriate sample wells. 100 µL of PBS was added to control and blank wells. After 2 h of incubation, 10 µL of AAPH was added to sample and AAPH positive control wells. Cells were incubated for another 4 h. After that, MTT reagent was added to all except the blank wells and the plate was incubated for 3 h until the intracellular punctate purple precipitate was visible under the microscope. Detergent reagent was then added to all except the blank wells. The plate was swirled gently and the absorbance at 560 nm was recorded (Opsys MR 96-well plate reader, DYNEX Technologies, Chantilly, VA, USA) after standing overnight at room temperature. The cytoprotective activity of C-IDF alkaline extracts was represented by cell viability (%). Result from six replicates run in three separate experiments.

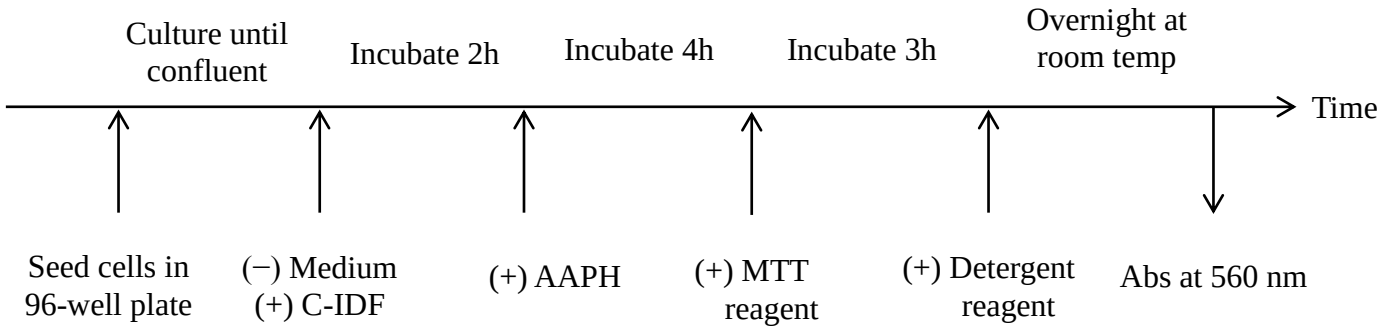


Figure 3.3 Flow chart of cytoprotective activity assay (AAPH induced oxidation).

Table 3.4 Arrangements of treatments in cytoprotective activity assay (AAPH induced oxidation).

Sample wells	Positive control wells	Negative control wells	Blank wells
Cells	Cells	Cells	Cells
Sample	PBS	PBS	PBS
AAPH	AAPH	-	-
MTT	MTT	MTT	-
Detergent reagent	Detergent reagent	Detergent reagent	-

3.2.5.4 Cytoprotective activity against xanthine-xanthine oxidase (X-XO) induced oxidation

The method in evaluating C-IDF extracts protection against xanthine-xanthine oxidase (X-XO) induced human intestinal cell oxidation was adapted from Graziani et al. (2005) as displayed in **Figure 3.4** and the arrangements of treatments are summarized in **Table 3.5**. Specifically, intestinal cell suspension at a density of 2×10^4 was seeded in a 96-well plate and cultured until confluent. The culture medium in all wells was then discarded. Various concentrations of C-IDF alkaline extracts were loaded to sample wells and PBS was added to control and blank wells. After 2 h per-incubation, 100 µL of X (prepared in PBS with sonication) and XO (dissolved in PBS) was added to sample and positive control wells. Cells were incubated for another 4 h. After that, MTT reagent was incubated with cells for 3 h to produce visible purple precipitates, and then 100 µL of detergent reagent was loaded. The plate was left overnight and the absorbance was measured at 560 nm (Opsys MR 96-well plate reader, DYNEX Technologies, Chantilly, VA, USA). The cytoprotective activity of C-IDF

alkaline extracts was represented by cell viability (%). Results were from six replicates run in three separate experiments.

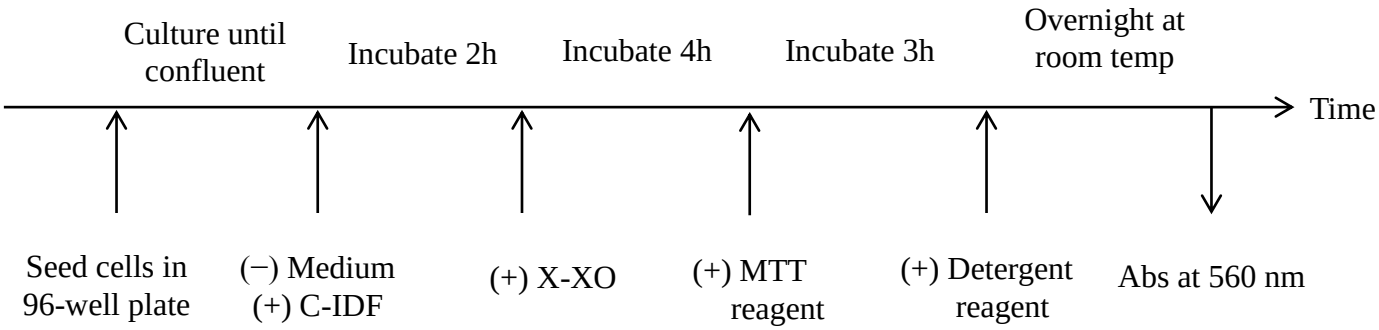


Figure 3.4 Flow chart of cytoprotective activity assay (X-XO induced oxidation).

Table 3.5 Arrangements of treatments in cytoprotective activity assay (X-XO induced oxidation).

Sample wells	Positive control wells	Negative control wells	Blank wells
Cells	Cells	Cells	Cells
Sample	PBS	PBS	PBS
X	X	-	-
XO	XO	-	-
MTT	MTT	MTT	-
Detergent reagent	Detergent reagent	Detergent reagent	-

3.2.6 Statistical analysis

The results were reported as mean ± standard deviation (SD). Data were analyzed by a one-way analysis of variance (ANOVA) test using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). Significant differences in fibre yields and antioxidant properties of extracts in chemical-based assays and cell-based assays were evaluated by using Tukey's test at $p < 0.05$.

3.3 Results and Discussion

3.3.1 Preparation of insoluble and soluble DF

Enzymatic-gravimetric method has been widely used to determine DF in foods and food products (Larrauri et al. 1997; Renger & Steinhart, 2000; Bunzel et al., 2001; Picolli da Silva & de Lourdes Santorio Ciocca, 2005). The principle involves removing starch and protein followed by separating IDF precipitate and SDF suspension. The final IDF and SDF are weighted and corrected for protein and ash content. In the present study, insoluble and soluble DF from seven species of WG were prepared according to a sequential enzymatic digestion (heat stable α-amylase, protease, and amyloglucosidase). The yields of IDF and SDF were expressed on a 100-gram WG basis. Total dietary fibre (TDF) yield was reported as a sum of IDF and SDF content.

Table 3.6 Extraction yields of insoluble, soluble and total dietary fibre from whole-grain cereals (g/100 g WG).[1]

Sample	IDF yield (g IDF/100 g WG)	SDF yield (g SDF/100 g WG)	TDF yield (g TDF/100 g WG)
Barley	18.05 ± 1.28 ^{b,B}	5.15 ± 0.83 ^{a,C}	23.20 ^{b,A}
Purple barley	23.71 ± 2.51 ^{a,A}	4.56 ± 0.13^{ab,B}	28.26 ^{a,A}
Wheat	16.93 ± 1.15^{bc,A}	2.95 ± 0.21^{cd,B}	19.88 ^{c,A}
Purple wheat	14.84 ± 1.20^{cd,B}	2.91 ± 0.45^{cd,C}	17.76 ^{c,A}
Yellow Corn	17.60 ± 0.73^{bc,B}	2.28 ± 0.10 ^{d,C}	19.88 ^{c,A}
Red rice	11.73 ± 0.97 ^{d,A}	2.29 ± 0.01 ^{d,B}	14.02 ^{d,A}
Oats	13.15 ± 2.59 ^{d,B}	3.89 ± 0.55^{bc,C}	17.04 ^{c,A}

[1] Values are expressed as mean ± standard deviation of two measurements.

Cereal species are compared by small letters (a-d): values in the same column without similar letter differ significantly at $p < 0.05$ by using Tukey's test.

Cereal fractions are compared by capital letters (A-C): values in the same row without similar letter differ significantly at $p < 0.05$ by using Tukey's test.

As seen in **Table 3.6**, differences ($P < 0.05$) in the levels of IDF, SDF, and TDF content were found among seven species of cereals. In general, the content of IDF were 3 to 8 times higher than that of SDF. IDF content ranged from 11.73 to 23.71 g/100 g WG, while SDF content varied between 2.28 and 5.15 g/100 g WG. The highest IDF content was found in purple barley, followed by barley, yellow corn, wheat, and purple wheat. Oats and red rice showed the lowest values of IDF. The highest SDF values were found in barley varieties (5.15 g/100 g in barley and 4.56 g/100 g in purple barley), followed by oats (3.89 g/100 g), and a lower group comprising wheat, purple wheat, yellow corn, and red rice. A similar trend as IDF yield was observed in TDF. Purple barley displayed the highest TDF content, while the lowest TDF value was found in red rice. The high IDF yields in barley varieties are probably due to

their thicker aleurone layer, which is a part of cereal bran fraction and rich in DF. Barley aleurone layer is made up of two to three layers of cells, while the aleurone layer in wheat, corn, rice, and oats is of single-celled thickness (Hoseney, 1994). The high SDF levels in barley and oats are likely due to their high concentrations of β-glucans, which are major components of SDF. The high β-glucan content in barley and oats was demonstrated earlier (Henry, 1987; Havrlentová & Kraic, 2006; Havrlentová et al., 2011). The ratios of SDF to IDF based on 100 g WG are shown in **Figure 3.5.** The ratios varied from 0.130 to 0.295, which agreed with the results reported previously (Picolli da Silva & de Lourdes Santorio Ciocca, 2005). Barley and oats exhibited the highest SDF to IDF ratio, while corn showed the lowest ratio.

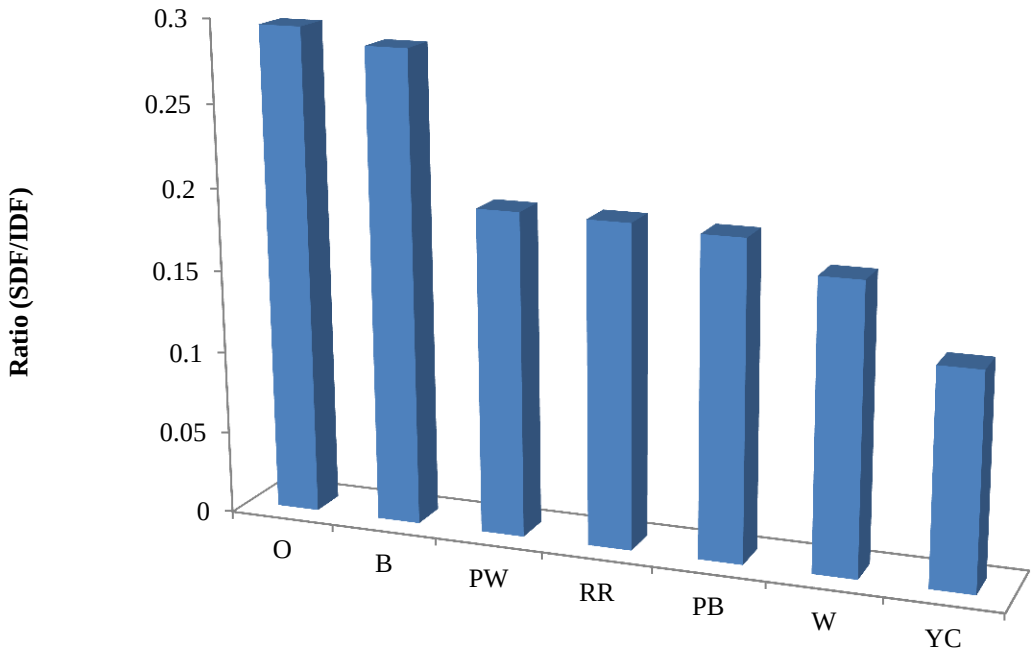


Figure 3.5 Ratios of SDF to IDF in WG. O, oats; B, barley; PW, purple wheat; RR, red rice; PB, purple barley; W, wheat; YC, yellow corn.

3.3.2 HPLC and HPLC-MS/MS analyses

Monomeric phenolic acids in WG and fibre alkaline extracts were identified by using HPLC and HPLC-MS/MS in comparing their retention times with phenolic acid standards and typical mass loss with available literature. The quantification of monomeric phenolic acids was accomplished by using the standard curves generated by phenolic acid standards.

3.3.2.1 Confirmation of monitor wavelength

According to Robbins (2003), the maximum absorbance wavelength is in the range of 200 to 290 nm for hydroxybenzoic acids and 270 to 360 nm for hydroxycinnamic acids. To choose a suitable wavelength for phenolic acid detection, the monitor wavelength in the present study was compared at 280 nm and 320 nm. Eleven commercial phenolic acids, which are widely found in cereals, were used as standards.

As seen in **Figure 3.6 (A)**, at 280 nm, all eleven phenolic acid standards were detected, which included five hydroxybenzoic acids (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, and syringic acid) and six hydroxycinnamic acids (caffeic, *p*-coumaric, ferulic, sinapic, *iso*-ferulic, and *o*-coumaric acid). Compared with **Figure 3.6 (A)**, three hydroxybenzoic acids (*p*-hydroxybenzoic, vanillic, and syringic acid) were absent at 320 nm in **Figure 3.6 (B)**, and the other two hydroxybenzoic acids (gallic and protocatechuic acid) were under the detection limit. However, all six hydroxycinnamic acids presented higher absorbance response at 320 nm. Therefore, it can be concluded that the wavelength at 320 nm is more favourable for hydroxycinnamic acids detection, while 280 nm is more capable for detecting all phenolic acids. In the present study, for the purpose of reflecting all the phenolic acid constituents in alkaline extracts, 280 nm was chosen as the monitoring wavelength.

