ANTIOXIDANT POTENTIAL OF BEANS

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By

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ABSTRACT

Four different bean varieties with different coat colours (red, brown, black and white) were dehulled and the antioxidant compounds in the hulls and whole seeds extracted separately with 80 % acetone. The total phenolic content of the red, brown and black were higher than that of white beans both in the hull and in the whole seed extracts. Total phenolic contents of red and brown hulls were 2.3 times higher than those of whole seeds while that of the hulls of black beans were 7 times higher than black bean whole extract. Total antioxidant capacity of the extracts was evaluated using Trolox equivalent antioxidative capacity (TEAC) assay, β-carotene-linoleate and bulk stripped corn oil model systems. Extracts were used at 50 and 100 ppm phenolics as catechin equivalents. Scavenging efficacies of the whole bean extracts for reactive oxygen species (ROS) were evaluated using hydrogen peroxide, superoxide and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Furthermore, inhibition of DNA-supercoiled strand scission and prevention of human low density lipoprotein (hLDL) oxidation by the extracts were evaluated. TEAC values of red whole bean extract (RWE), brown whole bean extract (BWE) and black whole bean extract (LWE) were 8.84, 7.46 and 4.64 while those of red bean hull extract (RHE), brown bean hull extract (BHE) and black bean hull extract (LHE) were 46.68 42.90, and 40.74, respectively. TEAC assay revealed that the antioxidant capacity of red, brown, and black bean hulls were in the same order of magnitude with little variation. Retention of β-carotene in a β-carotene-linoleate model system in the presence of RWE, BWE and LWE after 2 h of the assay was 46, 45, and 28% at 50 ppm and 52, 51, and 33% at 100 ppm, respectively, as compared to 2% in the control devoid of any extract. In a bulk corn oil model system, the order of efficacies of the extracts and

reference antioxidants as measured by the formation of conjugated dienes (CD) was catechin > BHA > RWE = BWE = LWE > α-tocopherol. Inhibition of formation of hexanal in corn oil after 7 days was catechin > RWE = BWE = BHA > LWE > α-tocopherol. The efficacy of RWE, BWE and LWE at 100 ppm in scavenging hydrogen peroxide was 76, 73 and 65%, respectively. The corresponding values for superoxide radical scavenging were 54, 53 and 60%. Red, brown and black whole and hull seed extracts showed total scavenging of DPPH radical when used at 100 ppm. The Fe^{2+} chelation capacities of RWE, BWE and LWE were 58, 56 and 39% at 50 ppm and 72, 69, and 60% at 100 ppm, respectively. Red, brown and black whole seed extracts exhibited total inhibition of hLDL oxidation when used at 25 and 50 ppm. Red, brown and black whole seed extracts exerted total protection against peroxyl-induced DNA scission at 50 and 100 ppm levels while they did not show any protection towards hydroxyl radical induced-DNA scission. HPLC analyses revealed the presence of cyanidin in LHE while RHE and BHE contained both cyanidin and delphinidin. White bean hull extracts did not contain any of the two anthocyanidins. Ferulic, caffeic, sinapic, *p*-coumaric and vanillic acids were identified as major phenolic acids in bean hull extracts.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Aerobic life utilizes large amounts of oxygen for maintenance of its activities. A great percentage of oxygen inspired is utilized in oxidative phosphporylation to generate bio-energy, while some (3-10%) is converted to reactive oxygen species (ROS) (Hiramatsu *et al.*, 1997) as by products of normal functions such as electron transport, peroxisomes as well as lipid peroxidation (Ames and Shigenaga, 1993). At normal rate of generation, some ROS are useful in the human body, while the rest are neutralized by enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (Halliwell and Gutteridge, 1999) and/or by endogenous antioxidants including α-tocopherol, β-carotene and ascorbic acid (Ames *et al.*, 1981). There is mounting evidence that ROS are underlying the pathogenesis of a number of diseases including cell injury, atherosclerosis, and cancer (Shahidi, 1997; Yoshikawa *et al.*, 1997).

As the endogenous antioxidants synthesized by aerobes do not completely prevent the damage by ROS *in vivo* (Halliwell, 1999), efficient repair systems are required to lower the damage in the form of dietary antioxidants (Shi *et al.*, 2001). Lipid peroxidation is inseparable from free radical damage as it contributes to the generation of ROS and also ROS in turn enhance lipid peroxidation in biological systems and food.

The addition of antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (tBHQ) and propyl gallate (PG) has been

in practice by the food industry as a means of increasing shelf life of lipids and lipid containing foods (Tsuda *et al.*, 1994a). However, the safety concerns of synthetic antioxidants such as carcinongenicity (Branen, 1975; Ito, 1983) and organ toxicity (Sun *et al.,* 1996) have led food manufacturers to seek alternative antioxidants.

It is well known that diets rich in fruits and vegetables are protective against cardiovascular diseases, cancer and other age-related disease conditions (Block and Langseth, 1994). The protective effects have been attributed in large, to the antioxidants such as dietary phenolic compounds which include flavonoids and phenylpropanoids (Rice-Evans *et al.,* 1996). Flavonoids are known to exert their antioxidant activity by inhibiting the activity of enzymes including xanthine oxidase, lipoxygenase and cycloxygenase (Hout *et al.*, 1994), by chelating metal ions (Morel *et al.,* 1994), and by scavenging free radicals (Hanasaki *et al.,* 1994).

Beans are reported to contain strong antioxidant (Tsuda *et al.,* 1993; Tsuda *et al.,* 1994a; Tsuda *et al.,* 1997) and antigenotoxic (de Meja *et al.,* 1999) activities. There are several bean species such as *Phaseolus vulgaris, P. multiflorus, P. atropurpures, P. coccineus* out of which *P. vulgaris* is commonly cultivated all over the world for pods and seeds. Depending on the cultivar, beans contain a variety of flavonoids and may have different colour of seed coats. Four colours are most common; red, brown, black and white (Mazza and Miniati, 2000). Great Nothern, black turtle, pink, dark red kidney, white kidney, large red kidney, pinto, Dutch brown and small red beans are some of the commonly cultivated varieties of *P. vulgaris*. The bean production in Canada is 276

thousand metric tonnes (Agriculture and Agri-Food Canada, 2000). Although beans are cultivated all over the world and consumed in both eastern and western dishes, little attention has been paid to their antioxidant and antigenotoxic potential.

Beans contain significant quantities of polyphenolic compounds such as flavonoids, phenolic acids and lignans but are typically low in ascorbic acid, β-carotene and α-tocopherol (Ganthavorn and Hughes, 1997). Phenolic compounds present in seed coat of beans include different anthocyanidin pigments that may belong to the delphinidin, cyanidin, pelargonidin, malvidin and petunidin (Mazza and Miniati, 2000) families and phenolic acids such as ferulic, caffeic and sinapic acids, among others (Drumm *et al.,* 1990). Anthocyanins such as delphinidin and cyanidin possess very strong antioxidative and antiradical activities (Rice-Evans *et al.,* 1996). In addition, anthocyanin pigments are known to bear several other therapeutic properties (Detre *et al.*, 1986; Kamei *et al.*, 1995).

The objectives of the present study were to investigate the antioxidative efficacies of four types of beans with different coat colours (red, brown, black and white). Thus, free-radical scavenging assays along with different model systems were used for this purpose. In addition, the capacities of bean extracts in inhibition of oxidation of human low density lipoprotein (hLDL) and inhibition of damage to DNA *in vivo* were investigated. In order to shed light on the molecular behaviour of active constituents of beans, the active phenolic compounds involved were also identified.

CHAPTER 2

LITERATURE REVIEW

The importance of the antioxidants contained in foods is well appreciated for both preserving the foods themselves and supplying essential antioxidants *in vivo* (Shi and Noriko, 2001). Antioxidants are known to act at different levels in the oxidative sequence involving lipid molecules (Shahidi, 1997). There is mounting interest in natural antioxidants due to safety concerns relating to synthetic antioxidants. In this context, it is important to investigate the antioxidative properties of beans, which is a legume grown and consumed in large quantities all over the world. The following sections describe the importance of lipid oxidation and, its implications in food and health, the role of reactive oxygen species (ROS), and synthetic and natural antioxidants.

2.1 Lipid oxidation

Lipids occur in nearly all foods and biological materials with the major classes being triacylglycerols, which occur in fat storage cells of plants and animals, and phospholipids in biological membranes. Lipids in foods and biological systems are important substrates for oxidative reactions, which produce off flavours in foods. Oxidation of lipids is a common and undesirable chemical change that may affect the flavour, aroma, texture as well as nutritional quality of food. Products of lipid oxidation are known to disturb many vital biological reactions, and there is overwhelming evidence to indicate that free radicals cause oxidative damage to lipids, proteins, and nucleic acids, thus leading to a number of diseases including cardiovascular and neurodegenerative diseases, cancer, and aging (Halliwell, 1994; Yu, 1994).

2.1.1 Autoxidation

Autoxidation is a natural process that takes place between molecular oxygen and unsaturated lipids in the environment. In food systems, autoxidation of unsaturated fatty acid moieties brings about deterioration of food lipids. Autoxidation proceeds in three distinctive steps of initiation, propagation and termination. The first step is initiation in which lipid radicals are formed from lipid molecules. Abstraction of a hydrogen atom by a reactive species such as a hydroxyl radical may lead to initiation of lipid oxidation. However, it is possible to have traces of preformed hydroperoxides due to the action of lypoxygenase enzyme during extraction of plant oils (Gordon, 2001a). The production of free radicals in the initiation step needs activation energy of about 35 kcal/mole. As the initiation reaction (Reaction 1) is thermodynamically unfavourable, production of the first few radicals is necessary to begin the propagation reaction (Nawar, 1995). The reaction of a lipid with molecular oxygen, in its excited singlet oxygen state ($^1O_2$), or by metal catalysts or by exposure to light can form lipid peroxyradicals (Shahidi and Wanasundara, 1992).

After initiation, propagation reaction (reactions 2 and 3) occurs in which one lipid radical is converted into a different lipid radical. These reactions commonly involve abstraction of a hydrogen atom from a new lipid molecule or addition of oxygen to an alkyl radical. The enthalpy of this reaction is relatively low compared with that of the initiation reaction, so propagation reactions occur rapidly compared with initiation reactions. The oxygenation reaction is very rapid, having almost zero activation energy; therefore, the concentration of peroxyl radical (ROO·) is much higher than that of alkyl

radical (R·) in foods in which oxygen is present (Shahidi and Wanasundara, 1992). Abstraction of hydrogen takes place preferentially at carbon atoms where the bond dissociation energy is low. Since dissociation energy of the C-H bond is reduced by neighbouring alkene functionality, abstraction of hydrogen takes place most rapidly at the methylene group between two alkene groups i.e. diallylmethylene in a polyunsaturated fatty acid. The reaction is propagated by further abstraction of hydrogen atoms from other unsaturated fatty acids. During the formation of hydroperoxides, a shift in the position of double bond could occur in order to take advantage of the resultant resonance stabilization.

Termination reactions combine two free radicals to form molecules with a full complement of electrons and these are low energy reactions that are limited by the low concentration of radicals and by the requirement for radicals with the correct orientation for collision reactions (Reactions 4 through 6). Hydroperoxides formed during propagation reactions decompose spontaneously at 160°C (Chang *et al.*, 1976) and the peroxy radical concentration can become relatively high leading to the formation of polymers (Gordon, 1990). Hydroperoxide decomposition may also lead to the formation of alkoxy radicals which can decompose to release volatile hydrocarbons, alcohols and aldehydes among others.

Initiation

$$RH \longrightarrow R^{\cdot} + H^{\cdot} \quad (1)$$

Propagation

$$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot} \quad (2)$$

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot} \quad (3)$$

Termination

$$R^{\cdot} + R^{\cdot} \longrightarrow RR \quad (4)$$

$$R^{\cdot} + ROO^{\cdot} \longrightarrow ROOR \quad (5)$$

$$ROO^{\cdot} + ROO^{\cdot} \longrightarrow ROOR + O_2 \quad (6)$$

2.1.2 Photooxidation

Photooxidation provides an alternate route leading to the formation of hydroperoxides instead of free-radical mechanism. Basically there are two types of photooxidation; type I (lipid is excited) and type II (oxygen is excited) (Chan, 1977). Type I photooxidation occurs in the presence of photosensitizers such as riboflavin and is characterized by hydrogen atom- or electron- transfer between excited triplet sensitizer and a substrate such as polyunsaturated fatty acids in order to produce free radical or free radical ions (Gordon, 2001a). Sensitizer after absorbing light, reacts with the substrate to form an intermediate that may thereafter react with ground state (triplet) oxygen to yield oxidative products (Reactions 7 and 8).

In type II photooxidation, molecular oxygen rather than the substrate is excited upon absorption of light (Reactions 9 and 10). Oxygen in the environment is in the triplet

state, which has the lowest energy level in which the two highest energy electrons have parallel spins and are in two different molecular orbital. However, triplet oxygen can be excited by light to singlet oxygen in the presence of sensitizers such as chlorophyll. Singlet oxygen is more active and faster in reacting with unsaturated lipids.

Type I

$$\text{Sens} + A + h\nu \longrightarrow \text{Intermediate-1} \quad (7)$$

$$\text{Intermediate-1} + O_2 \longrightarrow \text{Products} + \text{Sens} \quad (8)$$

Type II

$$\text{Sens} + O_2 + h\nu \longrightarrow \text{Intermediate-2} \quad (9)$$

$$\text{Intermediate-2} + A \longrightarrow \text{Products} + \text{Sens} \quad (10)$$

2.2 Factors affecting lipid oxidation

The extent to which oxidation of fatty acids and their esters occurs in food depends on the chemical structure of the fatty acids involved and minor constituents present in the oxidizing system as well as environmental conditions where the food is being processed and stored.

