Phenolic Compounds of Selected Lentil Cultivars and their Contribution to Antioxidant Activity

By

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ABSTRACT

Free, esterified, and insoluble-bound phenolics were extracted from selected lentil cultivars. Total phenolics, flavonoids and proanthocyanidins contents were determined. Catechin, epicatechin and procyanidins B were predominant in all fractions of all tested samples as evaluated using HPLC-DAD-ESI-MS^n. Methyl vanillate, procyanidin dimer A, and prodelphinidin dimer A were identified and quantified in lentils for the first time. Procyanidin dimer A was detected only in the insoluble-bound form, methyl vanillate was present in the esterified and insoluble-bound one and prodelphinidin dimer A was found in all forms. The presence of unrevealed compounds present only in the insoluble-bound form shows that ignoring the presence of phenolics linked to the cell wall of lentils may underestimate their potential health benefits. The *in vitro* antioxidant activities and the inhibition of cupric ion-induced human low-density lipoprotein peroxidation and peroxyl radical induced supercoiled plasmid DNA strand scission demonstrated that lentils may be considered as a functional food.

Keywords: *Lens culinaris*, HPLC-DAD-ESI-MS^n, hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, proanthocyanidins, bioactivity.

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ABBREVIATIONS

- TPC — Total phenolic content
- TFC — Total flavonoid content
- CTC — Condensed tannin (proanthocyanidin) content
- RP — Reducing Power
- TEAC — Trolox equivalent antioxidant capacity
- DRSC — DPPH radical scavenging capacity
- HRSC — Hydroxyl radical scavenging capacity
- EPR — Using electron paramagentic resonance
- FRAP — Ferric reducing antioxidant power
- ORAC — Oxygen radical absorbance capacity
- HPLC-DAD-ESI-MS^n — High pressure liquid chromatographic- diode array detector-electrospray ionization- mass spectrometer
- CCC — Counter-current chromatography
- APCI — Atmospheric-pressure chemical ionization
- MALDI — Matrix-assisted laser desorption/ionization
- DPPH — 2,2-diphenyl-1-picrylhydrazyl
- DMPO — Hydrogen peroxide, 5,5-dimethyl-l-pyrroline-*N*-oxide
- EDTA — Ethylenediminetetraacetic acid
- GAE — Gallic acid
- CE — Catechin
- SET — Single electron transfer
- HAT — Hydrogen atom transfer
- ROS — Reactive oxygen species
- HBA — Hydroxybenzoic acids
- HCA — Hydroxycinnamic acids
- ABTS — 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
- TCA — Trichloroacetic acid
- $[K_3Fe(CN)_6]$ — Potassium ferricyanide
- PUFA — Polyunsaturated fatty acids

CHAPTER 1

INTRODUCTION

Phenolic compounds, commonly found in both edible and non-edible plants, have been reported to possess multiple biological effects, including antioxidant activity (Kähkönen et al., 1999). In the human diet, polyphenols make the most important contribution to antioxidant activity (Manach et al., 2004). The beneficial health effects of phenolics are mainly because of their antioxidant activity (Fang et al., 2002). As antioxidants, phenolic compounds are able to decrease oxygen concentration, intercept singlet oxygen, prevent first chain oxidation initiation by scavenging initial radicals such as hydroxyl radicals, decompose primary products of oxidation to non-radical species, and chelate prooxidant metal ions (Shahidi and Naczk, 2004) such as ferrous ion (de Camargo et al., 2014a). Reactive oxygen species (ROS) and metal ions are detrimental to food and biological systems (de Camargo et al., 2015).

Numerous publications have reported the health benefits of phenolic compounds in disease risk reduction, for example in the protection against cancer (Mirali et al., 2014), including those of breast, colon, prostate, and stomach as well as cardiovascular disease (CVD) (Hertog *et al.,* 1995), and various inflammatory disorders (Andriantsitohaina *et al.,* 1999). These various beneficial health effects illustrate the importance of analyzing phenolic compounds in food resources (Mirali et al., 2014). The user industry has already noted the importance of phenolics in food preservation and for preventing chronic

diseases. Thus, the food industry is paying attention to phenolics in food as such and also in food fortification (de Camargo et al., 2014b).

Lentils (*Lens culinaris*) being one of the oldest crops cultivated in human history are widely consumed throughout the world (Xu and Chang, 2010). Lentils play an important nutrition function, render significant health promoting effects and serve as a good source of protein, carbohydrates, dietary fiber, minerals, vitamins, and phenolic compounds. Simple phenolic compounds, phenolic acids, flavan-3-ols, proanthocyanidins, anthocyanidins, flavonols, stilbenes, flavones, and flavanones are the major sub-classes of phenolic compounds found in lentil seeds. In the lentil seed coat, phenolic compounds are more abundant and diverse than in the cotyledon, and mainly consist of proanthocyanidins which are oligomeric and polymeric in nature (Mirali et al., 2014). In addition, Xu and Chang (2007) have reported that lentils have a high content of phenolic compounds. However there is little information available in the literature related to phenolic compounds in lentils.

Most studies have previously been carried out on crude soluble (free and esterified) phenolic extracts of lentils and their antioxidant activities (Xu and Chang, 2009), while ignoring the insoluble-bound phenolics, which may result in the underestimation of the total phenolic content and actual health benefits due to consumption of lentils. However, there are several studies focused on the fractionation of the phenolic compound extracted from lentils into soluble esters and insoluble-bound forms (Sosulski and Dabrowski, 1984), and soluble free and insoluble-bound forms (Han and Baik, 2008; Xu and Chang, 2009).

To the best of my knowledge, this is the first study reporting the separate contribution of soluble phenolics (free and esterified ones) and those in the insoluble-bound form, and subsequent evaluation of their overall antioxidant potential using different methods. In the current investigation, phenolic compounds of lentils were fractionated into their respective free, esterified, and insoluble-bound forms; the last two were released by alkali hydrolysis. By using different chemical assays and HPLC-DAD-ESI-MS^n, the identification and quantification of the various phenolic compounds were achieved. The antioxidant activity of 6 lentil cultivars with different coat colors (green, brown, tan, Black, and some of them were solid or speckled) was also determined with respect to their total phenolic, flavonoid, and condensed tannin (proanthocyanidin) contents. Furthermore, their antioxidant potential in biological model systems was investigated in order to highlight, for example, the ability of phenolics from lentils in scavenging radicals, reducing metal ions, inhibiting the oxidation of low-density lipoprotein (LDL) and DNA damage in *in vitro* models.

CHAPTER 2

LITERATURE REVIEW

Legumes

Legumes are dicotyledonous seeds of plants that belong to the family *Leguminosae* [16000-19000 species in ~750 genera] (Hoover et al., 1997). The annual production of legumes ranks fifth in the world after wheat, rice, maize, and barley (Zhao et al., 2014). Legumes are grown in India, Pakistan, Sri Lanka, Bangladesh, Mexico, Canada, South America and Africa. The global production of edible legumes was about 275 million tons in 2010 (Zhao et al., 2014). These crops are largely cultivated for their grains and utilized as valuable ingredients of various products for human consumption as well as for animal feed. Legume seeds are consumed extensively by humans in different areas of the world (Zhao et al., 2014). Legumes are an important food source and are rich in bioactives (Geil and Anderson, 1994) such as phenolics and polyphenolics, including flavonoids, as well as dietary fiber, protein, polypeptides, amino acids, vitamins, and carotenoids (Geil and Anderson, 1994; Zhao et al., 2014). Due to the presence of these compounds, legumes have long been recognized as functional foods that promote health and have therapeutic properties (Geil and Anderson, 1994; Xu and Chang, 2007).

There has been considerable research on the consumption of legumes and their health benefits (Mitchel et al., 2009). Legumes are known to reduce the risk of coronary heart disease, diabetes and obesity, as well as lowering serum cholesterol concentration (Geil

and Anderson 1994). Additionally, legumes are important for prevention of breast cancer (Adebamowo et al., 2005), and contribute to bone health (Alekel et al., 2000).

2.1 Lentils (*Lens culinaris*)

Lentil (*Lens culinaris*) are pulse crops (i.e., an annual leguminous crop yielding from one to twelve grains or seeds of variable size, shape, and color within a pod) (Amarowicz et al., 2010), and are commonly grown in rain fed, dry land and the agro-pastoral systems in many parts of the world (Boudjou et al., 2013). The origin of lentils dates back to ancient times in the Middle Eastern countries such as Turkey, and starting in the 1970s they began to play a significant role in Western Canada (Der, 2010). After India, Canada is the world's second largest producer of pulses, followed by Myanmar, China and Brazil [Food and Agricultural Organization of the United Nations (FAO) 2012]; however, as of 2010 Canada has been the largest exporter of pulses in the world (Carew et al., 2103). The consumption of lentils has been wide spread in many regions of the world due to their high nutritional value (Xu and Chang, 2007; 2010). The perceived nutritional characteristics of pulses and their culinary benefits have increased opportunities for the food processing industries, and have led to greater consumption of pulse ingredients in bakery and meat products (Carew et al., 2103). Generally, in retail sales, lentils are found as canned or dry-packaged, whole or split. In addition, they can be processed into flour to enrich several kinds of food; such as, cereal flour to make bread, cakes, and baby foods. They may also be used in soups, stews, salads, snack foods, and vegetarian dishes (Xu and Chang, 2010).

2.1.1 Nutritional quality

Lentils can play a major role in human nutrition and are considered to be a good source of both macronutrients and micronutrients (Xu and Chang, 2007; 2010). Wang and Daun (2006) reported the proximate composition of Canadian lentils (non-dehulled) having 24.3-30.2% protein, 54.2-62.5% carbohydrate, 1.0-1.3% fat, and 2.3-3.5% ash, on a dry weight basis. Additionally, lentils are a good source of minerals such as calcium, potassium, magnesium, phosphorus and zinc (Wang and Daun, 2006). Furthermore, as mentioned above, legumes serve as an important source of dietary fiber with a total dietary fiber content of 19.2% in lentil seeds (Perez-Hidalgo et al., 1997). Lentils as a good source of high quality protein are used as meat extenders or substitutes (Xu and Chang, 2010). On the other hand, the digestibility of protein may decrease because of the presence of certain anti-nutritional factors such as trypsin inhibitors, phytic acid, certain oligosaccharides and tannins (Costa et al., 2006; Der, 2010). Trypsin inhibitors are low molecular weight proteins capable of binding to and inactivating the digestive enzyme (Salunkhe and Kadam, 1989; Wang and Daun, 2006). Trypsin inhibitor activity in the raw lentil samples was 1.91-2.77 mg/g sample (Wang et al., 2009). Phytic acid lowers the bioavailability of minerals (Reddy et al., 1984) and its content varied from 6.2 to 8.8 mg/g dry matter in the raw seeds (Wang et al., 2009). Oligosaccharides raffinose, stachyose and verbascose are flatulence causing sugars (Fleming, 1981) and their contents in raw lentils are 4.5- 6.0, 7.4- 9.8 and 23.3- 29.9 g/kg dry matter, respectively (Wang et al., 2009). Tannins that are mostly present in the coat of lentils seed are known to form complexes with proteins, which are reported to be responsible for low protein digestibility and decreased amino acid availability (Salunkhe and Kadam, 1989; Wang et al., 2009). The phenolic groups of tannins bind very tightly with the –NH groups of

peptides and proteins, they prevent their hydrolysis and digestion in the stomach (Shahidi and Naczk, 2004). The mean tannin content in raw lentil seeds is 4.7 g/kg dry matter (Wang et al., 2009). Wang and Daun (2006) reported that cooking could substantially inactivate these anti-nutritional factors in lentils. Nevertheless, some studies have reported that several non-nutritive phytochemicals such as phytates, saponins, and oligosaccharides might also play a role in cancer prevention (Mitchel et al., 2009). In addition, Kabagambe et al. (2005) reported that phytic acid, saponins, tannins and other polyphenols in legumes may prevent chronic diseases. Midorikawa et al. (2001) and Rocio Campos-Vega et al. (2010) also reported that phytic acid has antioxidant and DNA protective effects.

Lentils have numerous potential health-promoting benefits, such as reducing the risk of cardiovascular disease, reducing cholesterol and blood lipids, managing blood-sugar disorders, and cancer (Xu and Chang, 2010). Protein and fiber have been shown to have potential roles in the prevention of heart disease and possibly certain types of cancers (Mitchel et al., 2009). The importance of dietary fiber in normal and therapeutic diets has been acknowledged by numerous authors (Perez-Hidalgo et al., 1997; Wang et al., 2009). To reduce blood cholesterol levels and prevent coronary heart disease the recommendation is to increase the intake of high fiber, carbohydrate foods (Jenkins et al., 1993). Numerous studies have shown that people with high fiber intake have low blood pressure (Wang et al., 2009). Studies have indicated that dietary fiber may protect against cardiovascular diseases, diabetes, obesity, colon cancer and other diverticular diseases (Perez-Hidalgo et al., 1997). Additionally, It is well known that phenolics and polyphenolics in food aid in the prevention of certain types of cancer and cardiovascular disease. It has also been reported that polyphenols act as potential anti-inflammatory

compounds which have a positive *in vivo* effect in decreasing visceral fat and in the reduction of obesity (Terra et al., 2007; Yao et al., 2012; Xuan et al., 2013; de Camargo et al., 2015). In addition, polyphenols play a positive role in the management of pre-diabetic and/or diabetic conditions (Roopchand et al., 2013; de Camargo et al., 2015). The phenolic compounds are believed to promote human health through a number of mechanisms, including enhancing antioxidant activity, anti-platelet aggregation, impacting cellular processes associated with apoptosis, blood vessel dilation, and enzyme activities related to carcinogen activation/deactivation (Shahidi and Wanasundara, 1992; Xu and Chang, 2009; Yao et al., 2010; Zhang et al., 2010). Due to the presence of the macronutrients, micronutrients and phytochemicals, which are important contributors to health promotion lentils are reported to be an excellent source of phenolic compounds, including condensed tannins (Zhang et al., 2015).

As a result of the ability of phenolic compounds to scavenge free radicals by donating an electron or a hydrogen atom (Heim et al., 2002), they are able to delay or inhibit oxidative damage, which prevents the onset of oxidative stress related diseases in the human body (Zhang et al., 2015). Due to the importance of phenolic compounds as antioxidants in lentils, which was the main interest in this study, the following section provides a more in depth review of phenolic compounds, their classifications, biological effects, antioxidant mechanisms, and reviews legumes and lentils as a source of phenolic antioxidants. Extraction methods are also discussed in detail.

2.2 Phenolics and Polyphenolics

Phenolic compounds, including polyphenols, have more than 8000 chemical structures and are among the most numerous and widely distributed groups of substances in the plant kingdom (Bravo, 1998). Phenolic compounds in foods belong to one of the main classes of secondary metabolites in plants (Naczk and Shahidi, 2004). Produced from phenylalanine and to a lesser extent tyrosine (via the removal of ammonia), phenolic compounds have much morphological and physiological importance in plants, and perform a myriad of vital roles (Baidez et al., 2007).

There has been increased research on plant product and they serve as antioxidants, because of their ability to protect against many diseases caused by free radicals (Hou et al., 2003). The activities of antioxidants are numerous; these include reducing hydroperoxides into stable hydroxyl derivatives, inactivating metal catalysts by chelation, interacting synergistically with other reducing compounds, and also scavenging free radicals (Frankel and Finley, 2008). The antioxidant activity of phenolics is associated with their chemical structures (Rice-Evans et al., 1996), and details about their mechanisms of action are discussed below.

Phenolics can chemically be defined as substances containing an aromatic ring with one or more hydroxyl groups and include their functional derivatives (Shahidi and Naczk, 2004). Plants and foods contain a large variety of phenolic derivatives such as flavonoids, phenolic acids, tannins, lignans, tocopherols, as well as terpenes, phospholipids and polyfunctional organic acids, among others, and these are all examples of naturally-occurring antioxidative compounds (Wanasundara and Shahidi, 1996;

Shahidi and Naczk, 2004). Plant phenolics are the primary sources of natural antioxidants and may be found in all parts of plants. In addition, they can also occur in fruits, vegetables, leaves, roots, barks, nuts, seeds, and flours (Wanasundara et al., 1995; Shahidi and Naczk, 2004). Figure 2.1 illustrates the classification of phenolic antioxidants.

2.2.1 Phenolic acids (hydroxybenzoic and hydroxycinnamic acids)

Phenolic acids, which are extensively prevalent in the plant kingdom, are known for their multiple bioactivities. The predominant phenolic acids found in plants are hydroxybenzoic and hydroxycinnamic acids; the most common being the hydroxycinnamic acids. These derivatives have in their aromatic rings different patterns of hydroxylation and methoxylation (Shahidi and Naczk, 2004; Mattila and Hellström, 2007). The basic pathway for synthesis of phenolic acids in plants starts from sugars through to aromatic amino acids phenylalanine, and, in some rare cases, tyrosine. Trans-cinnamic acid formation from phenylalanine and *p*-hydroxycinnamic acid from tyrosine is catalyzed by phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), respectively (Shahidi and Wanasundara, 1992). Phenolic acids present in plant foods occur in the free, soluble ester and insoluble-bound forms; the insoluble-bound form generally predominates in cereals, legumes and oilseeds (Herrmann, 1989).

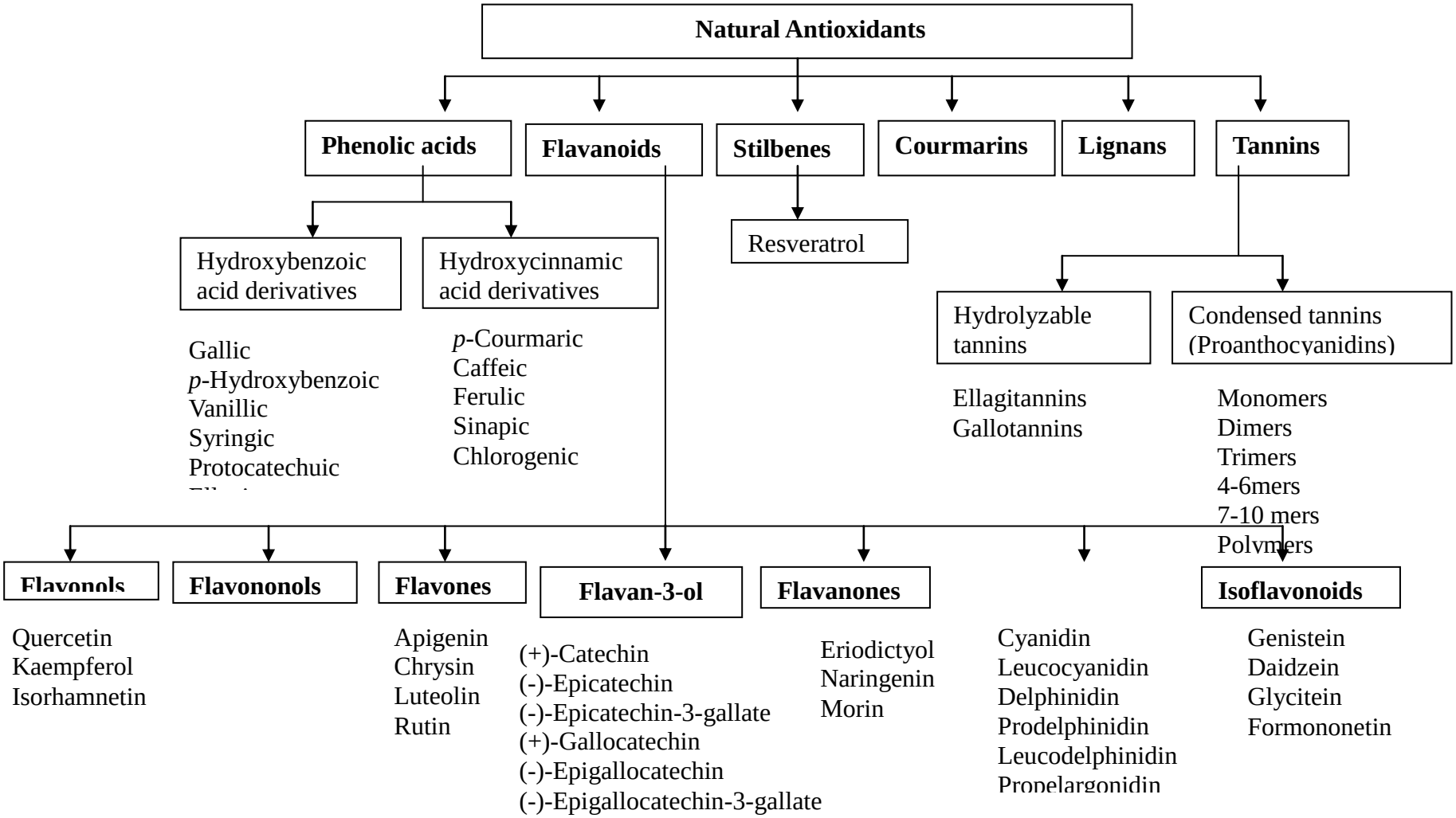
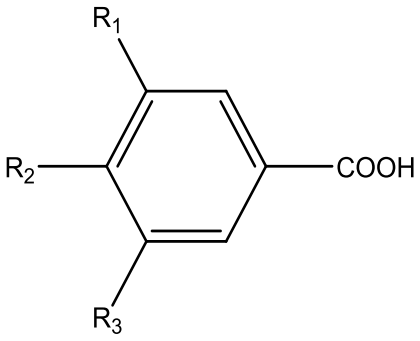


Figure 2.1 Classification of phenolic antioxidant (Adapted from Shahidi and Ho, 2007)

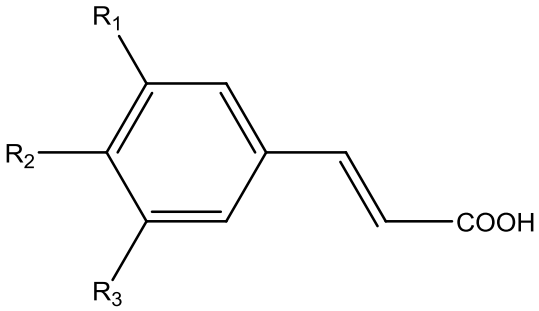
Hydroxybenzoic acid derivatives (HBA; C6–C1) (Figure 2.2) are mainly present in foods in the form of glucosides; *p*-hydroxybenzoic, gallic, vanillic, syringic and protocatechuic acids are the most common forms **(**Herrmann, 1989**;** Bravo, 1998; Shahidi and Naczk, 2004; Mattila and Hellström, 2007). Hydroxycinnamic acids (HCA; C6–C3) include caffeic, ferulic, *p*-coumaric and sinapic acids (Bravo, 1998) (Figure 2.2), which regularly occur in foods as simple esters (with quinic acid or glucose). Chlorogenic acid, which is actually combined caffeic and quinic acids, is likely the most popularly known bound hydroxycinnamic acid **(**Herrmann, 1989**;** Shahidi and Naczk, 2004; Mattila and Hellström, 2007).