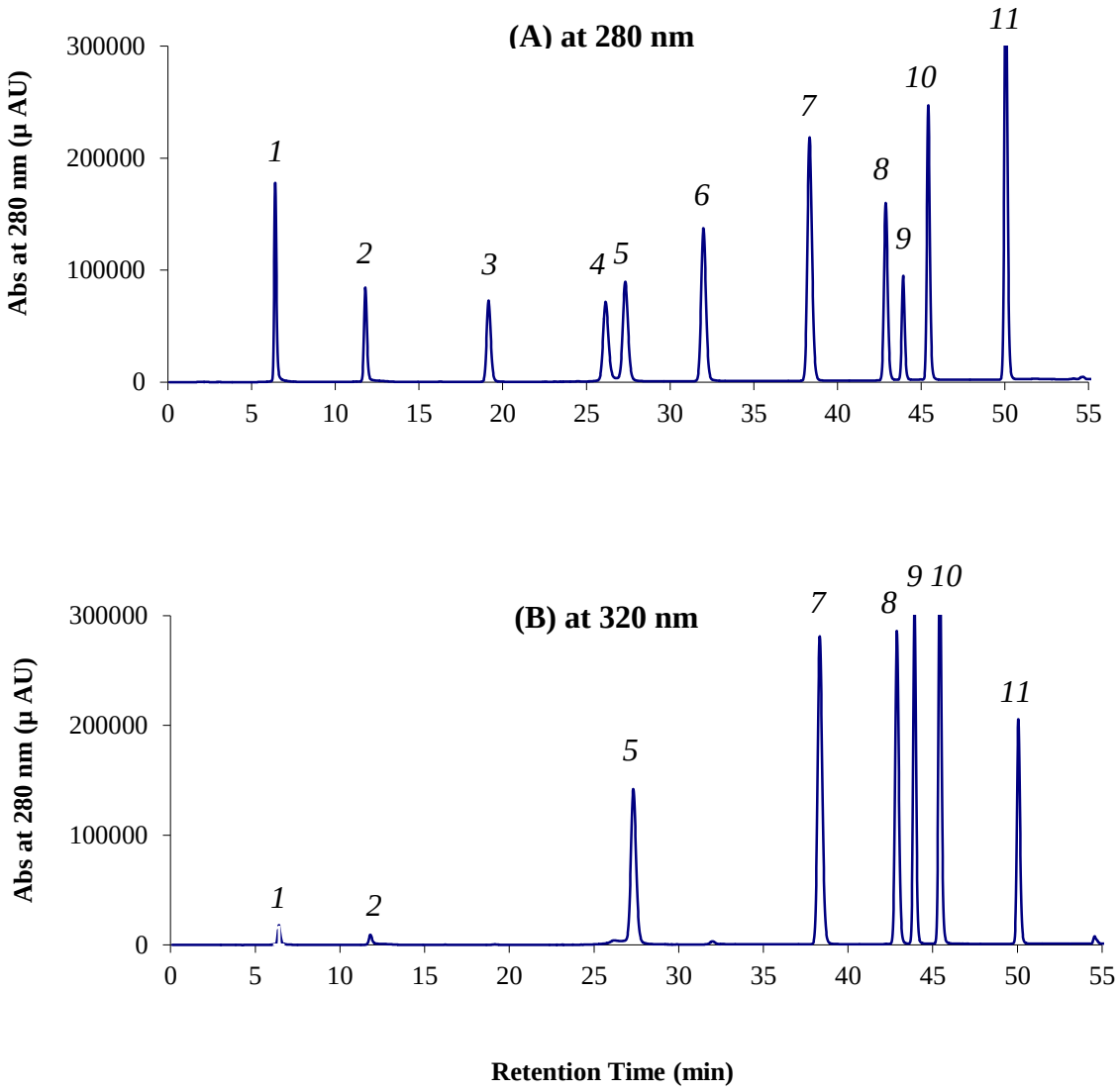
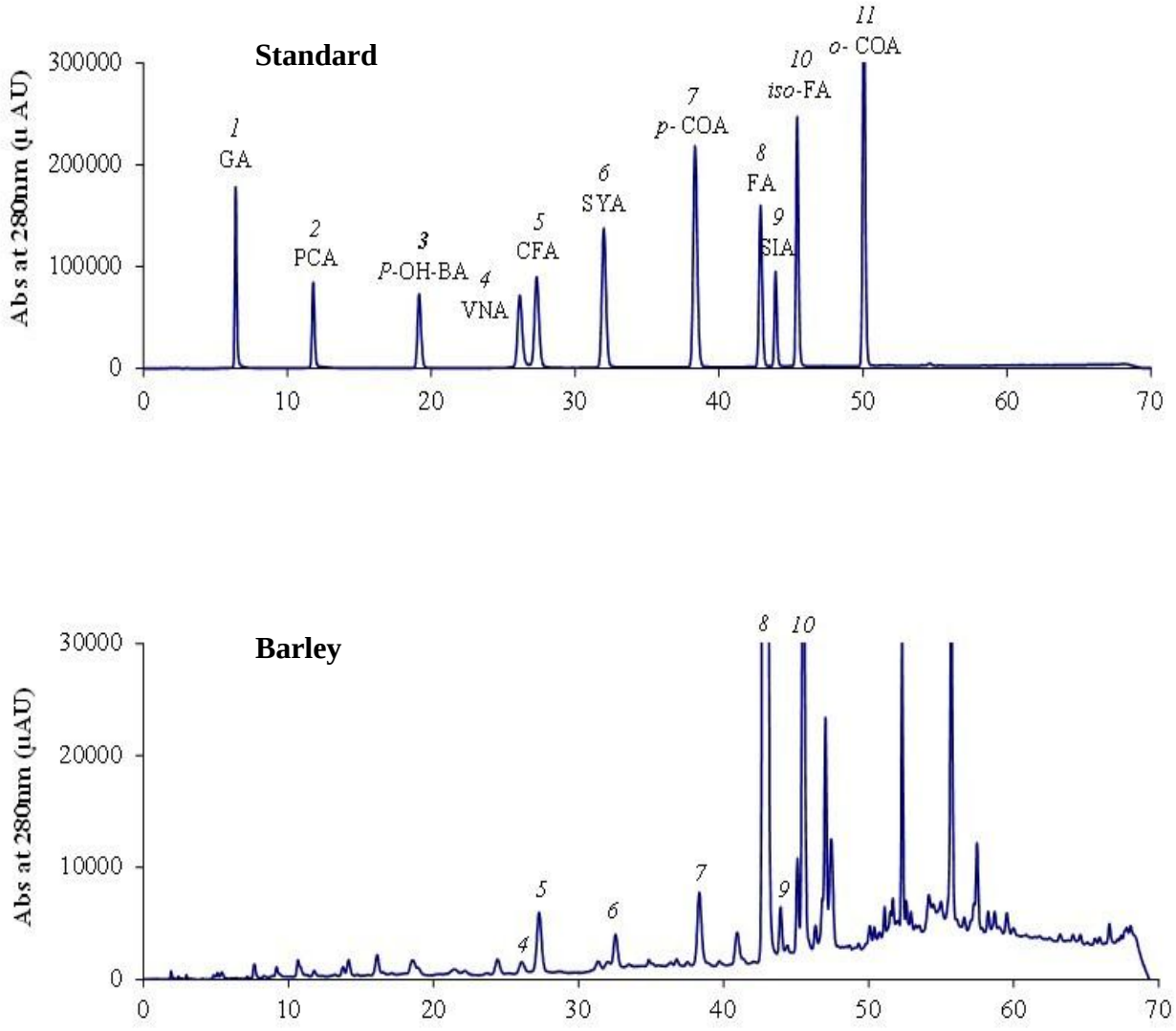
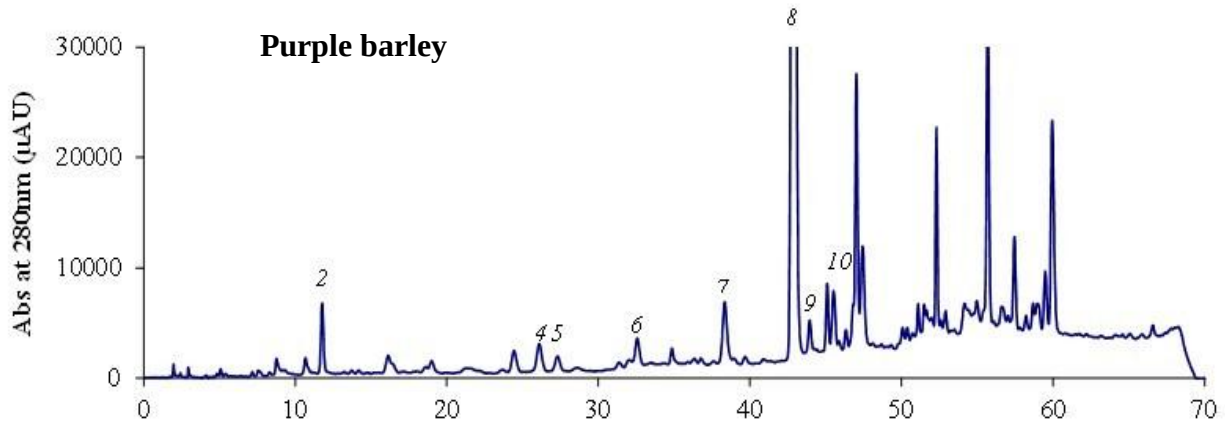


Figure 3.6 HPLC chromatogram of a mixture of phenolic acid standards with detection wavelength at (A) 280 nm and (B) 320 nm. (A) at 280 nm: *1* gallic acid, *2* protocatechuic acid, *3 p*-hydroxybenzoic acid, *4* vanillic acid, *5* caffeic acid, *6* syringic acid, *7 p*-coumaric acid, *8* ferulic acid, *9* sinapic acid, *10 iso*-ferulic acid, *11 o*-coumaric acid; (B) at 320 nm: *1* gallic acid, *2* protocatechuic acid, *5* caffeic acid, *7 p*-coumaric acid, *8* ferulic acid, *9* sinapic acid, *10 iso*-ferulic acid, *11 o*-coumaric acid.

3.3.2.2 Identification and quantification of monomeric phenolic acids

The HPLC chromatograms of IDF alkaline extracts from barley, purple barley, wheat, purple wheat, yellow corn, red rice, and oats are shown in **Figure 3.7**, with the chromatogram of phenolic acid standards as a reference.




Purple barley
Abs at 280nm (µAU)
30000
20000
10000
0
0
10
20
30
40
50
60
70
2
4
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7
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9
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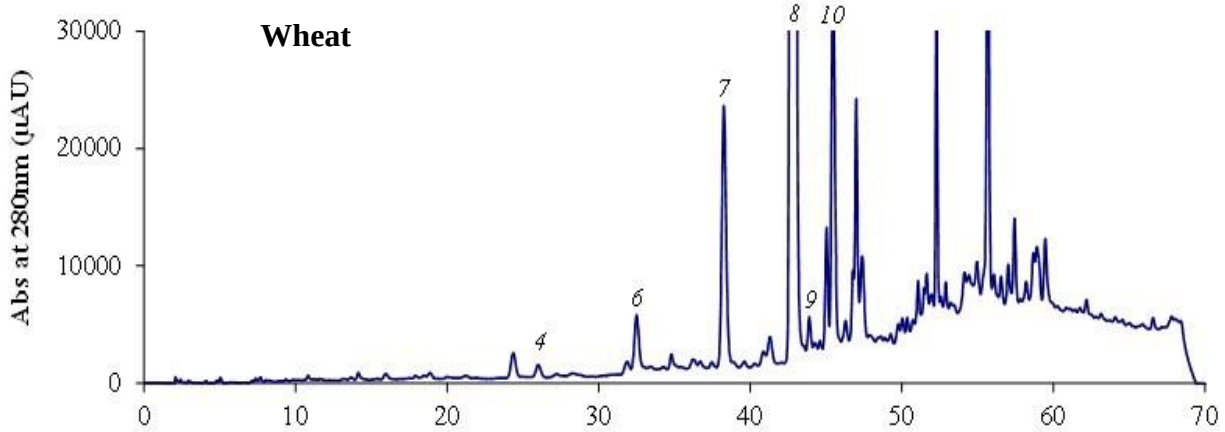
Wheat
Abs at 280nm (µAU)
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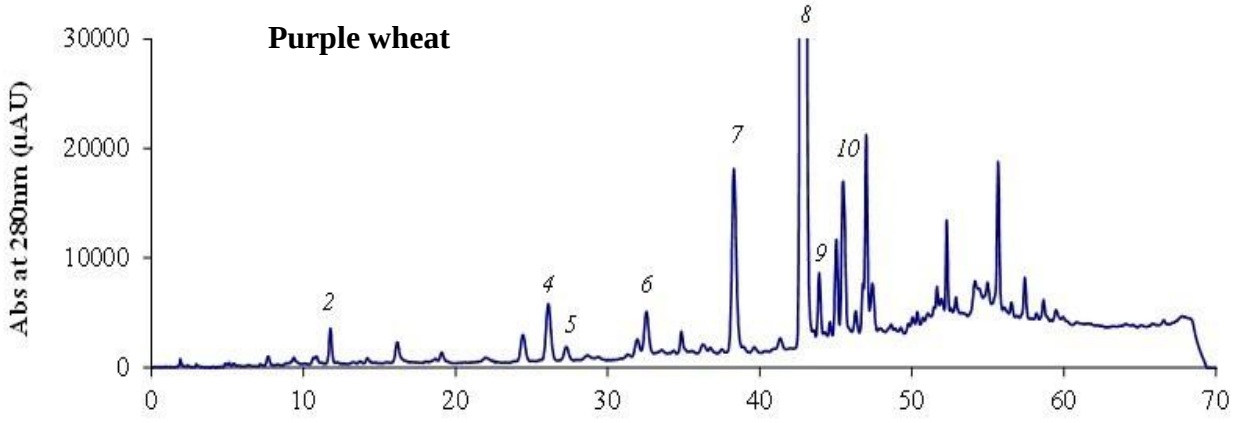
Purple wheat
Abs at 280nm (µAU)
30000
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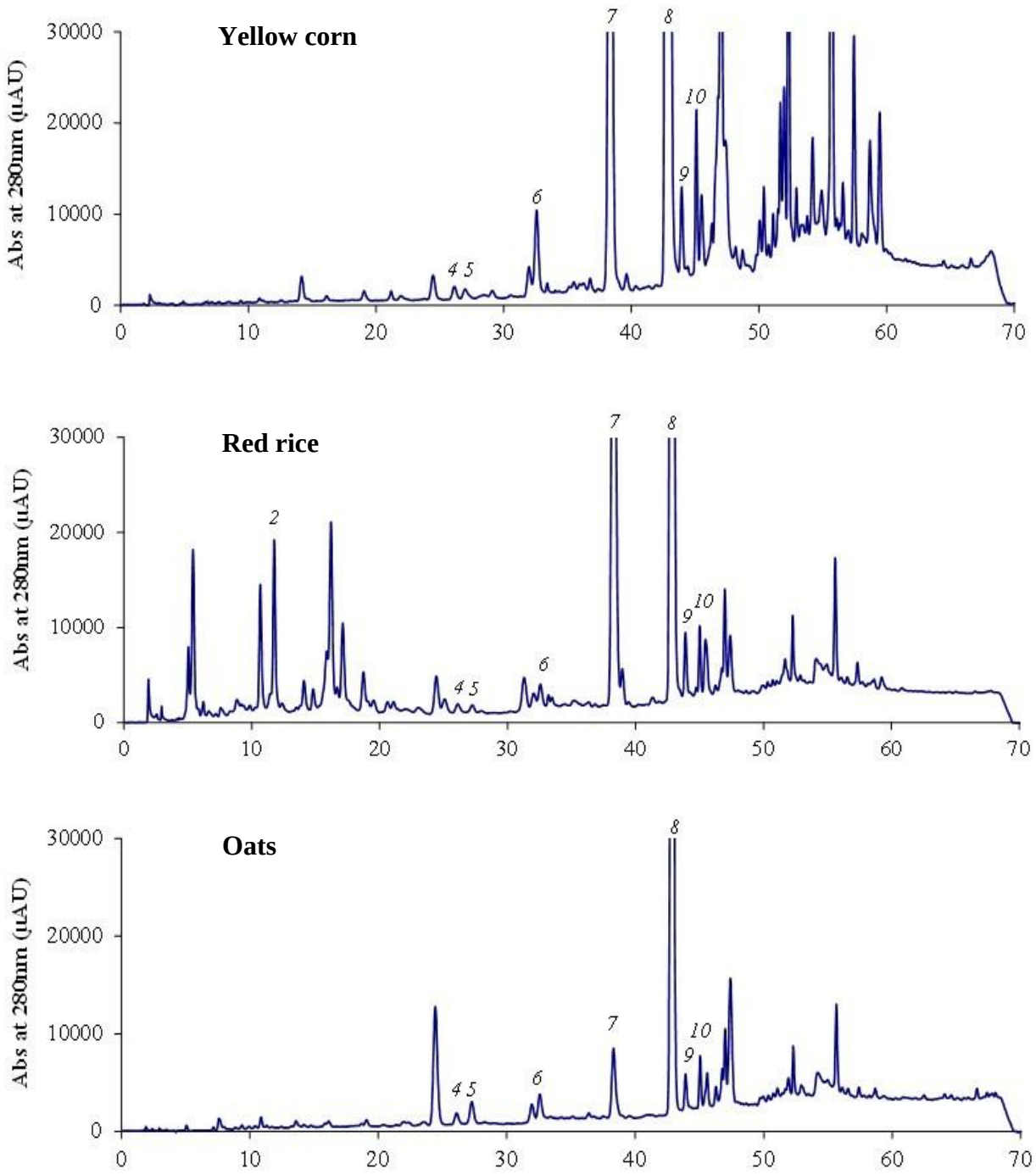
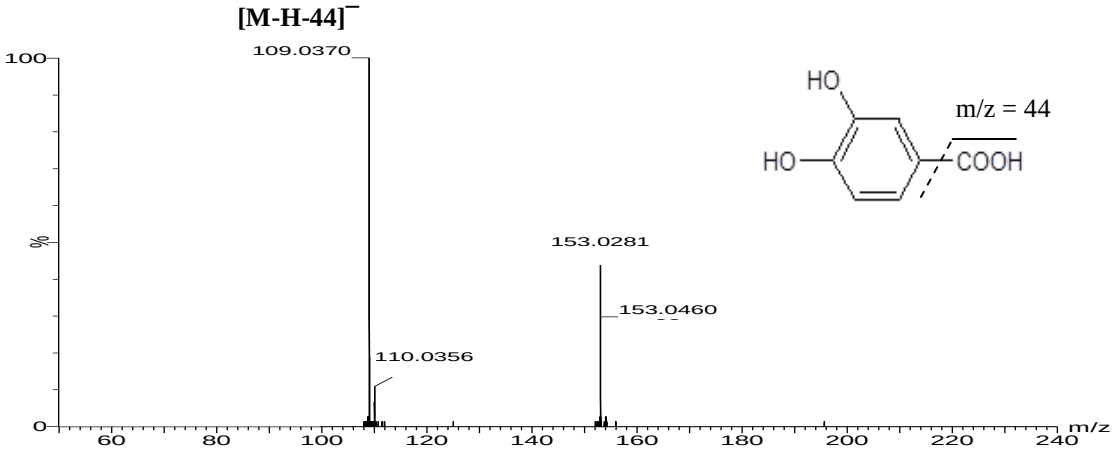