2.2.1 Role of Enzymes

Several enzyme systems are capable of initiating lipid oxidation. Lipoxygenase (LOX), a group of non-haem iron deoxygenase, catalyzes the formation of lipid hydroperoxide from unsaturated fatty acids (Hammarberg, 2001). Lipoxygenase stereospecifically abstracts a hydrogen atom from an active methylene group in the 1,4-

pentadiene structure of PUFA and releases a stereospecific conjugated dienes hydroperoxy fatty acid product (Erickson, 2002). Although LOX is well known for off-flavour development in vegetables and legumes, it has also been found in animal tissues like fish and chicken. The presence of LOX has been reported in liver, lung, kidney and testes of chicken (Goetzl *et al.,* 1980) and in muscle gastroanemius (Grossman *et al.*, 1988). Four isozymes of LOX have been identified in soybean (Gordon, 2001a).

The enzymes in oil-bearing seeds such as soybean can be an important source of hydroperoxides formed during oil extraction. In vegetables, oxidative changes due to the enzymes may lead to off flavours during storage (Gordon, 2001a).

2.2.2 Role of oxygen species in lipid oxidation

Triplet oxygen gives rise to singlet oxygen, which is more electrophillic than triplet state oxygen and hence reacts rapidly with unsaturated compounds. Lipid peroxidation is reported to be initiated by a number of oxygen species. Singlet oxygen is known to react with lipids according to an "ene" addition mechanism to produce hydroperoxides (Foote, 1976).

2.2.3 Role of metal ions in lipid oxidation

Transition metal ions such as cobalt, iron, manganese, copper, zinc and nickel are abundantly found in both living organisms and foods. It has been suggested that oxidative deterioration of the lipids in meat is caused by catalysis of haem compounds such as metmyoglobin (Kwoh, 1971). These metal-containing compounds catalyze

hydroperoxide decomposition, thus producing free radicals, which initiate further chain reactions (Pokorny, 1987). The redox-active transition metals transfer single electrons during changes in oxidation states (Reische *et al.*, 2002). Two mechanisms of oxidation promotion by metals have been proposed. Metals are believed to either interact with hydroperoxides or to react directly with lipid molecules producing lipid radicals.

$$M^{(n-1)+} + RH \longrightarrow M^{n+} + H^{+} + R^{\cdot} \quad (11)$$

Metals are also known to promote lipid oxidation by interacting with hydroperoxides formed in propagation reactions. Transitional metal ions in their lower valence state react very quickly with hydroperoxides. It is also believed that metal-hydroperoxide complexes are formed and subsequently decomposed to free radicals (Reische *et al.*, 2002). Metals enhance the rate of decomposition of hydroperoxides and generation of free radicals. The two possible reactions are shown below.

$$M^{(n-1)+} + ROOH \longrightarrow M^{n+} + H^{+} + ROO^{\cdot} \quad (12)$$

$$M^{(n-1)+} + ROOH \longrightarrow M^{(n+1)+} + OH^{-} + RO^{\cdot} \quad (13)$$

Even trace amounts of transition metal ions can promote electron transfer from lipids or hydroperoxides as the above reactions are cyclical, generating lower valence state of metal ions (Reische *et al.*, 2002). According to Reische and coworkers (2002) it is not clear whether redox-active transition metals promote lipid peroxidation directly through the formation of metal-lipid complexes or by forming peroxy and oxy radicals.

The redox potential of metals such as manganese and cobalt is too low to cause hydroperoxide decomposition in aqueous systems, but it might catalyze hydroperoxide decomposition in non-polar systems through a metal-hydroperoxide complex (Kanner *et al.*, 1987). Ferritins, the main molecules containing iron in biological systems, have been reported to promote lipid oxidation of muscle foods during initiation and propagation (Kanner and Doll, 1991). Ferrous ions can be released from ferritin by reductants small enough to pass through channels in the protein shell that surrounds certain iron core of the ferritin molecule (Kanner and Doll, 1991).

Ferrous ions in aerobic aqueous solutions can produce superoxide, hydrogen peroxide and hydroxyl radicals by Fenton reactions (Reactions 14 through 17). These reactions can be cycled either by superoxide radical (Harber-weiss) or by reducing agents like thiols or ascorbic acid (Redox-cycle). Ferrous ions can stimulate lipid peroxidation by generating hydroxyl radical from hydrogen peroxide but also by the breakdown of preformed lipid peroxides to form alkoxy radical ($RO^{\cdot}$) (Reaction 17).

$$Fe^{2+} + O_2^{\cdot -} \longrightarrow Fe^{3+} + O_2 \qquad (14)$$

$$2O_2 + 2H^{+} \longrightarrow H_2O_2 + O_2 \qquad (15)$$

$$Fe^{2+} + H_2O_2 \longrightarrow HO^{\cdot} + HO^{-} + Fe^{3+} \qquad (16)$$

$$ROOH + Fe^{2+} \longrightarrow RO^{\cdot} + HO^{-} + Fe^{3+} \qquad (17)$$

In muscle tissue, iron exists in a protein-bound form in myoglobin, haemoglobin, ferritin, transferrin, ovotransferrin, lactotransferrin and haemosderin all of them being important

in catalyzing lipid oxidation (Love, 1987; Decker and Hultin, 1992; Wettasinghe and Shahidi, 1996)

2.2.4 Role of water activity in lipid oxidation

The role of water activity in lipid peroxidation has been examined extensively. Water activity has been manipulated as a means to control lipid oxidation in susceptible food products and to explain the relationship between lipid oxidation rates and moisture content (Nelson and Labuza, 1992).

As water activity is decreased from 1, the rate of oxidation initially increases, reaching a maximum of 0.6 – 0.8 water activity range and then decreases again as water activity range reaches a minimum in the range of 0.3-0.4 and tends to increase afterwards (Figure 2.1).

2.2.5 Role of ionizing radiation in lipid oxidation

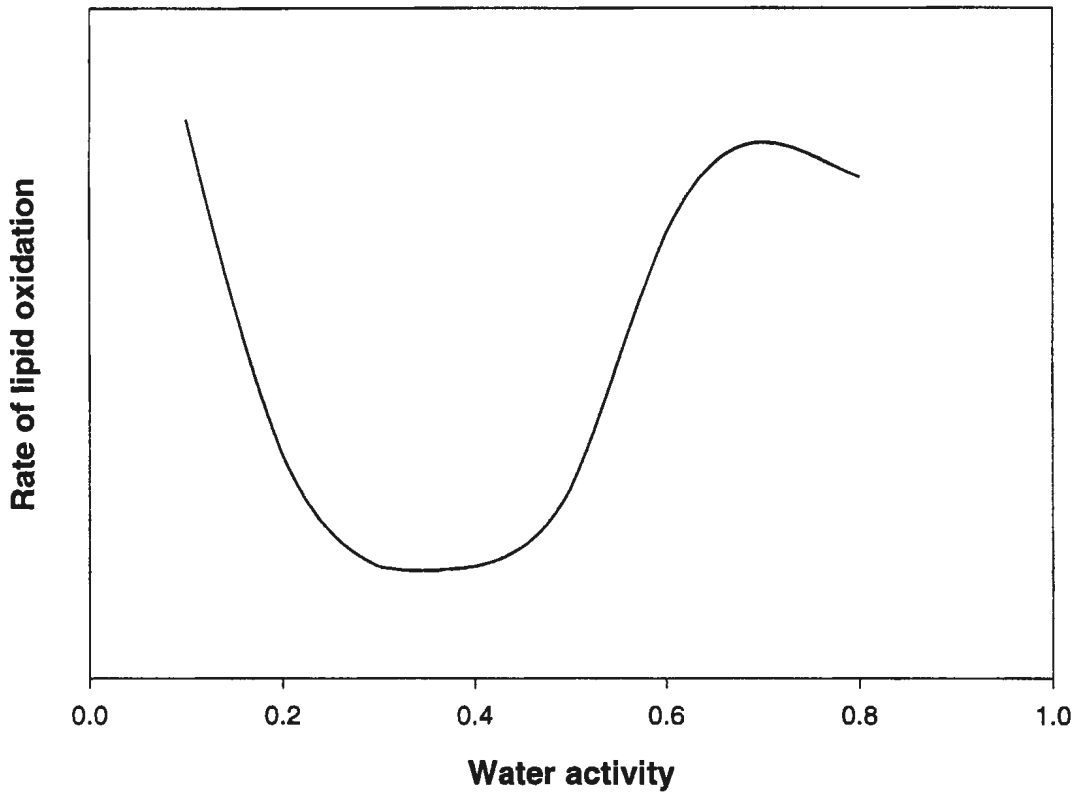
Ionizing radiation (x-and γ-rays, high energy electron and particles) generates free radicals via ionization of molecules (Simic *et al.*, 1992). These particles and electromagenetic waves can ionize atoms and molecules by ejecting electrons from them, thus forming positively charged species in the parent material. Electrons ejected by them possess sufficient energy to produce further ionization and excitation (Schaich, 1980).

2.3 Effect of lipid oxidation on food quality

Quality deterioration due to oxidation of lipids can take place in all types of food, including meat, seafood, spray-dried milk powder, vegetables, fruits, and oils (Decker and Hultin, 1992; Perkins, 1992; Roozen and Linssen, 1992; Spanier *et al.*, 1992). The changes in quality can be manifested by deterioration of flavour, colour, texture, nutritive value and production of toxic compounds (Kanner *et al.*, 1987; Kanner, 1992). Oxidation products of cholesterol are considered to be mutagenic and carcinogenic (Watanabe *et al.,* 1988; Paniangvait *et al.*, 1995) as well as harmful to the cells in blood vessels such as macrophages and endothelial cells leading to cardiovascular and pulmonary diseases (Kumar and Singhal, 1991).

Hydroperoxides are formed in the initial stage of autoxidation and these are also formed in lipoxygenase-catalysed oxidation. Although hydroperoxides are non-volatile and odourless, they are relatively unstable compounds that decompose either spontaneously or upon catalyzed reactions to form volatile aroma compounds, which are perceived as off-flavours (Gordon, 2001a). A variety of compounds, such as hydrocarbons, alcohols, aldehydes, ketones, furans, and acids can be formed during oxidation. Aliphatic carbonyl compounds, such as alkanals, trans,trans-2,4-alkadienals, trans,cis-2,4-alkadienals, and vinyl ketones, have the lowest threshold values which can affect the quality of edible oils (Min and Boff, 2002). Furthermore, oxidative deterioration of lipids can lead to bleaching of foods due to the reaction of lipid

Figure 2.1 Effect of water activity on lipid oxidation.
(Adapted from Nelson and Labuza, 1992)


Rate of lipid oxidation
0.0
0.2
0.4
0.6
0.8
1.0
Water activity

breakdown intermediates with pigments such as carotenoids. Figure 2.2 depicts formation of secondary oxidation products upon hydroperoxide decomposition.

A major factor involved in the loss of flavour or quality in meat is production of warmed-over flavour (WOF) described by Tims and Watts (1958). Meat flavour deterioration (MFD) is believed to be due to oxidation of membrane phospholipids catalyzed by iron. Dupuy *et al.* (1987) reported that WOF in meat is mainly due to the production of propanal, pentanal, hexanal, 2,3-octanedione, nonanal, heptanal, octanal, tetradecane and phenyl acetaldehyde. Off-flavour development due to lipid oxidation can occur in all types of raw, cooked, cured, uncured, canned and frozen meats and meat products (Dupuy *et al.*, 1987). It is also responsible for the changes in colour, texture and nutritional value as well as wholesomeness of muscle food (Shahidi, 1992).

The rate of MFD is primarily determined by the degree of unsaturation of the fat. Thus, meat flavour deterioration and rancidity development are faster in fish lipids followed by chicken, pork and beef (Tichivangana *et al.*,1985). The degradation of texture, flavour and odour of stored seafood is attributed to the oxidation of unsaturated lipids. Flick *et al.* (1992) reported that the skin and dark muscle of fish are more susceptible to lipid oxidation compared with that of white muscle.