Phenolic acids are one of the major group of phenolic compounds present in legumes (Shahidi and Naczk, 2004; López-Amorós et al., 2006). Ferulic acid is the most abundant phenolic acid present in common beans, whereas *p*-coumaric acid and sinapic acid were detected at intermediate levels in bean samples (Luthria and Pastor-Corrales, 2006). Madhujith et al. (2004b) reported vanillic, caffeic, *p*-coumaric, ferulic, and sinapic acids as the main phenolic acids in bean hull extracts. Amarowicz and Troszynska (2004) also determined vanillic, caffeic, *p*-coumaric, ferulic, and sinapic acids in the crude extract of red beans. Additionally, the contents of vanillic, caffeic, *p*-coumaric, sinapic, and ferulic acids in pea crude extract were 70, 20, 60, 320, and 70 μg /g extract (d.w.), respectively (Amarowicz and Troszynska, 2003).



Acids	R_1	R_2	R_3
p-Hydroxybenzoic	H	OH	H
Protocatechuic	OH	OH	H
Vanillic	OCH_3	OH	H
Syringic	OCH_3	OH	OCH_3
Gallic	OH	OH	H

Hydroxybenzoic acids



Acids	R_1	R_2	R_3
p-Courmaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH_3	OH	H
Sinapic	OCH_3	OH	OCH_3

Hydroxycinnamic acids

Figure 2.2 Chemical structures of naturally occurring phenolic acids and related compounds (Adapted from Shahidi and Naczk, 2004)

Sosulski and Dabrowski (1984) reported the phenolic compounds in defatted flours and hulls of ten different legume cultivars namely mung bean, smooth field pea, yellow lentil, small faba bean, pigeon pea, navy bean, white lupine, baby lima bean, chickpea, and cow pea; they were fractionated into soluble free and esterified, and insoluble-bound fractions. The flours contained only soluble esters; hydrolysis of these showed the presence of the following phenolic acids (*p*-hydroxybenzoic, protocatechuic, syringic, trans-*p*-coumaric, and trans-ferulic acids) in almost all of the legumes examined. The lowest amount of phenolic acids was found in mung bean, field bean, lentil, faba bean, and pigeon pea, with 2–3 mg of phenolic acids per 100 g of flour. The highest level of phenolic acids was determined in navy bean, lupine, lima bean, and cowpea. The leguminous hulls contained *p*-hydroxybenzoic, protocatechuic, syringic, gallic, trans-*p*-coumaric, and trans-ferulic acids in the soluble ester fraction; also they were identified in the insoluble-bound fraction of the yellow lentils extract except trans-*p*-coumaric, and trans-ferulic acids.

Xu and Chang (2010) identified the hydroxybenzoic derivatives, namely gallic acid, protocatechuic acid, 2,3,4-trihydroxybenzoic acid, *p*-hydroxybenzoic acid and vanillic acid, and the hydroxycinnamic derivatives, namely; caffeic, chlorogenic, *p*-coumaric, and sinapic acids, in the crude extract of 11 different lentil cultivars grown in Northern United States. The authors quantified these phenolic acids which namely above in high amounts (Xu and Chang, 2010). Additionally, phenolic acids, namely; sinapic, *p*-hydroxybenzoic, trans-*p*-coumaric, and trans-ferulic acids as well as gallic aldehyde were identified in red lentils, and their contents were 0.06, 73.46, 38.84, 15.99, and 13.45 μg /g extract (d.w.), respectively (Amarowicz et al., 2009). The identification of trans-ferulic acid (10.1 μg/g extract (d.w.)), and trans-*p*-coumaric acid (37.3 μg/g extract (d.w.)) was also reported by Amarowicz et al. (2010), the latter was the dominant phenolic in the

extract of green lentils. However, the authors quantified them in a lower quantity in the green lentils in comparison with those in read lentil seeds (Amarowicz et al., 2009). Boudjou et al. (2013) analyzed the phenolic extract of lentil hull; they identified syringic (550.81 μg/g), trans-*p*-coumaric (199.81 μg/g), ferulic (201.67 μg/g), and trans-cinnamic (1948.65 μg/g) acids. The authors reported that the lentil hull extract was rich in trans-cinnamic acid (Boudjou et al., 2013). In previous studies these phenolic acids in lentil hulls were reported; however, in lower quantities (Sosulski and Dabrowski, 1984; Dueñas et al., 2003). Additionally, in the most recent research by Zhang et al. (2015), analysis of phenolic compounds in 20 Canadian lentil cultivars was reported. The authors identified and quantified phenolic acids in the crude extracts of these cultivars as dihydroxybenzoic, *p*-hydroxybenzoic, syringic, trans-*p*-coumaroyl malic, trans-*p*-coumaric and sinapic acids, and their contents were in the range of 1.40-3.56, 2.93-5.80, 1.53-9.18, 0.62-1.78, 4.24-11.88, and 0.48-1.16 μg/g (d.w.), respectively (Zhang et al., 2015).

There has been much interest in the potential biological benefits of simple phenolic acids as well as their antioxidant activity (Robbins, 2003). Consumption of phenolic acids occurs daily due to their widespread presence in plant-based foods. Depending on the diet consumed (fruits, vegetables, spices, grains, teas, or coffees) the consumption range is estimated between 25 mg and 1 g per day (Clifford, 1999). The health benefits of phenolic compounds have commonly been attributed to their antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, antimicrobial, as well as other biological properties (Xu et al., 2008). One of the most naturally occurring and prominent cinnamic acids is caffeic acid which selectively blocks the biosynthesis of leukotrienes, components involved in immunoregulation diseases, allergic reactions, and asthma (Koshihara et al., 1984). Additionally, caffeic acid and some of its esters may possess

antitumor activity against colon carcinogenesis (Olthof et al., 2001; Robbins, 2003). It was reported that caffeic acid inhibits oxidation of LDL *in vitro* suppressing the spread of HepG2 tumor xenografts in nude mice *in vivo* (Nardini et al., 1995). In addition, Kawabata et al. (2000) reported that ferulic acid elevates the activities of detoxifying enzymes (glutathione S-transferase) and decreases the rate of occurrence of colonic carcinomas induced by azoxymethane.

2.2.2 Flavonoids and condensed tannins (proanthocyanidins)

Flavonoids are the predominant class of phenolics which account for approximately two-thirds of the dietary phenols (Robbins, 2003). Flavonoids are cyclized diphenylpropanes (chalcones) which occur in plants and particularly plant foods (Cao et al., 1997). They exist in plants as either glycones or aglycones (Harborne, 1989; Gharras, 2009). Some flavonoids are responsible for imparting color, flavor, and odor to the flowers, fruits and leaves (Harborne, 1989; Gharras, 2009). More than 6000 flavonoids have been identified (Harborne and Williams, 2000). Flavonoids have the characteristic C_6-C_3-C_6 carbon skeleton, and their derivatives vary in the structure around the heterocyclic oxygen ring (Figure 2.3) (Shahidi and Naczk, 2004; Yao et al., 2004). Flavonoids are classfied into isoflavones, flavones, flavonols, flavanones, flavanonols, flavanols and anthocyanidins (Das, 1994). Flavanones undergo a series of transformations affecting the heterocyclic carbon ring to give rise to anthocyanins and catechins (Das, 1994). In general, all flavonoids are derivatives of the 2-phenylchromone parent compound composed of three phenolic rings (A, B and C, Figure 2.3), which exhibit various levels of hydroxylation and methoxylation (Yao et al., 2004).

Isoflavonoids are found chiefly in the leguminoseae family, and consist of a phenyl ring (A-ring, Figure 2.3) being fused with the six-membered heterocyclic C-ring and another phenyl ring (the B-ring) at the C-3 position, while in the case of flavonoids, the B-ring is substituted at the C-2 position. However, some isoflavonoids have been found, despite having subtle structural differences, to be more active as antioxidants than their corresponding flavonoids (Han et al., 2009). Flavones and flavonols occur as aglycones in foods; approximately 200 flavonols and some 100 flavones have been identified in plants. These compounds have a double bond between C-2 and C-3. Flavonols differ from flavones in that they contain a hydroxyl group in the 3-position and can be considered as 3-deoxyflavonols (Figure 2.3) (Shahidi and Naczk, 2004). Characteristic of flavonones and flavononols is the presence of a saturated C_2-C_3 bond along with an oxygen atom (carbonyl group) in the 4-position. Thus, flavonones may be referred to as dihydroflavones. Flavononols are different from flavonones due to having a hydroxyl group in the 3-position and are commonly identified as 3-hydroxyflavonones or dihydroflavonols (Shahidi and Naczk, 2004). Among flavonoids, anthocyanins and catechins, known collectively as flavans because of lack of the carbonyl group in the 3-position, are important; flavan-3-ols and flavan-3,4-diols belong to this category (Shahidi and Naczk, 2004).

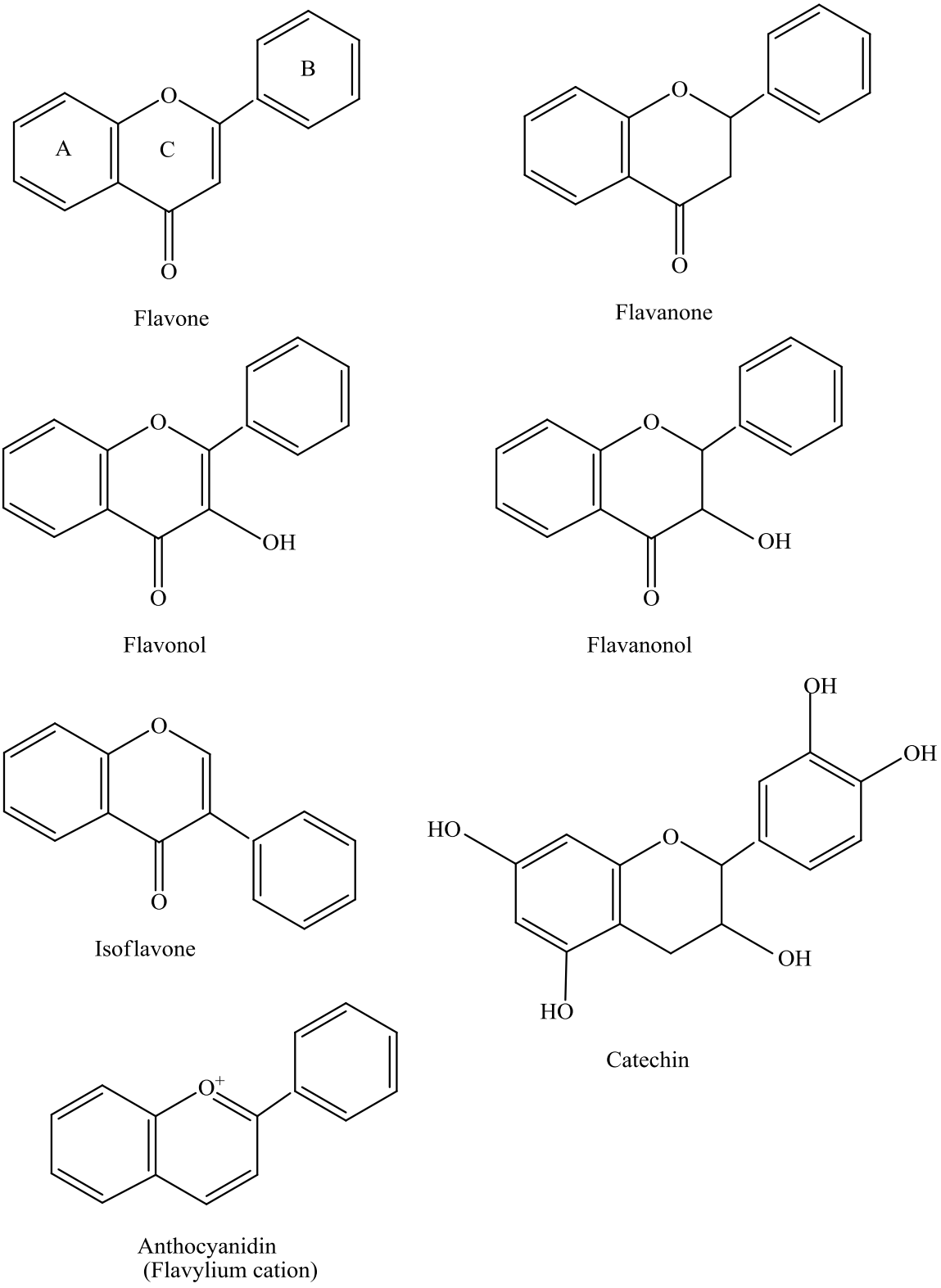
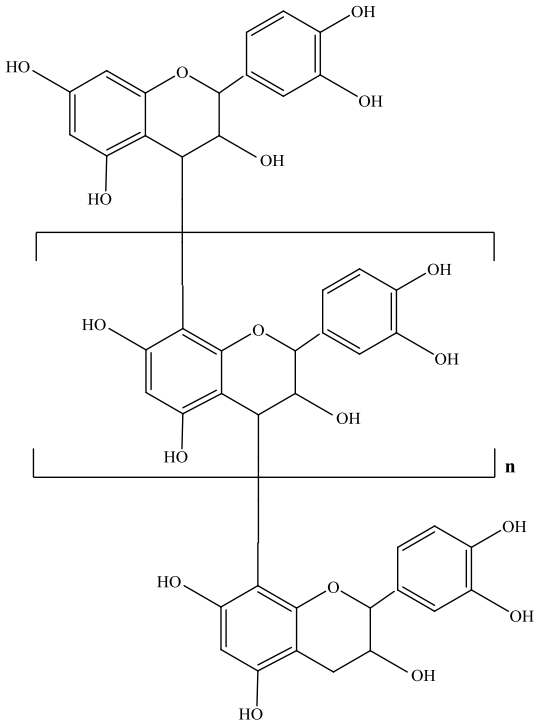


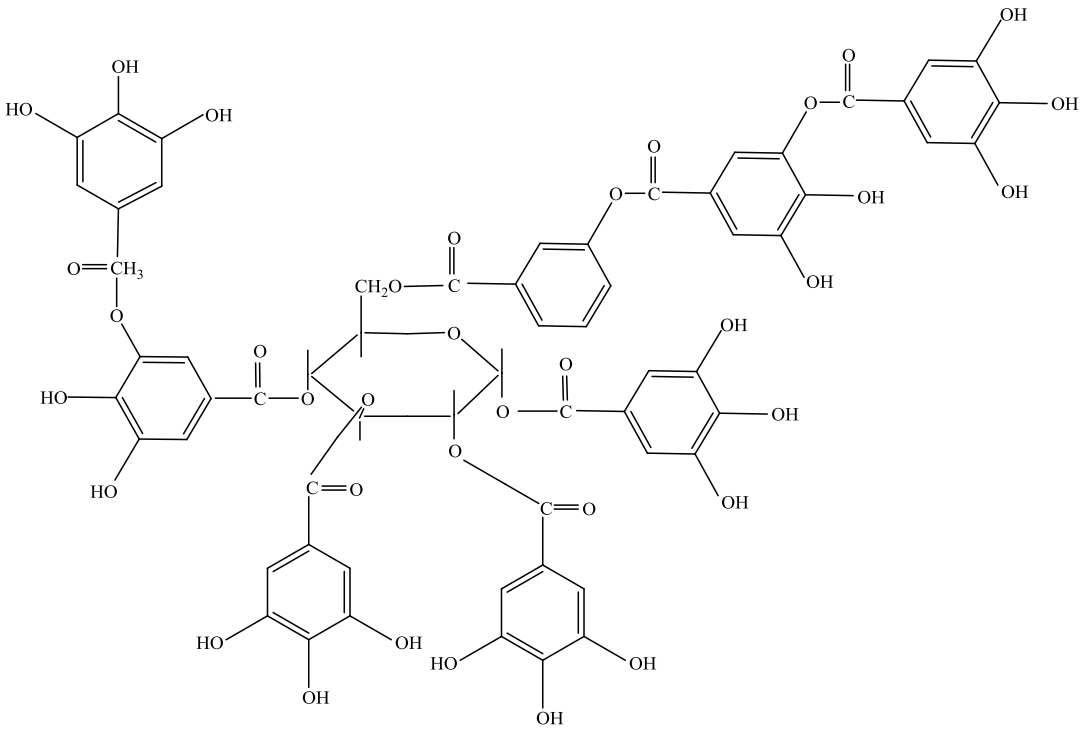
Figure 2.3 Chemical structures of major classes of flavonoids (Adapted from Shahidi and Naczk, 2004).

Tannins are defined as hydrolyzable or condensed (proanthocyanidins), depending on their chemical structures (Figure 2.4) (Shahidi and Naczk, 2004). Tannins, which are located mainly in the testa, have an important role in the system of defense for seeds, which are exposed to oxidative damage due to many environmental factors (Troszynska and Ciska, 2002). The condensed tannins are oligomers and polymers of flavonoids, specifically flavan-3-ols, while hydrolyzable tannins are glycosylated gallic acid (Ferreira and Li, 2000; Khanbabaee and van Ree, 2001). Tannins in beans are linear polymers of flavan-3-ol (catechin and gallocatechin) and flavan-3,4-diol (leucocyanidin and leucodelphinidin) units (Martin-Tanguy et al., 1977).

Flavonoids, procyanidins are the dominant phenolic compounds present in leguminous seeds (Choung et al., 2003; López-Amorós et al., 2006), and their seeds coats are also rich in anthocyanidins (Choung et al., 2003). Flavonol glycosides, tannins and anthocyanins are responsible for the color of the seed coat in dry beans (Shahidi and Naczk, 2004). Xu and Chang (2007) determined the total content of flavonoids in leguminous seeds (green pea, yellow pea, chickpea, lentil, red kidney bean and black bean), and found the highest quantity of total flavonoids was present in black bean, red kidney and lentil seeds in the range of 1.19-3.21, 0.85-2.93, and 0.72-2.21 mg catechin equivalents/g sample, respectively. Flavonoids present in leguminous seeds belong to flavanols, flavan-3-ols, flavones, flavanones, flavonols, anthocyanidins and proanthocyanidins (Dueñas et al., 2004; Díaz-Batalla et al., 2006; Mirali et al., 2014). The majority of them, however, are present as glycosides in the seeds (Díaz-Batalla et al., 2006). Lin et al. (2008) analyzed twenty-four common bean samples, and reported that the flavonoid components showed distinct differences.



Condensed Tannin



Hydrolysable Tannin

Figure 2.4 Chemical structures of condensed and hydrolysable tannins (Adapted from Shahidi and Naczk, 2004)

Pinto beans contained kaempferol and its 3-*O*-glycosides, while black beans contained primarily the 3-*O*-glucosides of delphinidin, petunidin and malvidin. Pink and dark red kidney beans contained the diglycosides of quercetin and kaempferol, however, light red kidney beans had traces of quercetin 3-*O*-glucoside and its malonates. Small red beans possessed kaempferol 3-*O*-glucoside and pelargonidin 3-*O*-glucoside, but no flavonoids were found in alubia, great northern, and navy beans (Lin et al., 2008).