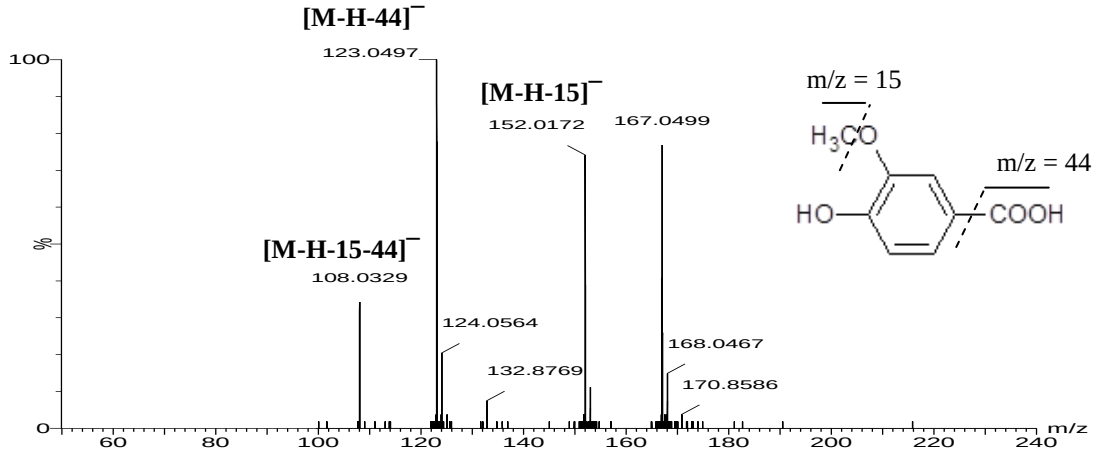
Figure 3.7 HPLC chromatograms (280 nm) of IDF alkaline extracts from cereal grains: *1* gallic acid, *2* protocatechuic acid, *3 p*-hydroxybenzoic acid, *4* vanillic acid, *5* caffeic acid, *6* syringic acid, *7 p*-coumaric acid, *8* ferulic acid, *9* sinapic acid, *10 iso*-ferulic acid, *11 o*-coumaric acid.

As seen in **Figure 3.7**, eight peaks were identified and assigned as protocatechuic acid (peak 2), vanillic acid (peak 4), caffeic acid (peak 5), syringic acid (peak 6), *p*-coumaric acid (peak 7), ferulic acid (peak 8), sinapic acid (peak 9), and *iso*-ferulic acid (peak 10) by comparing the retention times with standards. Vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, sinapic acid, and *iso*-ferulic acid were widely detected in alkaline extracts. Caffeic acid was not detected in wheat, while protocatechuic acid was only found in purple barley, purple wheat, and red rice. Further confirmation of the above assignments by HPLC-MS/MS was performed under a collision energy of 10 V. By plotting typical molecular ions, the MS/MS spectra of HPLC-identified phenolic peaks are shown in **Figure 3.8**. According to the parent ions and unique product ions, the previous assignments were verified. The commonly observed mass loss of 15 Da, 44 Da, and 59 Da were due to the loss of -CH_3 group, -COOH group or both of the two groups from the parent ion.

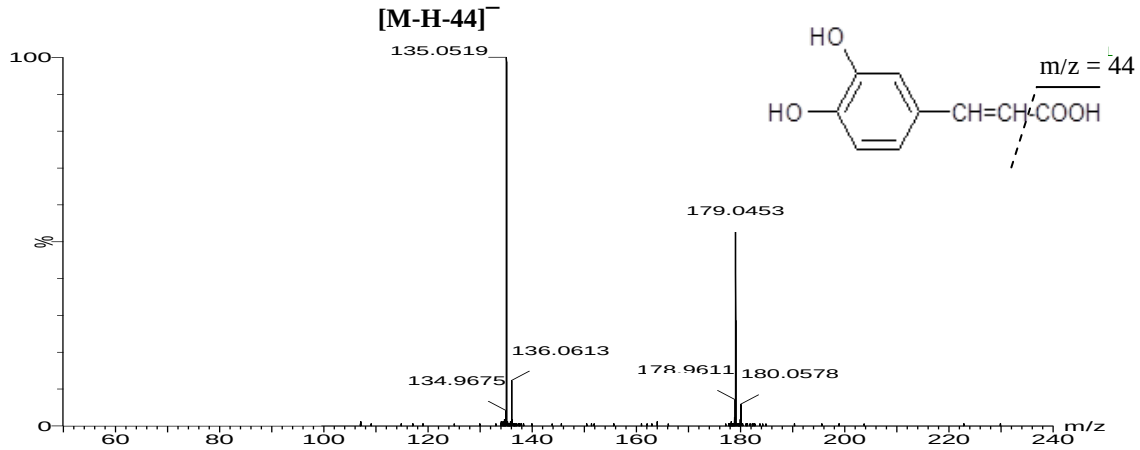
(A) protocatechuic acid



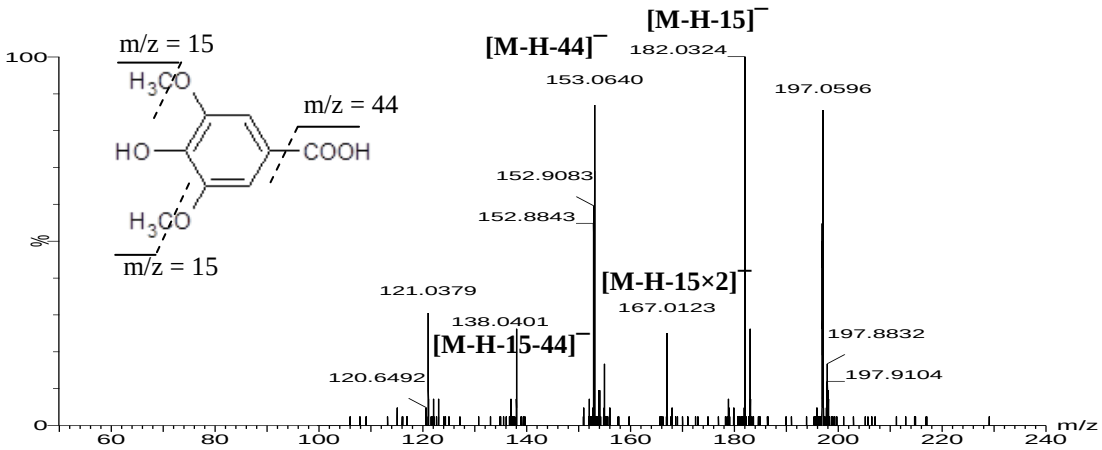
(B) vanillic acid



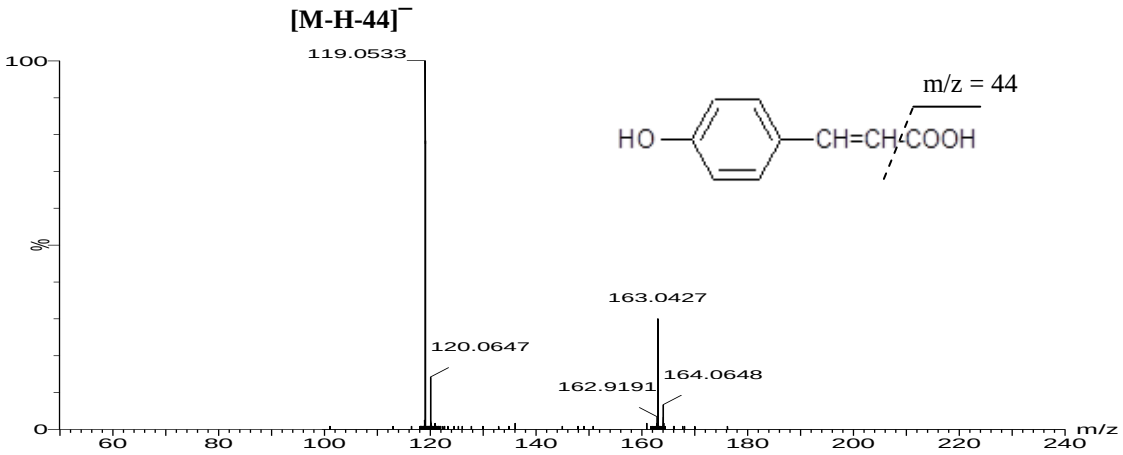
(C) caffeic acid



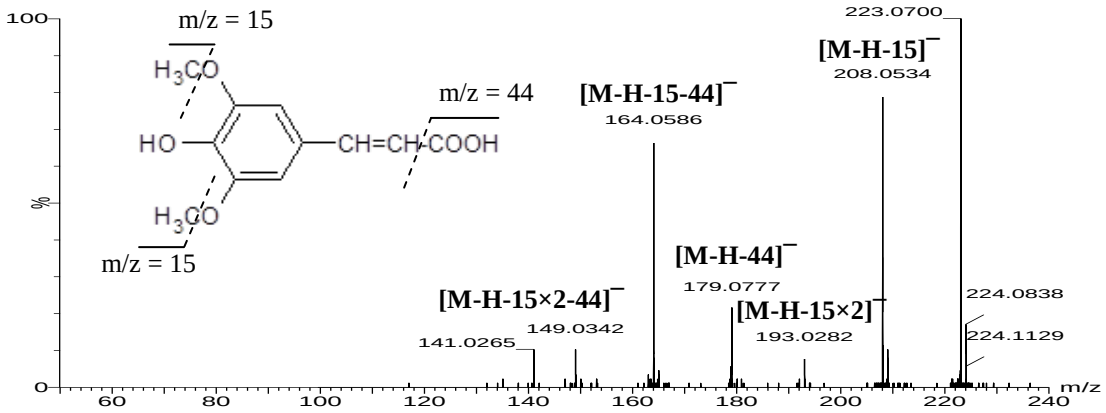
(D) syringic acid



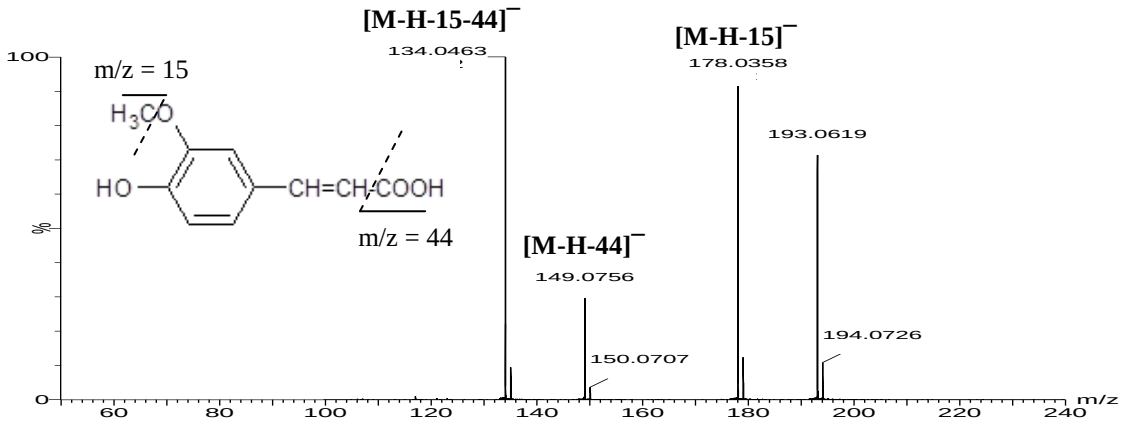
(E) *p*-coumaric acid



(F) sinapic acid



(G) ferulic acid



(H) *iso*-ferulic acid

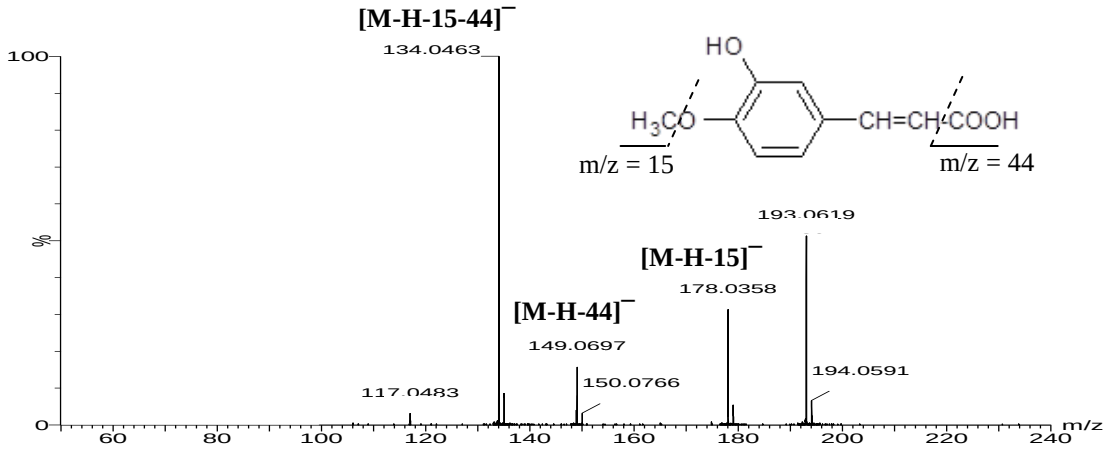


Figure 3.8 HPLC-MS/MS spectra (10 V) of identified peaks in IDF alkaline extracts and the possible fragmentation pathways. Typical molecular ion ($[M-H]^-$) was set as (A) m/z = 153, for protocatechuic acid; (B) m/z = 167, for vanillic acid; (C) m/z = 179, for caffeic acid; (D) m/z = 197, for syringic acid; (E) m/z = 163, for *p*-coumaric acid; (F) m/z = 223, for sinapic acid; (G) m/z = 193, for ferulic acid; (H) m/z = 193, for *iso*-ferulic acid.