Lipid oxidation in fish continues even at freezing temperatures, especially in fatty fish. Fish oils produce ketones, aldehydes and hydroxyacids (Flick *et al.*, 1992). Oxidation of $\omega 6$ fatty acids can produce hexanal during the oxidation of terrestrial animal

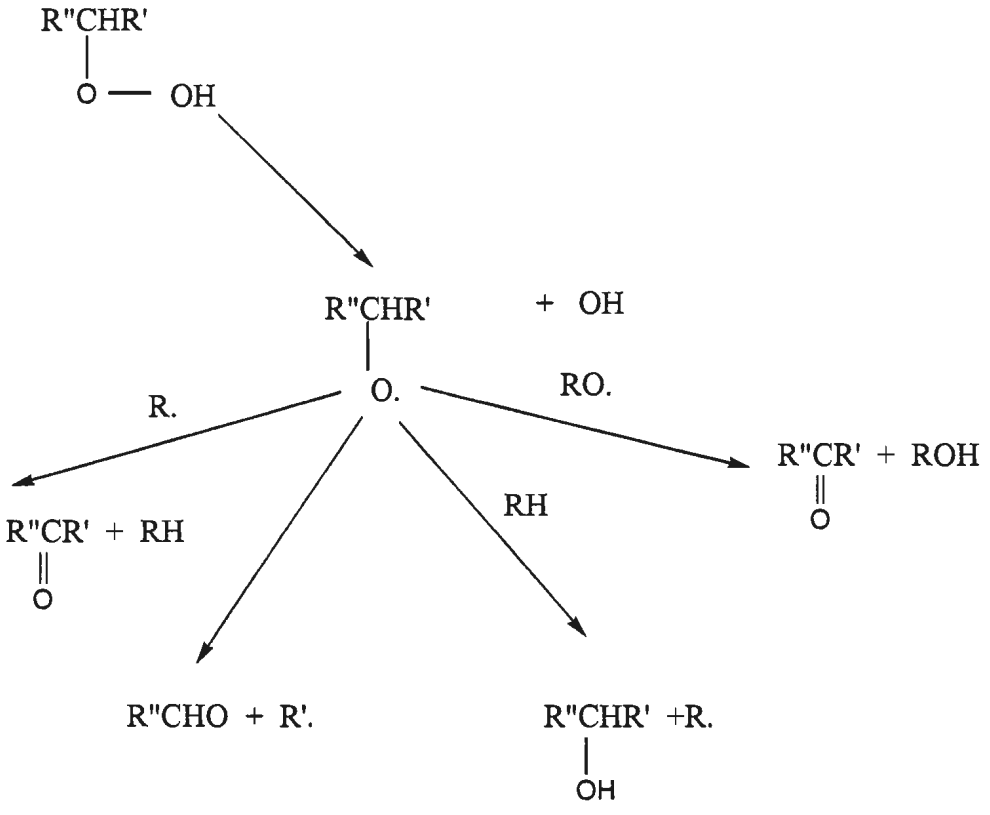
and plant lipids (Frankel *et al.,* 1989). Hexanal formation leads to the characteristic grassy flavour note (Shahidi and Pegg, 1992). Loss of ω3 fatty acids during oxidation can be minimized by judicious use of antioxidants, controlled exposure to oxygen and lower temperatures (Flick *et al.*, 1992).

Roozen and Linssen (1992) reported that spray-dried infant milk formulas which are enriched with PUFA and minerals are very sensitive to autoxidation. Hydroperoxides formed interact with milk proteins causing chemical changes including protein-protein cross-linking, protein scission, and amino acid damage (Gardner, 1979).

2.4 Impact of lipid oxidation on human health

Interest in the biochemistry of lipid peroxidation products, especially free radicals, has grown exponentially in the last two decades. There is an abundance of evidence that lipid peroxidation products are involved in the pathogenesis of certain human diseases and many tissue injuries. Lipid peroxidation is a very damaging process that is widely investigated in biological systems. Lipid peroxidation damages cells directly by attacking membrane structure and indirectly by releasing reactive products. Lipid oxidation products are broadly classified into two categories of primary and secondary. Lipid hydroperoxides, which are formed in the propagation steps, are considered as primary products. Aldehydes, ketones, hydrocarbons, acids and alcohols are among the secondary oxidation products

Figure 2.2 Formation of secondary lipid oxidation products upon hydroperoxide decomposition.


R"CHR'
O — OH
R"CHR'
O.
+ OH
R.
RO.
R"CR' + RH
O
R"CR' + ROH
O
RH
R"CHO + R'.
R"CHR' +R.
OH

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

The compound 4-hydroxynonenal (HNE) is known to have lethal effects on cells and inhibition effects on DNA synthesis (Cheeseman, 1993). HNE is biologically very active and causes severe disturbance of cell functions (Esterbauer *et al.*, 1991b). Siems and coworkers (1996) have reported that HNE effectively inhibits Na^+ and K^+-ATPase and is involved in transport inhibition following tissue injury.

Fluidity is one of the important features in biological membranes. Peroxidation of membrane lipids can reduce the fluidity and electrical resistance leading to leaky cells with impaired barrier properties (Cheeseman, 1993). Oxidative attack on cell membranes is well known to decrease the activity of their associated enzymes. Other than the direct effects from lipid oxidation, indirect effects caused by peroxidation products are also of great importance. The significance of these products is that they are biologically active and can diffuse from the site of production, therefore they can be considered important toxic agents (Cheeseman, 1993). Carr *et al.* (1996 and 1997) reported that HOCl, which

is generated in substantial amounts in cells, reacts with cholesterol leading to the formation of cholesterol chlorohydrins that could be potentially disruptive to cell membranes. Zavodnik *et al.* (2001) reported that HOCl treatment of erythrocyte membranes resulted in inhibition of Na^+, K^+ and Mg^{2+}-ATPase activities, oxidation of SH groups, tryptophan residues, formation of chloramine, and changes of membrane fluidity and surface area and membrane morphological transformations.

2.5 Reactive oxygen species and human health

In recent years, it has been shown that reactive oxygen species (ROS) are closely involved in various biological reactions. ROS and related oxidants are by-products of normal biological functions such as electron transport, oxygen utilizing systems, peroxisomes as well as lipid peroxidation (Ames and Shigenaga, 1993). Reactive oxygen species include not only the oxygen radicals but also some non-radical derivatives of oxygen. These oxygen radicals include superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxy, ($RO^{\cdot}{}_2$), alkoxy, ($RO^{\cdot}$) and hydroperoxy ($HO^{\cdot}{}_2$) radicals. Non-radicals belonging to ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and peroxynitrite (ONOO), among others. At normal rate of generation, some free radicals are useful in the human body and some are essential to many intracellular metabolite reactions and are considered critical for phagocytes to kill ingested microorganisms. However, when oxygen free radicals generation exceeds the capacity of antioxidant defense of the body results in oxidative stress, which makes a significant contribution to the pathogenesis of a number of diseases. Superoxide and hydroxyl radical are believed to mediate a large portion of the tissue damage produced

after inflammation, ischemia, and ischemia/perfusion of the small intestine, stomach, heart, kidney, liver, and skin (Shahidi, 1997; Yoshikawa *et al.,* 1997). In addition, ROS metabolites and free radicals are involved in many pathogenic conditions via DNA damage, inactivation of nitric oxide, oxidation of LDL and activation of adhesion molecules. Polidori and coworkers (2002) found high concentrations of malonaldehyde (MA) in patients affected by congestive heart failure (CHF) concluding that lipid peroxidation has a close relationship with CHF. Figure 2.3 shows the role of ROS in various pathological conditions.

The first recognized free radicals were those produced by homolytic dissociation of water brought about by ionizing radiation to form hydrogen atom and hydroxyl radical. Superoxide can be formed from various enzymatic reactions such as those catalyzed by flavin oxidase, xanthine oxidase and monoamine oxidase, as well as from autoxidation of various thiols such as glutathione, catecholamine, and ascorbic acid, among others (Knight, 1999). Another source of *in vivo* superoxide formation is the "respiratory burst" that occurs in phagocytic cells (neutrophils, monocytes and macrophages). Upon invasion by microorganisms, the cells increase oxygen uptake and elevate the production of superoxide and hypochlorous acid. Superoxide is converted to hydroxyl free radical, which is important in the killing of bacteria (Knight, 1999). Oxygen derived free radicals generated from eosinophils play a critical role in asthma and are closely linked with bronchial hyperresponsiveness (Yoshikawa *et al.,* 1997).

Hydrogen peroxide, although not reactive by itself, is produced along the electron transport as well as through autoxidation of small molecules and dismutation of hydroxyl radical by superoxide dismutase (SOD). In the presence of reduced metal ions it generates the highly reactive $OH^{\cdot}$ via Fenton reaction.

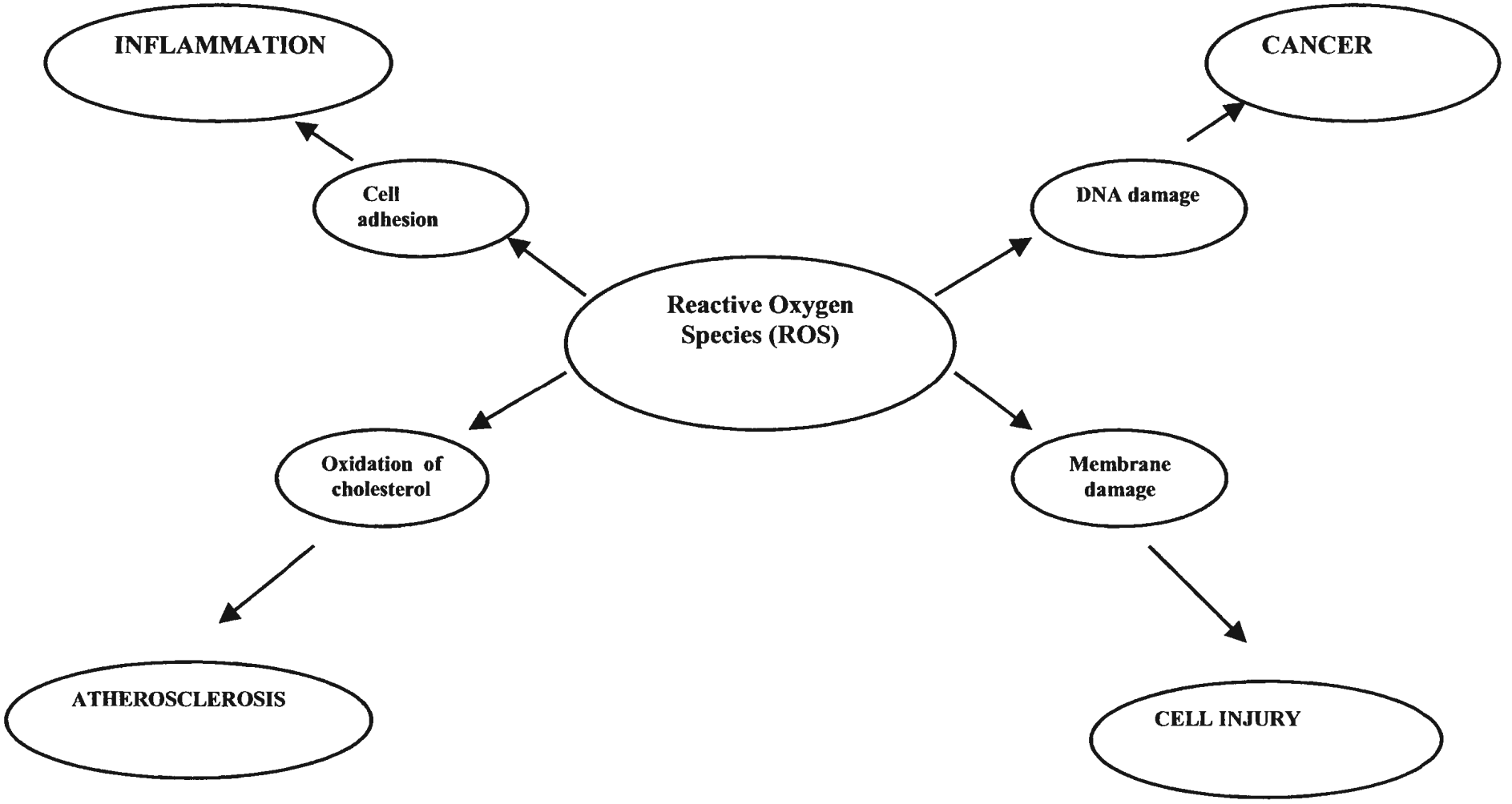
When ROOH is generated in membranes, peroxyl radical can react with amino acid residues of membrane proteins and impair their function. They can accept hydrogen atoms from the adjacent PUFA side chains, thus propagating the free radical chain reactions of lipid peroxidation. Therefore, a single initiating event can result in the conversion of hundreds of fatty acid side chains into lipid peroxides which alter the structural integrity and biochemical functions of membranes (Yoshikawa *et al.*, 1997), thus resulting in membrane lipid peroxidation, decrease in membrane fluidity, loss of enzyme and receptor activity, and damage to membrane proteins leading to cell inactivation (Dean *et al.*, 1993).

2.5.1 Reactive oxygen species and DNA damage

There is abundant evidence that ROS attack DNA base pairs leading to mutagenesis, carcinogenesis and aging (Shigenaga *et al.*, 1989; Halliwell and Gutteridge, 1990; Ames and Shigenaga, 1993; Dizdaroglu, 1993). ROS play a role in neurodegenerative diseases such as Parkinson and Alzheimer (Salles *et al.*, 1999). Oxidant-induced DNA damage and mitogenesis are two important causes of cancer. Oxidants that are produced by normal aerobic metabolism contribute to extensive DNA damage that has been measured in rodents and humans (Ames and Shigenaga, 1993).

ROS start attacking DNA when the capacity of intracellular ROS scavengers and antioxidants go down. The oxidative damage rate in mammalian species such as rats, with a high metabolic rate, short life span, and high age-specific cancer rate is much higher than the rate in species such as humans, with a lower metabolic rate, long life span and a lower age-specific cancer rate (Ames and Shigenaga, 1993). ROS can lead to DNA damage by either direct chemical attack on DNA or by indirect mechanisms such as the activation of calcium-dependent endonuclease (Halliwell and Gutteridge, 1999). Among different ROS, hydroxyl radical is the most reactive one towards biological molecules and generates a large number of modifications in DNA such as base and sugar damage, strand breakage and DNA-protein cross-linking (Dizdaroglu, 1993). Superoxide, nitric oxide and hydrogen peroxide, at physiologically relevant levels, do not appear to react with any of the DNA or RNA bases or with ribose or deoxyribose sugars at any measurable rate (Halliwell and Gutteridge, 1999). By contrast, hydroxyl radical exposure to DNA generates a multitude of products by attacking sugars, purines and pyrimidines. Hydroxyl radical can add onto guanine at positions 4, 5 or 8 in the purine ring. Addition to C-8 produces a C-8 OH adduct radical that can be reduced to 8-hydroxy-7, 8-dehydroguanine, oxidized to 8-hydroxyguanine or undergo opening of the imadazole ring, followed by one-electron reduction and protonation, to afford 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Halliwell and Gutteridge, 1999). Similarly, $^{\cdot}OH$ can add onto C-4, C-5 or C-8 of adenine.