In a crude extract of adzuki bean, catechin and epicatechin glucosides, quercetin glucoside, myricetin, and procyanidin dimers were the main phenolic compounds (Amarowicz et al., 2008a). The contents of procyanidin dimers and trimers ranged from 15.9 to 213 μg/g (Amarowicz et al., 2008a). The glucosides of flavones and flavonols in the cotyledon of peas (*Pisum sativum L.*) have been identified (Dueñas et al., 2006). The content of quercetin-3-O-glucoside and myricetin-3-O-glucoside (11.45, 9.64 μg/g extract (d.w.), respectively) was also quantified in raw cowpeas (*Vigna sinensis L.*) (Dueñas et al., 2005). Additionally, the presence of procyanidin B1 and B3 in adzuki bean was reported (Ariga and Hamano, 1990; Ariga et al., 1988), and procyanidins B2, C1, and C2 were identified in red, brown, and black bean hull extracts (Madhujith et al., 2004a).

In terms of flavonoid and tannin presence in lentils, Xu and Chang (2010) reported flavan-3-ols, namely (+)-catechin and (−)-epicatechin, in the range of 266.9-1898.9, and 2535.1-4946.7 μg/g of lentils, respectively, as the main phenolic compounds in the crude extract of 11 different lentils cultivars grown in Northern United States. However, Amarowicz et al. (2009) detected them in lower quantities in the crude extract of the red lentils with (+)-catechin (36.02 μg/g extract, (d.w.)), and (−)-epicatechin (98.21 μg/g

extract, (d.w.) being present. Zhang et al. (2015) reported that kaempferol glycosides were dominant phenolic compounds in lentils, with the tetraglycoside and triglycoside having the highest concentrations ranging from 210.05 to 297.15 μg/g (d.w.), respectively, followed by catechin glucoside, catechin gallate, and epicatechin glucoside. The most abundant proanthocyanidins in the lentil seed coat were polymers with a mean degree of polymerization (mDP) of 7–9, followed by oligomers with an mDP of 4–5 (Dueñas et al., 2003). The content of procyanidin B2, B3, and procyanidin tetramer in lentils was in the range of 0.1-0.5 μg/100 g (d.w.) (López-Amorós et al., 2006). Zhang et al. (2015) detected procyanidin dimer 1, 2, 3 in the range of 1.30-48.33 μg/g (d.w.). Zou et al. (2011) detected procyanidin dimer and trimer in lentils.

The important health benefits of common beans are associated with the presence of phenolic compounds that possess antioxidant properties (Campos-Vega et al., 2010). Consumption of beans and legumes has been associated with reduced risk of many diseases, as mentioned above, and here are examples of the biological effects of some flavonoid types. For instance, Ikeda et al. (1992) reported the significant benefit of catechin to reduce cholesterol absorption from rat intestine, and Mangiapane et al. (1992) showed the inhibition of oxidation of LDL induced by the mouse transformed macrophage cell line, 1774, human monocyte derived macrophages, and vascular endothelial cells isolated from umbilical cords. Furthermore, luteolin inhibited alpha-glucosidase and amylase (Kim et al., 2000). Quercetin plays an important role in the inhibition of mitogen activated protein (MAP) kinase in human epidermal carcinoma cells (Bird, et al., 1992), and induction of cell cycle arrest and apoptosis in human breast cancer cells *in vitro* Choi et al. (2001). Additionally, Xing et al. (2001) reported that quercetin inhibits the expression and function of the androgen receptor in LNCaP prostate

cancer cells, prevents and protects against streptozotocin induced oxidative stress and β-cell damage in rat pancreas. Pruzanski and Vadas (1991) reported quercetin and luteolin affected the inhibition protein kinase C (PKC). Furthermore, the authors' study showed the inhibition of human recombinant synovial phospholipase A2 by kaempferol-3-O-galactoside (Pruzanski and Vadas, 1991). Another report of the biological effects of dietary flavonoids, robinetin, quercetin and myricetin illustrated the inhibition of the tumorigenicity of BP-7, 8-diol- 9,10-epoxide-2 on mouse skin and in newborn mice Chang et al. (1985). Due to their significance, the daily human intake of all flavonoids was estimated at a hundred milligrams, varying by nearly 50-fold, from 20 to 1,000 mg/day (Mullie et al., 2007).

As a consequence of the presence of phenolic compounds and particularly flavonoids and tannins in lentil seeds, these compounds are thought to play a major role in the protection against major diseases as mentioned above. There are numerous studies to illustrate the nutritional benefits of lentils in maintaining and improving human health (Boudjou et al., 2013). Adebamowo et al. (2005) reported that the consumption of flavonol-rich beans and lentils was associated with a lower incidence of breast cancer (Zhang et al., 2015). Furthermore, most polyphenols, such as tannic acid, gallotannin, catechin and proanthocyanidin play a vital role in enzyme inhibition and protein precipitation that lead to the formation of various complexes (He et al., 2007). Phenolic compounds may also play a key role in the inhibition of alpha-glucosidase and lipase activities (Zhang et al., 2015). Zhang et al. (2015) reported that phenolic compounds extracted from lentils significantly inhibited alpha-glucosidase, and the IC_{50} values ranged from 23.08 to 42.15 mg/mL. These compounds, especially flavonols such as kaempferol and quercetin glycosides, are the major contributors to the inhibitory activity

of alpha-glucosidase (Zhang et al., 2015). The inhibition of alpha-glucosidase reduces intestinal glucose absorption and digestion, leading to minimization of the postprandial hyperglycemic response, and is vital in controlling type 2 diabetes (Zhang et al., 2010; 2015). Similarly, the soluble lentil extracts are effective in the inhibition of lipase activity, which varied from 6.26 to 9.26 mg de-sugared extract per mL solvent (mg/mL) (Zhang et al., 2015). The inhibition of lipase (a key enzyme involved in triacylglycerol digestion) is thought to be one of the best strategies for controlling obesity (Xu et al., 2005; Zhang et al., 2015). The authors reported that phenolic extracts of lentils had a much stronger activity in inhibition against pancreatic lipase than alpha-glucosidase. Thus, lentils are good for inhibition of pancreatic lipase and hence an excellent diet for controlling obesity (Zhang et al., 2015). Additionally, Boudjou et al. (2013) reported that lentil hulls exhibited high anti-inflammatory activities, which were measured using the lipoxygenase (15-LOX) inhibitor screening assay kit and the inhibition of cyclooxygenase (COX) enzymes using a colorimetric COX (ovine) inhibitor screening kit. The lentil hulls extract showed an important inhibition of 15-LOX (IC_{50}, 55 μg/mL), with moderate COX-1 (IC_{50}, 66 μg/mL) and COX-2 (IC_{50}, 119 μg/mL) inhibitory effects on the COX pathway (Boudjou et al., 2013). This effect can be associated with the presence of flavonoid compounds, overwhelmingly procyanidins, found exclusively in the seed coats of lentils (Dueñas et al., 2003). This inhibition might influence the process of inflammation since 15-LOX participates in oxidative modification of low-density lipoproteins (LDL) and in the development of atherosclerotic lesions (Boudjou et al., 2013). Chronic inflammation, which is the root cause of a number of serious degenerative diseases, might be reduced by use of the preferential LOX inhibition of the lentil hulls (Boudjou et al., 2013).

2.3 Phenolic antioxidants and their mechanism of action

Phenolic compounds have antioxidant potential which depends on the arrangement and number of the hydroxyl groups in the molecules of interest (Cao et al., 1997). Phenolic compounds can be classified into two groups, the first group being primary or chain breaking antioxidant and the second group is called secondary antioxidants or preventive antioxidants (Madhujith, 2002). The secondary antioxidants function by numerous mechanisms to retard the rate of autoxidation of lipids such as metal chelating, absorbing UV radiation or deactivating singlet oxygen, decomposing hydroperoxides and scavenging oxygen (Gordon, 1990).

In the first group (primary antioxidants) the compounds are mainly free radical scavengers that are able to react with lipid radicals to convert them to more stable products (Shahidi and Wanasudara, 1992). Phenolic antioxidants (AH) have the ability to donate hydrogen atoms to lipid radicals (Reaction V). The resultant antioxidant phenoxyl radical ($A^{\cdot}$) is more stable, and less reactive (Kiokias et al., 2008). Furthermore, the phenoxyl radicals may interfere with the chain-propagation reactions (Reactions VI and VII).

$$R^{\cdot} / RO^{\cdot} / ROO^{\cdot} + AH \longrightarrow A^{\cdot} + RH / ROH / ROOH \quad (V)$$

$$RO^{\cdot} / ROO^{\cdot} + A^{\cdot} \longrightarrow ROA / ROOA \quad (VI)$$

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot} \quad (VII)$$

As bond energy of hydrogen decreases in phenolic antioxidants, the hydrogen transfer to the free radical is more suitable energetically and is more rapid (McClements and Decker, 2007).

Any compound is capable of donating its hydrogen atom to that of a free radical as long as the compound has a reduction potential lower than that of a free radical (or oxidized species), unless the reaction is kinetically unfeasible (McClements and Decker, 2007). Stabilization of the phenoxyl radical occurs by delocalization of its unpaired electron around the aromatic ring (Figure 2.5).

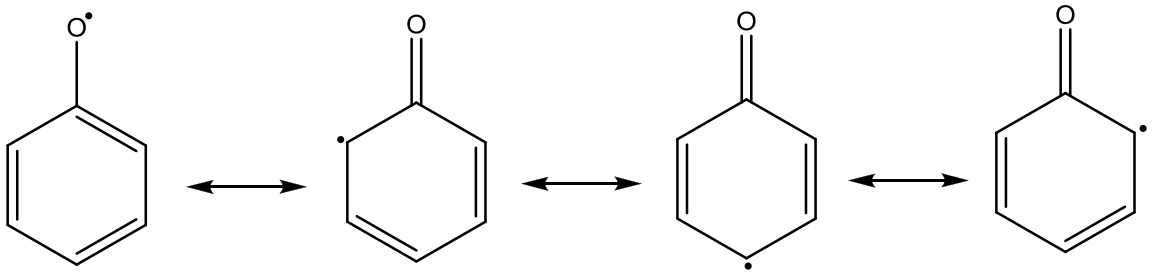


Figure 2.5 Resonance stabilization of phenoxyl radical

Generally food contains small quantities of metal ions originating from heme pigments or wear and tear of equipment, and these metals act as pro-oxidants at very low concentrations. The pro-oxidative effect of the metal ions is reduced by chelation of ions by food components. This also raises the activation energy of the initiation reaction. Some examples of good metal chelators are ethylenediminetetraacetic acid (EDTA), citric acid, malic acid, tartaric acid, and phosphoric acid. The chemical structures of phenolics influence their ability to chelate metal ions. It is known, for example, that the activity of flavonoids requires the presence of the 3' ,4'-dihydroxy configuration and C-4 carbonyl and a C-3 or C-5 OH group (Madhujith, 2002).

2.4 Legumes and lentils as sources of phenolic antioxidants

As mentioned above, legumes contain several phenolic compounds that are considered to be natural antioxidants (López-Amorós et al., 2006), and the major phenolic compounds presented in them are phenolic acids, flavonoids and tannins. One of the main parameters that dictate the potential antioxidant capacity of legume seeds, or antioxidant activity of extracts is the total phenolic content (TPC) in the seeds or extracts prepared from such plant materials (Shahidi and Naczk, 2004; Amarowicz and Pegg, 2008b).

According to Xu and Chang (2007) who compared the phenolic profile and antioxidant activities of different kinds of legumes, results were affected by solvent extraction systems employed. Their results showed that acidic 70% acetone afforded the highest total phenolic contents (TPC). In the study reported in this thesis, the same solvent system was used to extract the phenolic acids, the description of which is detailed in the following section. Xu and Chang (2007) reported that lentils have the highest phenolic, flavonoid and condensed tannin contents (CTC) (7.53 mg gallic acid equivalents/g, 2.21 and 8.70 mg catechin equivalents /g, respectively), followed by black beans and red kidney beans. Cultivars with the highest total phenolic content (lentil, black, and red kidney beans) exerted the highest antioxidant capacity as evaluated by DPPH radical scavenging, ferric reducing antioxidant power (FRAP), and the oxygen radical absorbance capacity (ORAC) (Xu and Chang, 2007) (Table 2.1). Additionally, Zhao et al. (2014) reported that lentils had the highest phenolic content, total reducing power, total antioxidant activity, and DPPH radical scavenging activity in comparison with different kind of legumes in their report. Additionally, Boudjou et al., 2013 found that lentil seeds had a higher total phenolic and flavonoids contents (total phenolics 11.42

mg GAE/g and the total flavonoids 0.25 mg quercetin eq/g), than whole faba beans when extracted under the same conditions (Boudjou et al., 2013). Furthermore, Xu and Chang (2007) reported that lentils and red bean are a very rich source of tannins (Table 2.1). Furthermore, Madhujith et al. (2004) study showed that condensed tannin contents (CTC) of an extract of common bean hulls was several times greater than that of the whole bean extract. In a study by Zhang et al. (2015), the TPC varied significantly among 20 Canadian lentil cultivars tested in their study, they were in the range of 4.56-8.34 mg GAE/g (d.w.). The CTC determined to be the main phenolic compound in their lentil samples tested, ranging from 3.00 to 7.80 mg catechin/g (d.w.), which accounted for 59.52-93.53% of the TPC (Zhang et al., 2015). Additionally, Amarowicz et al. (2010) fractionated the crude phenolic extract from green lentils to their low-molecular-weight fraction and tannin fraction. The authors' results showed the TPC was in the order of tannin fraction >>> crude extract > low-molecular-weight fraction. The CTC, total antioxidant activity, DPPH scavenging activity, and reducing power *in vitro* assays followed the same trend. Han and Baik (2008) also reported that lentils had the highest TPC amongst other legumes, and the insoluble-bound phenolic fraction showed a higher total antioxidant activity (TEAC) than the free phenolic fraction.

From the above summary, it can be seen that very little work has been done in the genotype of the Canadian variety of lentils and the existing literature has not fully examined the fractionation of the phenolic compounds extracted from lentils to soluble (free and esterified), and insoluble-bound fractions. Most of the existing literature discussed above has focused only on the identification and quantification of the phenolic compounds present in the soluble form in the crude extracts of lentils. However, in the current study the focus was on filling the existing gap in the literature to illustrate the

significance of fractioning the phenolic compounds extracted from lentils to soluble (free and esterified), and insoluble-bound forms; furthermore, to show the difference in their antioxidant activities measured by different assays. The current study also identified and quantified the phenolic compounds in each fraction.

Table 2.1 Phenolic content of the extracts (using acidic 70% acetone) and antioxidant activity of legumes

Legumes	Total phenolics[a]	Total flavonoid[b]	Condensed tannins[c]	DPPH scavenging[d]	FRAP[e]	ORAC[f]
Green pea	1.13	0.26	0.91	0.59	1.03	6.17
Yellow pea	1.28	0.29	1.02	1.36	1.34	13.30
Chickpea	1.57	2.87	1.21	0.94	0.81	7.41
Lentil	7.53	2.21	8.70	19.09	10.65	38.60
Yellow soybean	2.23	0.41	0.85	1.40	0.34	44.08
Black soybean	6.18	2.57	4.09	17.58	9.93	122.75
Red kidney	5.90	2.93	5.37	18.94	9.22	23.26
Black bean	6.89	3.21	6.74	18.33	11.03	51.54

[a] mg gallic acid eq/g, [b]mg catechin eq/g, [c] mg catechin eq/g, [d] μmol Trolox eq/g, [e]mmol Fe^{2+} eq/100 g, [f] μmol Trolox eq/g (Adapted from Xu and Chang, 2007)

2.5 Extraction of polyphenolics

The structural differences in different classes of phenolics influences their extraction. Thus, solvent system, extraction method, sample particle size, storage time, and the presence of other substances affect the extraction of phenolics from their source material (Naczk and Shahidi, 2004). Phenolics' extraction can be improved by adjusting / optimizing sample-to-solvent ratio, and the solvents used in the extraction of phenolics such as ethanol, acetone, water, methanol, propanol, ethyl acetate and their various combinations (Naczk and Shahidi, 1991). Tannin recovery from dry beans was significantly influenced by sample particle size (Deshpande, 1985). Variation in the polyphenolics polarity and biochemical modifications (glycosylation and esterification) impacts optimization of polyphenolic extraction (Pellegrini et al., 2007). Better extraction of polyphenolics can be achieved at higher solvent-to-sample ratios as evaluated in various solvent to sample ratios, and the conditions proposed by (Michiels et al. (2012) for extraction (acetone-water-acetic acid mixture (70:28:2, v/v/v) with solvent-to-solid ratio of 20:1 (v/w) and extraction for 1 h at 4^{o}C). Furthermore, it was found that hydrolyzed medicinal plant extracts (using 60% ethanol and 5 mL of 2 M hydrochloric acid) had a total phenolic content greater than non-hydrolysed extracts (Komes et al., 2010). Krygier et al. (1982) extracted free, and esterified phenolic acids from oilseeds using a mixture of methanol-acetone-water (7:7:6, v/v/v) at room temperature. First, the free phenolics were extracted with diethyl ether, and then the extract was treated with 4M NaOH under nitrogen. The hydrolyzate was acidified and the liberated phenolic acids were extracted with diethyl ether. After exhaustive extraction with a mixture of methanol-acetone- water, the left-over sample was treated with 4M NaOH under nitrogen to liberate insoluble bound phenolic acids. Similarly, phenolic compounds from lentil

seeds were extracted in this work under the same conditions as will be explained in the following chapter.

2.6 Separation and identification of polyphenolics

Polyphenolic extracts always contain a mix of various classes of phenolic and non-phenolic substances. To isolate the desired phenolic compounds from the crude extract additional purification may be required. Numerous obstacles are encountered because there is no universal method to isolate all phenolics. Flavonoids, being the prominent class of phenolics, have the same basic structure as flavones, flavonols, flavonones, flavanols, isoflavonoids, and anthocyanins. Various gas-solid and liquid-solid phase adsorption techniques have been utilized to adsorb target phenolics (Zagorodni, 2007; LeVav and Carta, 2007). For optimizing the recovery of different phenolics, Kammerer et al. (2010) studied non-polar adsorbent and ion-exchange resins. By using acidic resin cation-exchange chromatography, successful recovery of phloridzin and rutin was achieved (Kammerer et al., 2010). Polyphenolic purification is achieved using conventional methods such as reversed-phase liquid chromatography and ion-exchange resins. It was recently discovered that counter-current chromatography (CCC) qualifies as an excellent alternative for the isolation of various classes of phenolics (Pauli et al., 2008). In CCC, the partition ratio between stationary liquid phase and mobile liquid phase is the basis for achieving the separation of compounds. In high-speed CCC, the multilayer coil counter-current chromatography and centrifugal partition chromatography are two types of modern commercial CCC. Flavanols and proanthocyanidins from green tea (Cao et al., 2000), and anthocyanins from wine (Salas et al., 2005; Schwarz et al., 2003) have been fractionated and isolated using CCC. The most commonly utilized

analytical technique for separation and characterization of polyphenolics is high performance liquid chromatography (HPLC) (Carrasco-Pancorbo et al., 2007; Naczk and Shahidi, 2006; Valls et al., 2009). Separation of different compounds was enhanced by reversed-phase columns with C18 stationary phase (Gruz et al., 2008). Electrochemical, UV-visible, fluorescent, and photodiode array detectors are most commonly used with HPLC methods for analyzing food phenolics. The identification and quantification of different classes of phenolic compounds has been achieved using mass spectrometry coupled to HPLC. Characterization and identification of phenolic compounds is improved by mass spectrometry because it is selective (Nicoletti et al., 2007; Buiarelli et al., 2007). Isomeric flavonoid glycosides are not identified by mass spectrometry, instead ion-trap mass spectrometry which is based on the principle of sequential fragmentation of molecular ions, is used due to being a highly sensitive as an advanced technique (Prasain et al., 2004). Atmospheric-pressure chemical ionization (APCI), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry are among the other techniques used for analyzing polyphenolics (Valls et al., 2009). By utilizing HPLC coupled with photodiode array detector, separation and quantification of different polyphenolics from water samples was achieved (Liu et al., 2008). Additionally, using nano ESI-MS and LC-ESI, elucidation of the structures of polyphenolics present in carob fibre was obtained (Owen et al., 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Five different varieties of lentils, namely CDC Green Land, CDC Invincible, 3493-6, CDCSB-2, and Maxim which were kindly provided by Professor Albert (Bert) Vandenberg of the experimental farm of the University of Saskatchewan, Saskatoon, Canada, were growing under the same climatic conditions in different parts of the same plot during the harvest of 2012. The samples of Black lentil seeds were purchased from Bulk Barn store in St. John's, NL, Canada. Sodium carbonate, sodium chloride, sodium hydroxide, methanol, hexane, hydrochloric acid, acetone, acetic acid, diethyl ether, ethyl acetate, petroleum ether, and Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Fisher Scientific Co (Nepean, ON).