Iso-ferulic acid (3-hydroxy-4-methoxycinnamic acid) is a positional isomer of ferulic acid (4-hydroxy-3-methoxycinnamic acid). They share the same molecular weight and shape and display similar polarity in HPLC separation. Therefore, the identification of ferulic acid and *iso*-ferulic acid solely based on their retention time or UV-Vis spectra may lack evidence. In the present study, by setting an identical molecular ion at m/z 193, comparable MS/MS spectra of ferulic acid (**Figure 3.8 (G)**) and *iso*-ferulic acid (**Figure 3.8 (H)**) with unique fragmentation behaviours were observed. The deprotonated molecules of ferulic acid and *iso*-ferulic acid (m/z 193) all eliminated methyl group (-CH_3) and carboxyl group (-COOH) to form fragment ions at m/z 178 and m/z 149, respectively. However, the fragment ion of m/z 178 and m/z 149 were more abundant in ferulic acid (90% and 30%) than found in *iso*-ferulic acid (30% and 15%). This is explainable in terms of the position of hydroxyl group (-OH) on benzene ring. The -OH in *para* position (*p*-OH) activates the benzene ring and stabilizes the charge in the resulting ions (Smith, 2005) thus producing higher intensities of ion fragments at m/z 178 and m/z 149, as seen in ferulic acid MS/MS spectrum (**Figure 3.8 (G)**). By comparison, the -OH group in *meta* position (*m*-OH) lacks the ability to stabilize the intermediate product ions, so m/z 134 formed by the elimination of both -CH_3 and -COOH dominated the *iso*-ferulic acid MS/MS spectrum (**Figure 3.8 (H)**). Moreover, benzene rings with *p*-OH group are more polar than those with *m*-OH group. Therefore, in the RP-HPLC system, ferulic acid with *p*-OH structure was eluted ahead of *iso*-ferulic acid with *m*-OH structure (**Figure 3.7**). As discussed above, ferulic acid and *iso*-ferulic acid can be confidently distinguished by HPLC-MS/MS in the present study.

Table 3.7 shows the content of detected monomeric phenolic acids in cereals. The dominant phenolic acid in alkaline extracts was ferulic acid, which accounted for 67-87% of

total monomeric phenolic acids in WG, 71-92% in IDF, and 57-79% in SDF. High levels of ferulic acid in the IDF alkaline extracts demonstrated that esterified ferulic acid was concentrated in the IDF fraction. Our finding was in agreement with Rybka et al. (1993), who pointed out that 85-90% of the alkaline-soluble ferulic acid in grains is localized in the IDF fraction. In our study, the highest concentration of ferulic acid was found in the corn IDF (8322 µg/g), which was 2 to 10 times higher than other cereal IDF extracts. Besides ferulic acid, *p*-coumaric acid, sinapic acid, and *iso*-ferulic acid were also widely detected in all fractions, but their contents were much lower in comparison with ferulic acid. In addition, small amounts of vanillic acid and syringic acid were detected in WG and IDF extracts. The SDF fraction displayed much lower levels of phenolic acids compared with WG and IDF. Such a finding implied that bound phenolic acids rarely exist in SDF. It is known that phenolic acids in cereals are linked to cell-wall materials, mainly arabinoxylan (AXA) and lignin, which are abundant in IDF. However, as the major component of cereals SDF, β-glucan links no phenolic acids (Autio, 2006). Ahluwalia and Fry (1986) fractionated barley cell walls into their β-glucan and AXA components and found that AXA carried virtually all the ferulate groups, while no ferulic acid was associated with β-glucan. Therefore, in our study, the SDF fraction with lower levels of AXA displayed lower bound phenolic acid content. The detected phenolic acids in SDF are mainly the ones that link with water-soluble AXA or the ones that exist as free types in SDF alkaline extracts.

Protocatechuic acid has rarely been reported as a major phenolic acid component in cereals, although it has been found in some studies (Onyeneho & Hettiarachchy, 1992; Yu et al., 2001; Mattila et al., 2005). In our study, protocatechuic acid was observed in coloured cereals (purple barley, purple wheat, and red rice) with red rice showing the highest

protocatechuic acid concentration (188.61 μg/g). However, the reasons for such findings are unclear, since extraction methodology, HPLC condition, and sample varieties could all vary the levels of phenolic acids in cereal extracts. *p*-Hydroxybenzoic acid has widely been reported in wheat, barley, oat, rice, and sorghum in previous studies (Sosulski et al., 1982; Yu et al., 2001; Mattila et al., 2005; Tian et al., 2005; Kim et al., 2006; Qiu et al., 2010). However, no detectable levels of *p*-hydroxybenzoic acid were observed under the conditions used in the present study. This may be caused by the extraction method. Yu et al. (2001) pointed out that different extraction methods could yield significantly different levels of *p*-hydroxybenzoic acid in barley. *Iso*-ferulic acid is known as a main phenolic acid in cell walls of monocots (Smith & Hartley, 1983). However, there have been rare reports of *iso*-ferulic acid in cereal studies. It is possible that *iso*-ferulic acid is occasionally misidentified as *trans*-ferulic acid or *cis*-ferulic acid due to their similar polarity on HPLC separation. In our study, *iso*-ferulic acid was successfully identified by HPLC-MS/MS and firstly reported as one of the major phenolic acids in cereal alkaline extracts.

Table 3.7 Contents of detected monomeric phenolic acids in whole-grain cereals (WG), insoluble dietary fibre (IDF), and soluble dietary fibre (SDF) (μg/g sample).[a]

Class	Spl.	PCA	VNA	CFA	SYA	*p*-COA	FA	SIA	*iso*-FA	TPA
WG	B	-[b]	5.96±0.35	13.98±1.02	6.81±0.40	11.13±0.07	601.85±14.68	31.54±1.08	79.74±1.59	749
	PB	18.38±0.08	17.05±0.16	-	5.95±0.34	11.69±0.18	469.35±8.99	18.80±2.60	11.56±1.46	550
	W	-	6.05±0.38	-	4.21±0.36	18.89±0.52	565.12±45.88	22.36±3.22	36.95±2.93	652
	PW	-	34.02±1.19	-	7.12±0.68	17.78±0.63	562.96±18.50	42.00±0.54	18.39±0.86	682
	YC	-	10.29±0.68	-	23.17±2.25	113.33±1.68	1387.46±4.61	52.85±4.42	8.67±0.56	1594
	RR	28.09±2.35	-	-	-	66.53±0.91	301.74±9.29	47.23±1.82	5.46±0.25	449
	O	-	11.42±0.87	-	17.89±0.94	13.81±0.15	249.44±7.94	28.91±0.54	5.81±0.22	327
IDF	B	65.90±0.35	13.22±0.65	71.72±2.70	4.58±0.16	31.29±1.15	2287.56±63.58	43.37±2.17	186.70±16.24	2638
	PB	-	35.61±0.79	22.12±0.64	4.11±0.09	29.37±1.06	1412.16±27.45	28.88±0.04	27.54±0.57	1626
	W	34.63±0.63	13.91±0.11	-	6.07±0.04	101.07±0.44	3543.82±12.71	32.71±1.82	155.00±9.10	3851
	PW	-	72.75±4.82	20.14±0.13	10.21±0.45	81.66±6.39	3081.57±222.07	66.14±3.68	75.54±11.46	3440
	YC	188.61±21.58	18.29±0.41	-	20.44±0.55	576.52±7.32	8322.16±255.06	107.56±1.82	47.38±2.34	9092
	RR	-	11.32±1.11	13.86±0.18	10.90±0.80	425.29±12.91	1814.34±92.26	70.33±0.85	31.11±0.93	2565
	O	-	14.29±0.65	45.67±3.78	9.97±0.64	36.74±1.71	829.17±14.41	37.45±0.62	15.17±1.39	988
SDF	B	-	-	-	-	15.29±0.32	146.72±0.93	12.00±0.77	13.05±0.63	186
	PB	-	-	-	-	21.45±0.45	140.06±4.25	15.63±2.42	10.39±0.21	181
	W	-	-	-	-	15.03±1.45	94.87±1.08	5.90±1.65	9.65±0.35	126
	PW	-	-	-	-	15.43±2.52	99.46±7.86	3.99±1.07	13.07±1.36	131
	YC	-	-	-	-	31.96±2.52	103.59±0.15	32.27±0.58	13.10±0.28	181
	RR	-	-	-	-	31.94±0.20	100.67±1.83	26.31±1.26	14.72±0.00	174
	O	-	-	-	-	20.59±0.22	107.41±2.65	8.64±0.10	8.71±0.35	145

[a] Values are expressed as mean ± standard deviation of two measurements.

[b] - Not detected.

PCA, protocatechuic acid; VNA, vanillic acid; CFA, caffeic acid; SYA, syringic acid; *p*-COA, *p*-coumaric acid; FA, ferulic acid; SIA, sinapic acid; *iso*-FA, *iso*-ferulic acid; TPA, total phenolic acid.

3.3.2.3 Identification and quantification of dimeric ferulic acids

The identification of diferulic acid was achieved by comparing the HPLC eluting sequence and HPCL-MS/MS fragment ion pattern with literature (Garcia-Conesa et al., 1997; Dobberstein & Bunzel, 2010; Callipo et al., 2010; Bauer et al., 2012). *Trans*-cinnamic acid was used as a standard to quantify the content of diferulic acid in alkaline extracts.

Figure 3.9 shows the HPLC chromatogram of IDF alkaline extracts from yellow corn. It can be seen that some peaks other than monomeric phenolic acids were observed after 44 min. By performing a scan at m/z = 358, which is a typical m/z value for ferulic acid dehydrodimer diagnostication, four diferulic acid peaks were detected (**Figure 3.10**). Their retention times were at 46.80, 52.30, 55.46, and 57.17 min, respectively. The MS/MS fragment ion patterns of detected diferulic acids were obtained under a series of collision energies: 10, 20, and 30 V. The MS/MS product ion m/z values are summarized in **Table 3.8** and the MS/MS spectra with possible fragmentation pathways are shown in **Figure 3.11**.

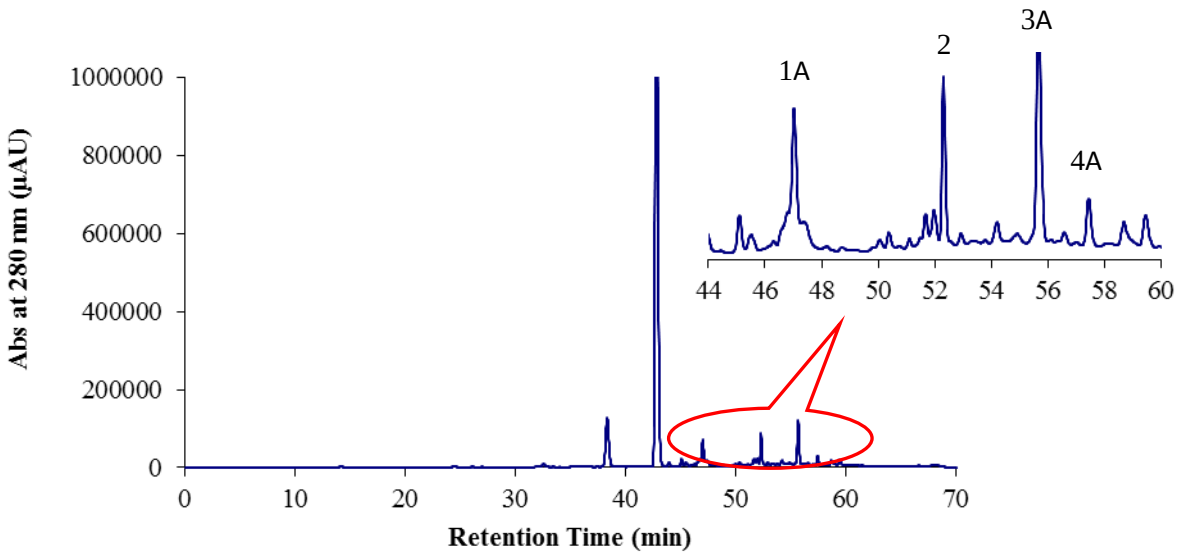


Figure 3.9 HPLC chromatogram (0-70 min and 44-60 min) of corn IDF alkaline extracts.

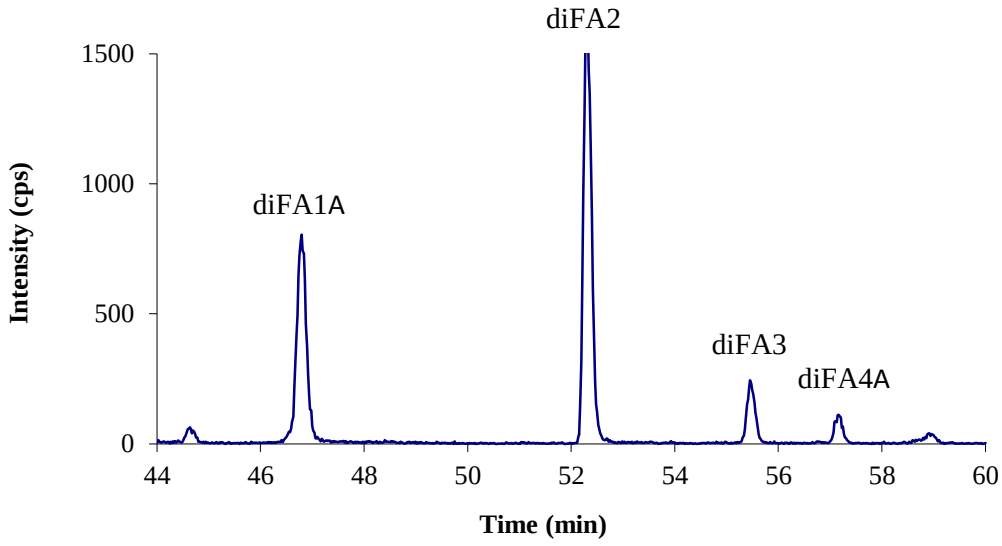


Figure 3.10 Extracted ion chromatogram (EIC) at m/z=385.

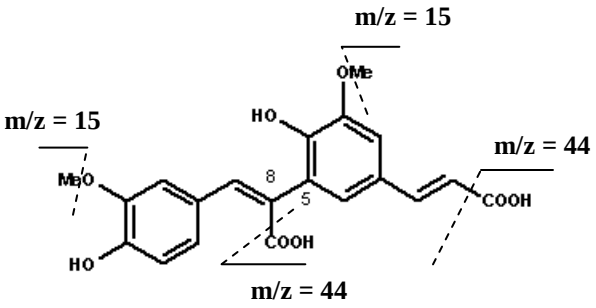
Table 3.8 HPLC-MS/MS product ions of detected diferulic acids (diFA) under different collision voltages.

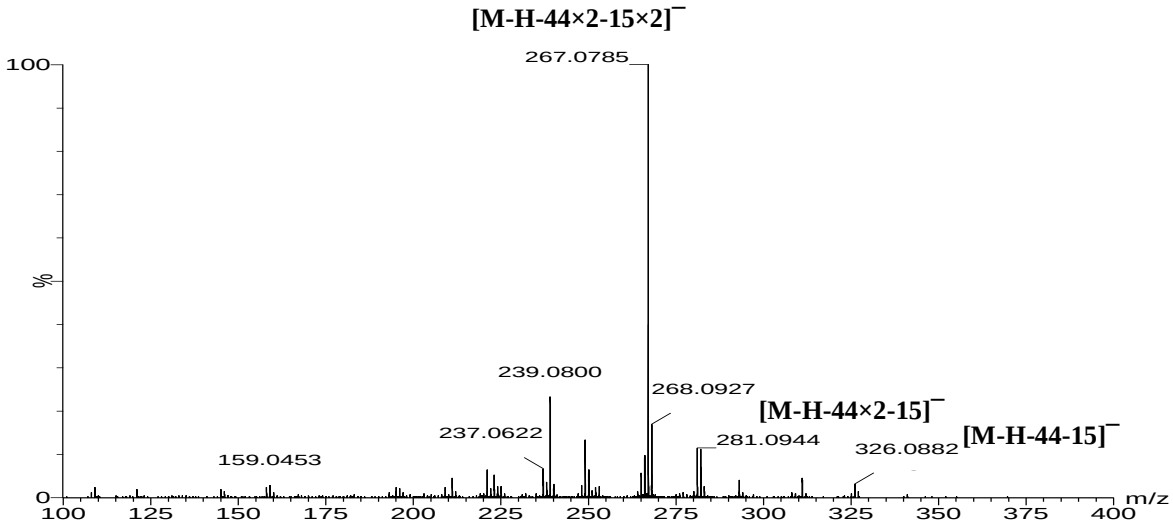
Peak	Product ions (m/z) under different collision energy (V)			Identification
	10 (V)	20 (V)	30 (V)	
diFA1	**385**, 341, 326	385, 326, **282**, 267, 235	326, 281, **267**, 239 , 237	8-5’
diFA2	**385**, 341, 309	385, 326, **282**, 265, 235, 193, 149	325, **281**, 267, 239, 178*, 149*, 134*	5-5’
diFA3	385, 313, 282, 194, **193**	326, 298, 239, **193**, 178, 134	325, 283, 267, 239, 193*, 178*, 149*, **134***	8-*O*-4’
diFA4	**341**, 297	341, 326, 282, **267**, 239, 223, 179	326, 281, **267**, 239, 225	8-5’ (BF)

Values in bold indicate the highest intensity;

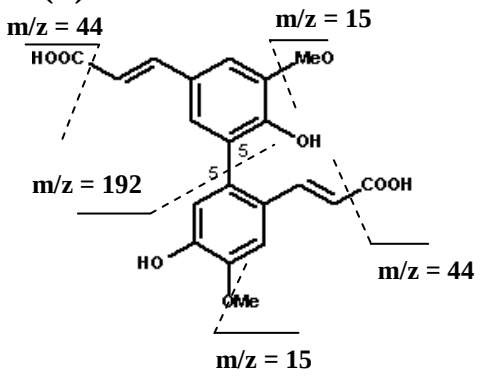
Values with star represent the typical product ion.