Figure 2.3 Role of reactive oxygen species in various pathological conditions (Adapted from Yoshikawa *et al.,* 1997)


INFLAMMATION
CANCER
Cell adhesion
DNA damage
Reactive Oxygen Species (ROS)
Oxidation of cholesterol
Membrane damage
ATHEROSCLEROSIS
CELL INJURY

Halliwell and Gutteridge (1999) reported that pyrimidines are also attacked by hydroxyl radical to afford a multitude of products. Hydrogen peroxide can arise from superoxide anion by catalyzed or spontaneous dismutation, and thus it is generated during intracellular autoxidation of many reduced xenobiotics or following enzymatic formation of $O_2^{\cdot-}$. In addition, several oxidases produce hydrogen peroxide without the intermediacy of $O_2^{\cdot-}$. According to the findings of Meneghini and Martins (1993), hydrogen peroxide is of extreme importance in terms of DNA damage because it can cross cell membranes easily whereas $O_2^{\cdot-}$ cannot. This is supported by the findings of Halliwell and Gutteridge (1999) who reported that the addition of hydrogen peroxide to many mammalian cell types produces increased strand breakage within a few minutes and brings about an increase in the production of DNA base modification products. The chemical pattern of the damage is consistent with the attack by hydroxyl ion. Hydrogen peroxide itself, like superoxide anion radical, does not damage DNA. Furthermore, Halliwell and Gutteridge (1999) reported that damage to DNA by peroxides could be prevented by pre-treating the cells with certain metal chelating agents such as o-phenanthroline. This shows that hydroxyl ions available in the nucleus or the metal ions released during oxidative stress can contribute to the formation of hydroxyl ion (Halliwell and Gutteridge, 1999).

Hydroxyl radical reacts with the sugar moiety of DNA by abstracting a hydrogen atom from each of the carbon atoms. Further reactions of the so formed carbon-centred sugar radicals generate various sugar products, DNA strand breaks and base-free sites by a variety of mechanisms (Dizdaroglu *et al.*, 2002). Modified sugars are either released

from DNA or bound to DNA with one or both phosphate linkages. 2-Deoxypentose-4-ulose, 2,5-dideoxypentose-4-ulose, erythrose, 2-deoxytetrodialdose, and glycolic acid are some of the free-radical induced products of the sugar moiety in the DNA (Dizdarogle *et al.*, 2002).

2.5.2 Reactive oxygen species and atherosclerosis

It is postulated that modification of LDL to a form recognized by the acetyl-LDL receptor is required for lipid loading of macrophage-derived foam cells (Goldstein *et al.*, 1979). Acetylated-LDL is rapidly taken up by scavenger receptors resulting in cholesterol accumulation in macrophages with the resultant formation of foam cells leading to atherosclerogenesis (Knight, 1999).

Oxidized LDL is chemotactic for monocytes and T cells, which can result in further recruitment into the developing atherosclerotic lesions. In addition, oxidized LDL is toxic to macrophages and thereby inhibits their mobility by preventing them from reentering the circulation (Knight, 1999). Cell injury, or any event which may disrupt endothelial integrity and permeability properties, may be involved in the early events leading to atherosclerotic lesion formation (Henning and Chow, 1998). Among factors contributing to endothelial cell injury or dysfunction are free radical-mediated reactions, including lipid peroxidation. The reactive oxygen species generated result in cell damage through readily oxidizable target molecules, especially membrane polyunsaturated fatty acids (PUFA) (Henning and Chow, 1998). Unsaturated fatty acids found in the membrane phospholipids, glycolipids and transmembrane proteins containing oxidizable

amino acids are susceptible to free radical damage (Henning and Chow, 1988). Because of its constant exposure to blood components, including prooxidants, the endothelium is constantly subjected to oxidative stress and free radical-mediated reactions. This susceptibility of the endothelial cells to oxidative stress is increased when the cellular antioxidant system is inadequate. Lipid peroxidation products can mediate plaque formation or atherosclerosis by directly injuring endothelial cells and causing membrane malfunction; enhancing the uptake of LDL by endothelial and smooth muscle cells; inactivating prostacyclin synthetase of endothelial cells and thereby enhancing the adhesion of neutrophils and platelets; increasing the susceptibility of platelets to aggregate and enhance the proliferation of smooth muscle cells and increasing platelet formation of chemotactic factors required for the migration of smooth muscle cells (Henning and Chow, 1988). To the contrary, Steinbrecher and coworkers (1990) reported that it has not been established if there is a causal relationship between oxidative modification of LDL and atherogenesis.

2.5.3 Lipid peroxidation and Alzheimer's disease

There are extensive evidence that lipid peroxidation has a strong link to Alzheimer's disease (AD). Amyloid β-peptide formed from proteolytic cleavage of the transmembrane amyloid precursor protein is reported to cause lipid peroxidation in the brain cell membranes (Mark *et al.*, 1999). This has been evidenced by high 2-thiobarbituric acid reactive substances (TBARS) values reported in frontal lobe, sensory and occipital cortices of AD affected brains (Subarao *et al.*, 1990).

PUFA, including arachidonic acid (AA) and docosahexaenoic acid (DHA) are abundant in the brain (Butterfield and Lauderbak, 2002). Several studies have shown that PUFA content is decreased in AD affected brains and that lipid peroxidation occurs at a significant level in the AD brains (Butterfield and Lauderbak, 2002). Furthermore, enzymes involved in regulating oxidative stress are altered in AD especially glutathione peroxidase activity in AD hippocampus (Butterfield and Lauderbak, 2002).

2.6 Control of lipid oxidation in food

Consequences of lipid oxidation in food have drawn attention of researchers to find out ways for controlling it. Optimum oxidative stability can be achieved by minimizing exposure of lipids and lipid-containing food products to air, light and higher temperatures during processing and storage. Theoretically, the most elegant way of preserving fatty foods from oxidation is to remove all the available oxygen from the food during manufacturing and from the packaging container (Yanishlieva-Maslarova, 2001). However, it is not practical to remove traces of oxygen from food. Liquid oils are traditionally packaged in clear glass containers and brown or green bottles are sometimes used to protect unstable oils from photooxidation. Plastic containers now generally replace glass bottles. Polyvinyl chloride is preferred due to its superiority to polyethylene, which is more permeable to oxygen (Yanishlieva-Maslarova, 2001).

Over the decades, researchers have developed a number of means to retard lipid oxidation in foods; vacuum packaging (*sous vide*) or packing in an inert gas (Coppen, 1983), hydrogenation of unsaturated fatty acids (Nawar, 1996) and removal of metal ions

(Kanner, 1994) are among strategies employed for this purpose. In addition, use of superoxide scavengers such as glucose oxidase and ascorbic acid (Hsieh and Kinsella, 1989), refrigeration and freezing (Coppen, 1983), and addition of synthetic and natural antioxidants may be practiced (Shahidi and Wanasundara, 1992; Simic *et al.*, 1992).

The main justification of using antioxidants in foods is to extend the shelf life of foodstuffs and to reduce wastage and nutritional loss by inhibiting or delaying oxidation. The antioxidants can be defined as compounds capable of delaying, retarding or preventing autoxidation processes, when present at low concentrations compared to that of an oxidizable substrate (Halliwell and Gutteridge, 1999). The antioxidants regularly incorporated into food by manufacturers are of either synthetic or natural origin. Synthetic antioxidants include phenolics such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), propyl gallate (PG) and non-phenolics such as erythrobic acid and ascorbyl palmitate (Shahidi *et al.,* 1987; Frankel, 1996). Natural antioxidants include β-carotene naturally occurring ascorbic acid, amino acids, citric acid, certain nucleotides and dipeptides (Chen *et al.*, 1995), inorganic salts (Wettasinghe and Shahidi, 1996), tocols and their derivatives (Shahidi and Wanasundara, 1992; Hall, 2001), carotenoids, antioxidant enzymes and a large number of phenolic compounds of mainly plant origin. The importance of antioxidants contained in food is well appreciated for both preserving foods themselves and supplying essential antioxidants *in vivo*.

2.7 Types of antioxidants

Antioxidants are referred to substances that protect foods against autoxidation. The free radical chain process of autoxidation can be retarded by two groups of inhibitors; the first group suppresses autoxidation by scavenging the free radicals thus inhibiting chain initiation and/or breaking the chain propagation while the second group prevents autoxidation by acting in the first defense line by suppressing the formation of free radicals and reactive oxygen species (Yanishelieva-Maslarova, 2001). The first group of compounds is often called primary or chain breaking antioxidants and the second group is known as secondary or preventive antioxidants. Based on their mechanism, antioxidants can be divided into three categories as the antioxidants capable of transferring the free radical characteristics with the formation of a reactive derived radical, the ones that can trap free radicals with the formation of a stable or inert free radical trap and the molecules which mimic antioxidant enzymes found in biological systems.

2.7.1 Primary antioxidants

Primary antioxidants can be defined broadly as the compounds, which can react with lipid radicals to convert them to more stable products. Antioxidants have the ability to donate a hydrogen atom to lipid radicals and neutralize them (Reactions 14 and 15).

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot} \quad (14)$$

$$RO^{\cdot} + AH \longrightarrow ROH + A^{\cdot} \quad (15)$$

$$ROO^{\cdot} + A^{\cdot} \longrightarrow ROOA \quad (16)$$

$$RO^{\cdot} + A^{\cdot} \longrightarrow ROA \quad (17)$$

$$R^{\cdot} + A^{\cdot} \longrightarrow RA \quad (18)$$

The resulting antioxidant phenoxy radical (A˙) does not initiate new free radicals and is not subject to rapid oxidation by a chain reaction. The antioxidant radicals may also participate in termination reactions of ROO˙, RO˙ and other antioxidant radicals, thus preventing propagation of chain reactions.

These reactions (19-21) are exothermic in nature and the activation energy increases with increasing A-H and R-H bond dissociation energy (Shahidi and Wanasudara, 1992). A molecule will be able to act as a primary antioxidant if it is able to donate a hydrogen atom to a lipid radical and if the radical derived from the antioxidant is much more stable than the lipid radical, or is converted to other stable products. Phenol itself is inactive as an antioxidant, but substitution of alkyl groups into the 2, 4 or 6 positions increases the electron density on the hydroxyl group by an inductive effect and this enhances the reaction with lipid radicals (Gordon, 1990).

$$A^{\cdot} + O_2 \longrightarrow AOO^{\cdot} \qquad (19)$$

$$AOO^{\cdot} + RH \longrightarrow AOOH + R^{\cdot} \qquad (20)$$

$$A^{\cdot} + RH \longrightarrow AH + R^{\cdot} \qquad (21)$$

The presence of bulky substituents in the 2, 6 positions also reduce the rate of reaction of the phenol with lipid radicals. The steric effect opposes the increased stabilization of the radical and both effects must be considered in assessing the overall activity of an antioxidant (Gordon, 1990). The introduction of a second hydroxyl or

methoxy group at the *ortho-* or *para-* positions of the hydroxy group of a phenol increases its antioxidant activity.

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

2.7.2 Secondary antioxidants

Secondary antioxidants are compounds which retard the rate of autoxidation of lipids by processes other than that of interrupting the autoxidation chain reaction by converting free radicals to more stable species. These may operate by a number of mechanisms including scavenging oxygen, decomposing hydroperoxides to radical species, metal chelating, absorbing UV radiation or deactivating singlet oxygen (Gordon, 1990).

Food generally contains trace amounts of metals originating from haem pigments, enzymes or processing equipment. Metals, even at low concentrations, act as pro-oxidants by electron transfer, liberating radicals from fatty acids or hydroperoxides. Chelation of metal ions by food components may reduce the pro-oxidative effect of the

ions and raises the energy of activation of the initiation reaction. Citric acid, EDTA, phosphoric acid and their derivatives as well as malic and tartaric acids are found to be good metal chelators. Citric acid, which is widely used in foods, is a weaker chelating agent than EDTA. Metal chelators can stabilize the oxide forms of metal, thus preventing metals from promoting oxidation. In addition, the metal chelators form complexes with the metals making them unavailable to promote oxidation. The metal chelation activity of food phenolics depends on their molecular structure. As an example, activity of flavonoids requires the presence of the 3',4'-dihydroxy configuration and C-4 quinone and a C-3 or C-5 OH group (Hudson and Lewis, 1983).

2.7.3 Synthetic antioxidants

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

The toxicology of antioxidants has become one of the most controversial areas in the continuing debate in the safety of food additives. In recent years, problems have arisen with the antioxidants; new long-term studies have shown that BHA and BHT could

produce tumors in animals (Barlow, 1990). In the case of BHA, the increased incidence of tumors was seen in forestomach of the rat (Barlow, 1990; Williams *et al.*, 1999). In the case of BHT, an increase in liver tumors was observed in rat studies (Barlow, 1990). However, these two antioxidants did not appear to be genotoxic and therefore, raised the question about the mechanism by which they cause tumors (Barlow, 1990). Thus, Barlow (1990) concluded that BHT is not genotoxic *in vivo* and so it is unlikely that the tumors seen in mouse lung and rat and mouse liver are induced by genotoxic mechanisms. Furthermore, the only toxicological problem with BHA concerns its effects on the rodent forestomach and possible effects on rodent tissues in other species. Tests with other phenolics have shown that structurally related compounds can also induce forestomach hyperplasia.