The compounds 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), Trizma base, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide, 5,5-dimethyl-l-pyrroline-*N*-oxide (DMPO), sinapic acid, caffeic acid, *p*-coumaric acid, gallic acid, catechin, epicatechin, ferrous sulfate, Folin Ciocalteu's phenol reagent, copper sulfate, as well as mono- and dibasic potassium phosphate, vanillin, ferric chloride ($FeCl_3$), trichloroacetic acid (TCA), potassium ferricyanide [$K_3Fe(CN)_6$], sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), ferrous sulfate ($FeSO_4$), ethylenediaminetetraacetic acid (EDTA), human low density lipoprotein (hLDL), pBR 322 plasmid DNA, agarose, Tris acetate, bromophenol blue, xylene cyanol,

and glycerol were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). SYBR safe gel stain was procured from Probes Invitrogen (Eugene, OR, USA).

3.2 Methods

Experiments were generally carried out in triplicates and results were reported as means ± standard deviation, unless otherwise indicated. The methodologies followed are described below.

3.2.1 Sample preparation and defatting

Lentil samples were ground using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc. Brockville, ON) to obtain a fine powder, which passed through mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH), and then defatted by blending with hexane (1:5 w/v, 5 min three times) in a Waring blender (Model 33BL73, Waring products Division Dynamics Co. of America, New Hartford, CT, USA) at room temperature (24 ^{0}C). Then the samples were suction filtered to remove the hexane. Defatted samples were vacuum packed in polyethylene pouches and stored at -20 ^{0}C until used for extraction of phenolics within one week.

3.2.2 Extraction of soluble phenolic compounds

Free and esterified phenolic compounds were extracted and fractionated according to the method described by Krygier *et al*. (1982) and Naczk and Shahidi (1989), with slight modification. Defatted meal was mixed with acetone/ H_2O/ acetic acid (70:29.5:0.5,

v/v/v) with a solid to solvent ratio of 1:10 (w/v) (Xu and Chang, 2007), and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) and sonicated at the maximum power for 25 min to extract soluble phenolics. After centrifugation of the resulting slurry for 5 min at 4000g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA), the supernatant was collected and extraction was repeated two more times. Combined supernatants were evaporated to a residue in water using a vacuum evaporator at 40 ^{0}C (Buchi, Flawil, Switzerland), which was adjusted to pH 2 with 6 M HCl. Free phenolics were extracted five times into diethyl ether and ethyl acetate (1:1, v/v). The free phenolic extract was evaporated to dryness under vacuum at 40 ^{0}C. The aqueous phase was used for alkali treatment to release esterified phenolics. To the water phase 4 M NaOH was added and the content hydrolyzed for 4 h at room temperature 24 ^{0}C under a nitrogen atmosphere. After acidification to pH 2 with 6 M HCl, phenolics compounds released from soluble esters were extracted from the hydrolysate five times with diethyl ether (1:1, v/v) and evaporated to dryness under vacuum and reconstituted in HPLC grade methanol and stored at -20 $^{\circ}$C until used for further analysis. The solid residues of defatted samples were then air-dried for 24 h and stored at -20 $^{\circ}$C until used for extraction of insoluble-bound phenolic compounds within a week. During all stages, extracts were protected from light by covering their containers with aluminum foil (Krygier *et al*., 1982; Chandrasekara and Shahidi, 2011b).

3.2.3 Extraction of insoluble-bound phenolic compounds

The extraction of insoluble-bound phenolic compounds was carried out according to Krygier *et al*. (1982) and as modified by Han and Baik (2008). The solid residue of the

whole grain lentils obtained after extraction of soluble phenolics was hydrolyzed with 4M NaOH at a solid to solvent ratio of 1:20 (w/v) at room temperature (24 ^{0}C) for 4 h with stirring under nitrogen. The resulting slurry was acidified to pH 2 with 6 M HCl, and centrifuged for 5 min at 4000g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA). Subsequently, petroleum ether (20 mL) was added to the acidified solution, stirred for 20 min to defat the sample. Addition of petroleum ether and stirring were repeated again. After removal of the petroleum ether layer, ethyl acetate (20 mL) was added and stirred for 30 min to extract and release insoluble-bound phenolics. Then the ethyl acetate layer was collected; addition of ethyl acetate and stirring was repeated twice. Subsequently, the insoluble-bound phenolic extract solution was evaporated to dryness under vacuum at 40 $^{\circ}$C. The insoluble-bound fraction was reconstituted in HPLC grade methanol and stored at -20 $^{\circ}$C until used for further analysis.

3.2.4 Determination of total phenolic content (TPC)

The TPC of each extract was determined using the method described by Singleton and Rossi (1965) with slight modifications as described by Chandrasekara and Shahidi (2011a). Phenolic extracts were dissolved in HPLC grade methanol and diluted appropriately (0.03-0.07 mg/mL). In centrifuge tubes, 0.5 mL of Folin-Ciocalteu's phenol reagent was added to 0.5 mL of diluted extract, and the contents were mixed thoroughly by vortexing. A saturated solution of sodium carbonate (1mL) was then added to each tube to neutralize the reaction. Subsequently, 8 mL distilled water were added followed by thorough mixing. Tubes were allowed to stand in the dark at room temperature (24 $^{\circ}$C) for 35 min. The contents were centrifuged for 10 min at 4000g.

Absorbance of the supernatant was read at 725nm (HP 8452A diode array spectrophotometer, Agilent Technologies, PaloAlto, CA, USA). The total phenolics contents in each extract was determined using a standard curve prepared with gallic acid and expressed as mg gallic acid equivalents (GAE)/g of defatted sample.

3.2.5 Determination of total flavonoid content (TFC)

The TFC was determined using a colorimetric method described by Kim *et al*. (2003) with slight modifications as described by Chandrasekara and Shahidi (2010). In a centrifuge tube, 1 mL of extract or standard solution dissolved in methanol was mixed with 4 mL of distilled water; and 0.3 mL of 5% solution of $NaNO_2$ was added to the tube. Then the tubes were allowed to stand for 5 min for completion of the reaction. Thereafter, 0.3 mL solution of 10% $AlCl_3$ was added to the reaction mixture and allowed to stand for a further 1 min period. Finally, 2 mL of 1 M NaOH and 2.4 mL of distilled water were added and mixed immediately. Tubes were allowed to stand in the dark at room temperature (24 C^0) for 15 min followed by centrifugation for 5 min at 4000g. Absorbance of the supernatant was read at 510 nm against a blank prepared in a similar manner by replacing the extract with distilled water. The TFC in the lentil samples were calculated using a standard curve prepared for catechin and the results were expressed as mg of catechin equivalents (CE)/g of defatted sample.

3.2.6 Determination of condensed tannin (proanthocyanidin) content (CTC)

Condensed tannins (Price et al., 1980) were evaluated according to the method

described by de Camargo et al. (2012). A 0.1 mL solution of soluble (free and esterified) and insoluble-bound phenolics extracts, dissolved in methanol, was added into tubes and then 5.00 mL of a 1:1 (v/v) solution prepared with 1% (w/v) vanillin in methanol and 8% (v/v) HCl in methanol were added to each tube. The tubes were then shaken in a water bath at 30 $^{\circ}$C, and the absorbance was subsequently read at 500 nm. The condensed tannin content was calculated from a standard curve for catechin and the results reported as mg catechin equivalents (CE)/ g of defatted sample.

3.2.7 Evaluation of antioxidant activity of lentil extracts *in vitro* assays

3.2.7.1 Reducing Power (RP)

Reducing power (Oyaizu, 1986) of soluble (free and esterified) and insoluble-bound phenolics extracts of lentils was determined according to the method described by Alasalvar *et al.* (2009). The extracts (1mL) were mixed with 0.2 M phosphate buffer solution pH 6.6 (2.5 mL) and 1% (w/v) solution of potassium ferricyanide [$K_3Fe(CN)_6$] (2.5mL). The mixture was then incubated in a water bath at 50 ^{0}C for 20 min and 10% (w/v) trichloroacetic acid (TCA) solution was added (2.5 mL). Following this, the mixture was centrifuged for 10 min. Subsequently, the supernatant (1 mL) was transferred into a tube containing distilled water (2.5 mL) and 0.1% (w/v) ferric chloride ($FeCl_3$, 0.5 mL). The absorbance was read at 700 nm. Reducing power was calculated using a standard curve of Trolox and the results were expressed as mmoles of Trolox equivalents (TE)/g defatted sample.

3.2.7.2 Trolox equivalent antioxidant capacity (TEAC)

The total antioxidant capacity of lentil extracts was determined according to the method described by Van den Berg *et al.* (1999) and modified by Siriwardhana and Shahidi (2002). The TEAC assay is based on scavenging of 2,2`azinobis-(3-ethylbenzothiazoline-6- sulfonate) radical cation ($ABTS^{\cdot+}$). All solutions were prepared in 0.1 M phosphate buffer pH 7.4 containing 0.15 M sodium chloride (PBS). An $ABTS^{\cdot+}$ solution was prepared by mixing 2.5 mM AAPH with 2.0 mM ABTS stock solution at 1:1 (v/v) . The solution was heated for 30 min at 60 ^{0}C, protected from light and stored at room temperature. The radical solution was used within 3 hours because the absorbance of the radical itself depletes with time, and was filtered before mixing with the extracts using medium-porosity filter papers (Fisher Scientific Co., Pittsburgh, PA, USA). A blank stock solution of $ABTS^{\bullet+}$ was used for each measurement to account for the decrease in the absorbance of the radical solution itself with time. The extracts were dissolved in PBS (pH 7.4, 0.15 M NaCl) and diluted to a concentration to fit within the range of values in the standard curve prepared using different concentrations of Trolox. Phenolic extracts (40 μL) were mixed with $ABTS^{\bullet+}$ solution (1.96 mL) to determine their TEAC values. The absorbance of the reaction mixture was read at 734 nm after 6 min because the extracts needed a minimum of 6 min for completion of the reaction. TEAC values were expressed as mmoles of Trolox equivalents (TE)/g of defatted sample.

TEAC values were calculated using the following equation.

$$\text{TEAC (\%)} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

where $Abs_{control}$ is the absorbance of ABTS radical cation + PBS; Abs_{sample} is the absorbance of ABTS radical cation + phenolic extract or Trolox.

3.2.7.3 DPPH radical scavenging capacity (DRSC) using electron paramagentic resonance (EPR)

DRSC assay was conducted using EPR spectroscopy following the method described by Madhujith and Shahidi (2006) and John and Shahidi (2010). One milliliter of 0.125 mM solution of DPPH in methanol was mixed with 250 µL of appropriately diluted free, esterified and insoluble-bound phenolics extracts in methanol. Contents were mixed well, and after 10 min the mixture was passed through the capillary tubing which guides the sample through the sample cavity of a Bruker E-scan EPR spectrophotometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA, USA). The spectrum was recorded on a Bruker E-scan food analyzer (Bruker Biospin Co.). The parameters were set as follows: 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.62s sweep time, 8 scans, 100 G sweep width, 3495 G centre field, 5.12 ms time constant, 9.79 GHz microwave frequency, 86.00 kHz modulation frequency, 1.86 G modulation amplitude. DRSC of the extracts was calculated using the following equation:

DPPH radical scavenging capacity, % = 100 − (EPR signal intensity of extracts / EPR signal intensity of control) ×100.

From the standard curve plotted for the DRSC of Trolox, the scavenging activity of lentil extracts was expressed as mmoles of Trolox equivalents (TE)/g of defatted sample.

3.2.7.4 Hydroxyl radical scavenging capacity using electron paramagentic resonance (EPR)

Hydroxyl radical scavenging capacity was determined using the method described by John and Shahidi (2010) with modifications reported by de Camargo et al. (2015). Hydroxyl radical was generated via Fe (II)-catalyzed Fenton reaction and spin-trapped with DMPO. The resultant DMPO-OH adduct was detected using a Bruker E-scan EPR (Bruker Biospin Co., Billercia, MA, USA). Free, esterified and insoluble-bound phenolic extracts were prepared in 75 mM phosphate buffer (pH 7.0). The extracts (200 µL) were mixed with 10mM H_2O_2 (200 µL), 17.6 mM DMPO (400 µL), and 0.1 mM $FeSO_4$ (200 µL) After 3 min, the EPR spectrum of the mixture was recorded at 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.62 s sweep time, 8 scans, 100 G sweep width, 3465 G center field, 5.12 ms time constant, 9.79 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. Hydroxyl radical scavenging capacities of lentil extracts were calculated and expressed as mmole catechin equivalents (CE)/g defatted sample, using the following equation.

Hydroxyl radical scavenging capacity, % = 100 − (EPR signal intensity of extracts / EPR signal intensity of control) ×100.

3.2.8 High pressure liquid chromatographic (HPLC) analysis of phenolic compounds

The soluble (free and esterified), and insoluble-bound phenolic compounds of lentils were identified and quantified using high-performance liquid chromatography-diode array detection-electrospray ionization multi-stage mass spectrometry according to the method described by Zou et al. (2011) with slight modifications. The phenolic compounds were analyzed using an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1311A quaternary pump, a G1379A degasser and a G1329A ALS automatic sampler, a G1330B ALS Therm, a G1315B diode array detector (DAD) and a system controller linked to Chem Station data handling system (Agilent Technologies). Separations were conducted with a SUPERLCOSILTM LC-18 column (250× 4.6 mm inner diameter, 5-µm particles, Supelco, Bellefonte, PA, USA) at 40 °C. The detection of compounds was performed at 270 nm. All samples were filtered through a 0.45µm PTFE membrane syringe filter (Whatman Inc., Florham Park, NJ, USA) before injection. The injection volume was 20 µL. The elution solvents were A (100% methanol) and B (aqueous 0.1% formic acid aqueous solution) and the flow rate was adjusted to 0.7 mL/min. The gradient elution was used as follows: 5-30% A over 50 min. The solvent gradient was held at 30% A for an additional 15 min and increased to 100% A at 66 min. The solvent gradient was held at 100% A for an additional 10 min to clean up the column, followed by re-equilibration of the column for 5 min with 95% A and 5% B before the next run.

For HPLC-MS analysis, an Agilent 1100 SL LC/MSD ion trap mass spectrometer (Agilent Technologies) was connected to the Agilent 1100 HPLC system via an ESI

interface in the negative ion detection mode. The MS revealed the negative molecular ions; MS-MS broke down the most abundant one with dependent collision-induced dissociation. The selected spray chamber parameters were as follows: capillary potential, 3500 V; gas temperature, 350 ^{o}C; drying gas flow, 13 L/min; and nebulizer pressure, 414 kPa. For full scan MS analysis, the mass spectrometer was operated in a scan range from m/z 100 to 1500.

Identification of compounds by HPLC–MS analysis was made by comparing retention times (RT) and mass spectral data of the unknown peaks with those of the available reference standards, and the remaining compounds were tentatively identified based upon mass spectral characteristics and information available in the literature. Quantification of compounds was on the basis of their peak areas, comparison with a calibration curve obtained with corresponding standards (gallic acid, protocatechuic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic cacid, catechin and epicatechin). The remaining compounds, whose identification was tentative, were quantified using the regression equation of gallic acid for methyl vanillate and catechin for the remaining flavonoids. The results were expressed as μg/g defatted sample.

3.2.9 Inhibition of cupric ion-induced human low-density lipoprotein (LDL) peroxidation

The methods described by Andreasen et al. (2001) and Chandrasekara and Shahidi (2011a) were followed to determine the human LDL cholesterol oxidation inhibitory activities of lentils extracts. Human LDL cholesterol (in PBS, pH 7.4, with 0.01%

EDTA) oxidation inhibition was dialyzed against 10 mM PBS (pH 7.4, 0.15 M NaCl) for 12 h under nitrogen at 4 ^{0}C, and EDTA-free LDL was subsequently diluted to obtain a standard protein concentration of 0.04 mg/mL with PBS. The diluted LDL cholesterol solution (0.8 mL) was mixed with 100 µL of extract (0.02 and 0.003 mg/mL) in an Eppendorf tube. Oxidation of LDL cholesterol was initiated by adding 100µM $CuSO_4$ solution (0.1 mL). The mixture was incubated at 37 ^{0}C for up to 22 h. The initial absorbance (time zero) was read at 234 nm immediately after mixing. The formation of conjugated diene (CD) hydroperoxides was evaluated after 12 and 22 h. The corrected absorbance at 12 and 22 h against time zero was employed to calculate the percentage inhibition of CD formation using the following equation

% Inhibition of CD formation = $(Abs_{oxidized} - Abs_{sample} / Abs_{oxidized} - Abs_{native}) \times 100$

Where $Abs_{oxidized}$ = absorbance of LDL mixture and PBS with $CuSO_4$ only; Abs_{sample} = absorbance of LDL with extract and $CuSO_4$; and Abs_{native} = absorbance of LDL with PBS.

3.2.10 Inhibition of peroxyl radical induced supercoiled plasmid DNA strand scission

The methods described by Hiramoto et al. (1996) and Chandrasekara and Shahidi (2011a) were adopted to evaluate the inhibition activity of lentil phenolic extracts against supercoiled strand DNA scission induced by peroxyl radical. Supercoiled plasmid DNA (pBR 322 from *Escherichia coli* RRI) solution (50 µg/mL) and phenolic extracts (0.1 mg/mL) were prepared in 0.5 M, pH 7.4, phosphate buffer solution (PBS). In an eppendorf tube, 2 µL of supercoiled plasmid DNA solution were added to 2 µL of extract, 2 µL of PBS, and 4 µL of 7mM AAPH, in the order stated for peroxyl radical-induced

oxidation. The mixture was incubated at 37 ^{0}C for 1 h. The loading dye (2 μL), composed of 0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol in distilled water, was added to the reaction mixture after completion of the incubation period. A control with DNA alone and a blank devoid of phenolic extracts were prepared.

The samples were loaded onto a 0.7% (w/v) agarose gel prepared in Tris-acetic acid-EDTA (TAE) buffer (40 mM Tris – acetate, 1 mM EDTA, pH 8.5) containing SYBR safe DNA gel stain (100 μL/L). Gel electrophoresis was run at 80 volts for 120 min using a model B1A horizontal mini gel electrophoresis system (Owl Separation systems Inc., West Chester, PA, USA) in TAE buffer. The bands were visualized under UV light using Alpha-Imager^{TM} gel documentation system (Cell Biosciences, Santa Clara, CA, USA). The images were analyzed using ChemiImager 4400 software (Cell Biosciences) to quantify DNA scission. The protective effect of lentil extracts was calculated using retention percentage of the normalized supercoiled DNA using the following equation.

DNA retention (%) = (Intensity of supercoiled DNA with the oxidative radical and extract/intensity of supercoiled DNA in control) ×100.

3.3 Statistical analysis

Unless otherwise stated, the experimental design was randomized with three replications. The results were analyzed (ANOVA) using the F-test. Mean comparisons were tested based on Tukey ($p < 0.05$) by SAS software. Correlation analyses were carried out with ASSISTAT 7.6 program.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Total phenolic, flavonoid, and condensed tannin (proanthocyanidins) contents

Natural phenolic compounds, which are widely distributed in food plants, have beneficial health effects mainly through their antioxidant activities (Zhang et al., 2015), because of their ability to donate a hydrogen atom or an electron and form stable radical intermediates (Zhao et al., 2014). However, beneficial effects of phenolic compounds may also be rendered by other mechanisms. Flavonoids and condensed tannins are the predominant phenolic compounds in legume seeds and are found in lentils, peas, soybeans and common beans (Zhang et al., 2015). Xu and Chang (2007) have reported acidic 70% acetone (0.5% acetic acid) as the best solvent for the extraction of phenolic compounds from lentils, as this rendered the highest total phenolic content (TPC) and the total flavonoid content (TFC).

In the present study, The TPC of selected lentil samples and their fractions (free, esterified and insoluble-bound) is shown in Table 4.1. TPC in the free, esterified and insoluble-bound forms of lentil phenolics was in the range of 1.37-5.53, 2.32-21.54, and 2.55-17.51 mg GAE/g of defatted samples, respectively. In general, the TPC was higher in the fraction containing esterified or insoluble-bound phenolics. However, it is worth noting that the percentage of soluble phenolics (free plus esterified) was higher than that of the insoluble-bound ones in all samples, except for CDC Green Land, which had the highest percentage in the insoluble-bound form. The percentage of esterified phenolics

in the total phenolics of CDC SB-2, 3494-6, CDC Invincible, CDC Green Land, Maxim, and Black was 37.2, 25.0, 64.6, 24.7, 65.7, and 66.0%, respectively, and TPC in the free form was in lower concentration, and its percentages in CDC SB-2, 3494-6, CDC Invincible, CDC Green Land, Maxim, and Black were 22, 28.5, 25.5, 12.2, 12.4, and 17.0%, respectively. The TPC of the fraction containing esterified phenolics of CDC SB-2, CDC Invincible, Maxim and Black lentil cultivars were significantly higher ($p<0.05$) than those of 3494-6 and CDC Green Land cultivars. However, these two cultivars (3494-6 and CDC Green Land) displayed a different trend, showing higher TPC in their insoluble-bound form. The insoluble-bound phenolic content of CDC Green Land was significantly higher ($p<0.05$) than that of 3494-6, which was the highest among all samples tested.