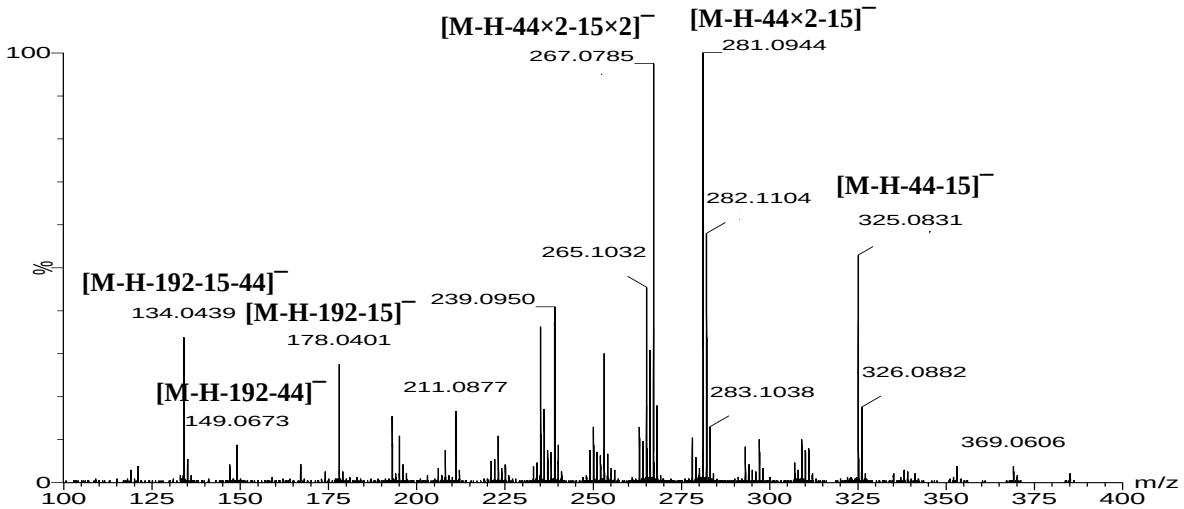
(A) 8-5’ diFA





(B) 5-5’ diFA





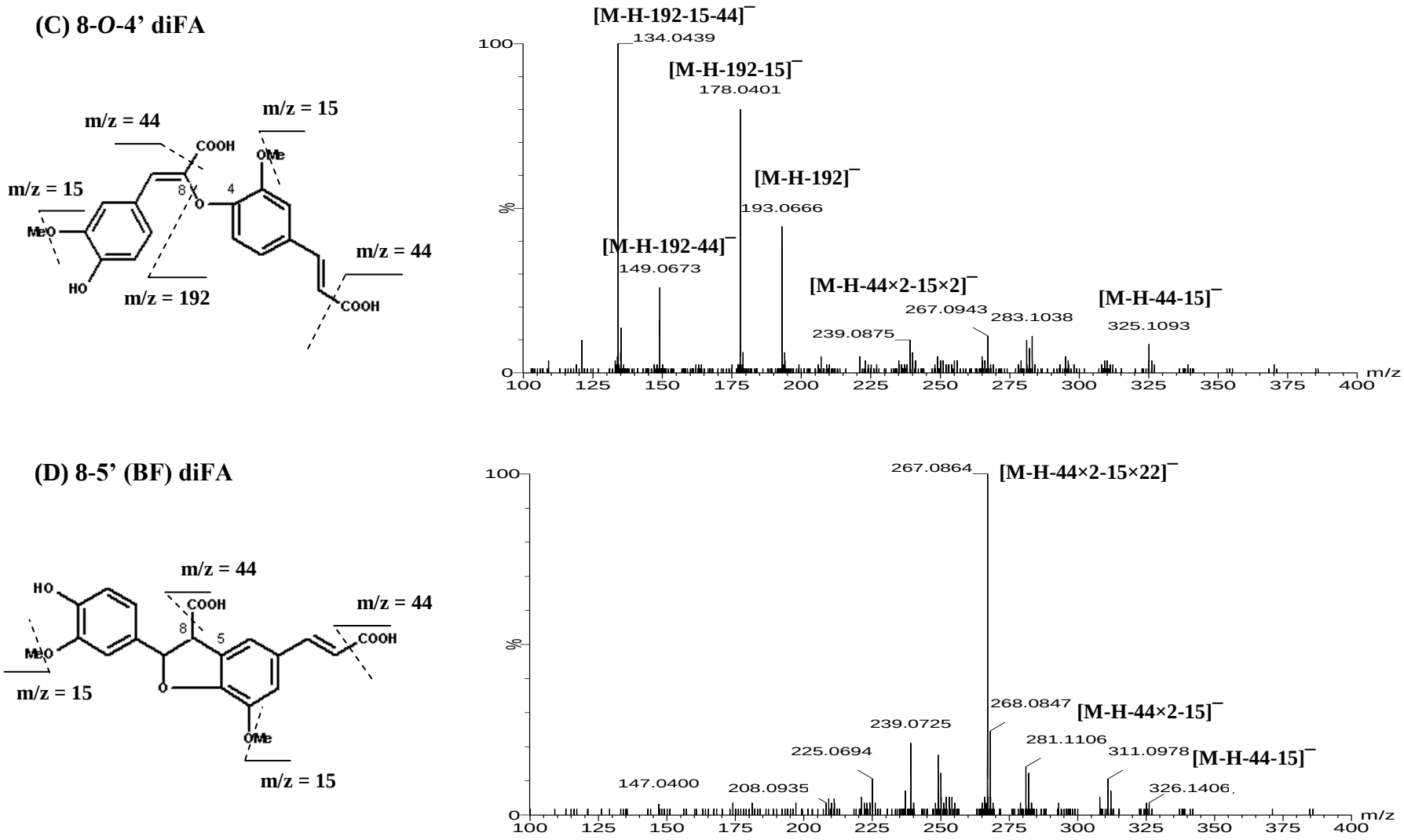


Figure 3.11 HPLC-MS/MS spectra (30 V) of detected diferulic acids (diFA) and the possible fragmentation pathways. Typical molecular ion ($[M-H]^-$) was set as m/z = 385.

As seen in **Table 3.8**, 10 V of collision energy could not provide sufficient ion fragments to distinguish different diferulic acids. The prevalent product ion at m/z 341, m/z 326, and m/z 282, were due to the loss and rearrangement of -COOH ($[M-H-44]^-$), -COOH and $-CH_3$ ($[M-H-44-15]^-$), -COOH × 2 and $-CH_3$ ($[M-H-44\times2-15]^-$) from the deprotonated molecule $[M-H]^-$ 385. By increasing the collision energy, more ion fragments were observed. At collision voltage of 30 V, four diferulic acids with characteristic product ions were assigned as 8-5'-diFA, 5-5'-diFA, 8-*O*-4'-diFA, and 8-5' (benzofuran)-diFA, respectively (**Figure 3.11**). 8-*O*-4'-diFA was confirmed by the typical product ion at m/z 193, which was produced from the cleavage of -O- bound between two ferulic acid monomer structures. Further fragments at m/z 178, m/z 149, and m/z 134 were due to the elimination of $-CH_3$ (15 Da), -COOH (44 Da), $-CH_3$ plus -COOH (59 Da) from m/z 193, respectively. 5-5'-diFA displayed similar fragmentation pathways as 8-*O*-4'-diFA, but the intensities of their product ions are different. Although 8-5' linear form and 8-5' benzofuran form diFA showed similar MS/MS fragmentation pattern, their retention times on HPLC differed greatly. Such assignments precisely agreed with the eluting sequence and content difference reported by Dobberstein & Bunzel (2010), who separated diferulic acid in insoluble fibre from whole corn grains. 8-5'-diFA, 5-5'-diFA, and 8-*O*-4'-diFA as the most abundant diferulic acids were used for quantification.

The contents of 8-5'-diFA, 5-5'-diFA, and 8-*O*-4'-diFA in WG and IDF alkaline extracts are shown in **Table 3.9**. The highest diferulic acid content was found in the corn IDF extracts. No diferulic acids were detected in SDF. Bunzel et al. (2001) explained that the low degree of cross-linking between diferulic acids and AXA in SDF is responsible for the trace amounts of diferulic acid in SDF.

Table 3.9 Contents of detected diferulic acids (diFA) in whole-grain cereals (WG) and insoluble dietary fibre (IDF) (μg/g sample).[a]

Class	Sample	diFA (μg/g)			In total (μg/g)
		8-5’	5-5’	8-*O*-4’	
WG	Barley	5.00±0.69	3.93±0.47	9.01±0.85	17.94
	Purple barley	8.28±0.42	3.42±0.24	9.84±0.70	21.54
	Wheat	-[b]	-	-	-
	Purple wheat	3.95±0.98	1.29±0.39	1.32±0.03	6.56
	Yellow corn	10.88±1.32	4.84±0.17	4.27±0.05	19.99
	Red rice	1.74±0.19	0.74±0.02	1.35±0.13	3.83
	Oat	2.40±0.18	1.13±0.16	0.49±0.03	4.02
IDF	Barley	30.79±0.43	27.68±0.48	46.55±0.89	105.02
	Purple barley	31.46±2.51	16.49±0.72	35.59±3.84	83.54
	Wheat	43.35±16.13	27.90±0.65	55.96±0.41	127.21
	Purple wheat	28.94±2.77	13.41±1.97	37.75±2.21	80.10
	Yellow corn	112.30±19.93	79.17±7.51	140.61±16.05	332.08
	Red rice	16.04±0.06	9.16±0.86	18.52±0.95	43.72
	Oat	17.14±2.65	8.52±0.55	27.80±1.81	53.46

[a] Values are expressed as mean ± standard deviation of two measurements.

[b] - Not detected.

3.3.3 Chemical model assays

The *in vitro* antioxidant potential of the alkaline extracts from WG, IDF, and SDF was evaluated by two chemical-based assays: the total phenolic content (TPC) measured by Folin-Ciocalteu assay and the antioxidant activity (AOA) measured by DPPH radical scavenging activity assay.

3.3.3.1 Total phenolic content (TPC) of alkaline extracts

The total phenolic content (TPC) of alkaline extracts from WG and their insoluble and soluble DF fractions was assayed by Folin-Ciocalteu method. The principle of Folin-Ciocalteu assay is that phenolic compounds (or other reducing substance) can react with Folin-Ciocalteu reagent to produce a blue coloured complex. The absorbance of the resulting blue colour is proportional to the number of reacting phenolic hydroxyl groups and can be measured spectrophotometrically (Karadag et al., 2009; Qiu, 2009). In the present study, ester-bound phenolic acids in cereal samples were prepared by alkaline hydrolysis. Therefore, ferulic acid as the predominant bound phenolic acid in cereal grains was used as a standard. The TPC results were expressed as milligrams of ferulic acid equivalent per gram of sample (mg FAE/ g sample).

As shown in **Table 3.10**, IDF displayed significantly ($p < 0.05$) higher levels of TPC than WG and SDF. The TPC of IDF were 2 to 6 times higher than that of WG and 4 to 13 times higher than that of SDF. The higher TPC of IDF alkaline extracts demonstrated that esterified phenolic acids are mainly concentrated in cereal IDF fraction. In IDF, yellow corn alkaline extracts exhibited the highest TPC (18.35 mg FAE/g IDF), followed by red rice (15.04 mg FAE/g IDF). The TPC of other IDF extracts were 2 (9.09 mg FAE/g IDF in purple wheat)

to 5 (3.97 mg FAE/g IDF in oat) times lower than that of corn IDF extracts. In WG, a similar trend was observed. Yellow corn showed the highest TPC (4.18 mg FAE/g WG), followed by red rice (2.82 mg FAE/g WG), while the lowest TPC was found in oats (1.18 mg FAE/g WG). Consistent with HPLC results, SDF alkaline extracts displayed significantly ($p < 0.05$) lower TPC contents (< 2 mg FAE/g SDF), which further certified that bound phenolic acids rarely exist in SDF fraction. The phenolic acids detected by HPLC analyses are the major contributors to the phenolic content of the SDF extracts.

Table 3.10 Total phenolic content (TPC) of alkaline extracts from whole-grain cereals (WG), insoluble dietary fibre (IDF), and soluble dietary fibre (SDF).[1]

Sample	TPC (mg FAE*/g sample)		
	WG	**IDF**	**SDF**
Barley	$2.74 \pm 0.09^{b,B}$	$7.11 \pm 0.17^{d,A}$	$0.85 \pm 0.02^{e,C}$
Purple barley	$2.55 \pm 0.06^{c,B}$	$6.16 \pm 0.06^{e,A}$	$1.33 \pm 0.05^{c,C}$
Wheat	$1.55 \pm 0.05^{e,B}$	$8.81 \pm 0.02^{c,A}$	$0.88 \pm 0.03^{e,C}$
Purple wheat	$1.95 \pm 0.06^{d,B}$	$9.09 \pm 0.05^{c,A}$	$1.05 \pm 0.04^{d,C}$
Yellow corn	$4.18 \pm 0.10^{a,B}$	$18.35 \pm 0.57^{a,A}$	$1.45 \pm 0.02^{b,C}$
Red rice	$2.82 \pm 0.02^{b,B}$	$15.04 \pm 0.38^{b,A}$	$1.81 \pm 0.05^{a,C}$
Oat	$1.18 \pm 0.05^{f,B}$	$3.97 \pm 0.03^{f,A}$	$1.13 \pm 0.00^{d,B}$

* Ferulic acid equivalent.

[1] Values are expressed as mean ± standard deviation of two measurements.

Cereal species are compared by small letters (a-f): values in the same column without similar letter differ significantly at $p < 0.05$ by using Tukey's test.

Cereal fractions are compared by capital letters (A-C): values in the same row without similar letter differ significantly at $p < 0.05$ by using Tukey's test.

3.3.3.2 Antioxidant activity (AOA) of alkaline extracts

DPPH radical scavenging activity assay is widely used to evaluate the *in vitro* antioxidant activity (AOA) of dietary antioxidants. It measures the activity of sample against DPPH, which is a stable organic nitrogen radical, induced oxidation (Karadag, 2009). The results of DPPH assay are usually expressed as 1) percentage scavenging of DPPH radical (DPPH %/weight of sample), which means the percentage of DPPH radical scavenged by sample in a certain period of time; 2) half maximal inhibitory concentration (IC_{50}), which means the concentration of sample required to decrease the initial absorbance of DPPH by 50%; 3) trolox equivalent (TE/weight of sample), which means the DPPH radical scavenging activity of sample as compared to a trolox standard. In our study, the *in vitro* AOA of alkaline extracts from whole-grain cereals and their insoluble and soluble dietary fibre fractions was evaluated by DPPH method and the results were expressed as micromole of trolox equivalent per gram of sample (μmol TE/g sample), as shown in **Figure 3.12**.