Approximately 40 countries reportedly permit the use of BHT. Food grade BHA, referred to as 2(3)-tert-butyl-4-hydroxyanisole, is generally a mixture of greater than 85% of 3-tert-butyl-4-hydroxyanisole (3-BHA) and 15 % or less 2-tert-butylhydroxyanisole (2-BHA), while food-grade BHT which is 3,5-di-tert-butyl-4-hydroxytoluene is not less than 99% (w/w) pure (Williams *et al.,* 1999). Neither BHA nor its metabolites have shown adduct formation in genotoxicity studies (Williams *et al.*, 1999). BHA is metabolized to tert-butylhydroquinone (TBHQ) and tert-butylquinone (TBQ) in the liver. DNA damage has been reported with TBQ but not with BHA or TBHQ (Morimoto *et al.*, 1991). It has been found that BHA at high doses above 3000 ppm induces forestomach squamous cell carcinoma in rodents, but not glandular cell or other types of neoplasms in the glandular stomach. Humans do not have a forestomach and therefore are less

sensitive to exposures to BHA damage than rodents (Williams *et al.,* 1999). Moreover, these exposures to humans are well below those producing the epigenetic effects in rodents such as cell proliferation (Williams *et al.*, 1999). Whysner and Williams (1996) reported that human exposures to BHA (0.1 mg/kg/day) is far below the level reported for developing hyperplasia in rats (230 mg/kg/day) which is the most sensitive effect in rodents. Based on the latest evidences, BHA is not genotoxic or reproducibly carcinogenic although it can be at high doses (> 250 mg/kg/day) (Williams *et al.*, 1999).

BHT is a phenolic antioxidant that has been widely used in USA since its approval in 1954 by the FDA. BHT which is frequently added to various foods and animal feed is readily excreted in some animal species. However, in humans, it has been found at high concentrations in adipose tissues (Collings and Sharratt, 1970; Lambert *et al.*, 1996).

No reports are found to the effect that BHT is causing cancer in humans. Moreover, BHT is known to inhibits UV-carcinogenesis in animals (Black *et al.,* 1978; Lambert *et al.*, 1996), provides protection to some other UV-evoked physiological conditions, inhibits lipid and cholesterol oxidation which may produce possible carcinogen, and also acts as an antiviral agent by changing the membrane fluidity (Snipes *et al.*, 1975). The mechanism by which BHT exerts its antioxidative activity involves the quenching of ROS (superoxide, hydroxyl, peroxyl radical and singlet oxygen) and lipid soluble radicals.

Synthetic phenolic antioxidants are always substituted by alkyl groups to improve their solubility in fats and oils. The most suitable antioxidant for vegetable oils is TBHQ while BHA and BHT are often used for stabilization of fats in baked and fried products (Yanishlieva-Maslarova, 2001). TBHQ is regarded as the best antioxidant for protecting frying oils against oxidation. It is adequately soluble in fats and does not complex with iron. TBHQ can be used alone or in combination with either BHA or BHT at a maximum amount of 200 ppm based on the fat content (Shahidi and Wanasundara, 1992).

The efficiency of antioxidants depends on many factors including water activity, storage conditions, amount and the nature of lipid as well as physical state of the food (Loliger, 1991). However, volatile antioxidants such as BHA and BHT are readily lost during heat processing. TBHQ and gallate esters can be used in situations where food processing induces a great loss of BHA and BHT e.g. evaporation of water during drying (Loliger, 1991). BHA, BHT, trihydroxybutyrophenone (THBP), 4-hydroxymethyl 2,6-di-tert-butylphenol, thiodipropionic acid, and TBHQ may be used provided that the total antioxidant content does not exceed 200 ppm of the fat or oil content according to good manufacturing practices (GMP) (Mikivo, 2001). A list of some of the antioxidants reviewed in *Codex Alimentarius* is listed in the Table 2.1

Table 2.1 List of some of the antioxidants reviewed in *Codex Allimentarius*

INS	Chemical name of the antioxidant
220	sulphur dioxide
223	sodium metabisulphite
226	calcium sulphite
228	potassium bisulphite
300	ascorbic acid
301	sodium ascorbate
302	calcium ascorbate
303	potassium ascorbate
304	ascorbyl palmitate
305	ascorbyl stearate
307	alpha-tocopherol
308	synthetic gamma-tocopehrol
309	synthetic delta-tocopherol
310	propyl gallate
311	octyl gallate
312	dodecyl gallate
313	ethyl gallate
314	guaiac resin (gum guaiac)
315	isoascorbic acid (erythorbic acid)
317	potassium isoascorbate
318	calcium isoascorbate
319	tertiary butylhydroquinone (TBHQ)
320	butylated hydroxyanisole (BHA)
321	butylated hydroxytoluene (BHT)
322	lecithins
323	anoxomer
324	ethoxyquin
330	citric acid
384	isopropyl citrate
385	calcium disodium ethylenediamnietetraacetate
386	disodium ethylenediamnietetraacetate
387	oxystearin
388	thiodipropionic acid
391	phytic acid
512	stannous chloride
539	sodium thiosulphate
1102	glucose oxidase

INS - International numbering system for food additives

Adapted from Mikova, 2001

Only antioxidants which satisfy the requirements, laid down by the scientific committee for food may be used in foodstuff. With regard to the most recent scientific and toxicological information on these antioxidants, only the suitable and safe ones are permitted for some foods under certain conditions. In general antioxidants are not permitted in foods such those for infant foods, dairy products and egg products (Mikiova, 2001). However, a few antioxidants are permitted in infant weaning foods under strict regulations set forth by the European Union (Directive 89/389/EEC) (Makivo, 2001). Table 2.2 lists the antioxidants permitted for foods for infants and young children.

The use of antioxidants in foods is governed by regulations of individual countries as well as international bodies. For instance the use of antioxidants in the USA is governed by regulations of the federal drug and cosmetic act, meat inspection act, poultry inspection act and various state Laws; in Australia it is governed by the National Food Authority (NFA) (Mikova, 2001). Even though many natural and synthetic compounds have antioxidant properties only a few are accepted as generally recognized as safe (GRAS) international bodies such as joint FAO/WHO expert committee on food additives (JECFA) and the European community's scientific committee for food (SCF) (Mikova, 2001).

Table 2.2 Antioxidants permitted in foods for infant and young children.

Antioxidant	**Food stuff**	**Maximum permitted level**
Citric acid	weaning food	*quantum satis*
L-Ascorbic acid & Sodium L-ascorbate	baby foods	300 ppm
Calcium L-ascorbate	infant cereals & Biscuits	200 ppm
Tocopherol	biscuits, rusks & Baby foods	100 ppm
Lecithin	biscuits, rusks, Infant cereals & Baby foods	1000 ppm

Adapted from Mikova, 2001

An antioxidant is considered safe if its LD_{50} value does not fall below 1000ppm based on the body weight and the antioxidant does not have any significant effect on the growth of the experimental animals in long-term studies at a level of 100 times greater than that proposed for human consumption (Lehman *et al.*, 1951). A good antioxidant should be soluble in fats, should not impart a foreign colour, odour or flavour even over a long storage period; should be effective for at least one year at room temperature; should be stable to heat during processing and protect the finished product; should be easy to incorporate and should be effective at low concentrations (Mikova, 2001). Antioxidants can be added directly to vegetable oils and melted fats. Antioxidants can also be administered in diluents such as propylene glycol, sprayed with, or dipped in solutions or suspensions, or incorporated into the packaging film.

Sulphur dioxide, which is generally classified as a preservative against microbial contamination can be regarded as an antioxidant when the risk of oxidation is greater than the risk of microbial spoilage. Sulphur dioxide is allowed in the range of 15-2000 ppm, depending on the foodstuff (Makivo, 2001).

In some circumstances, phenolic antioxidants exhibit prooxidant and genotoxic effects. Aruoma *et al.* (1993) reported that gallic acid and its derivatives can exert a prooxidant effect towards DNA and carbohydrates, presumably by reacting with iron and this can be attenuated by the presence of protein, but resulting in protein damage. According to Li and coworkers (2000), 1,2,4-benzenetriol, caffeic acid, gallic acid and gossypol can cleave DNA strands by exerting a prooxidant effect in biological systems.

According to Aruoma (1991), the antioxidants such as PG, myricetin and quercetin as well as other antioxidants being developed for food use cannot simply be classified as antioxidants on the basis of experiments performed in lipid systems. The attention must also be paid to the potential pro-oxidant activities of food additives.

2.7.4 Natural antioxidants

The investigation of natural antioxidants for food preservation has received much attention due to the general resistance to synthetic food additives by consumers and manufacturers. Natural antioxidants include plant polyphenolics that may occur in many parts of the plants. Plant phenolics are multifunctional and can act as reducing agents, metal chelators, and singlet oxygen quenchers (Shahidi and Wanasundara, 1992).

Natural antioxidants occurring in foods may be used as a component of composite food formulations in order to stabilize them or may be extracted and added to food. For instance extracts of green tea, rosemary, sage can be added to various foods in order to increase their shelf life (Hall, 2001). Tocopherols found in many plants can be used in bulk oils to prevent their oxidation.

The overall effectiveness of natural antioxidants depends on the involvement of the phenolic hydrogen in radical reactions, the stability of natural antioxidant radical formed during the reaction, and the substituents present in the structure. The substitutions on the structure are probably the most significant contribution to the ability

of a natural antioxidant to participate in the control of radical reactions and the formation of resonance stabilized natural antioxidant radicals (Hall, 2001).

Epidemiological studies have shown that increased consumption of vegetables and fruits are related to reduced risk of many disease conditions including cardiovascular diseases and cancer. Vegetables such as root and tuber crops, cruciferous vegetables, green leafy vegetables, onions, tomatoes and other vegetables have been screened for their antioxidative activities (Pratt and Watts, 1964; Ramarathnam *et al.*, 1997; Vinson *et al.*, 1998; Arts *et al.*, 2000; Yansihlieva-Maslarova, 2001;). Similarly, herbs (Hu and Kitts, 2000), cereals (Maillard *et al.,* 1996; Duh *et al.*, 2001) and pulses (Shahidi *et al.,* 2001) have been studied. The following section describes the function and chemistry of natural antioxidants from different sources.

2.8 Sources of natural antioxidants

2.8.1 Spices and Herbs

Although spices and herbs have been added to food since ancient times for flavour improvement and modification, their antioxidative activities were first revealed in 1943 by Dubois and Tressler (1943) who showed the antioxidative action of sage, mace and black pepper. Since then herbs and spices have become one of the most important targets in search for natural antioxidants. Man has used spices not only for flavouring foods but also for antiseptic and medical purposes since the prehistoric era.

Rosemary is one of the most effective spices that is widely used in food processing. It is commercially available for the use as an antioxidant in the Europe and the USA (Yanishleiva-Maslarova, 2001). Rosemary is considered to be a lipid antioxidant capable of scavenging superoxide radicals as well as a metal chelator (Basaga *et al.*, 1997). Rosemary is commercially available as a fine powder. Depending on the content of active substances, it is recommended to use these natural antioxidant preparations at levels between 20-1000 ppm of finished products. Generally, the powders are dispersible in oils and fats, insoluble in water, but soluble in organic solvents. Due to their powdery nature they can also be used by dry mixing in powdered foods (Schuler, 1990).

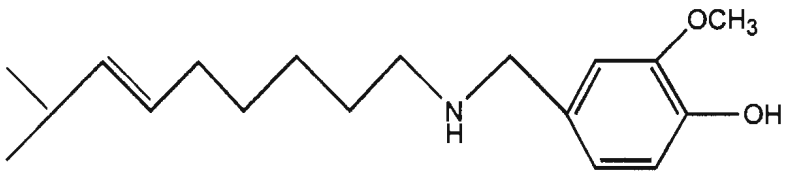
Spice extracts have attracted a great deal of interest in recent years because they can easily be added to fats and oils in bulk. Rosemary antioxidants are suitable in deep frying oils (Gordon and Lourismka, 1995) and in sausages (Barbut *et al.*, 1985). In rosemary, carnosic acid has been described as the most effective antioxidant constituent while the activity of rosemaric acid is comparable to that of caffeic acid (Schuler, 1990). Turmeric (*Cucurma longa* L) has been used as a spice and a colorant in foods since ancient times. It is also popular for its antiseptic activities. Turmeric contains tetrahydrocurcumin, a heat resistant colourless antioxidant (Osawa *et al.,* 1989). Chili pepper (*Capsicum frutescence*) and red pepper (*C. annum*) contain capsicin, which is mainly responsible for their pungency and exhibits significant antioxidative properties. A new antioxidant capsaicinol was also isolated from chili pepper (Matsufuji *et al.*, 1998). Ripe fruit of paprika, widely used as a vegetable and fruit colorant, is rich in carotenoid

pigments. The red carotenoids are mainly capsanthin and capsorubin which account for 30-60% of total carotenoids in fully ripe fruits. Matsufuji and co-workers (1998) found that capsanthin is the most effective antioxidant in capsicum. These esterified capsanthin are limited to hydrophobic regions such as lipophilic globules of chromoplates (Matsufuji *et al., 1998*). Capsicin and dihydrocapsicin present in capsicum fruit also show antioxidative properties (Nakatani *et al.,* 1988). The chemical structure of capsicum antioxidants are depicted in Figure 2.4

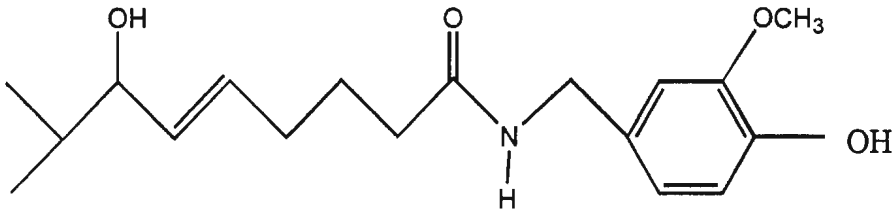
Black pepper (*Riper nigum*) contains five phenolic acid amides with antioxidant properties. Ferulic acid amide of tyramine and piperine related compounds with an open methylenedioxy ring have been reported to contain strong antioxidative properties while bearing no taste or objectionable odour (Nakatani *et al.*, 1986) (Figure 2.5A). Mint contains a number of antioxidants; carvone, menthol, linalool, neoisomenthol, pulegeone (Hilton *et al.*, 1995) (Figure 2.5B).