According to Xu and Chang (2007) the TPC of lentils was in the range of 4.96-9.60 mg GAE/g, which was higher than that found for peas, green peas, soybeans and chickpeas. Other studies also found higher TPC for lentils amongst other legumes (Zhao et al., 2014; Han and Baik, 2008).

Table 4.1 Total contents of phenolics, flavonoids and condensed tannins of defatted lentil cultivars[1]

Lentil Cultivar	Free	Esterified	Bound
Total phenolics (mg GAE[2] eq/g defatted sample)			
CDC SB-2	1.37 ± 0.03 ^{Bc}	2.32 ± 0.14 ^{Ae}	2.55 ± 0.12 ^{Ad}
3494-6	5.94 ± 0.37 ^{Ba}	5.21 ± 0.46 ^{Bd}	9.68 ± 0.59 ^{Ab}
CDC Invincible	3.10 ± 0.12 ^{Bb}	7.86 ± 0.46 ^{Ac}	1.21 ± 0.05 ^{Cd}
CDC Green Land	3.38 ± 0.04 ^{Cb}	6.85 ± 0.34 ^{Bc}	17.51 ± 1.50 ^{Aa}
Maxim	2.91 ± 0.17 ^{Cb}	15.45 ± 0.65 ^{Ab}	5.16 ± 0.08 ^{Bc}
Black	5.53 ± 0.06 ^{Ba}	21.54 ± 0.95 ^{Aa}	5.54 ± 0.20 ^{Bc}
Total flavonoids (mg Catechin eq/g defatted sample)			
CDC SB-2	0.35 ± 0.004 ^{Bb}	0.36 ± 0.003 ^{Be}	0.44 ± 0.02 ^{Ad}
3494-6	0.80 ± 0.04 ^{Ba}	0.80 ± 0.01 ^{Bd}	1.34 ± 0.02 ^{Ab}
CDC Invincible	0.10 ± 0.01 ^{Bc}	1.47 ± 0.06 ^{Ac}	0.08 ± 0.02 ^{Be}
CDC Green Land	0.01 ± 0.002 ^{Cd}	1.42 ± 0.13 ^{Bc}	2.95 ± 0.07 ^{Aa}
Maxim	0.08 ± 0.003 ^{Cc}	2.84 ± 0.06 ^{Ab}	0.72 ± 0.04 ^{Bc}
Black	0.01 ± 0.003 ^{Cd}	4.13 ± 0.22 ^{Aa}	0.80 ± 0.05 ^{Bc}
Condensed tannin content (mg Catechin eq/g defatted sample)			
CDC SB-2	1.09 ± 0.04 ^{Bd}	0.64 ± 0.03 ^{Cf}	1.41 ± 0.08 ^{Acd}
3494-6	2.67 ± 0.05 ^{Ba}	1.74 ± 0.04 ^{Be}	4.28 ± 1.02 ^{Ab}
CDC Invincible	1.74 ± 0.08 ^{Bc}	4.20 ± 0.08 ^{Ac}	0.03 ± 0.002 ^{Ce}
CDC Green Land	0.71 ± 0.09 ^{Ce}	2.71 ± 0.09 ^{Bd}	5.57 ± 0.10 ^{Aa}
Maxim	2.10 ± 0.06 ^{Bb}	6.79 ± 0.25 ^{Ab}	0.65 ± 0.06 ^{Ced}
Black	0.4 ± 0.12 ^{Cf}	10.48 ± 0.11 ^{Aa}	2.07 ± 0.27 ^{Bc}

[1] Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column are not significantly different ($p > 0.05$).[2] GAE, gallic acid equivalent.

However, Zhao et al. (2014) reported remarkably higher phenolic content than the current study in their lentils' crude extract (47.6 ± 5.3 mg GAE/g sample). This may be attributed to the differences in the raw materials in terms of growing location and harvest period. Furthermore, extraction techniques may also impact the results. Other studies (Amarowicz et al., 2009; 2010) have reported different concentrations of TPC in different fractions of red and green lentils, respectively. The authors found a noticeable TPC difference between the fraction containing tannins and that consisting of sugar and low molecular weight phenolics, the first one showing the highest TPC.

In the present study, the total flavonoid content (TFC) exhibited a similar trend as that observed for TPC. In general, soluble TFC (free plus esterified) was higher than that found for the fraction containing insoluble-bound phenolics. CDC Green Land had higher TFC in its insoluble-bound form, and 3494-6 varieties had its TFC in similar percentages of soluble and insoluble-bound forms. The TFC of the fraction containing phenolics released from their insoluble-bound form from CDC Green Land (2.95 ± 0.07 mg catechin eq/g defatted sample) was two times higher than 3494-6 (1.34 ± 0.02 mg catechin eq/g defatted sample). The TFC released from the insoluble-bound form of CDC SB-2

3494-6, CDC Invincible, CDC Green Land, Maxim, Black was 38.3, 45.6, 4.85, 67.4, 19.8 and 16.2%, respectively. These results show that the insoluble-bound flavonoid content of CDC Green Land was the highest (2.95 ± 0.07 mg catechin eq/g defatted sample) among all tested samples, and that of CDC Invincible (4.85%) was 37 times lower than CDC Green Land (67.35%). These findings are comparable to the results obtained by HPLC analysis in this study (Table 4.7), where the total of flavonoids and derivatives showed the highest quantity in the bound form of CDC Green Land.

Furthermore, the TFC was higher in the esterified form than the free form. Free and esterified flavonoids were in the range of 0.01–0.80 and 0.36–4.13 mg catechin eq/g defatted sample, respectively. The TFC in the present study (soluble plus esterified) was in good agreement with the TFC ranges reported by Xu and Chang (2007) and Zhang et al. (2015). The observed minor differences may be due to different fractionation techniques (e.g. free, esterified, and insoluble-bound phenolics) used in the present study, different solvent systems or sample sources.

The total condensed tannin content (CTC) or proanthocyanidins' content was evaluated using the vanillin/HCl method. CTC followed the same trend as that for TPC and TFC. The soluble (free plus esterified) fraction had the highest concentration of condensed tannin than that found for insoluble-bound phenolics from CDC Green Land and 3494-6. Furthermore, soluble phenolics released from the ester form of Black lentils showed a positive color reaction with vanillin/HCl, due to their high content of condensed tannins (Zou et al., 2011). The esterified phenolic fraction of Black lentils had the highest concentration, among others, in all fractions (10.48 ± 0.11 mg catechin eq/g defatted meal). The content of condensed tannins of insoluble-bound fraction was in decreasing order, CDC Green Land > 3494-6> Black> CDC SB-2> Maxim, and CDC Invincible. The current results are in good agreement with those of Zhang et al. (2015) except for esterified Black lentils, which reported the highest CTC (10.48 ± 0.11 mg catechin eq/g defatted meal). However, this exception matched the CTC reported by Xu et al. (2007). These authors reported a higher CTC in all lentil cultivars in comparison with other legumes such as yellow peas, green peas, soybeans and chickpeas.

4.2 Antioxidant activity assays

Phenolic compounds display multifunctional properties by acting as reducing agents, hydrogen donating antioxidants, iron chelators and singlet oxygen quenchers. Antioxidants play significant roles to protect the body against reactive oxygen species (ROS) and may have an additive or synergistic effect with endogenous antioxidants (Shahidi and Naczk, 1995; Albishi et al., 2013b). In the present study, the antioxidant activities of the lentil extracts were evaluated using different assays such as reducing power, Trolox equivalent antioxidant capacity (TEAC), DPPH radical scavenging capacity (DRSC), and hydroxyl radical scavenging activity (HRSA). There are two main mechanisms for antioxidants in scavenging of free radicals: hydrogen atom transfer (HAT) and single electron transfer (SET). In the antioxidant activity assays used in the current study, reducing power is believed to measure antioxidant activity by SET mechanism and DPPH scavenging and TEAC assays follow a mix of both HAT and SET (Albishi et al., 2013b).

4.2.1 Reducing power

The reducing power of soluble (free and esterified) and insoluble-bound phenolic extracts of lentils was evaluated using the potassium ferricyanide method. The results of reducing power of lentil extracts are presented in Table 4.2. The phenolic compounds that have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferrous ion complex that has an absorption maximum at 700 nm (Jayanthi and Lalitha, 2011). In other words, the ferric chloride ($FeCl_3$) and potassium ferricyanide [$K_3Fe(CN)_6$] system offers a sensitive method for evaluation of the lentil phenolic extracts as they take part in redox

reactions (Amarowicz et al., 2010). These phenolic compounds with reducing power showed that they could donate electrons thus reducing oxidized intermediates produced during lipid peroxidation processes, acting as primary and secondary antioxidants (Jayanthi and Lalitha, 2011). In this assay, the yellow color of the test solution changes to various shades of green depending on the reducing power of the extract (Jayanthi and Lalitha, 2011). The absorbance increase by increasing the phenolic contents in the extracts shows a stronger reducing capacity.

The data are presented in Table 4.2 indicate that, in general, free phenolics from lentil cultivars had lower reducing power than either esterified or insoluble-bound ones. The fractions containing free, esterified and insoluble-bound phenolics had reducing power in the range of 0.02-0.07, 0.03-0.55, and 0.02-0.27 mmoles Trolox eq/g defatted sample, respectively. Extracts containing free or esterified phenolics from Black lentils exhibited the highest reducing power in comparison with the other cultivars. Furthermore, the fraction containing esterified phenolics from Black lentils had the highest reducing power, thus lending support to the results for TPC, TFC, and CTC. CDC Green Land phenolics released from their insoluble-bound form rendered the highest reducing power as compared to those of the remaining cultivars. The correlations between TPC ($r = 0.9447$), TFC ($r = 0.9467$) or CTC ($r = 0.9348$) and the reducing power were significant ($p < 0.01$). This finding is in agreement with those previously reported, showing a high correlation between reducing power in lentils and legumes and their phenolic content (Amarowicz and Troszynska, 2004; Zhao et al., 2014). Zhao et al. (2014) found the highest reducing power for lentils in comparison with other legumes.

4.2.2 Trolox equivalent antioxidant capacity (TEAC)

The antioxidant activity of lentil extracts was determined using the TEAC assay. TEAC assay measures the ability of antioxidant compounds from the lentil extracts to scavenge the 2,2`-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\cdot+}$) using Trolox as a standard (John and Shahidi, 2010). The total antioxidant activity of soluble (free and esterified) and insoluble-bound phenolics of the lentil extracts as mmoles Trolox equivalents/g defatted sample is shown in Table 4.2. Generally, cultivars having higher TPC were most effective in scavenging the ABTS radical cation. TEAC values of fractions containing esterified phenolics, which ranged from 0.14 to 1.49 mmoles Trolox eq/g of defatted sample, were higher than those of fractions containing free phenolics and insoluble-bound phenolic counterparts with values in the range of 0.02-0.18, 0.09-0.93 mmoles Trolox eq/g of defatted sample, respectively. The TEAC value of extracts containing phenolics released from their esterified forms from Black lentil was significantly ($p<0.05$) higher than those from free and esterified phenolics forms. TEAC value of Black esterified phenolics was 75 and 17 times higher than those of free and insoluble-bound phenolics of Black lentil, respectively.

Table 4.2 Antioxidant activity of defatted lentil cultivars reflected in their reducing power and free radical scavenging activity by different methods[1]

Lentil Cultivar	Free	Esterified	Bound
Reducing power (mmoles Trolox eq/g defatted sample)			
CDC SB-2	0.02 ± 0.003 ^{Cd}	0.03 ± 0.001 ^{Bd}	0.03 ± 0.001 ^{Ad}
34946	0.05 ± 0.003 ^{Cb}	0.05 ± 0.001 ^{Bd}	0.09 ± 0.001 ^{Ac}
CDC Invincible	0.04 ± 0.002 ^{Bc}	0.13 ± 0.005 ^{Ac}	0.02 ± 0.001 ^{Ce}
CDC Green Land	0.05 ± 0.0001 ^{Cb}	0.12 ± 0.002 ^{Bc}	0.27 ± 0.003 ^{Aa}
Maxim	0.04 ± 0.003 ^{Cc}	0.29 ± 0.021 ^{Ab}	0.09 ± 0.002 ^{Bc}
Black	0.07 ± 0.003 ^{Ca}	0.55 ± 0.012 ^{Aa}	0.11 ± 0.007 ^{Bb}
TEAC[2] (mmoles Trolox eq/g defatted sample)			
CDC SB-2	0.13 ± 0.008 ^{Bb}	0.14 ± 0.002 ^{Be}	0.20 ± 0.004 ^{Ac}
34946	0.18 ± 0.004 ^{Ba}	0.26 ± 0.006 ^{Ade}	0.26 ± 0.006 ^{Ab}
CDC Invincible	0.05 ± 0.001 ^{Cc}	0.91 ± 0.007 ^{Ab}	0.10 ± 0.001 ^{Bd}
CDC Green Land	0.05 ± 0.0004 ^{Cc}	0.68 ± 0.006 ^{Bc}	0.93 ± 0.026 ^{Aa}
Maxim	0.03 ± 0.002 ^{Bd}	0.37 ± 0.175 ^{Ad}	0.23 ± 0.001 ^{ABbc}
Black	0.02 ± 0.005 ^{Bd}	1.49 ± 0.050 ^{Aa}	0.09 ± 0.022 ^{Bd}
DPPH radical scavenging activity (mmoles Trolox eq/g defatted sample)			
CDC SB-2	0.01 ± 0.001 ^{Cc}	0.07 ± 0.010 ^{Bf}	0.13 ± 0.011 ^{Ac}
34946	0.13 ± 0.006 ^{Bb}	0.12 ± 0.005 ^{Be}	0.24 ± 0.029 ^{Ab}
CDC Invincible	0.06 ± 0.002 ^{Bbc}	0.27 ± 0.008 ^{Ac}	0.04 ± 0.002 ^{Cd}
CDC Green Land	0.05 ± 0.0001 ^{Cbc}	0.22 ± 0.008 ^{Bd}	0.42 ± 0.018 ^{Aa}
Maxim	0.05 ± 0.001 ^{Cbc}	0.33 ± 0.014 ^{Ab}	0.12 ± 0.003 ^{Bc}
Black	0.48 ± 0.090 ^{Aa}	0.44 ± 0.008 ^{Aa}	0.24 ± 0.006 ^{Bb}
Hydroxyl radical scavenging activity (mmoles catechin eq/g defatted sample)			
CDC SB-2	0.05 ± 0.023 ^{Aab}	0.02 ± 0.003 ^{Ac}	0.07 ± 0.07 ^{Ab}
34946	0.08 ± 0.020 ^{Aa}	0.09 ± 0.02 ^{Abc}	0.08 ± 0.03 ^{Ab}
CDC Invincible	0.04 ± 0.013 ^{Bbc}	0.17 ± 0.02 ^{Abc}	0.06 ± 0.01 ^{Bb}
CDC Green Land	0.02 ± 0.001 ^{Bbc}	0.21 ± 0.17 ^{ABbc}	0.30 ± 0.09 ^{Aa}
Maxim	0.06 ± 0.004 ^{Bbc}	0.34 ± 0.13 ^{Aab}	0.04 ± 0.01 ^{Bb}
Black	0.01 ± 0.008 ^{Bc}	0.52 ± 0.07 ^{Aa}	0.06 ± 0.01 ^{Bb}

[1] Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column are not significantly different ($p > 0.05$)

[2] TEAC, Total antioxidant capacity by Trolox equivalent antioxidant capacity.

As found in the reducing power assay, the higher TEAC value of the fraction containing esterified phenolics in Black lentil as compared with all fractions from all cultivars may be explained by its higher TPC, TFC, and CTC. The TEAC value of the extract containing phenolics released from insoluble-bound forms from CDC Green Land was the highest among other varieties (0.93 ± 0.026 mmoles Trolox eq/g of defatted meal), being up to 10-fold higher than those found for the remaining cultivars. However, as can be noted in Table 4.1 the content of insoluble-bound phenolics (TPC, TFC, and CTC) was up to 185-fold higher than those of the remaining cultivars, showing that the TEAC value is dependent not only on the phenolic concentration but also on the identity and reaction mechanism operative. Furthermore, the highest correlation ($p < 0.01$) existed between TFC and TEAC, suggesting that flavonoids from lentils contributed most to the antioxidant activity as evaluated by TEAC, which evaluates radical scavenging activity of hydrophilic and lipophilic compounds.

4.2.3 DPPH radical scavenging capacity (DRSC)

DPPH radical is more stable than hydroxyl and superoxide radicals; as a result, DPPH assay has been used to evaluate the scavenging activities of antioxidant compounds (Siriwardhana and Shahidi, 2002). In the present study, DPPH assay confirmed that lentil extracts with higher phenolic and flavonoid contents had strongest antiradical action (Table 4.1). However, extracts containing free phenolics from Black lentils that had lower TPC, TFC and CTC than the fraction containing the esterified ones showed no differences between their DRSC ($p>0.05$). This may be due to the existing differences in the chemical constituents contributing to the scavenging activity (Albishi et al., 2013c).

The effectiveness of phenolic compounds as antioxidants, extracted from plant foods, frequently varies, however this does not always depend on their quantities, but may well be dictated by the chemical structures of their constituents (Shahidi and Naczk, 2004; Albishi et al., 2013c). Overall the DRSC of extracts containing free phenolics, and those released from their esterified and insoluble-bound forms were in the range of 0.01-0.48, 0.07-0.44, 0.04-0.42 mmoles Trolox eq/g defatted sample, respectively. It is interesting that the DRSC of all tested fractions were approximately in the same range; however, the other antioxidant assays in the current study afforded noticeably different values among the tested fractions. The DRSC of phenolics released from insoluble-bound form from CDC Green Land lentils was the highest, followed by 34946 ≥ Black > CDC SB-2 ≥ Maxim > CDC Invincible, and differences among the higher and the lower DRSC differed 11 times.

In general, the results for the fraction containing free phenolics in the present study were in good agreement with those reported by Zhang et al. (2015). The few differences found may be due to fractionation techniques or related to different varieties and/or samples used (e.g. crop year, stress condition, soil quality). According to Amarowicz et al. (2009) DPPH scavenging activity of the fraction consisting of tannins from red lentils was several times greater than those of the crude extract and the fraction containing low-molecular-weight phenolics. Overall, the fractions containing higher amounts of condensed tannins in the present study (Table 4.1) also showed higher DRSC.

4.2.4 Hydroxyl radical scavenging activity (HRSA)

Hydroxyl radical is a highly reactive species, having a very short life span (10^{-9} s). Hydroxyl radicals can be generated in cells by a variety of processes such as Fenton reaction, and may attack all biological molecules such as DNA, proteins, and polyunsaturated fatty acids (PUFA) in the membranes, among others (Rafat Husain et al., 1987; Chandrasekara and Shahidi, 2011b). The decomposition of hydrogen peroxide produces hydroxyl radical in the presence of iron, is considered as the main source of hydroxyl radical *in vivo*, and is a major mechanism of biological damage. The short lifetime of the hydroxyl radical means that its direct measurement may not be possible. To overcome this problem in the present study, the generated hydroxyl radicals were spin-trapped with DMPO. The intensity of the characteristic 1:2:2:1 EPR signals decreased with increasing antioxidant concentration. This may be due to either scavenging of the hydroxyl radical by the extract or chelation of Fe (II) by phenolics present in the extracts. However, a combination of both mechanisms may also be operative (John and Shahidi, 2010).

The HRSA of free, esterified and insoluble-bound phenolics from lentil extracts were in the range of 0.01-0.08, 0.02-0.52, 0.04-0.30 mmoles catechin eq/g defatted sample, respectively (Table 4.2). The results obtained from HRSA of the free phenolics from Black lentils were 52 times lower than that of phenolics released from the esterified phenolic form. This finding is contradictory to the result obtained in DPPH scavenging assay; however it is in agreement with other antioxidant activity tests in the current study. It is worthwhile to note that the HRSA for esterified fraction was generally higher than those for the free and insoluble-bound forms. On the other hand, the CDC Green Land

insoluble-bound phenolics form showed the highest HRSA, among others (0.30 ± 0.09 mmoles catechin eq/g defatted sample). Generally, these two cultivars had the most prominent capability in terms of antioxidant activity (Black and CDC Green Land lentil cultivars).

The better hydroxyl radical scavenging of the fraction containing esterified phenolics of Black lentils could be related to the high concentration of flavonoid and derivatives, which were determined by the HPLC analysis in the present study and summarized in (Table 4.7). The flavonoids also acted as strong superoxide radical anion ($O_2^{\cdot -}$) scavengers and singlet oxygen ($^1O_2$) quenchers. Depending upon their structures, flavonoids display a different hydroxyl radical scavenging trait. Furthermore, the number of hydroxyl groups substituting ring B, especially at C-3', directly affected the radical scavenging activity. When the number of hydroxyl groups decreases the OH quenching ability falls rapidly (Husain et al., 1987). It is worthwhile to note that hydroxyl radical scavenging activity followed the same trend as the antioxidant activities in this study.