Consistent with TPC results, IDF alkaline extracts exhibited 4-6 times and 6-15 times greater DPPH radical scavenging activity than WG and SDF, respectively. The highest DPPH radical scavenging activity was also found in yellow corn IDF extracts (18.72 μmol TE/g IDF) and a similar DPPH value was observed in red rice IDF extracts (18.55 μmol TE/g IDF). Barley, purple barley, wheat, and purple wheat IDF extracts showed intermediate levels of DPPH radical scavenging activities ranging from 9.45 to 12.47 μmol TE/g IDF. Oats showed the lowest DPPH radical scavenging activity at 6.77 μmol TE/g IDF. In WG, yellow corn extracts exhibited the highest DPPH radical scavenging activity (3.62 μmol TE/g WG) followed by red rice extracts (2.96 μmol TE/g WG) and barley (2.81 TE/g WG), while the

lowest DPPH radical scavenging activity was observed in oats (1.47 TE/g WG). Lower DPPH radical scavenging activity found in SDF was consistent with HPLC and TPC results.

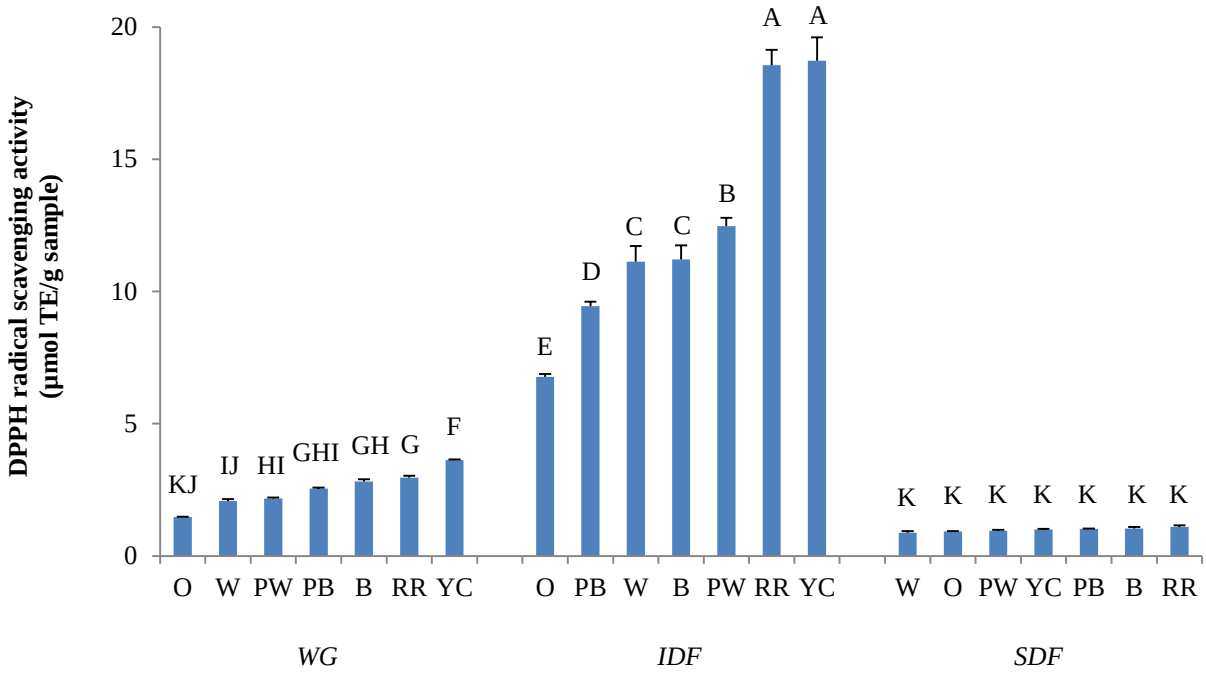


Figure 3.12 DPPH radical scavenging activity of alkaline extracts from whole-grain cereals (WG), insoluble dietary fibre (IDF), and soluble dietary fibre (SDF). B, barley; PB, purple barley; W, wheat; PW, purple wheat; YC, yellow corn; RR, red rice; O, oats. Bars marked by different letters are significantly different ($p < 0.05$).

3.3.4 Correlation analyses

3.3.4.1 Correlation analyses between antioxidant activity and phenolic content in alkaline extracts

The antioxidant activity (AOA) evaluated by DPPH assay was correlated with the content of total phenolics (TPC) measured by Folin-Ciocalteu method and the level of total phenolic acids (TPA) assayed by HPLC.

Figure 3.13 (A) demonstrated that two chemical antioxidant capacity assays agreed with each other. Excellent correlation (R^2 = 0.9499) was shown between TPC measured by Folin-Ciocalteu method and AOA measured by DPPH assay. The high correlation between TPC-DPPH assays can be explained by the similar mechanism occurring in both assays: electron transfer reaction (Hung et al. 2005). A less strong correlation (R^2 = 0.7174) was found between DPPH assay and TPA measured by HPLC method. After removing an unexpected observation representing red rice, a good correlation (R^2 = 0.851) was displayed. Such a result is coincident with the findings described above: red rice alkaline extracts exhibited relatively high TPC (**Table 3.10**), strong AOA (**Figure 3.12**), but low FA and TPA content (**Table 3.7**). Thus, it is speculated that some antioxidant components with phenol structures other than phenolic acids contribute to the AOA of red rice. Oki et al. (2002) pointed out that polymeric procyanidins are the major components responsible for the high DPPH radical scavenging activity of red rice. Similar results were also reported by Min et al. (2011). In our study, the presence of proanthocyanidins in red rice alkaline extracts is unknown. To confirm this, further analysis on red rice need to be conducted.

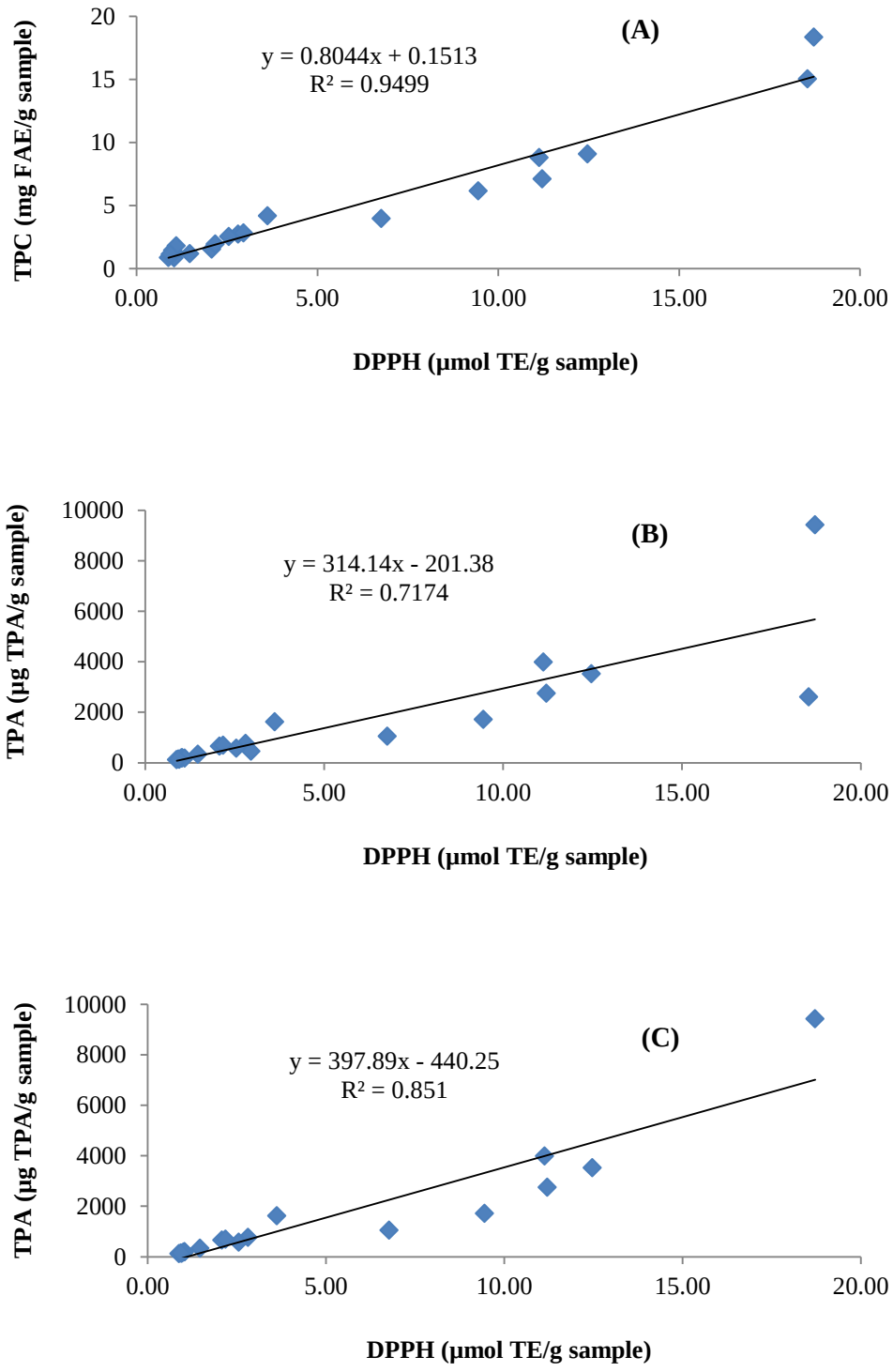


Figure 3.13 (A) Correlation of DPPH value (DPPH) with total phenolic content (TPC); (B) Correlation of DPPH value (DPPH) with total phenolic acid content (TPA); (C) Correlation of DPPH value (DPPH) with total phenolic acid content (TPA) without red rice.

3.3.4.2 Correlation analyses between IDF and ferulic acid content in whole-grain cereals

It is known that ferulic acid and diferulic acid are linked to fibre components, including cellulose, hemicellulose, and lignin, in cereal cells walls (Selvendran, 1984). In order to reveal the relationship between the levels of fibre and fibre-linked phenolic acids, correlation analyses were performed between the yield of IDF and the content of ferulic acid and diferulic acid of WG.

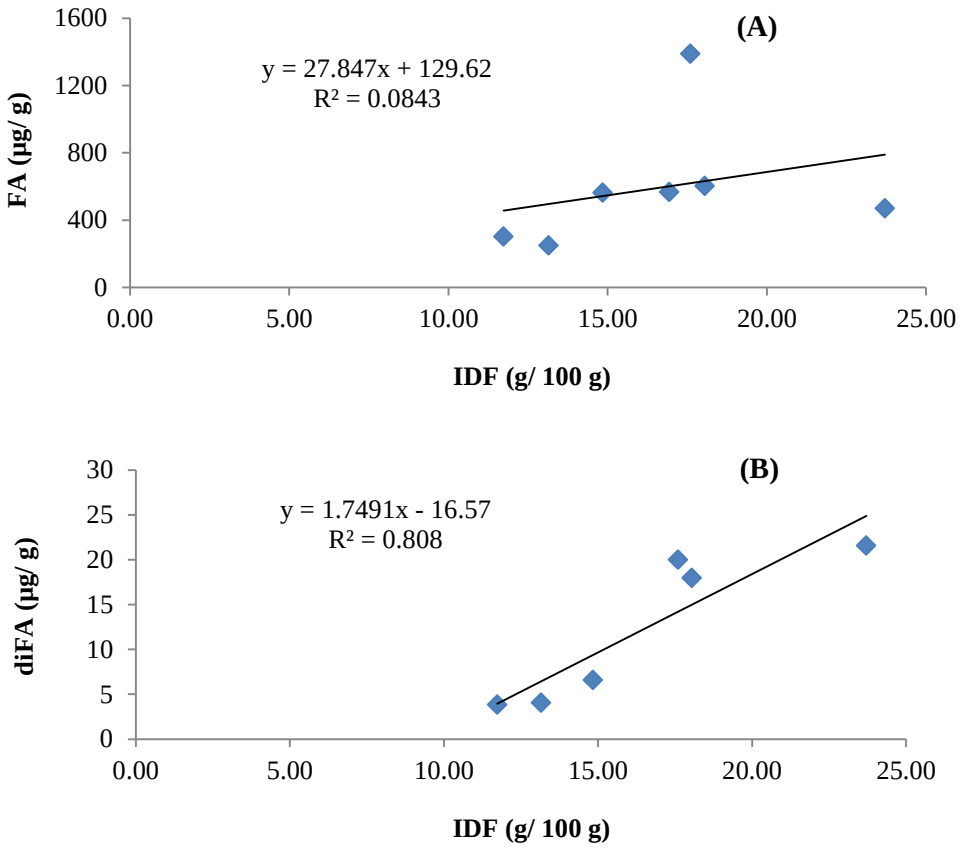


Figure 3.14 (A) Correlation of insoluble dietary fibre yield (IDF) with ferulic acid content (FA); (B) Correlation of insoluble dietary fibre yield (IDF) with diferulic acid content (diFA).

According to **Figure 3.14**, no relationship (R^2 = 0.0843) could be found between IDF and ferulic acid content, but a good correlation (R^2 = 0.808) between IDF and diferulic acid content was observed. This could be explained by the extraction method and the linkage forms of ferulic acid and diferulic acid in cereals. In our study, alkaline hydrolysis was used to cleave ester linkages in releasing bound ferulic acid and diferulic acid from cell wall components. However, it is reported that alkaline hydrolysis is also efficient in the release of free phenolic acids (Kim et al., 2006). Thus, the ferulic acid in WG alkaline extracts contains both free FA and DF component-linked FA, with the latter as the dominant form. Therefore, it is likely to observe a poor correlation between DF content and total ferulic acid level in cereal alkaline extracts. Uncorrelated relationship between the levels of DF and ferulic acid in methanol extracts was also reported previously (Gorinstein et al., 2002; Ragaee et al., 2006). Unlike ferulic acid, diferulic acids do not freely exist in cereals. They serve as structural components in plant cell walls by cross-linking AXA chains (Renger & Steinhart, 2000). Therefore, the levels of diferulic acids in alkaline extracts depend on fibre content, as displayed in **Figure 3.14 (B)**. Moreover, the composition of DF and the linkage types between phenolic acids and DF components are complex. The content of ferulic acid and diferulic acid-attached hosts (e.g., lignins and hemicelluloses), the degree of heteroxylan branching, and the level of crosslinking act together in deciding the amount of ferulic acid and diferulic acid in cereals.

3.3.5 Cell culture assays

Due to the higher antioxidant potential, corn IDF (C-IDF) alkaline extracts at the concentrations of 0.2, 0.5, 1, 2, 3, 5, 10, 20 mg/mL (ferulic acid content was 2, 4, 8, 17, 25, 42, 83, and 166 μg/mL, respectively) were applied in cell culture models. The cytotoxicity and

cytoprotective activity of C-IDF alkaline extracts in Caco-2BBE and FHs 74 Int cells was evaluated by MTT cell viability assay.

3.3.5.1 Caco-2BBe and FHs 74 Int

Caco-2BBe is a subclone of Caco-2 human colon carcinoma cell line. When this cell line is grown in culture, it differentiates spontaneously to form a polarized monolayer with an apical brush border morphologically and functionally comparable to that of the human colon (Caco-2BBe cell comments, ATCC). Therefore, differentiated Caco-2BBe cells that perform similar to human enterocytes and retain the infinitive propagation property, are suitable for evaluating the physiological response of human intestine. FHs 74 Int cells, although were derived from human fetal small intestine, they are morphologically and characteristically like normal mature epithelial cells (Kawamura et al., 1994; Wagner et al., 1998).

3.3.5.2 Cytotoxicity of corn IDF alkaline extracts

Cytotoxicity assay measures the toxic effect of treatments on cells. In this study, the cytotoxic effect of alkaline extracts from C-IDF was evaluated by means of MTT cell viability assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) is a water soluble yellow coloured reagent. When incubated with cells, it can be decomposed to water insoluble purple formazan by metabolically live cells, whereas dead cells do not have this ability. Thus, the amount of formazan formed in live cells reflects the degree of cell viability. In the present study, the toxic effect of C-IDF at concentrations from 0.2 to 20 mg/mL was tested on Caco-2BBe and FHs 74 Int human intestinal cell lines.