Alpinia speciosa belonging to the Zingiberaceae family is widely cultivated and consumed in Japan and the South-East Asian countries. Masuda *et al.* (2000) have identified two new feruloyl esters together with epicatechin in *Alpinia* rhizome extracts. These newly identified esters, namely 4-O-feruloyl-β-glucoopyranoside, and 4-hydroxy-3-methoxyphenyl 4-O-feruloyl-β-glucopyranoside are reported to be more potent than epicatechin (Masuda *et al.*, 2000) (Figure 2.6).

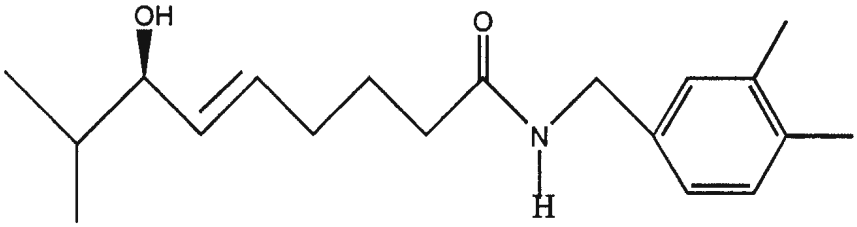
Figure 2.4 Chemical structures of capsicum antioxidants.



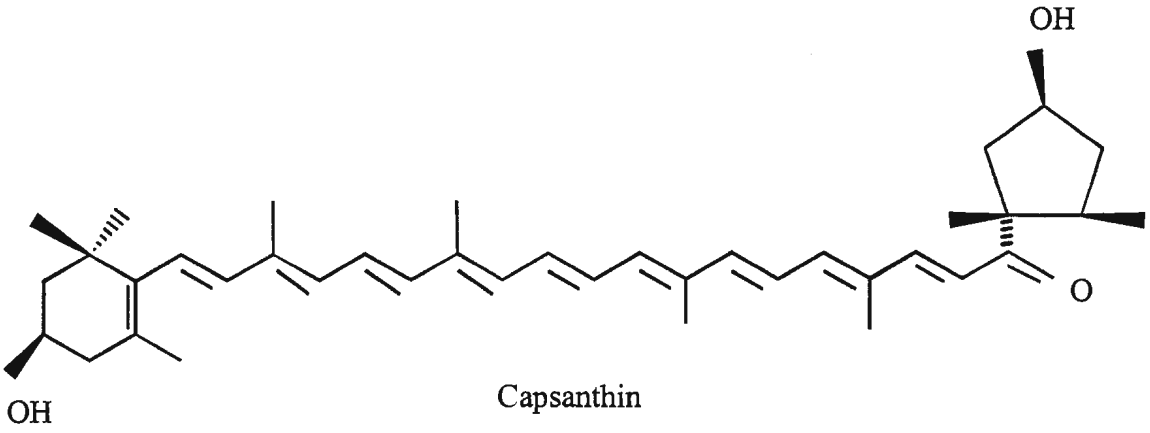
Capsicin



Hydroxycapsicin



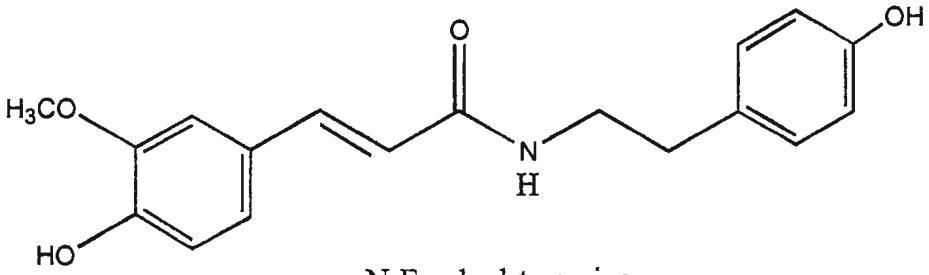
(-) Capsaicinol



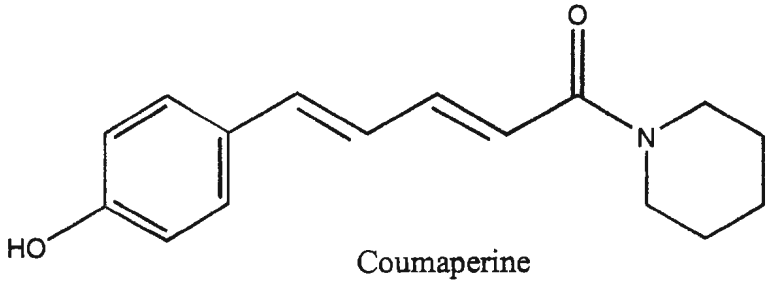
Capsanthin

Figure 2.5 Chemical structures of black pepper (A) and mint (B)

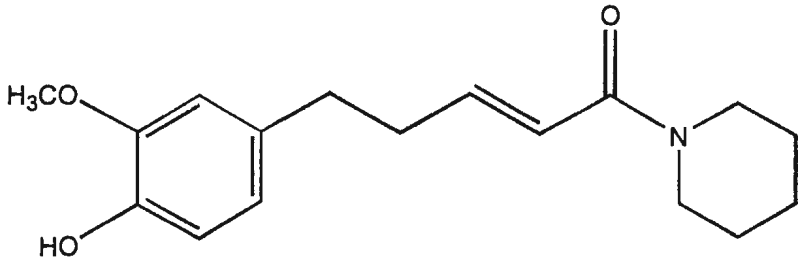
(A)



N-Feruloyl tyramine

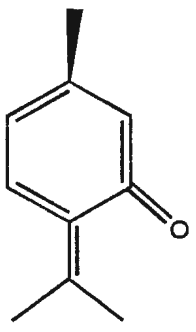


Coumaperine

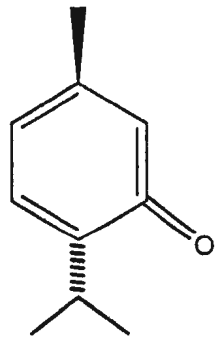


N-5-(4-hydroxy-3-methoxyphenyl)-2E-pentenoyl piperidine

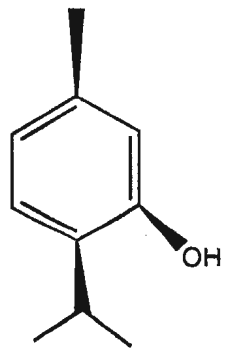
(B)



Pulegone

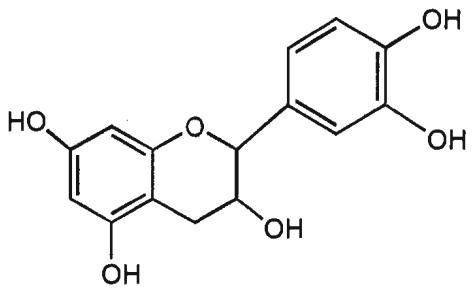


Menthone

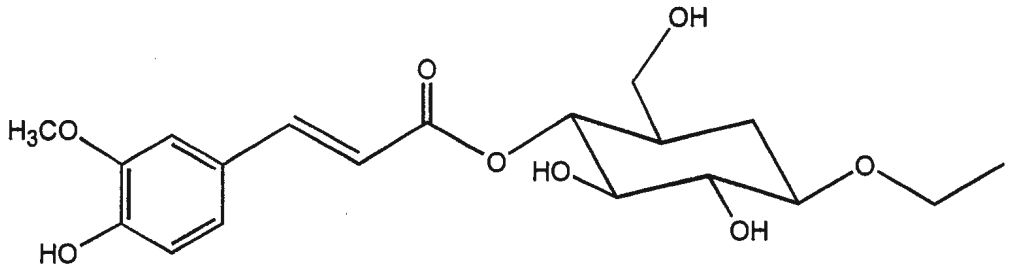


Menthol

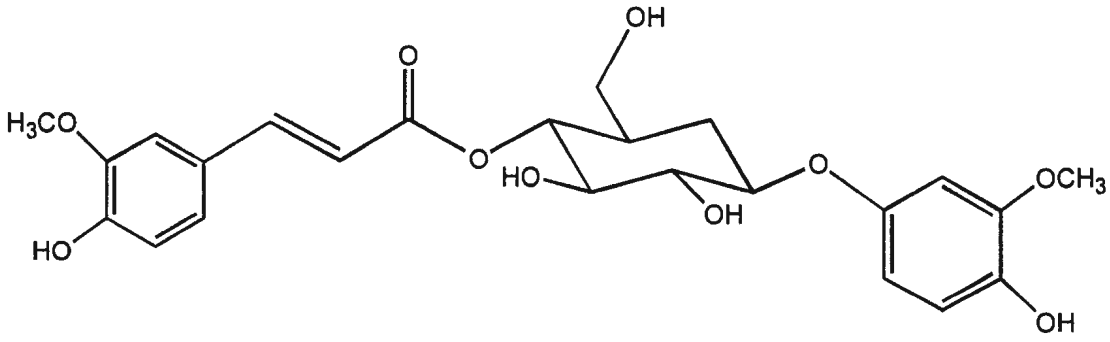
Figure 2.6 Chemical structures of *Alpinia speciosa*



(+) Epicatechin



4-O-feruloyl-beta-glucopyranoside



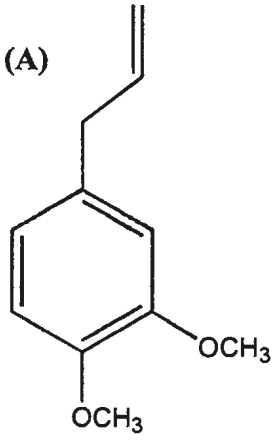
4-Hydroxy-3-methoxyphenyl 4-O-feruloyl-beta-glucopyranoside

Basil which is widely used as a spice has been reported to contain monoterpenoids sesquiterpenoids and phenyl propanoids, linalool, eugenol, methyl chavicol (Gayer *et al.*, 1996). (Figure 2.7A) Wang *et al.* (1998) isolated phenolic compounds from butanol fraction of sage extracts and identified a novel compound; 4-hydroxyacetophenone-4-O-β-D-apiopyranosyl-(1- 6)-O-β-D-glucopyranoside. Among phenolic compounds identified, rosmarinic acid and luteolin-7-O-β-glucopyranoside were the most active ones (Wang *et al.,* 1998).

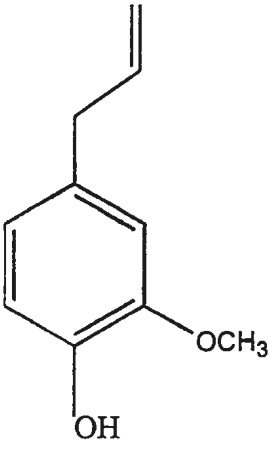
Echinacea, a native herb in the North America and the Europe, widely accepted for its immunostimulant medicinal usage, is known to contain several antioxidative compounds. Glowniak *et al.* (1996) reported that aerial parts of *Echinacea* species contain 70-1400 μg/g of total phenolics on a dry weight basis. Echinacoside and cichoric acids were the major antioxidative compounds found in *Echinacea* species (Hu and Kitts, 2000) (Figure 2.7B). Summer savoury (*Satureja hortensis* L.) is an annual herb widely used in the food industry. Antioxidative compounds isolated from summer savoury were rosemarinic acid, carnosol, carnosic acid, carvacrol and thymol (Yanishlieva-Maslaravoa, 2001) Yanishlieva-Maslarova and coworkers (1997) have investigated the antioxidative effects of summer savoury in sunflower oil. The addition of 0.1-0.5% of ethanolic extracts of summer savoury reduced oxidation of sunflower oil considerably during frying.