4.2.5 Antioxidant activities and correlation results

The contribution of total phenolic content to the antioxidant activity of plant extracts has been reported in previous studies (Zhang et al., 2015). As a result, the correlations between TPC, TFC and CTC and the antioxidant activities evaluated by different methods in the current study were analyzed using the Pearson correlation test in order to corroborate the above findings.

Correlation coefficients (R^2) are summarized in Table 4.3. Strong and positive correlations existed between the TPC, TFC and CTC and reducing power ($R^2 = 0.9447$, $p < 0.01$), ($R^2 = 0.9467$, $p < 0.01$), ($R^2 = 0.9348$, $p < 0.01$), respectively. These findings are in agreement with those reported by Xu and Chang (2010) and Zhang et al. (2015). However, Zhang et al. (2015) also found that condensed tannins were major contributors to the reducing power. TEAC assay positively and strongly correlated with TPC, TFC and CTC, with correlation coefficients of $R^2 = 0.8200$, 0.8783, 0.8357, respectively. It is worth noting that TPC, TFC and CTC made a significant contribution to the antioxidant activities of lentil extracts. However, the antioxidant assay measured by DPPH method showed moderate correlation with TPC, TFC and CTC. These findings are comparable to those reported by Zhang et al. (2015) and also similar to the existing literature for other foods (Albishi et al., 2013b). The moderate and low correlation can be explained by the fact that antioxidants other than phenolic compounds may also be present in lentil extracts (Zhang et al., 2015). These authors also tested commercial phenolic standards (e.g. catechin, epicatechin, procyanidin B1) and demonstrated their different antioxidant activities depending on the compound as well as the method used (DPPH or ORAC). A positive and strong correlation existed between TPC, TFC and CTC and hydroxyl radical scavenging assay. In this study in general hydroxyl radical exhibited the highest correlation with TPC ($r = 0.9205$), TFC ($r = 0.9658$), and CTC ($r = 0.9436$) as compared with the other antioxidant assays, suggesting that phenolics from lentil cultivars may have important role in neutralizing ROS.

Table 4.3 Pearson's correlation between total phenolic content (TPC), total flavonoid content (TFC), or condensed tannin content (CTC) of lentils and antioxidant activity by different assays

Antioxidant assays	TPC	TFC	CTC
Reducing power	0.9447**	0.9467**	0.9348**
TEAC	0.8200**	0.8783**	0.8357**
DPPH radical scavenging activity	0.7949**	0.6964**	0.6508**
Hydroxyl radical scavenging activity	0.9205**	0.9658**	0.9436**
TBARS in fish meat model system	0.5084*	0.5229*	0.2390ns

** Is significant ($p < 0.01$); ns is non-significant. Copper-induced LDL-cholesterol oxidation and supercoiled plasmid DNA strand breakage inhibition were not correlated with TPC, TFC or CTC.

4.3 Characterization and quantification of phenolic compounds by HPLC-DAD-ESI-MS^n

The mass spectral data and identification of free, esterified, and insoluble-bound phenolic compounds of selected lentil cultivars are summarized in Table 4.4. The identification of phenolic compounds present in the lentil extracts was achieved using HPLC-DAD and HPLC-MS analyses and by comparison of their retention times (RT) and mass spectral data with those of available reference compounds. The remaining peaks were tentatively identified based upon their mass spectral characteristics and information available in the literature. Furthermore, quantification of compounds of interest was on the basis of their peak areas and by using calibration curves of their corresponding standards.

In the present study, 17 phenolic compounds belonging to hydroxybenzoic acids, hydroxycinnamic acids, monomeric flavonoids and proanthocyanidins were tentatively or positively identified in lentil cultivars. The MS spectra of gallic acid and protocatechuic acid gave deprotonated ions $[M-H]^-$ at *m/z* 169 and 153, respectively. Their respective MS^2 signals at *m/z* 125 and 109 enabled their positive identification, which was further confirmed by using authentic standards. Furthermore, methyl vanillate was tentatively identified by comparison with the MS data in the literature (Chandrasekara and Shahidi, 2011b). The deprotonated ion $[M-H]^-$ for this compound showed an *m/z* at 181 and MS^2 fragment at *m/z* 166 due to the loss of a methyl group $[M-H-15]^-$. These compounds, which belong to the hydroxybenzoic acid subclass have also been detected in whole grains of lentils (Xu and Chang, 2010), and in pea cotyledon (Dueñas et al., 2004).

Table 4.4 Phenolic compounds identified in the fractions containing free, esterified and insoluble-bound fractions of defatted lentil cultivars using *HPLC-DAD-ESI-MS^n*

Lentil Cultivar						Phenolic Compound	Molecular weight	M-H(*m/z*)	ESI negative ions (*m/z*)
CDC SB-2	3494-6 F	CDC Invincible	CDC Green Land	Maxim	Black				
Hydrobenzoic acids derivatives									
E B	F E B	B	E	B	F E B	Gallic acid[a]	170	169	125
B	E B	E B	E B	E B	E	Methyl vanillate	182	181	166
E B	E B					Protocatechuic acid[a]	154	153	109
Hydroxycinnamic acids and derivatives									
F E B	E B	F E	F E	F E	E	Caffeic acid[a]	180	179	135
F E B	F E B	F E B	F E B	F E B	F E B	*p*-coumaric[a]	164	163	119
F E B	F E B	F E B	F E	F E B	F E	Ferulic acid[a]	194	193	134, 149, 178
E	F E B	F E	E	E	E	Sinapic acid[a]	224	223	149, 164, 179
Flavonoids and derivatives									

Flavan-3-ol monomers									
F E B	F E B	F E B	F E B	F E B	F E B	Catechin[a]	290	289	125, 161, 205, 245
F E B	F E B	F E B	F E B	F E B	F E B	Epicatechin[a]	290	289	125, 151, 161, 179, 205, 245
Flavan-3-ol dimers and trimers (Proanthocyanidins)									
F E B	F E B	F E B	F E B	F E B	F E B	Procyanidin dimer B	578	577	245, 289, 407, 425, 451, 559
			B	B		Procyanidin dimer A	576	575	285, 287, 435, 539
B	F E B	F E B	E B	E	E	Prodelphinidin dimer A	592	591	285, 303, 421, 465, 573
F	F B	F	F	F	F	Procyanidin trimer C1	866	865	287, 407, 451, 575, 577, 695, 739
F	F	F		F	F	Procyanidin trimer A	882	881	449, 559, 575, 737, 863
F B	F E B	F E B	F E B	F	F B	(+)-Catechin-3-glucoside	452	451	289
Flavone									
E B	E B	E	B	E	E	Luteolin 3'-7-diglucoside	610	609	285
Flavonol									
			F			Kaempferol dirutinoside	902	901	577, 285

F: Free Phenolics, E: Esterified phenolics, B: insoluble-bound phenolics.

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

Furthermore, Chandrasekara and Shahidi (2011b) also reported their presence in millet grains. To the best of our knowledge, methyl vanillate was identified in lentils for the first time. It is worth noting that this compound was present only in the esterified and insoluble-bound forms; thus, prior to hydrolysis, it was linked with other molecules through ester bonds or cross linked to the cell wall, which may explain the fact that methyl vanillate has not been reported before.

Hydroxycinnamic acids (caffeic acid, *p*-coumaric, ferulic, and sinapic acids) were positively identified by comparing their retention times and spectral data with those of the authentic standards. Caffeic acid and *p*-coumaric acid showed a deprotonated molecular ion $[M-H]^-$ at *m/z* 179 and 163, respectively, followed by *m/z* signals at 135 and 119 in MS^2. Furthermore, the deprotonated molecular ions $[M-H]^-$ at *m/z* 193, and 223 indicated the presence of ferulic acid and sinapic acid, respectively, consistent with their remaining MS^2 signals as compared with the commercial standards. Trans *p*-coumaric, trans-ferulic, and sinapic acids were reported by Amarowicz et al. (2009) in crude extract of red lentils.

Several compounds from different subclasses of flavonoids comprising flavan-3-ol (monomers, dimers, and trimers), flavones, and flavonols were positively or tentatively identified in the present study. Generally, flavonoids are conjugated with sugars and occur as *O*- or *C*-glycosides, but they also exist as free aglycones (Chandrasekara and Shahidi, 2011b). Both flavan-3-ol monomers, catechin and epicatechin, which were found in all fractions of all cultivars were positively identified by comparing their retention times and fragmentation patterns. Dimers and trimers of flavan-3-ol, commonly known as proanthocyanidins, were tentatively identified.

The HPLC-MS analysis gave a deprotonated molecular ion $[M-H]^-$ at *m/z* 865 suggesting an 866 molecular weight that corresponded to procyanidin trimer C1 Sarnoski et al., 2012; de Camargo et al., 2014a; 2015). The *m/z* signal at 577 indicated the presence of procyanidin dimer B. Several peaks were identified as procyanidin B, as they eluted in different retention times. However, this is common for such compounds, as procyanidins B1 to B8 are well known to exist (Saint-Cricq et al., 1999). Isomers of procyanidins have the same molecular weights and dissociation pattern but their different retention times in HPLC stems from their different stereoisomer configurations. Similar to catechin and epicatechin, procyanidins B were found in all fractions of all cultivars. Dimers and trimers of procyanidin B were tentatively identified by comparing the MS^2 fragmentation pattern (Aguilera et al., 2010; Chandrasekara and Shahidi, 2011b; Sarnoski et al., 2012; de Camargo et al., 2014a; 2015), and they have previously been identified in lentils (Amarowicz et al., 2009; Aguilera et al., 2010; Troszyńska et al., 2011).

Procyanidin dimer A was also tentatively identified as indicated by its deprotonated molecular ion $[M-H]^-$ at *m/z* 575; a similar MS^2 fragmentation of it has already been reported (Sarnoski et al., 2012; de Camargo et al., 2014a; 2015). In contrast with procyanidin dimer B, which is one of the major compounds in all cultivars, and present in the free, esterified and insoluble-bound forms, procyanidin A was found only in the insoluble-bound form of two cultivars (CDC Green and Maxim). Procyanidins consist exclusively of catechin and epicatechin units, while prodelphinidins have (epi)gallocatechin in their structures. In the present study, prodelphinidin dimer A was tentatively identified as indicated by its MS signal at *m/z* 591. Furthermore, its fragmentation pattern was similar to the literature data (Sarnoski et al., 2012; de Camargo

et al., 2014a; 2015). Procyanidin trimer A was tentatively identified on the basis of the deprotonated ion $[M-H]^-$ *m/z* at 881 followed by loss of water $[M-H-18]^-$ in MS^2 giving remaining signals at *m/z* 863, 737, 575,559, and 449, which are consistent with previous studies (Sarnoski et al., 2012; de Camargo et al., 2014a; 2015). As far as we know, the presence of procyanidin dimer A and prodelphinidin dimer A in lentils were not previously reported. Procyanidin dimer A was detected only in the insoluble-bound form, whereas prodelphnidin dimer A was found in all forms (free, esterified and insoluble-bound). The presence of unrevealed compounds present only in the insoluble-bound form shows that ignoring the presence of molecules linked to the cell wall of plant materials underestimate the content and potential health benefits of plant foods.

The deprotonated ion $[M-H]^-$ at m/z 451 and at *m/z* 289 in MS^2 indicate the loss of hexose $[M-H-162]^-$, therefore this compound was tentatively identified as (+)-catechin-3-glucoside. This compound was previously reported in several studies with different lentil varieties (Amarowicz et al., 2009, Aguilera et al., 2010; Troszyńska et al., 2011; Zhang et al., 2015). Furthermore, a compound belonging to the flavonol subclass was tentatively identified only in the free phenolics of the CDC Green Land cultivar. This compound showed an *m/z* at 901, and at *m/z* 755 and 285 in MS^2 showing loss of two rhamnoside-glucoside $[M-H-616]^-$, indicating its identity as kaempferol dirutinoside (Aguilera et al., 2010), which was also found in other studies (Troszyńska et al., 2011; Zhang et al. 2015). The flavone luteolin 3'-7-diglucoside was also tentatively identified due to $[M-H]^-$ signal at *m/z* 609, and *m/z* 285 in MS^2, in agreement with Aguilera et al. (2010).

The contents of hydroxybenzoic acids, hydroxycinnamic acids and flavonoids identified in lentil extracts are presented in Tables 4.5-4.7. The content of phenolic compounds positively identified (gallic acid, protocatechuic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, catechin and epicatechin) was quantified by HPLC using regression equations from their respective standard curves. Concentrations of the phenolic compounds were expressed as μg of authentic standard/g defatted sample. The remaining compounds, whose identification was tentative, were quantified using the regression equation of gallic acid for methyl vanillate and catechin for the remaining flavonoids. These results were expressed as μg of gallic acid or catechin equivalents/g defatted sample, respectively. The results showed differences between the content and type of phenolic compounds depending on the lentil variety and phenolic fraction. This lends support to varietal differences found by Zhang et al. (2015), who reported the soluble phenolics of 20 lentil cultivars, and the report by Xu and chang (2010).

Generally, hydroxybenzoic acids present in lentil cultivars were dominant in the esterified and insoluble-bound forms, as compared with the content of free phenolics. With the exception of two cultivars (34946 and Black), which showed gallic acid as the major phenolic, methyl vanillate was the main compound among hydroxybenzoic acids. Protocatechuic acid made the lowest contribution, being found only in the insoluble-bound form of CDC SB-2 and 34946 cultivars. Gallic acid is a potent antioxidant, thus even at a lower concentration this compound may make a significant contribution to antioxidant activities especially for Black lentils, whose gallic acid content accounted for 95% of total hydroxybenzoic acids.

Table 4.5 The content of hydroxybenzoic acids in lentil cultivars (µg/g defatted sample)[1]						
Phenolic Compound	**CDC SB-2**	**34946**	**CDC Invincible**	**CDC Green Land**	**Maxim**	**Black**
Gallic acid						
Free	nd	0.81 ± 0.55a	nd	nd	nd	*
Esterified	*	0.07 ± 0.09Ba	nd	*	nd	6.69 ± 0.09Aa
Insoluble-bound	0.04 ± 0.02B	0.28 ± 0.06Ba	*	nd	*	1.87 ± 0.15Ab
Methyl vanillate						
Free	nd	nd	nd	nd	nd	nd
Esterified	nd	0.11 ± 0.005Bb	0.54 ± 0.04Aa	0.14 ± 0.06Bb	0.14 ± 0.02Bb	0.44 ± 0.04A
Insoluble-bound	0.28 ± 0.01B	0.30 ± 0.02Ba	0.20 ± 0.01Bb	0.33 ± 0.02Ba	0.81 ± 0.09Aa	nd
Protocatechuic acid						
Free	nd	nd	nd	nd	nd	nd
Esterified	nd	nd	nd	nd	nd	nd
Insoluble-bound	0.13 ± 0.02B	0.26 ± 0.004A	nd	nd	nd	nd
Hydroxybenzoic acids and derivatives						
Free	nd	0.81 ± 0.55a	nd	nd	nd	nd
Esterified	nd	0.18 ± 0.09Ca	0.54 ± 0.04Ba	0.14 ± 0.06Cb	0.14 ± 0.02Cb	7.13 ± 0.14A

Insoluble-bound	0.45 ± 0.01C	0.83 ± 0.05Ba	0.20 ± 0.01Cb	0.33 ± 0.02Ca	0.81 ± 0.09Ba	1.87 ± 0.15A
Σ Hydroxybenzoic acids and derivatives (Free + Esterified + Insoluble-bound)						
Free + Esterified + Insoluble-bound	0.45 ± 0.01C	1.82 ± 0.41B	0.74 ± 0.03C	0.47 ± 0.08C	0.95 ± 0.07C	9.00 ± 0.29A

[1] Data represent the mean values for each sample ± standard deviations (n = 2). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

nd: Not detected

*: Compounds detected but concentration in sample not determined

Table 4.6 The content of hydroxycinnamic acids in lentil cultivars (µg/g defatted sample)

Phenolic compound	CDC SB-2	3494-6	CDC Invincible	CDC Green Land	Maxim	Black
Caffeic acid						
Free	*	nd	2.52 ± 0.03Aa	0.25 ± 0.24Cb	1.59 ± 0.12Bb	nd
Esterified	2.32 ± 0.50Ba	0.97 ± 0.23B	1.94 ± 0.28Ba	1.29 ± 0.09Ba	3.76 ± 0.53ABa	5.39 ± 1.57A
Insoluble-bound	4.44 ± 0.50a	*	nd	nd	nd	nd
***p*-coumaric acid**						
Free	2.29 ± 0.28Bb	1.94 ± 0.11Ba	5.90 ± 0.14Ab	2.46 ± 0.10Bc	2.13 ± 0.33Bb	0.63 ± 0.11Cb
Esterified	0.54 ± 0.04Ea	1.35 ± 0.15Db	12.0 ± 0.004Aa	5.64 ± 0.06Ba	5.89 ± 0.32Ba	2.52 ± 0.05Ca
Insoluble-bound	*	0.07 ± 0.05Cc	0.38 ± 0.03Bc	3.16 ± 0.11Ab	0.02 ± 0.03Cc	*
Ferulic acid						
Free	*	0.39 ± 0.24Ba	1.32 ± 0.01Aa	0.05 ± 0.02Bb	0.36 ± 0.09Bb	*
Esterified	*	0.33 ± 0.02Ba	1.50 ± 0.51Aa	0.41 ± 0.04Ba	1.28 ± 0.13ABa	1.70 ± 0.12A
Insoluble-bound	0.30 ± 0.14A	*	*	nd	0.18 ± 0.12Ab	nd

Sinapic acid						
Free	nd	2.63 ± 0.14Aa	0.62 ± 0.10Ba	nd	nd	nd
Esterified	nd	0.67 ± 0.53Aa	0.33 ± 0.11Aa	1.20 ± 0.04A	0.62 ± 0.32A	0.59 ± 0.52A
Insoluble-bound	nd	*	nd	nd	nd	nd
Hydroxycinnamic acids and derivatives						
Free	2.29 ± 0.28Db	4.97 ± 0.26Ba	10.4 ± 0.02Ab	2.76 ± 0.12Db	4.09 ± 0.12Cb	0.63 ± 0.11Eb
Esterified	2.86 ± 0.54Cab	3.32 ± 0.43Cb	15.8 ±0.12Aa	8.54 ± 0.12Ba	11.5 ± 0.40Ba	10.2 ± 2.03Ba
Insoluble-bound	4.74 ± 0.64Aa	0.07 ± 0.05Cc	0.38 ±0.03Cc	3.16 ± 0.11Bb	0.20 ± 0.08Cc	nd
Σ Hydroxycinnamic acids and derivatives (Free + Esterified + Insoluble bound)						
Free + Esterified + Insoluble bound	9.90 ± 1.46D	8.35 ± 0.22D	26.6 ± 0.11A	14.5 ± 0.12BC	15.8 ± 0.20B	10.8 ± 2.13CD

[1] Data represent the mean values for each sample ± standard deviations (n = 2). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

nd: Not detected

*: Compounds detected but concentration in sample not determined

Table 4.7 The content of flavonoids in lentil cultivars (µg/g defatted sample)

Phenolic compound	CDC SB-2	34946	CDC Invincible	CDC Green Land	Maxim	Black
(+) - Catechin-3-glucoside						
Free	36.3 ± 8.26BCa	30.2 ± 5.59BCb	23.2 ± 2.56BCb	77.2 ± 9.64Ab	20.0 ± 2.26C	45.9 ± 3.22Ba
Esterified	nd	28.2 ± 0.89Ab	34.1 ± 0.49Aa	29.7 ± 4.57Ac	nd	nd
Insoluble-bound	44.2 ± 0.63BCa	62.4 ± 1.32Ba	15.1 ± 0.69Dc	122 ± 11.52Aa	nd	33.3 ± 0.12CDb
luteolin 3'-7-diglucoside						
Free	nd	nd	nd	nd	nd	nd
Esterified	30.9 ± 1.20Ca	30.4 ± 0.21Ca	27.80 ± 5.21C	nd	45.3 ± 0.05B	58.8 ± 1.41A
Insoluble-bound	8.34 ± 0.07Bb	10.2 ± 0.64Bb	nd	49.3 ± 4.08A	nd	nd
Procyanidin dimer B						
Free	42.3 ± 0.04Da	54.5 ± 2.70Ca	74.6 ± 2.05Ba	26.5 ± 4.15Ec	88.9 ± 4.13Aa	85.8 ± 0.64Ab
Esterified	49.1 ± 5.30Ca	34.4 ± 2.17Ca	50.2 ± 0.24Cb	209 ± 21.9Aa	97.0 ± 7.89Ba	221 ± 3.04Aa
Insoluble-bound	50.2 ± 1.89BCa	46.8 ± 7.95BCa	35.7 ± 150Cc	167 ± 16.2Ab	52.84 ± 3.77BCb	70.4 ± 1.76Bc