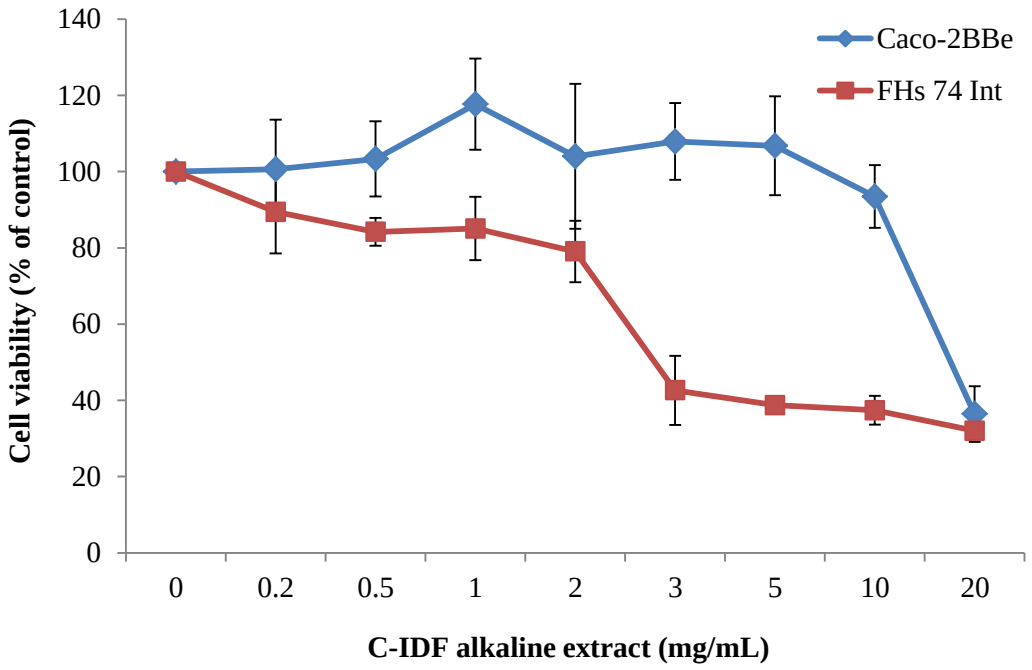


Figure 3.15 Cytotoxicity of C-IDF alkaline extracts at 0 to 20 mg/mL in Caco-2BBe and FHs 74 Int cells measured by MTT assay.

As seen in **Figure 3.15**, C-IDF extracts displayed different cytotoxicity to Caco-2BBe and FHs 74 Int cell lines. In Caco-2BBe cells, no toxic effect was demonstrated when C-IDF extracts were applied from 0.2 to 5 mg/mL. Cell proliferation was shown within such a range of treatments. However, when C-IDF extracts went up to 20 mg/mL, a sudden drop of cell viability was observed. Increasing concentration of C-IDF from 10 to 20 mg/mL decreased Caco-2BBe cell viability from 93.47% to 36.40%. In FHs 74 Int cells, there was a sharp decline of cell viability produced by C-IDF at 3 mg/mL, which resulted in the cell viability to

42.61%. Higher levels of C-IDF (3 to 20 mg/mL) that caused more than 50% of cell loss are considered as toxic treatments to FHs 74 Int cells.

Some phenolic acids have been reported as pro-oxidants in various evaluation systems, although their antioxidant properties are clear (Decker, 1997; Fukumoto & Mazza, 2000; Maurya & Devasagayam, 2010). The antioxidant or pro-oxidant activity of phenolic compounds intimately depends on their concentration and the presence of metal ions (Yordi et al., 2012). In cell culture, phenolic compounds may react with the metal ions in the cell culture medium and produce pro-oxidative effects to cultured cells (Lapidot et al., 2002). Since pro-oxidant activity could damage the biomolecules and induce a consequent cell death, the results shown in our cytotoxicity experiment may be explainable as the pro-oxidant effect of the ferulic acid concentrated IDF. Above the concentration limit, C-IDF extracts started behaving as pro-oxidants and causing oxidative damage to cells, but the concentration limit are various in two cell lines (10 mg/mL in Caco-2BBe cells and 2 mg/mL in FHs 74 Int cells). Lower concentration limit obtained from FHs 74 Int cells indicated their higher sensitivity to the presence of C-IDF extracts. Such a finding implied that specific cellular metabolism and subsequent proliferation of different cell types caused their different responses to treatments (Elisia, 2005). However, no theoretical explanations about these two human intestinal cell lines were discussed previously. Diehl-Jones et al. (2007) gave a clue that fetal cells exhibit an enhanced inflammatory response and may result in a higher response when exposed to oxidative stress.

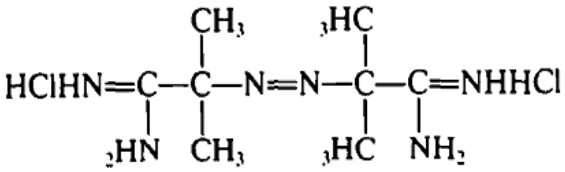
In consideration of the cytotoxicity of C-IDF extracts, in the following analyses, 0.2 to 10 mg/mL of C-IDF extract can be regarded as non-toxic doses to Caco-2BBE cells, while 0.2

to 2 mg/mL of C-IDF, which maintains at least 80% cell viability, is suitable to apply in FHs 74 Int cells.

3.3.5.3 Cytoprotective activity of corn IDF alkaline extracts against AAPH induced oxidation

3.3.5.3.1 Cytotoxicity of AAPH

AAPH (**Figure 3.16 (a)**) is known as a peroxyl radical generator. It generates free radicals at a constant and measurable rate by its thermal decomposition at 37 ℃ (Terao & Niki, 1986). The mechanism of AAPH initiated free radicals is displayed in **Figure 3.16 (b)** (Terao & Niki, 1986). AAPH decomposes to form two carbon radicals. The carbon radicals quickly react with oxygen molecules to yield peroxyl radicals. The active peroxyl radicals attack various biological molecules, induce cellular damage, and eventually cause a diverse array of pathological changes (Yokozawa et al., 2000). Therefore, an AAPH-intoxication experiment may be a promising *in vitro* assay for evaluating biological activities of antioxidants.



(a)

$$\mathbf{A{-}N{=}N{-}A \rightarrow (1 - e)\ A{-}A + 2eA\bullet + N_2}$$

$$\mathbf{A\bullet + O_2 \rightarrow AOO\bullet}$$

(b)

Figure 3.16 Structure of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (a) and its decomposition to produce peroxyl radicals (b). Where A is $HClHN{=}C(NH_2)C(CH_3)_2$, e is the efficiency of free radical production, and AOO• is peroxyl radical.

In the present study, to determine an optimal concentration of AAPH in inducing cellular oxidative stress, a preliminary cytotoxic effect of AAPH on Caco-2BBe and FHs 74 Int cell lines was conducted. AAPH at concentrations of 0, 3, 5, 10, 15, 20, 25, 30 mM (prepared in PBS) were incubated with confluent cells in 96-well plates for 4 h. The cell death caused by AAPH-induced oxidation was examined by MTT assay and expressed as cell viability (%).

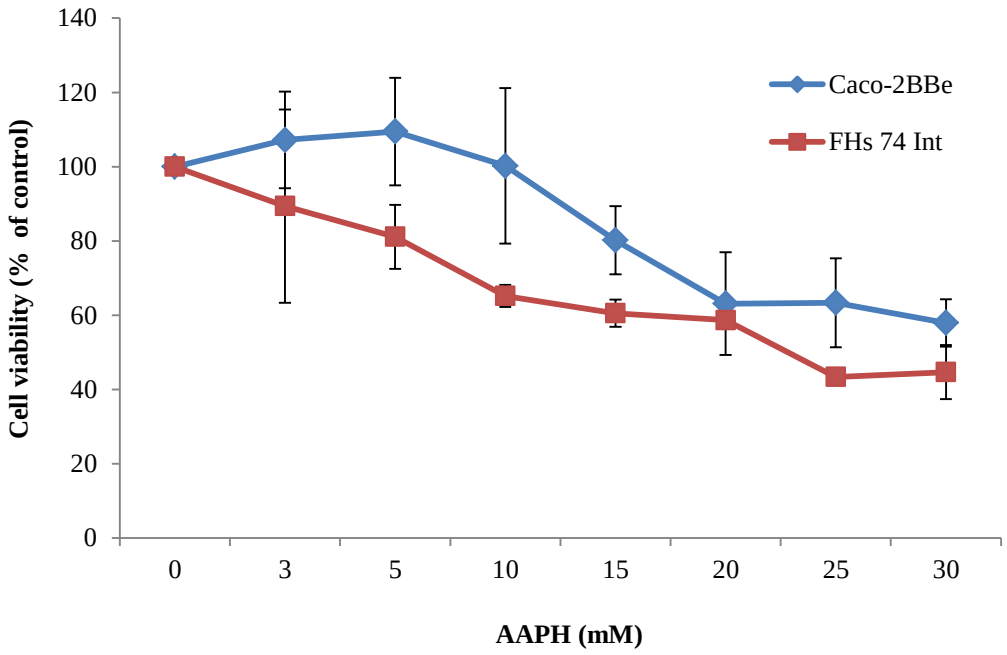


Figure 3.17 Cytotoxicity of AAPH peroxyl radical generator at 0 to 30 mM in Caco-2BBe and FHs 74 Int cells measured by MTT assay.

As seen in **Figure 3.17**, an escalating concentration of AAPH from 0 to 30 mM resulted in an increasing cell loss in both two cell lines. After 4 h of treatment with 30 mM AAPH, up to 42.08% and 55.32% loss of cell viability were observed in Caco-2BBe and FHs 74 Int cells, respectively. Different LC_{50} values (the concentration of drug results in a 50% cell viability reduction) obtained from concentration-response curves of two cell lines indicated that FHs 74 Int cells (LC_{50} = 32.60) are more sensitive than Caco-2BBe cells (LC_{50} = 23.35) in the presence of AAPH. On the basis of the results, 25 mM of AAPH was chosen to stimulate sufficient levels of oxidation in the subsequent studies.

3.3.3.5.2 Cytoprotection of corn IDF alkaline extracts against AAPH induced cytotoxicity

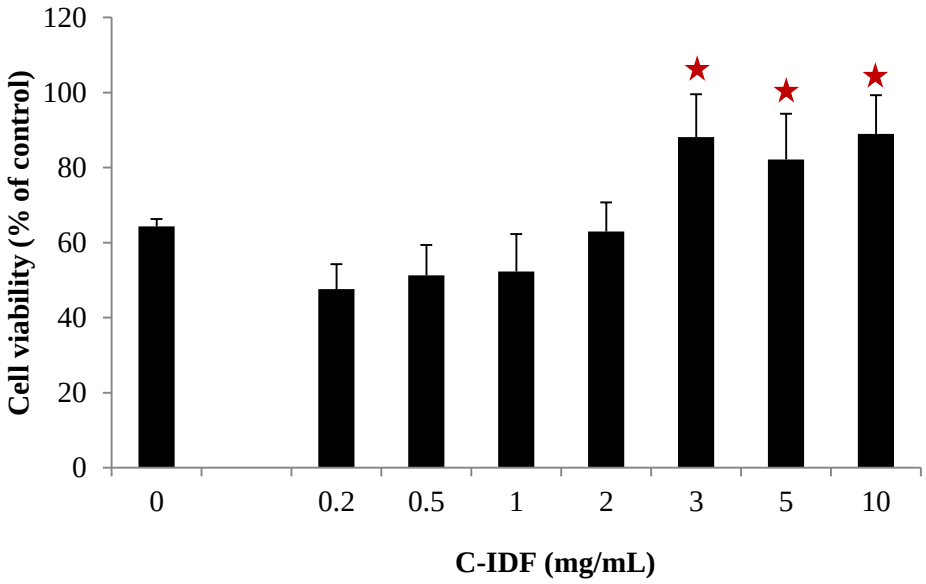
Based on the cytotoxicity results, 0.2 to 10 mg/mL and 0.2 to 2 mg/mL of C-IDF extracts was applied against 25 mM AAPH induced oxidative damage in Caco-2BBe and FHs 74 Int cells, respectively. As seen in **Figure 3.18**, C-IDF extracts behaved differently in two intestinal cell lines.

In Caco-2BBe cells (**Figure 3.18 (a)**), 3 to 10 mg/mL of C-IDF extracts significantly ($p < 0.5$) attenuated free radical toxicity. The cell viability increased from 64.33% (cells treated with 25 mM of AAPH only) to more than 80% (cells pre-treated with 3-10 mg/mL of C-IDF extracts followed by 25mM of AAPH). The highest cell viability was produced by Caco-2BBe cells pre-incubated with 10 mg/mL of C-IDF. However, no protective effect was observed when cells were treated with 0.2 to 2 mg/mL of C-IDF extracts, although a dose-dependent manner was demonstrated as C-IDF level increased within this concentration range.

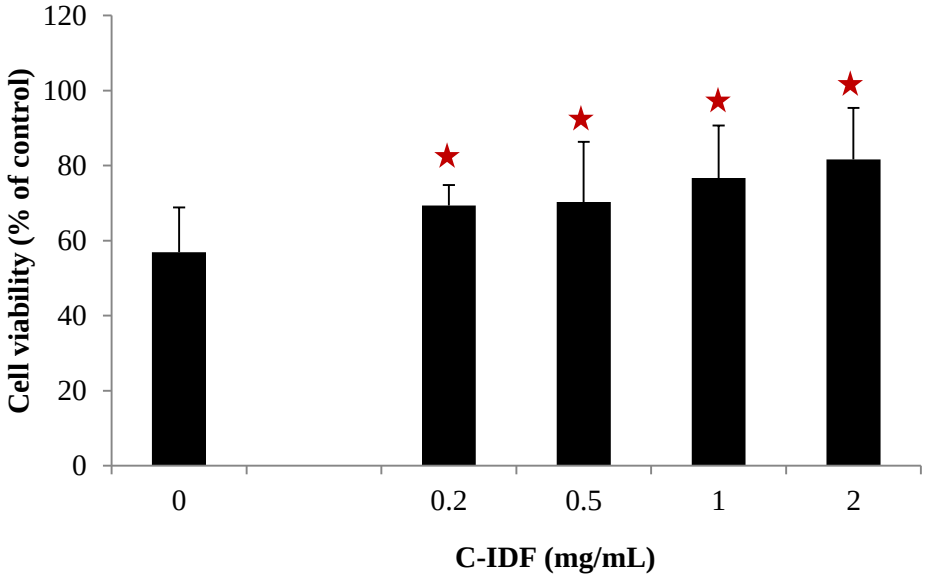
By contrast, all tested doses of C-IDF extracts suppressed the AAPH initiated oxidative damage in FHs 74 Int cells (**Figure 3.18 (b)**). Incubation of cells with escalating levels of C-

IDF from 0.2 to 2 mg/mL resulted in a concentration-dependent stimulation of cell viability that observed from 69.35% to 81.61%. Compared with C-IDF untreated cells, C-IDF caused a minimum increase of FHs 74 Int cell viability of 12.48% and a maximum increase of 24.74%.

According to a previous report (Elisia & Kitts, 2008), AAPH-initiated loss of cell viability in Caco-2 cells is partially attributed to the inductive effect of AAPH on cell apoptosis. AAPH under thermal decomposition produces peroxyl radicals in cultured cells. Accumulated intracellular peroxyl radicals increase lipid peroxidation, damage cell membrane integrity, and eventually induce cell apoptosis (Kulkarni et al., 2008). In this study, certain concentrations of C-IDF displayed effective protection in Caco-2BBe and FHs 74 Int cells against AAPH induced cell loss. Our findings therefore suggest that C-IDF extracts may function as free radical cleaners to reduce cellular ROS level and prevent the formation of apoptotic cells caused by AAPH oxidation. As the major antioxidant component in C-IDF alkaline extracts, ferulic acid may dominantly contribute to the cytoprotective effect of C-IDF. It is testified that ferulic acid is powerful in scavenging free radicals in different evaluation systems (Kanski et al., 2002; Ferguson et al., 2005). However, since there is presence of other phenolic acids and undetected phenolic compounds in C-IDF alkaline extracts, the cytoprotective function of ferulic acid need to be further confirmed. The cell viability of Caco-2BBe and FHs 74 Int cell lines as well as their per-incubation C-IDF concentrations are summarized in **Table 3.11.**



(a) Caco-2BBe



(b) FHs 74 Int

Figure 3.18 Cytoprotective activity of corn IDF alkaline extracts against AAPH induced oxidation. Bars marked by star are significantly higher than the control ($p < 0.05$).