Figure 2.7 Chemical structures of Basil (A) Echinacea (B)

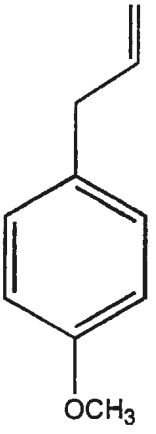
(A)



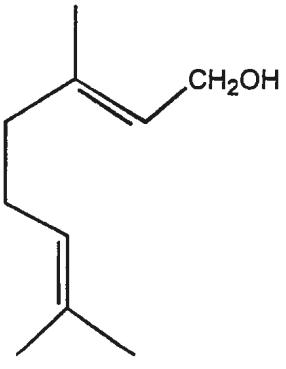
Methyl eugenol



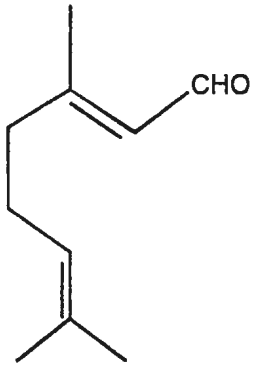
Eugenol



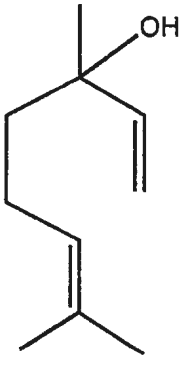
Methyl chavicol



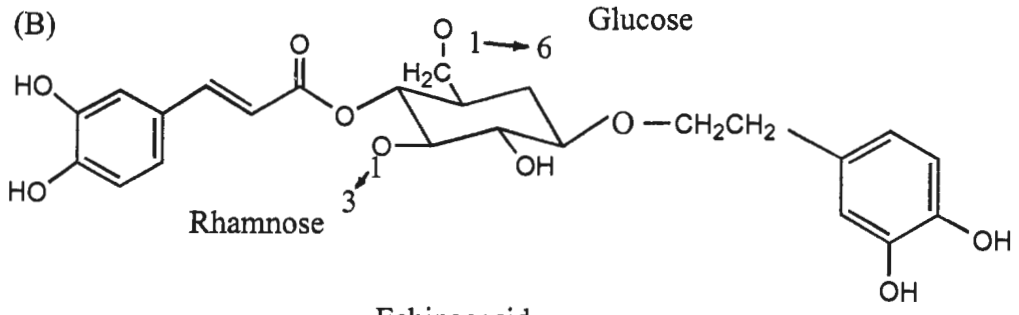
Geraniol



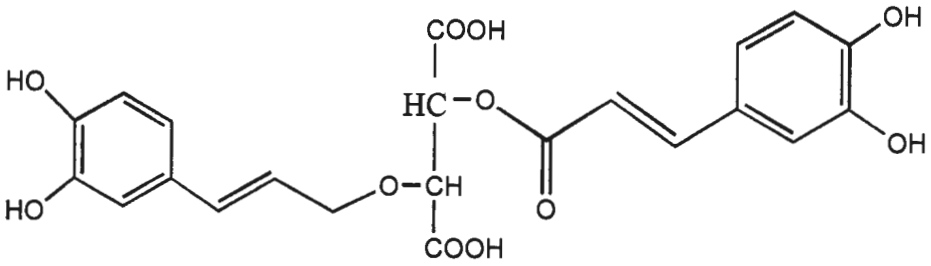
Geranial



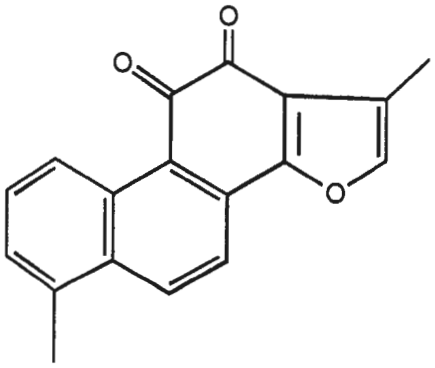
Lianlool



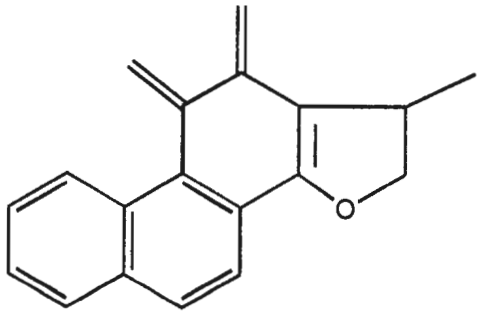
Echinacoside



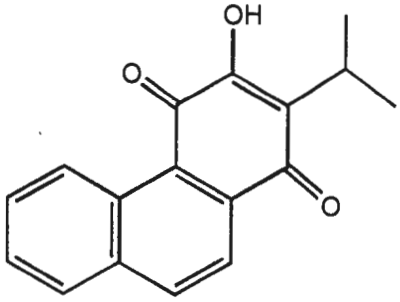
Cichoric acid



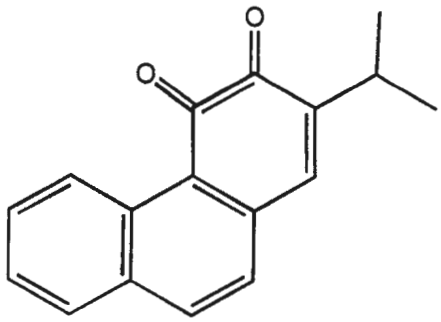
Tanshione



Dihydrotanshinone



Danshenxinkun B



Miltirone

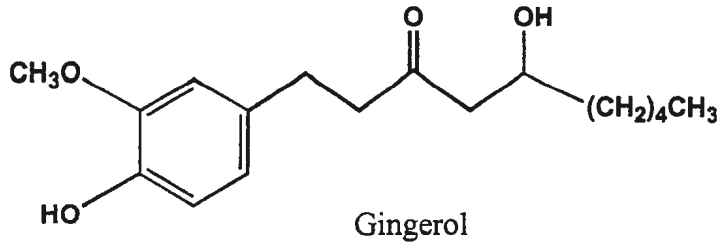
Ginger (*Zingiber officinale*) rhizome is widely used as a spice and food seasoning due to its sweet aroma and pungent taste. Kikiuzaki *et al.* (1994) reported that nonvolatile fractions of dichloromethane extracts of dried ginger contained 30 compounds out of which 16 were new. These compounds were classified into gingerol-related compounds and diarylheptanoids. Figure 2.8A depicts the chemical structures of ginger antioxidants. Tanshen (*Salvia miltiorrhiza* Bunge), a medicinal plant in China, is popular for its antioxidative properties. Figure 2.8B depicts the chemical structure of Tanshen antioxidants (Zhang *et al.*, 1990).

Roots of licorice plant (*Glycyrrhiza glabra*) have been consumed for a long time as flavouring and a sweetening agent as well as demulcents and expectorant in western countries. Glycyrrhizin and its aglycone, glycyrrhetinic acid, the main components in licorice plant root, are clinically used to treat hyperlipaemia, atherosclerosis, viral disease, and allergic inflammations (Kimura *et al.,* 1993). Vaya *et al.* (1977) have characterized some of the isoflavans, chalcones and isoflavone (Figure 2.9). The licorice root extracts have shown to be effective in depressing β-carotene bleaching and preventing LDL oxidation (Vaya *et al.,* 1997).

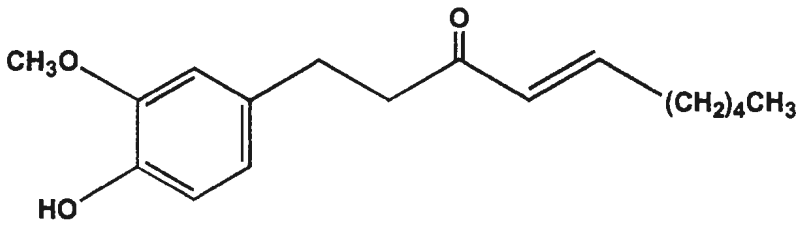
Garlic (*Allium spp*) is known to possess antioxidant activity (Yang *et al.,* 1993). Garlic is also well known for its ability to lower the risk of coronary heart diseases which may be due to its antioxidative properties (Dong *et al.,* 2001). Flavonoids, kaempferol-3-O-β-D-glucopyranose and isorhametin-3-O-β-D-glucopyranose, have been identified in garlic shoots (Mi *et al.*, 2000). Prasad *et al.* (1996) indicated that allicin is responsible

Figure 2.8 Chemical structures of (A) ginger and (B) *Tanshen* antioxidants

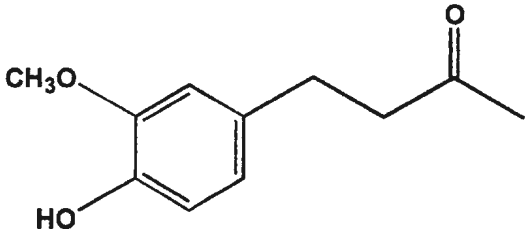
(A)



Gingerol

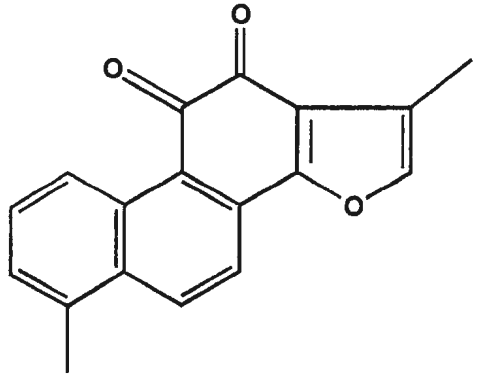


Shogaol

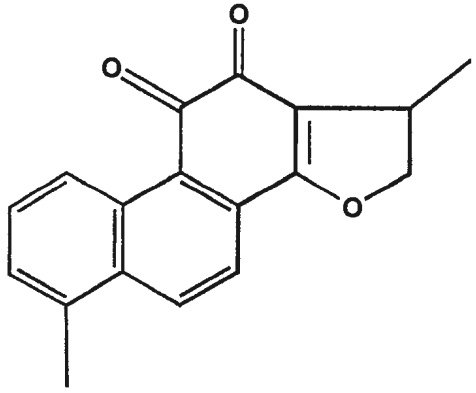


Zingerone

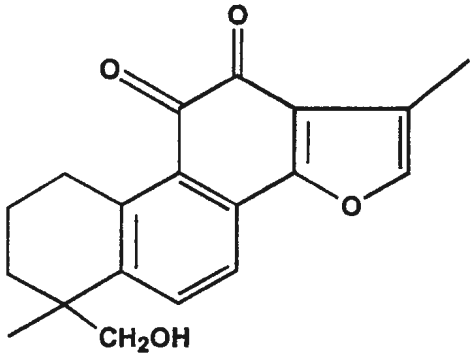
(B)



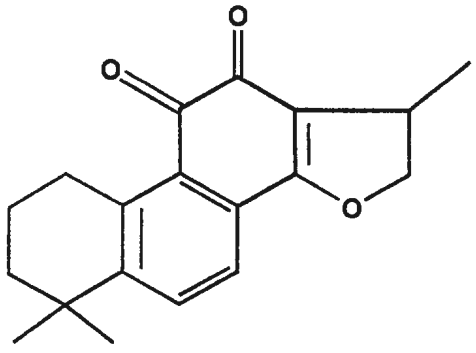
TanshinoneI



DihydrotanshinoneI

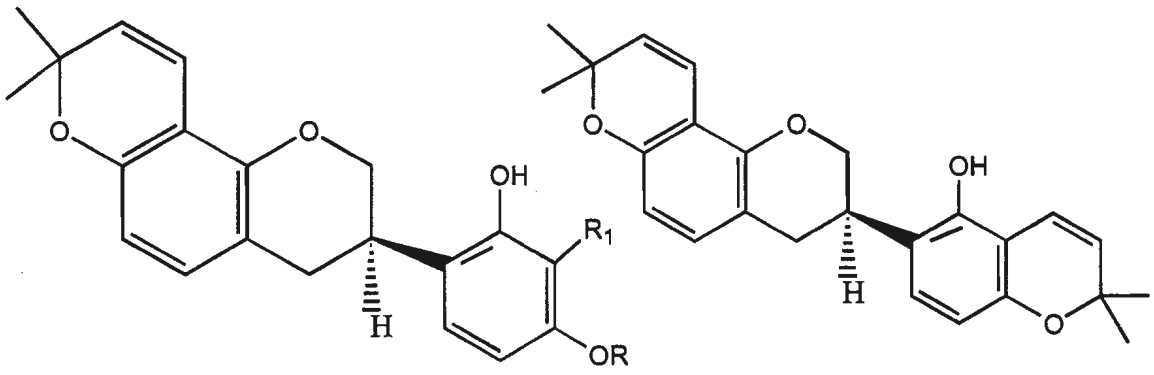


Tanshinone IIB



Cryptotanshinone

Figure 2.9 Chemical structures of *Licorice* antioxidants

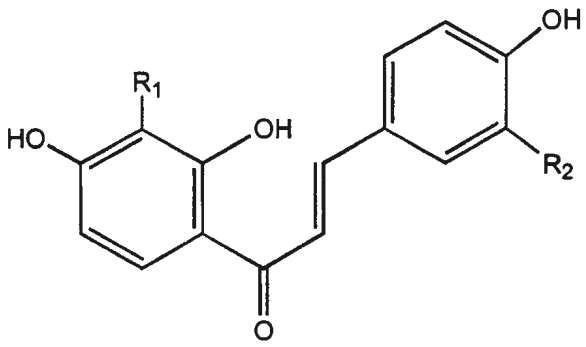


Hispaglabridin A, R=H, R1 = CH2CH=C(CH3)2

4-O-Methylglabridin, R=CH3, R1=H

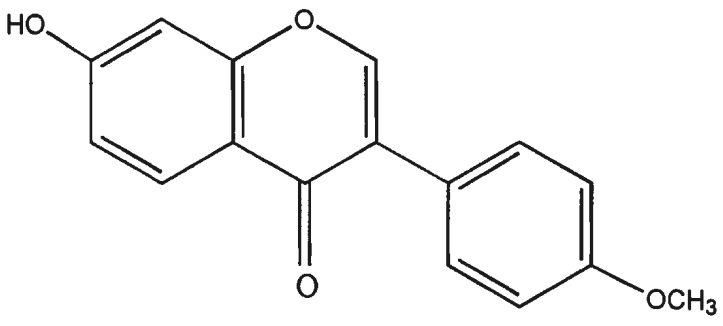
Glabridin, R=R1=H

Hispaglabridin B



Isoprenylchalcone, R1=R2=CCH2CH=C(CH3)2

Isoliquiritigenin R1=R2=H



Formononetin

for peroxy radical scavenging activity of garlic bulb. Allicin is produced when the bulb is injured. Yin and Chen (1998) observed that allicin concentration was not strongly correlated to the antioxidant activity of garlic suggesting that other compounds present might have be involved in antioxidant action of *Allium* foods. Heat treatment, acid treatment or their combination, may reduce the activity or even enhance the pro-oxidant activity in *Allium* plants (Yin and Chen, 1998).