Procyanidin dimer A						
Free	nd	nd	nd	nd	nd	nd
Esterified	nd	nd	nd	nd	nd	nd
Insoluble-bound	nd	nd	nd	13.8 ± 0.78A	17.8 ± 1.35A	nd
Catechin						
Free	18.1 ± 0.89Fb	44.0 ± 0.25Cb	66.6 ± 2.08Ab	25.6 ± 1.98Eb	53.5 ± 2.60Bb	36.0 ± 0.16Dc
Esterified	27.6 ± 3.91Eab	59.1 ± 2.64Da	118 ± 0.82Ba	81.6 ± 0.48Ca	126 ± 2.00Ba	178 ± 1.50Aa
Insoluble-bound	30.2 ± 1.95Ca	36.2 ± 1.76BCc	15.0 ± 0.21Dc	78.4 ± 4.77Aa	32.3 ± 1.23Cc	41.9 ± 0.09Bb
Prodelphinidin dimer A						
Free	nd	7.81 ± 2.10Bb	8.87 ± 0.33Bb	nd	nd	27.0 ± 0.68Aa
Esterified	nd	11.4 ± 0.28ABb	18.2 ± 1.08ABa	10.4 ± 1.46Bb	15.6 ± 2.17AB	18.5 ± 3.56Aa
Insoluble-bound	11.6 ± 0.28C	46.0 ± 0.61Aa	6.87 ± 0.03Db	18.8 ± 0.59Ba	nd	nd
Procyanidin trimer C1						
Free	61.3 ± 2.23A	30.1 ± 3.72Ba	16.7 ± 2.28C	7.45 ± 0.44D	25.4 ± 0.58B	27.2 ± 0.11B
Esterified	nd	nd	nd	nd	nd	nd

Insoluble-bound	nd	8.79 ± 1.88b	nd	nd	nd	nd
Procyanidin trimer A						
Free	24.8 ± 0.59C	32.3 ± 0.65B	16.7 ± 2.28D	nd	12.2 ± 0.90D	40.5 ± 0.40A
Esterified	nd	nd	nd	nd	nd	nd
Insoluble-bound	nd	nd	nd	nd	nd	nd
Epicatechin						
Free	0.59 ± 0.14Bb	1.64 ± 0.004Bb	7.13 ± 0.49Aa	0.62 ± 0.09Bc	5.00 ± 1.71Aa	7.91 ± 0.16Ab
Esterified	1.08 ± 0.30Db	3.28 ± 0.41CDa	4.17 ± 0.37BCb	4.04 ± 1.16BCb	5.84 ± 0.54Ba	9.39 ± 0.005Aa
Insoluble-bound	3.57 ± 0.28Ba	2.70 ± 0.02Ba	0.50 ± 0.01Cc	7.94 ± 0.47Aa	3.68 ± 0.02Ba	1.57 ± 0.39Cc
Kaempferol dirutinoside						
Free	nd	nd	nd	13.0 ± 0.005	nd	nd
Esterified	nd	nd	nd	nd	nd	nd
Insoluble-bound	nd	nd	nd	nd	nd	nd
Flavonoids and derivatives						
Free	183 ± 7.41Ca	201 ± 9.01BCab	214 ± 2.19Bb	150 ± 3.15Dc	205 ± 5.86BCb	270 ± 1.70Ab

Esterified	109 ± 0.49Fc	167 ± 6.05Eb	253 ± 2.95Da	335 ± 18.1Bb	290 ± 8.30Ca	486 ± 3.69Aa
Insoluble-bound	148 ± 0.21Cb	213 ± 10.3Ba	73.1 ± 0.58Dc	458 ± 19.8Aa	107 ± 3.87Dc	147 ± 1.94Cc
Σ Flavonoids and derivatives (Free + Esterified + Insoluble bound)						
Free + Esterified + Insoluble bound	440 ± 7.70E	580 ± 7.32C	540 ± 1.34D	943 ± 1.45B	602 ± 6.31C	904 ± 3.94A

[1] Data represent the mean values for each sample ± standard deviations (n = 2). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

nd: Not detected

*: Compounds detected but concentration in sample not determined

The hydroxycinnamic acids were found mainly in the free and esterified forms, but dominated the esterified phenolic fraction. CDC Invincible variety showed the highest content of the total hydroxycinnamic acids as compared with the other varieties; having *p*-coumaric acid as the main one, accounting for 69% of the total hydroxycinnamic acids. With the exception of CDC SB-2 variety, which had caffeic acid as the major contributor, *p*-coumaric acid was also the main compound among hydroxycinnamic acids for the remaining varieties. Amarowicz et al. (2010) also reported *p*-coumaric acid as the predominant free phenolic acid amongst the remaining phenolics they identified in their study.

The total content of flavonoids was up to 2000- and 84-fold higher than that found for hydroxybenzoic acids or hydroxycinnamic acids. The same trend was also found for individual fractions (free, esterified, insoluble-bound phenolics); though there was a difference in the contents among them. This result is in agreement with that of Amarowicz et al. (2009) and Zhang et al. (2015). Amarowicz et al. (2009) reported that flavan-3-ols, a subclass of flavonoids, were the most plentiful compounds (~50%) detected in crude red lentil extract; whereas the non-flavonoid phenolics (e.g. phenolic acids) were present in lower concentration (20%). In fact, flavonoids and procyanidins are the dominant phenolic compounds in leguminous seeds (Amarowicz and Pegg, 2008b). Lending support to the TFC (Table 4.1), the quantification of flavonoids using HPLC showed higher contents of insoluble-bound and esterified phenolics for CDC Green Land and Black lentils, respectively, as compared with the remaining lentil cultivars tested.

Catechin was the main monomeric flavonoid whereas procyanidin dimer B was the main proanthocyanidin contributing to the total flavonoids. Catechin, epicatechin and procyanidins B, considered as the predominant phenolic compounds, were found in all fractions of all cultivars in the current study. Catechin concentration was in the range of 18.1-66.6, 27.6-178, 15.0-78.4 μg catechin eq/g defatted sample in the free, esterified and insoluble-bound fractions, respectively; whereas procyanidin dimer B contents were in the range of 26.5-88.9, 34.4-221, 35.7-167 μg catechin eq/g defatted sample in the free, esterified and insoluble-bound fractions, respectively. These results showed flavonoids as being the major extractable phenolic compounds in the tested lentil cultivars.

4.4 Inhibition of cupric ion-induced human low-density lipoprotein (LDL) peroxidation

A high concentration of LDL-cholesterol has been associated with ischemic heart disease, and stroke. The level of oxidation of LDL-cholesterol has also been used as a biomarker for human cardiovascular diseases. Transition metal ions serve as catalysts in non-enzymatic LDL oxidation. In the present study, a cupric ion induced human low-density lipoprotein (LDL) peroxidation model system was used and the results are shown in Table 4.8. Free phenolics extracts from CDC SB-2 and 34946 varieties as well as insoluble-bound phenolics extracts from CDC Green Land variety inhibited the formation of human LDL-cholesterol oxidation up to 67.3% by using a very low concentration (0.003 mg/mL) of the phenolic extracts. Free phenolics fractions of CDC SB-2 and 34946 also showed the highest TFC (Table 4.1).

Table 4.8 Effect of free, esterified and insoluble-bound phenolic extracts (0.02 mg/mL) in preventing cupric ion induced human low density lipoprotein (LDL) peroxidation[1]

Lentil Cultivar	12h	22h
	Free	
CDC SB-2	*	*
3494-6	*	*
CDC Invincible	25.07 ± 7.05^{b}	37.06 ± 9.41^{a}
CDC Green Land	44.03 ± 4.95^{a}	47.88 ± 11.18^{a}
Maxim	37.05 ± 7.49^{ab}	46.11 ± 11.70^{a}
Black	24.19 ± 4.68^{b}	28.02 ± 5.21^{a}
	Esterified	
CDC SB-2	50.98 ± 7.84^{a}	50.03 ± 13.30^{ab}
3494-6	34.85 ± 2.41^{b}	34.97 ± 3.23^{ab}
CDC Invincible	40.17 ± 5.82^{ab}	42.37 ± 12.92^{ab}
CDC Green Land	55.89 ± 6.22^{a}	59.07 ± 11.83^{a}
Maxim	42.35 ± 7.28^{ab}	29.76 ± 1.21^{b}
Black	45.24 ± 2.10^{ab}	40.83 ± 9.95^{ab}
	Insoluble-bound	
CDC SB-2	63.92 ± 9.49^{a}	85.44 ± 10.47^{a}
34946	33.50 ± 7.49^{b}	40.23 ± 16.77^{b}
CDC Invincible	66.75 ± 2.90^{a}	89.66 ± 1.11^{a}
CDC Green Land	*	*
Maxim	48.71 ± 13.67^{ab}	67.36 ± 3.06^{a}
Black	60.31 ± 5.14^{a}	76.14 ± 5.68^{a}

[1] Data represent the mean values for each sample ± standard deviations (n=3). Means followed by the same lower case letters within a column are not significantly different ($p > 0.05$).

* Corresponding LDL values for free phenolics fraction of CDC SB-2 and 3494-6 lentil samples with (0.003 mg/mL) concentration are (41.01 ± 3.12 and 52.08 ± 0.65, respectively) 12 h. (50.36 ± 0.23 and 49.32 ± 10.0, respectively) 22h. Corresponding LDL values for insoluble-bound fraction of CDC Green Land with (0.003 mg/mL) concentration is 48.95 ± 3.17 (12 h), 67.30 ± 0.25 (22h).

Furthermore, the highest TFC released from the insoluble-bound form of phenolics was found in CDC Green Land cultivars. Thus, TFC results support the lowest concentration required for prevention of LDL-cholesterol oxidation. The remaining fractions showed inhibition of human LDL-cholesterol oxidation by up to 89.7%, however a higher concentration of the phenolic extract was required (0.02 mg/mL).

No correlation existed between TPC, TFC, CTC and the inhibition of human LDL-cholesterol oxidation; thus, these parameters were not good predictors for LDL oxidation. As discussed above, the procyanidin dimer B-type and catechin were major phenolics detected in all cultivars and fractions using HPLC analysis; however, epicatechin was not a major contributing compound in comparison with procyanidin dimer B-type and catechin. In general *p*-coumaric acid was also found in all cultivars and fractions, except for the insoluble-bound fractions of CDC SB-2 and Black lentil varieties (Tables 4.5-4.7). Positive correlations ($r = 0.8565$-0.9669; $p < 0.05$) existed between procyanidin dimer B-type, catechin, epicatechin, or *p*-coumaric acid released from their bound form and the inhibition of human LDL-cholesterol oxidation, showing these compounds as being possibly responsible for this potential human health benefit, thus preventing an eventual cardiovascular disease.

Interestingly the phenolics released from their insoluble-bound form were not the major contributors to TPC, TFC or CTC assays. In fact, for these assays, phenolics in the esterified form were present at higher concentrations. However, in general, phenolics released from their insoluble-bound form had the highest inhibition effect on human LDL- cholesterol oxidation, in comparison with free phenolics and those released from their esterified forms. Therefore, the present results also provide new evidences for the

bioactivity of insoluble-bound phenolics from lentils, which must be taken into account in future studies, with focus on functional foods.

A high LDL-cholesterol concentration is not an isolated issue. In general, it comes with other health problems such as diabetes and obesity. Zhang et al. (2015) demonstrated that phenolics from lentils had inhibitory effect on alpha-glucosidase, which may reduce glucose absorption and post-prandial hyperglycemia, being potentially beneficial in managing and/or preventing diabetes. These authors showed lentil phenolic extracts as pancreatic lipase inhibitors, which in turn has a potential management effect on body weight gain and obesity.

4.5 Inhibition of peroxyl radical induced supercoiled plasmid DNA strand scission

Peroxyl radicals are among ROS that possess deleterious effects on biological systems. Their negative effects may take place in both intra- and extracellular media. This effect stems from the relatively long half-life of peroxyl radicals. The supercoiled plasmid DNA strand breakage inhibition is a useful biological marker to evaluate the potential bioactivity of mixtures of compounds or isolated ones. In the process, the DNA conformation change was evaluated by electrophoresis (Figure 4.4 A-C). The DNA may be converted to nicked or linear forms due to single- or double-strand breaks, respectively. Phenolic compounds extracted from all lentils fractions (free, esterified, and insoluble-bound forms) in the present study were effective in inhibiting the DNA strand break and this was in the range of 28.1-91.7% (Table 4.11). In general, no difference in the activity was found among free, esterified and insoluble-bound phenolics, with the

exception of those extracted from 34946 and Black varieties. This may indicate that the mechanism of the reaction was influenced mostly by individual phenolics and/or their interactions rather than their total concentration. Modifications in DNA conformation may affect its replication and transcription, which may cause mutagenesis and cancer initiation.

The ability of phenolic extract to inhibit the DNA strand break may be explained by their scavenging capacity against peroxyl radicals as previously demonstrated (Boudjou et al., 2013) with crude phenolic extracts (free plus esterified phenolics) from lentils using the oxygen radical absorbance capacity (ORAC) assay, for which peroxyl radicals were generated by AAPH. These authors also demonstrated *in vitro* anti-inflammatory activities of crude phenolics from lentils by inhibiting the activity of cyclooxygenases (COX-1 and COX-2). Non-steroidal anti-inflammatory drugs (NSAIDs) also exhibited this inhibition effect. Furthermore, Boudjou et al. (2013) reported *in vitro* anti-inflammatory against lipoxygenase (15-LOX). This enzyme oxidizes arachidonic acid and produces leukotrienes, which are related to asthma and allergic rhinitis. In fact, Kandhare et al. (2013) demonstrated anti-asthmatic effects of procyanidine type A isolated from cinnamon bark in rat models. The above mentioned literature and the present results support the fact that lentil may be considered as a potential functional food.

Table 4.9 The inhibitory effect of lentil extracts in peroxyl radical induced DNA strand breakage (%)[1]

Lentil Cultivar	Free	Esterified	Insoluble-Bound
CDC SB-2	78.36 ± 7.85^{Ab}	86.29 ± 2.51^{Aa}	81.06 ± 7.22^{Aab}
3494-6	28.06 ± 0.58^{Bc}	83.60 ± 8.54^{Aa}	79.47 ± 7.80^{Aab}
CDC Invincible	81.03 ± 0.78^{Aab}	84.64 ± 4.62^{Aa}	88.01 ± 2.83^{Aa}
CDC Green Land	86.73 ± 2.10^{Aab}	84.18 ± 4.81^{Aa}	69.64 ± 7.73^{Bb}
Maxim	81.84 ± 5.91^{Aab}	77.39 ± 3.35^{Aa}	75.56 ± 5.23^{Aab}
Black	91.71 ± 0.58^{Aa}	75.21 ± 3.27^{Ba}	78.60 ± 3.47^{Bab}

[1]Data represent the mean values for each sample ± standard deviations (n=3). Means followed by the same capital case letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column are not significantly different ($p > 0.05$).

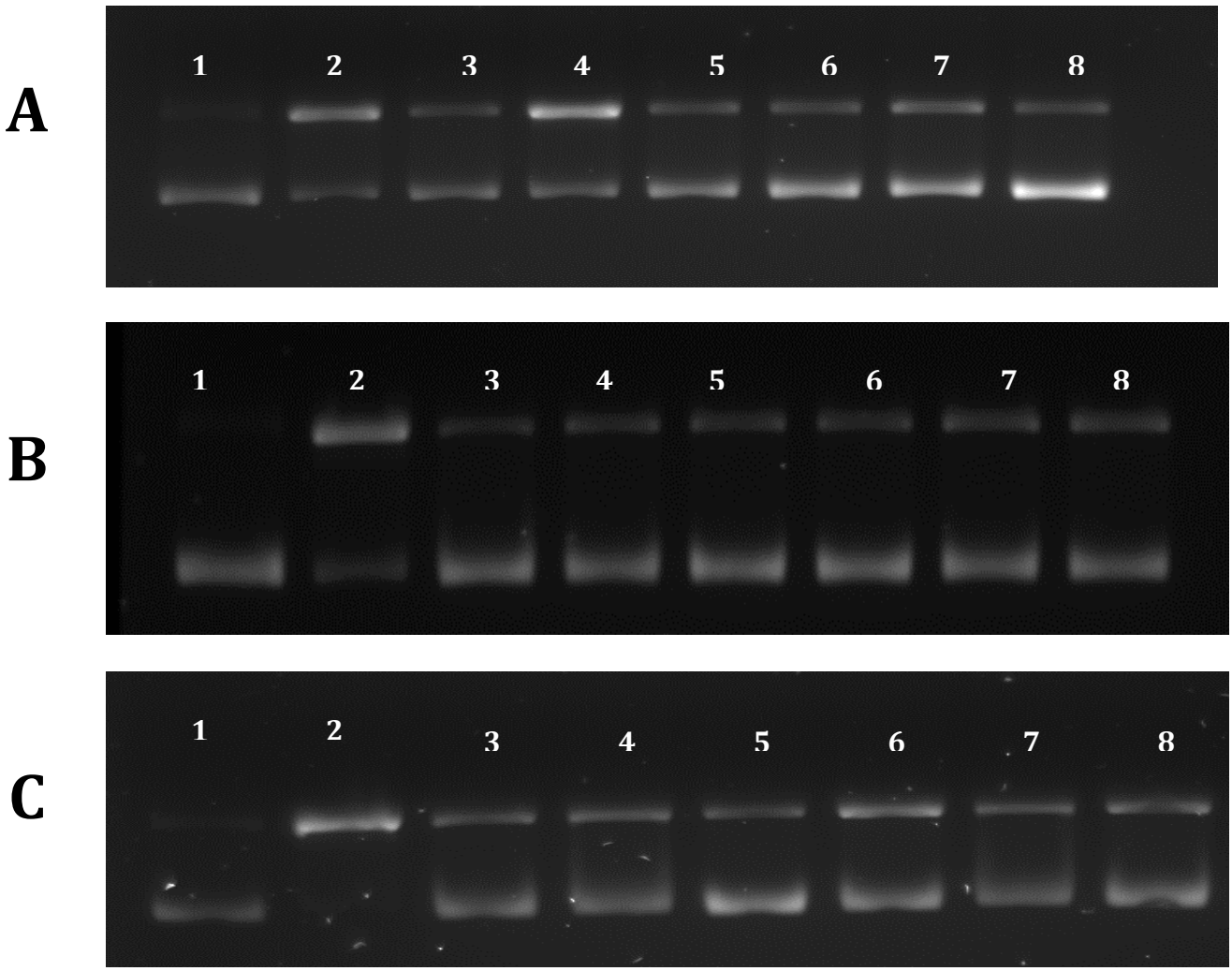


Figure 4.1 Effect of addition of free, esterified and bound phenolic extracts of Black lentils in peroxyl radical treated DNA system

(A) Lane 1: control (DNA only); lane 2: blank (DNA and AAPH); lane 3: CDC SB-2 free phenolic; lane 4: 3494-6 free phenolic; lane 5: CDC Invincible free phenolic; lane 6: CDC Green Land free phenolic; lane 7: Maxim free phenolic; lane 8: Black free phenolic.

(B) Lane 1: control (DNA only); lane 2: blank (DNA and AAPH); lane 3: CDC SB-2 esterified phenolic; lane 4: 3494-6 esterified phenolic; lane 5: CDC Invincible esterified phenolic; lane 6: CDC Green Land esterified phenolic; lane 7: Maxim esterified phenolic; lane 8: Black esterified phenolic.

(C) Lane 1: control (DNA only); lane 2: blank (DNA and AAPH); lane 3: CDC SB-2 bound phenolic; lane 4: 3494-6 bound phenolic; lane 5: CDC Invincible bound phenolic; lane 6: CDC Green Land bound phenolic; lane 7: Maxim bound phenolic; lane 8: Black bound phenolic.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The free, esterified, and insoluble-bound phenolics from selected lentil cultivars were positively or tentatively identified and quantified. Flavonoids and derivatives made the highest contribution, which were followed by hydroxycinnamic and hydroxybenzoic acids and their respective derivatives. Catechin was the major monomeric flavonoid, while procyanidin dimer B was the major proanthocyanidin present. Soluble phenolics (free plus esterified) made the major contribution; however, the insoluble-bound ones should not be ignored, especially for CDC Green Land, which showed 48% insoluble-bound phenolics while the free phenolics and esterified phenolics 16% and 35%, respectively, as evaluated by HPLC. Among soluble phenolics (free vs esterified), the esterified ones were generally present in higher concentration. Methyl vanillate, procyanidin dimer A, and prodelphinidin dimer A were identified and quantified in lentils for the first time. *p*-Coumaric acid, catechin, epicatechin, and procyanidin dimer B, were present in all fractions from all cultivars tested, thus these compounds must be investigated in future studies on lentils. Phenolic extracts showed reducing power and scavenging activity against DPPH and hydroxyl radicals and ABTS radical cation. The potential bioactivity of all fractions and cultivars was confirmed through inhibition of cupric ion induced human LDL peroxidation and peroxyl radical induced DNA strand breakage, thus lentils should be included in the diet due to their potential beneficial health effects. Despite their lower contribution to the phenolic content of lentils, insoluble-bound phenolics, compared with free and esterified phenolics, showed the highest effect

against LDL peroxidation, thus providing new evidences of their importance as functional food components with potential health beneficial properties. However, the bioavailability of the phenolics present in lentils as well as their *in vivo* antioxidant effects were not evaluated in the present study. Our study showed that phenolic compounds present in lentils have a significant antioxidant effects *in vitro*. Thus, biological trials using different approaches such as cell models, with animals and humans are still necessary. In addition, future studies should be also addressed towards the bioavailability of phenolics, also taking into account those in the insoluble-bound form with focus on development of functional foods. Futhermore, lentils must be cooked before consumption; Xu and Chang (2009) reported that raw legumes might be better material than cooked legumes for the nutraceutical industry to produce certain anticancer agents. Thermal processes affect the antioxidant and antiproliferation of legumes (Xu and Chang, 2009), thus further studies would be required to determine the effect of heat processing on antioxidant activity of lentils.