Table 3.11 Summary of the cell viability caused by corn IDF alkaline extracts in the presence of 25 mM AAPH.

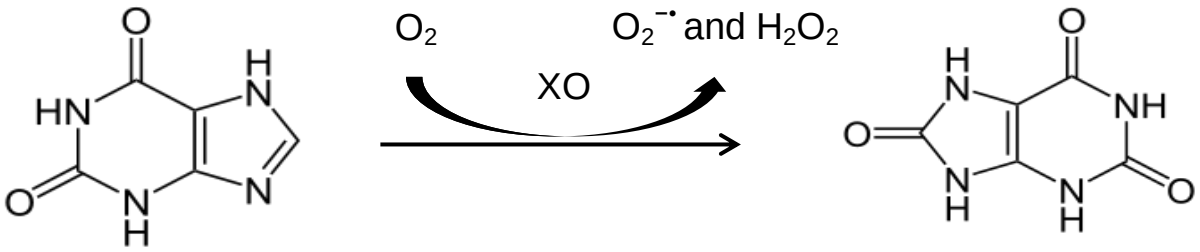
C-IDF alkaline extract (mg/mL)	0	0.2	0.5	1	2	3	5	10
ferulic acid content (μg/mL)*	0	2	4	8	17	25	42	83
ferulic acid content (uM)	0	9	21	43	86	129	214	429
Cell viability of Caco-2BBE (%)	64.33	47.58	51.27	52.26	62.94	88.17	82.14	88.97
Cell viability of FHs 74 Int (%)	56.87	69.35	70.33	76.71	81.61	-	-	-

* Ferulic acid content was calculated based on the HPLC results.

3.3.5.4 Cytoprotective activity of corn IDF alkaline extracts against X-XO induced oxidation

3.3.5.4.1 Cytotoxicity of X-XO

Xanthine (X; 3,7-dihydro-purine-2,6-dione) is a purine base found in blood, urine, and muscle tissue. In the presence of xanthine oxidase (XO; E.C. 1.1.3.22), xanthine can be degraded to uric acid while producing superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) as by-products (**Figure 3.19**). Both $O_2^{-\bullet}$ and H_2O_2 can further convert to hydroxyl (•OH) and peroxyl (ROO•), which are more reactive species (Dew et al., 2005). It testified that the reaction associated with X and XO is the major source of ROS in ischemic small intestine and is a primary inducer of cellular damage (Manna et al., 1996). Therefore, xanthine-xanthine oxidase (X-XO) model was chosen in investigating the ROS effect in human intestinal cells.



Xanthine **Uric acid**

Figure 3.19 Conversion of xanthine to uric acid by xanthine oxidase (XO).

Source: Tristan et al. (2005)

In order to determine the effect of oxidative stress on intestinal cell viability, both Caco-2BBe and FHs 74 Int cell lines were exposed to a series dose of XO (0, 10, 25, 50, 100, 200, 500 mU/mL) in the presence of X (1 mM). After 4 h incubation, cell viability (%) was measured by MTT assay.

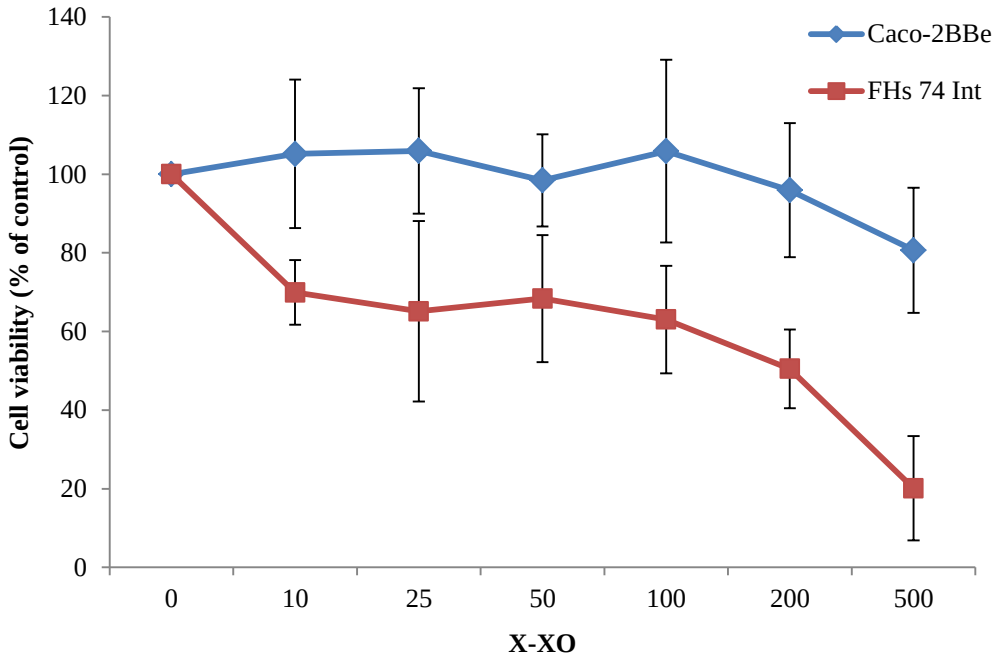


Figure 3.20 Cytotoxicity of X (1 mM) - XO (0 to 500 mU/mL) in Caco-2BBe and FHs 74 Int cells measured by MTT assay.

Results from the preliminary experiment indicated that X-XO caused FHs 74 Int cell viability to decline in a concentration-dependent manner (**Figure 3.20**). When the levels of XO increased from 10 to 500 mU/mL, the loss of cell viability went up from around 30% to 80%. The biggest decline in cell viability was found in cells that were treated with X (1 mM)-XO (500 mU/mL). In order to produce a moderate oxidative stress, X (1 mM)-XO (200 mU/mL),

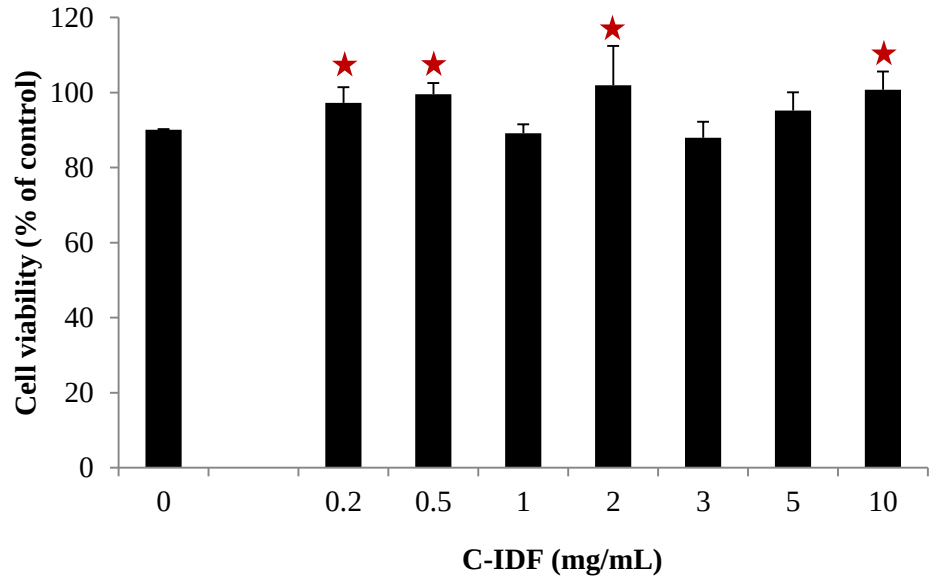
which led to around 50% cell viability, was applied in the following FHs 74 Int cytoprotection assays. No remarkable cell viability reduction was observed in Caco-2BBe cells under various amounts of X-XO. 10 to 200 mU/mL of XO maintained Caco-2BBe cell viability higher than 90%, so they can be regarded as non-toxic treatments. The highest cytotoxicity was produced by X (1 mM)-XO (500 mU/mL), which caused around 20% of cell death and yielded a cell viability at 80.66 %. Thus, for Caco-2BBe cells, X (1 mM)-XO (500 mU/mL) was selected in producing a slight cell loss.

3.3.5.4.2 Cytoprotection of corn IDF alkaline extracts against xanthine-xanthine oxidase induced cytotoxicity

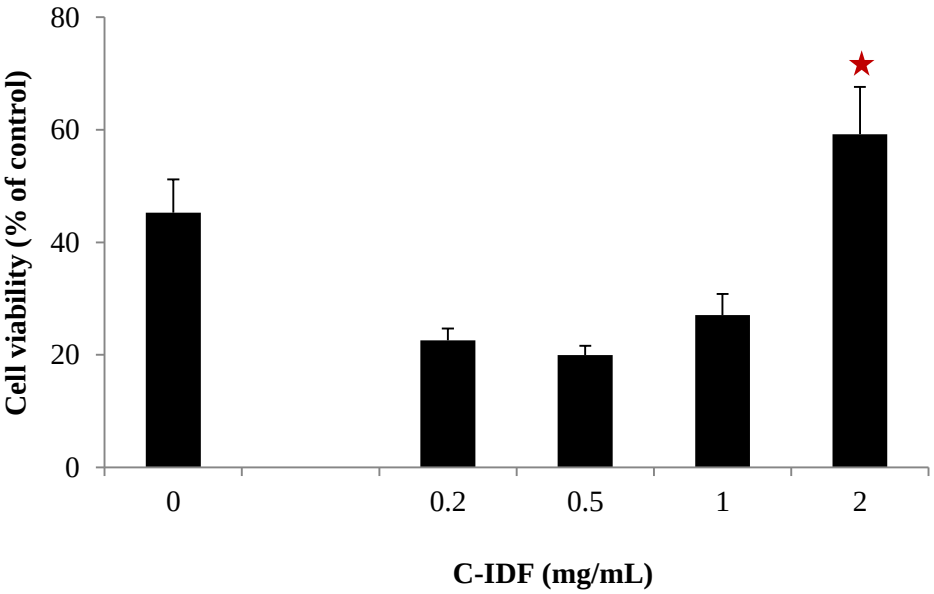
The efficacy of C-IDF extracts protection against X-XO initiated cytotoxicity was evaluated in Caco-2BBe and FHs 74 Int cells. For Caco-2BBe cells, 0.2 to 10 mg/mL of C-IDF was applied in reducing X (1 mM)-XO (500 mU/mL) caused cell damage. For FHs 74 Int cells, the concentration of C-IDF was set from 0.2 to 2 mg/mL in protecting against X (1 mM)-XO (200 mU/mL) induced cytotoxicity.

As seen in **Figure 3.21 (a)**, under the oxidative action of X-XO, a higher cell viability was produced by C-IDF treatments (except 1, 3 and 5 mg/mL of C-IDF), but the degrees of cytoprotection are various under different concentrations of extracts and no dose-dependent fashion was observed. In FHs 74 Int cells (**Figure 3.21 (b)**), the effective protection against X (1 mM)-XO (200 mU/mL) induced oxidative damage was produced by 2 mg/mL of C-IDF extracts. At this concentration, FHs 74 Int cell viability increased from 45.28% (cells exposed to X-XO only) to 59.19%. However, 0.2 to 1 mg/mL of extract pre-treatments did not alleviate FHs 74 Int cell death caused by X-XO oxidation.

The mechanism of C-IDF extracts protect against X-XO manipulated cell injury is possibly due to the free radical scavenging activity and lipid peroxidation prevention property of their phenolic compounds (Manna et al., 1997; Graziani et al., 2004). However, phenolic antioxidants can also indirectly alleviate oxidative stress through inhibiting the activity of free radical generating enzymes or enhancing the expression of intracellular antioxidant enzymes (Lü et al., 2010). It has been testified that a combined antioxidant activity from radical scavenging, XO inhibition, and chain breaking contributed to the protective effect of ferulic acid on rat intestinal injury, with the chain-breaking effect as a contributory role (Itagaki et al., 2009). Due to the complexity of the extracts and the different testing models in our study, whether the C-IDF extracts mainly function as free radical scavenger or enzyme activity inhibitor is unknown.



(a) Caco-2BBe



(b) FHs 74 Int

Figure 3.21 Cytoprotective activity of corn IDF alkaline extracts against X-XO induced oxidation. Bars marked by star are significantly higher than the control ($p < 0.05$).

Table 3.12 Summary of the cell viability caused by corn IDF alkaline extracts in the presence of X-XO.

C-IDF alkaline extract (mg/mL)	0	0.2	0.5	1	2	3	5	10
ferulic acid content (μg/mL)*	0	2	4	8	17	25	42	83
ferulic acid content (uM)	0	9	21	43	86	129	214	429
Cell viability of Caco-2BBE (%)	90.09	97.3	99.53	89.13	101.94	87.96	95.2	100.79
Cell viability of FHs 74 Int (%)	45.28	22.56	19.94	27.1	59.19	-	-	-

* Ferulic acid content was calculated based on the HPLC result.

3.4 Conclusion

The present study investigated the antioxidant properties of insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) extracts from selected whole-grain cereals (WG). In chemical model assays, the alkaline extracts of IDF showed better antioxidant capacities than WG and SDF in terms of total phenolic content (TPC), DPPH radical scavenging activity (DPPH), and total phenolic acid content (TPA). Excellent correlations found in DPPH-TPC and DPPH-TPA suggested that phenolic acids are the main compounds responsible for the antioxidant potential in cereal alkaline extracts. The most abundant phenolic acid found in alkaline extracts was ferulic acid, which accounted for more than 57% of monomeric phenolic acids in WG, IDF, and SDF alkaline extracts. *Iso*-ferulic acid was identified and reported for the first time as one of the major phenolic acids in a wide species of cereal extracts. Four ferulic dehydrodimers were detected in cereal samples and assigned as 8-5', 5-5', 8-*O*-4', and 8-5' (benzofuran) -coupled dimeric ferulic acids. Significant differences ($p < 0.05$) in TPC, DPPH radical scavenging activity, and TPA between IDF and SDF implied that the levels of phenolic acid hosts in DF, mainly cellulose, hemicellulose, and lignin, determine the degree of DF antioxidant capacity. The high values of IDF yield, TPC, DPPH radical scavenging activity, and TPA make yellow corn IDF preferable among the studied cereals for cellular antioxidant assays. The cell model studies revealed the protective function of corn insoluble dietary fibre (C-IDF) alkaline extracts containing esterified phenolic acids in cultured human intestinal cells. Although the C-IDF extracts performed differently in defending against various free radicals in two intestinal cell models, an efficient cytoprotection was observed in AAPH treated FHs 74 Int cells and xanthine-xanthine oxidase incubated Caco-2BBe cells. This effect was speculated to be associated with the antioxidant activity of C-IDF linked phenolic compounds, especially

the ferulic acid. In conclusion, C-IDF as an abundant source of ferulic acid exhibited excellent antioxidant properties and can be suggested as a functional food ingredient in lowering the risk of free radical mediated gastrointestinal disease. However, its bioavailability in intestinal tract need to be further confirmed.

3.5 Future research directions

Based on the results obtained from the present study, insoluble dietary fibre is an abundant source of phenolic acids and corn insoluble dietary can be regarded as a functional food ingredient. However, there are still additional investigations required to validate the antioxidant potential of cereal derived dietary fibre. In order to confirm the unidentified antioxidant activity contributors in red rice, further HPLC-MS/MS analyses are needed. The physiologically-relevant methods, such as biological enzymes digestion, need to be adopted in preparing dietary fibre and dietary fibre-antioxidants. Furthermore, more cell lines and cellular based antioxidant activity assay are useful in investigating the antioxidant mechanism of dietary fibre associated phenolic compounds in cell culture assays.

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