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APPENDIX

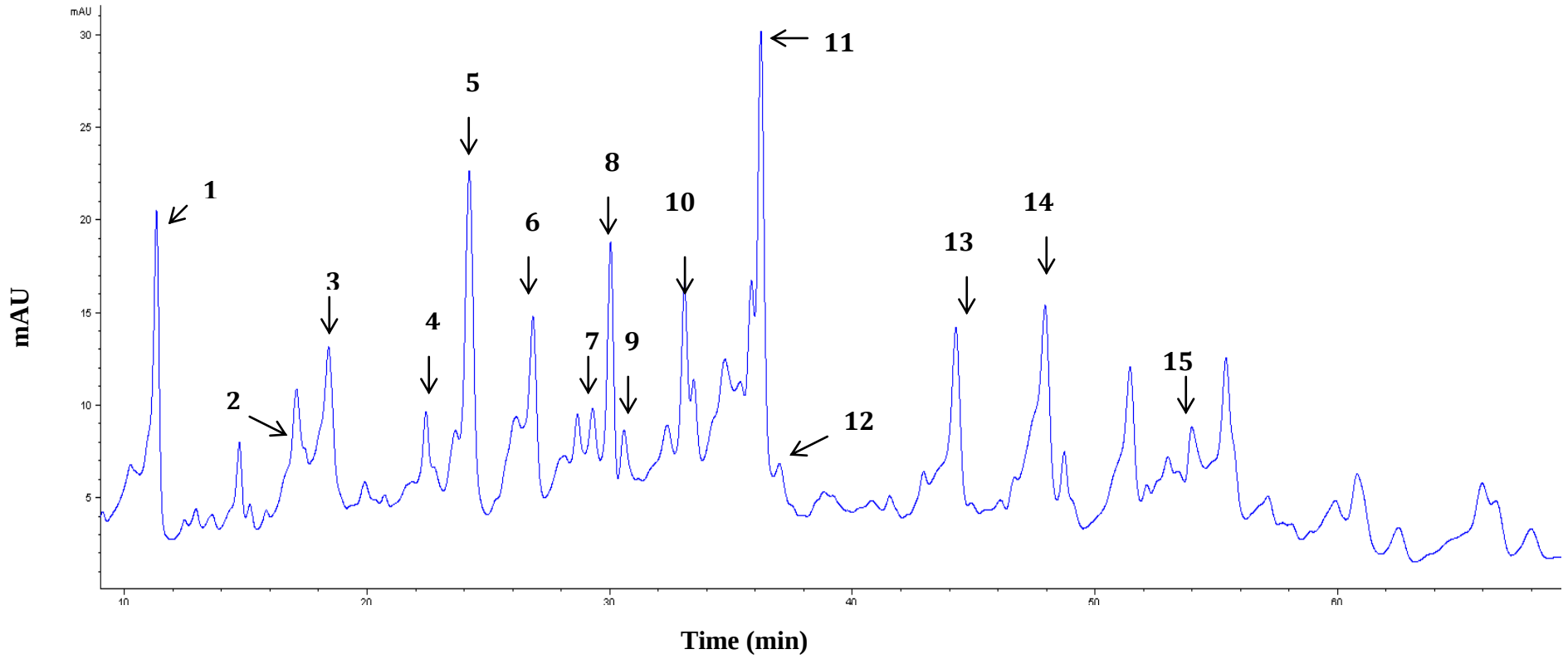

Figure A-1 HPLC Chromatograms at 270 nm of phenolic compounds extracted from free fraction of Black lentils. Peaks: 1, Gallic acid; 2, Procyanidin dimer B; 3, Procyanidin trimer A; 4, Procyanidin dimer B; 5, Procyanidin dimer B; 6, (+)-Catechin-3-glucoside; 7, Procyanidin dimer B; 8, Catechin; 9, Procyanidin trimer C1; 10, Procyanidin dimer B; 11, Prodelphinidin dimer A; 12, Epicatechin; 13, *p*-coumaric; 14, Ferulic acid.
The order of appearance of the compounds listed is similar up to those observed in the literature (Chandrasekara and Shahidi, 2011b; Ma et al., 2014; de Camargo et al., 2015).

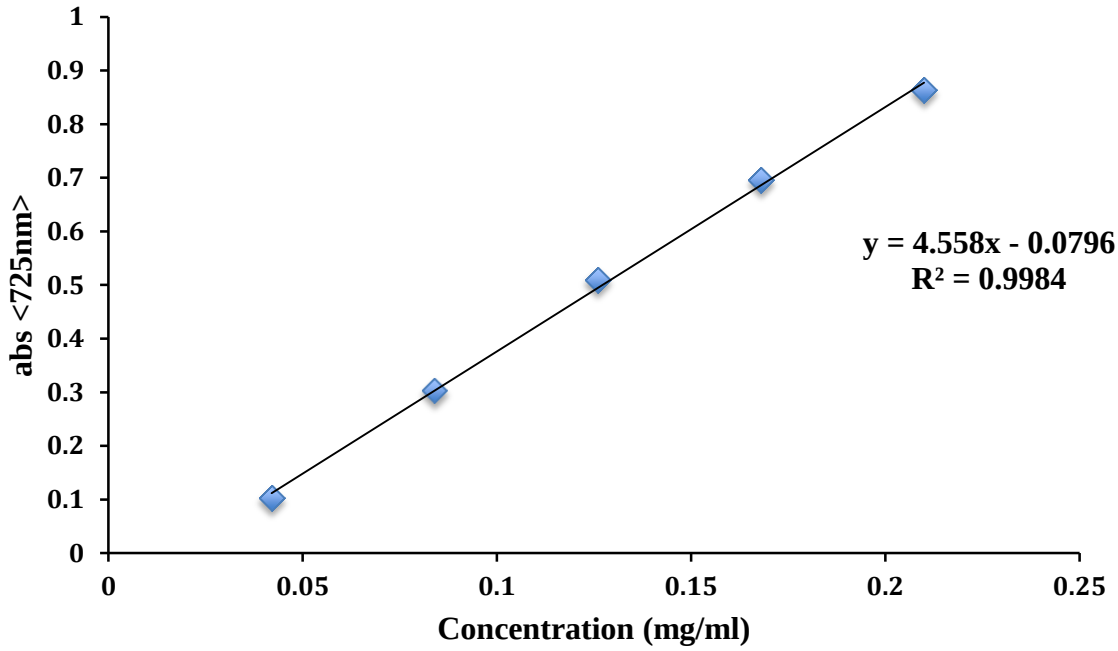

Figure A-2 Standard curve for the determination of total phenolic content (Gallic acid equivalent)

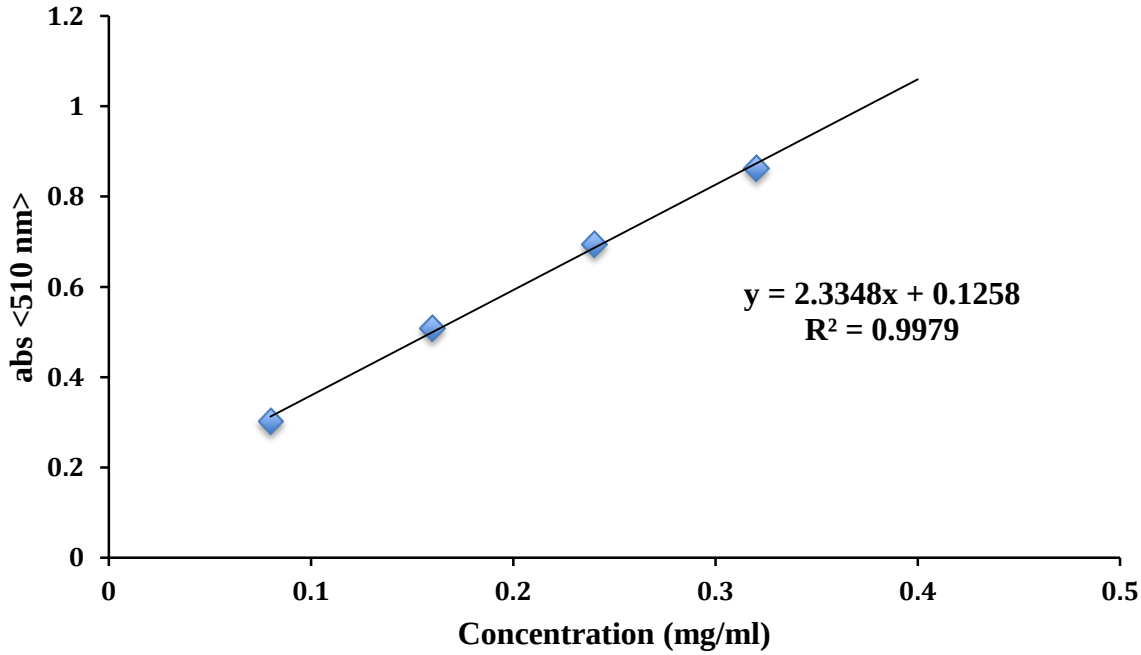

Figure A-3 Standard curve for the determination of total flavonoid content (Catechin equivalent)

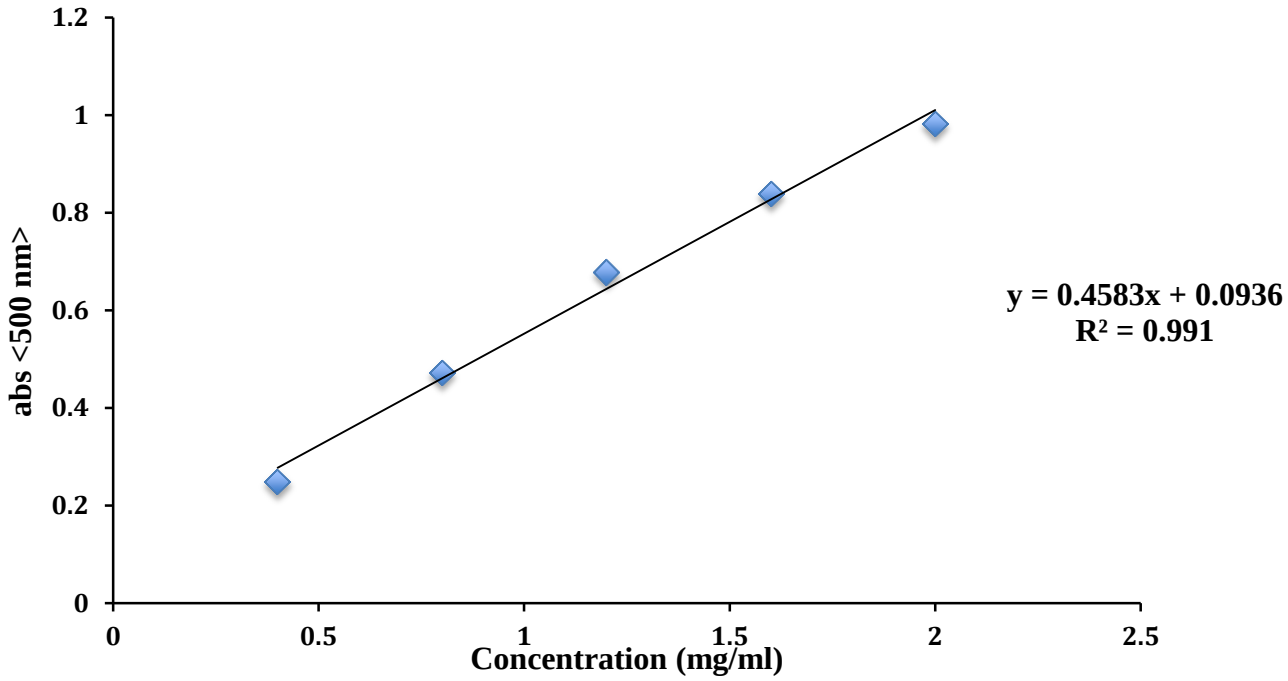

Figure A-4 Standard curve for the determination of condensed tannin (proanthocyanidin) content (Catechin equivalent)

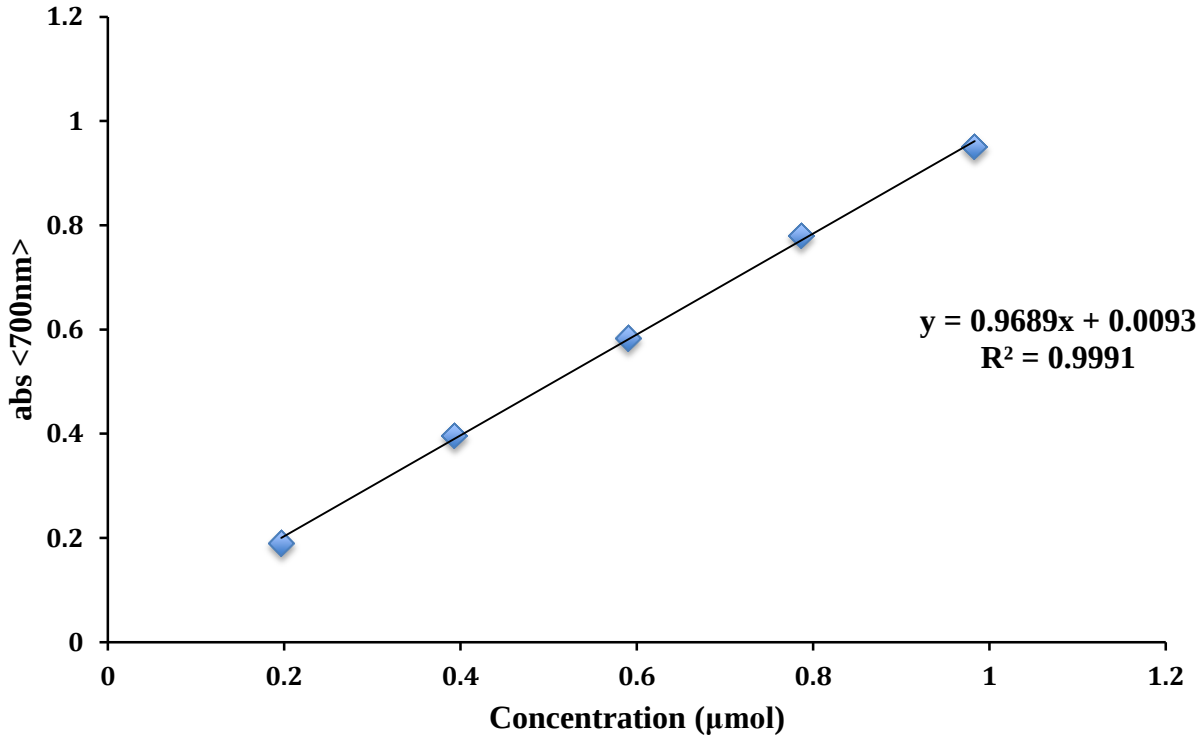

Figure A-5 Standard curve for the determination of reducing power (Trolox equivalent).

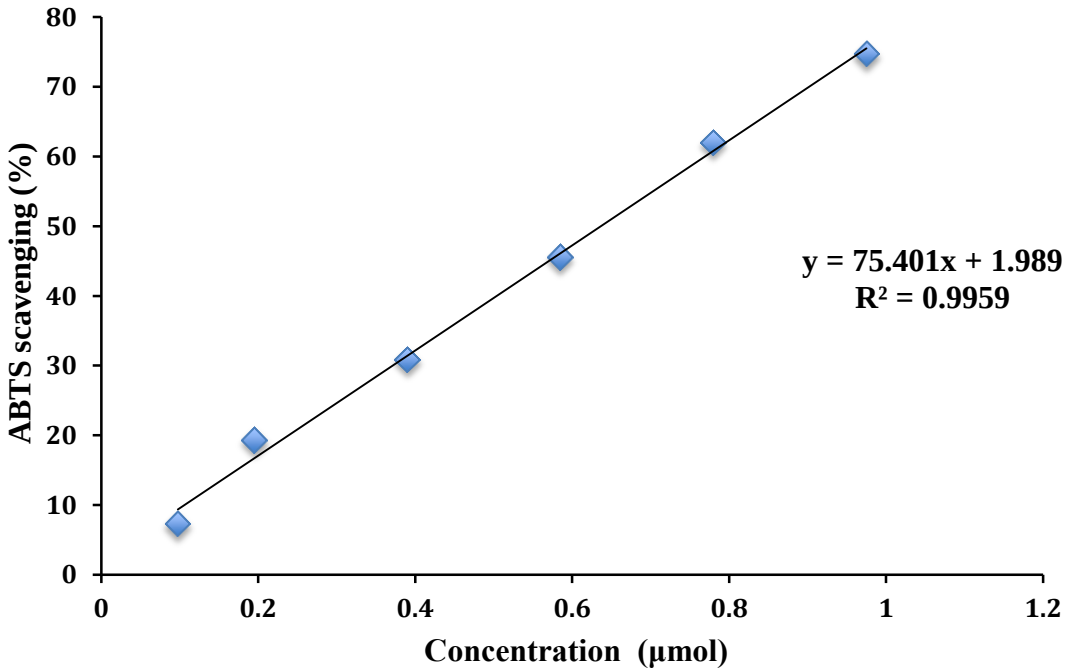

Figure A-6 Standard curve for the determination of Trolox equivalent antioxidant capacity (TEAC)

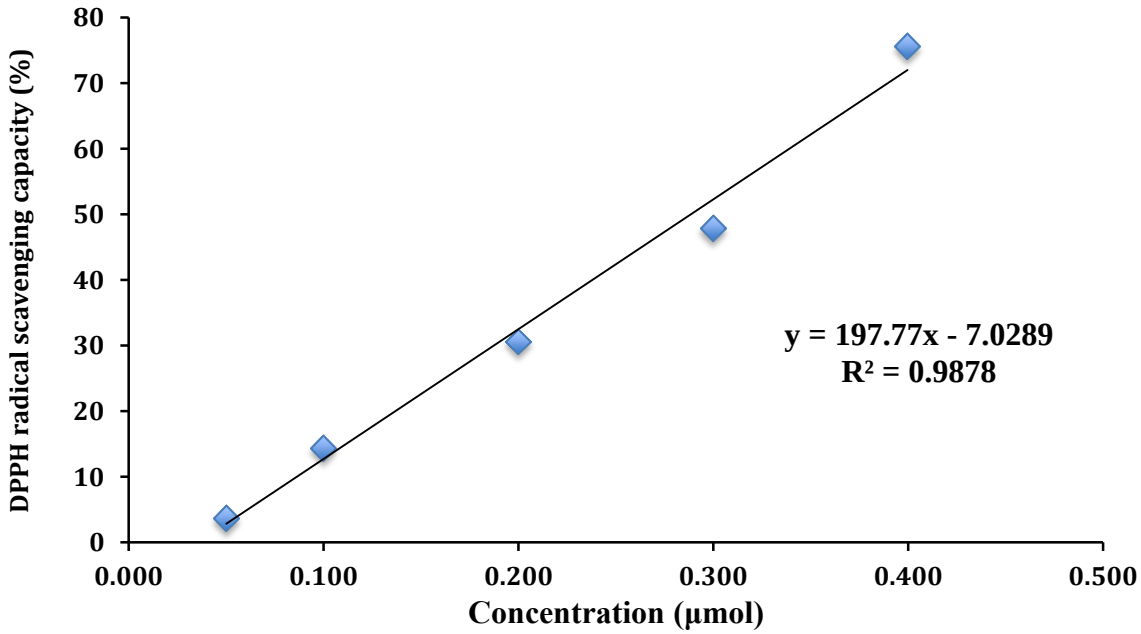

Figure A-7 Standard curve for the determination of DPPH radical scavenging capacity (Trolox equivalent)

PUBLICATION

The current work has been accepted for publication in the Journal of Functional Foods.

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