Fennel (*Foeniculum vulgare*) indigenous to Italy is used as a spice in many parts of the world. Kaempferol, quercetin, p-hydroxybenzoic, gentisic, vanillic, syringic, ferulic acids were among the other flavonoids identified in fennel (Umadevi and Daniel, 1990). The major anthocyanin pigments present in fennel are cyanidin-3-ferulylxylosylglucosyl galactoside and cyaniding 3-sinapyl-xylosylglucosyl galactoside (Harborne, 1976). *Cassia tora* L, a plant belonging to the Leguminosae family; has been used as a laxative and a herb in China and several other countries. Kim (1994) reported that *C. tora* exhibited strong antioxidative activity in a linoleic acid model systems. Yen and Chuang (2000) indicated that *C. tora* showed greater inhibition effect on peroxidation of linoleic acid than that of α-tocopherol. However, roasting reduced their antioxidant activity. *Cassia tora* exhibited good antioxidative activity in a liposome peroxidation system induced by Fenton reactions as well as in the enzymatic microsome peroxidation systems, thus may also inhibit oxidation of biological membrane *in vivo* (Yen and Chuang, 2000).

Various other herbs, spices and vegetables have been shown to possess antioxidative properties. For example, marjoram, dittany, peppermint, spearmint, common balm, allspice, nutmeg, caraway, cinnamon, bay leaves, dill, parsley, coriander, cumin, hyssop and juniper have been shown to prevent oxidation (Yanishlieva-Masralova, 2001). Recently, more than 700 Chinese herbs and spices were screened for their antioxidant efficacy. Out of 700 species, 64 showed good antioxidative properties while 24 showed very strong antioxidative properties (Yanishlieva-Masralova, 2001).

2.8.2 Oilseeds

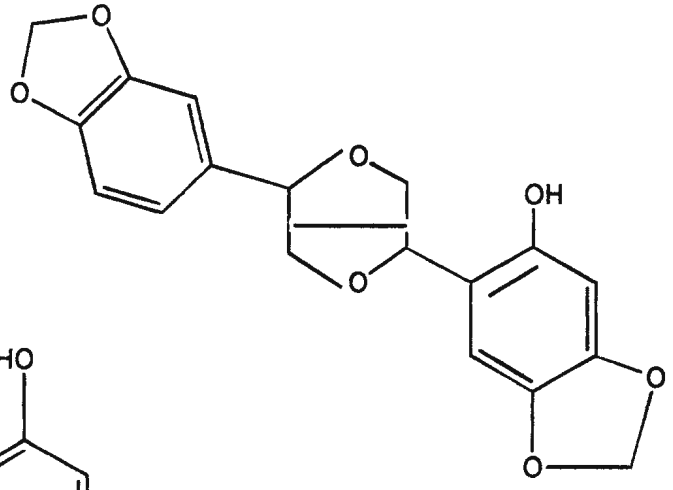
Antioxidant compounds have been isolated from oilseeds such as flax, sunflower, soybean, cottonseed, canola, evening primrose and others. Soybean is a source of a larger range of antioxidant compounds including tocopherols, flavonoids, isoflavone glycosides and their derivatives and synergists such as phopholipids, amino acids and peptides (Schuler, 1990). Aqueous extracts as well as organic solvent extracts from soybean, soy protein concentrate, and soy protein hydrolysates have shown antioxidant activity (Schuler, 1990). Hall (2001) reported that aqueous extracts of soybean contain isoflavones and phenolic acids which acted as the main antioxidant, while organic solvent extracts of soy contained tocopherols, sterols, phopholipids and other flavonoids; protein hydrolysates contained antioxidant amino acids and peptide. Soybean meal was found to contain antioxidants such as isoflavones, tocopherols, glycosides and derivatives, amino acids and peptides (Hayes *et al.,* 1977). Some of the isoflavone glycosides have been identified as genistein, glycitein, daidzein, purnetin, formonoetin.

Yokota and coworkers (1996) observed that antioxidant containing fractions from soybean fermented with *Bacillus subtilis* can prevent the development of atherosclerosis and cholesterolsis in cholesterol-fed rabbits through oral supplementation. The antioxidant fraction was found to lower the serum TBARS, total cholesterol, LDL cholesterol and triacylglycerols as well as the incidence of atherosclerotic lesions.

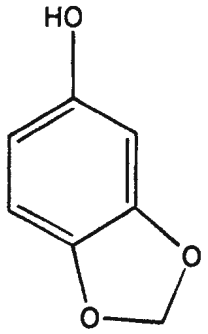
Sesame (*Sesamum indicum* L) seeds and oil are traditional foods and medicinal ingredients in several cultures. The main antioxidants in sesame seeds have been identified as sesamolin, sesamolinol, sesaminol, sesamol, pinoresinol, tocopherols, syringic acid and ferulic acid (Figure 2.10; Fukuda *et al.*, 1986). Canola (*Brassica napus*) seeds contain considerable amounts of phenolic compounds compared with other oilseeds (Krygier *et al.*, 1982). Canola hulls contain phenolic acids (Krygier *et al.,* 1982) and soluble and insoluble condensed tannins (Naczk *et al.*, 1994). Wanasundara *et al.* (1994) identified the most active phenolic antioxidant compound in canola meal as 1-O-β-D-glucopyranosyl sinapate.

Olive oil is reported to contain natural antioxidants such as tocopherols, carotenoids, sterols and phenolic compounds (Boskou, 1996). Gallic, caffeic, vanillic, syringic ferulic, homovanillic, protocatechuic acids, tyrosol and hydroxytyrosol were among the phenolic compounds present (Montedoro *et al.*, 1992). The polyphenolic compounds in olive (*Olea europaea* L) are relevant to the sensory quality of virgin and extra virgin oils and play a role in extending its shelf life (Motedoro *et al.,* 1993).

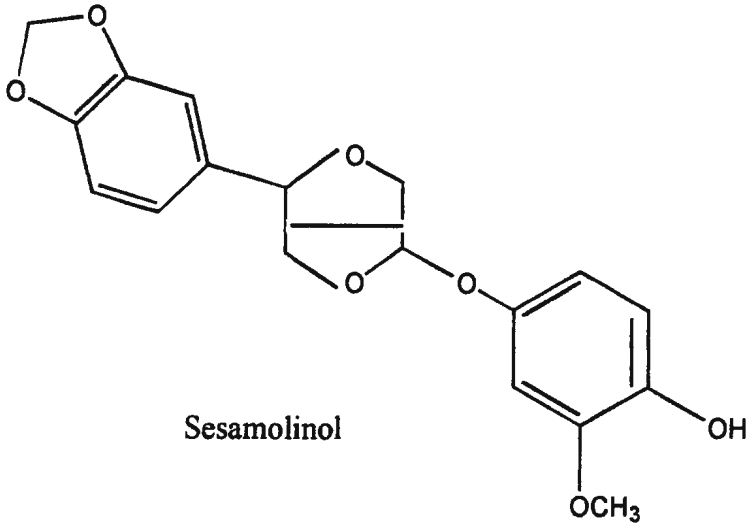
Figure 2.10 Chemical structures of sesame antioxidants.



Sesaminol



Sesamol



Sesamolinol

According to Aruoma *et al.* (1998), hydroxytyrosol, found in extra virgin olive oil strongly inhibited 2,2' azobis (2-amidinopropane) hydrochloride (AAPH) induced LDL oxidation.

Pratt and Miller (1984), have reported that peanut is effective in controlling the bleaching of β-carotene in a model system. The active compounds were identified as dihydroquercetin. The major phenolic compounds included trans-*p*-coumaric, syringic, trans-ferulic, trans-caffeic and *p*-hydroxybenzoic (Dabrowski and Sosulski, 1984). The low molecular weight phenolics were mainly flavan-3-ols of catechin and epicatechin (Hall, 2001).

2.8.3 Cereals

Nishiyama *et al.* (1993) isolated a novel antioxidant from young green barley leaves known as 2'(3')-*O*-glycosylisovitexin. Other flavonoids found in barley include anthocyanins, proanthocyanidins and flavonols which are located in the pericarp and aleurone layers of barley kernel (Sumere *et al.*, 1972). The phenolic acids in barley include sinapic, ferulic, *p*-, *m*- and-*O*-coumaric, and *p*-hydrobenzoic acids (Sumere *et al.*, 1972).

Duh *et al.* (2001) have evaluated the antioxidant activity of water extracts of barley. They reported that the antioxidative activity of water extracts of unroasted barley was comparable to those of BHA and tocopherol in a linoleic acid model system. Furthermore, they reported that barley extracts acted as good metal chelators, but this

effect was decreased with roasting. Goupy *et al.* (1999) indicated that catechin and its derivatives, tocopherols and arytenoids were the major antioxidative compounds in barley.

Ramarathnam *et al.* (1988 and 1989) have shown the antioxidant activity of methanolic extracts of rice hulls. Crude rice bran oil is reported to contain a mixture of ferulic acid esters of triterpenoid alcohols (Rogers *et al.*, 1993). Rice bran oil contained 608 ppm tocols or 157 ppm on a dried rice bran basis and 2847 ppm of oryzanols (Weicheng *et al.*, 1996). Ramarathnam *et al.* (1989) identified isovitexin, a glycosyl flavonoid in a long-term rice variety, Katakutara. Active compounds in rice hulls have been identified as 2,3,6-trimethylanisole, *m*-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde and 4-hydroxy-3, 5-dimethoxybenzaldehyde (syringaldehyde) (Asamarai *et al.*, 1996). Rice bran is a very rich source of vitamin E (up to 300 ppm); the major components being α-tocopherol and γ-tocotrienol (shin *et al.*, 1997).

Xu *et al.* (2001) reported that tocopehrols and γ-oryzanol components of rice bran reduced the production of toxic cholesterol oxidation production. Furthermore, they concluded that the antioxidant activity of γ-oryzanol was about 10 times higher than that of vitamin E in rice bran (Xu *et al.*, 2001). Wu *et al.* (1994) demonstrated that methanolic and ethanolic extracts of wild rice extracts had a significant antioxidant activity when added to ground beef or lard.

Furthermore, pulverized, cooked and uncooked wild rice substantially reduced rancidity in ground beef (Wu *et al.,* 1994). They suggested that antioxidant ingredients for common applications could be prepared from wild rice.

Fractionation of buckwheat groat extracts resulted in four catechin compounds; (-)-epicatechin, (-)-epicatechin 3-*O*-*p*-hydroxybenzoate, (-)-epicatechin 3-*O*-(3,4-di-*O*-methyl)-gallate, and (+)-catechin 7-*O*-β-D-glucopyranoside (Watanabe, 1998). The active components of the hull were identified as protocatechuic acid, 3,4-dihydroxybenzaldehyde, hyperin, rutin, quercetin, vitexin and isovitexin. Przybylski *et al.* (1998) reported that rutin was the major flavonoid present in buckwheat followed by quercetin and quercitrin.

Millet (*Pennisetum americanum* L) is known to possess good antioxidant activity. Polyphenols in millet grains are responsible for a gray pigmentation (Mazza and Miniati, 1993). Raju *et al.* (1985) have identified derivatives of cyanidin, delphinidin, and pelargonidin in millets.

2.8.4 Fruits and vegetables

Increased consumption of fruits and vegetables is associated with a lower risk of degenerative diseases including cardiovascular disease, cancer, cataracts and brain and immune dysfunction (Ames and Shigenaga, 1993). A number of phenolic compounds present in fruits and vegetables are responsible for their antioxidant activity. Tocopherols, flavonoids and phenolic acids, nitrogen compounds, carotenoids and

ascorbic acid are among the antioxidative compounds present in fruits and vegetables. Many of the natural antioxidants, especially flavonoids, exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergenic, antithrombic, and vasodilatory actions (Cook and Samman, 1996).

Gazzani *et al.* (1998), reported that at 2°C most vegetables show a prooxidant activity. The prooxidant activity is prominent in vegetables like egg plant, tomato and bell pepper. It was reported that upon boiling antioxidant activity of vegetable is increased suggesting that the initial pro-oxidant activity is due to the presence of lipoxygenase enzymes, which are possibly destroyed during boiling (Yanshlieva-Maslarova, 2001). Potato is considered a to serve as good source of antioxidants such as ascorbic acid, α-tocopherol and polyphenolic compounds. Pratt and Watts (1964) have shown that potato peels exhibit a high antioxidative activity. The antioxidant compounds isolated from potato peel were derivatives of caffeic acid and caffeolyquinic acid with sugar moieties (Onyeneho and Hettiarachchy, 1993).

Williams *et al.* (1978) have identified the composition of acylated anthocyanins in grapes which contain antioxidant activity. Tamura and Yamagani (1994) have evaluated the antioxidant activity of malvidin derivatives isolated from Muscat Bailey A grape. At a concentration of 10 μM, malvidin 3-(p-coumaroylglucosido)-5-glucoside was very effective as an antioxidant. Crude pigments in grape skin which are a mixture of acylated and nonacylated anthocyanins which have shown a better antioxidant effect than (+) catechin, naringenin and α-tocopherol (Tamura and Yamagami, 1994). Berries are rich in

flavonoids and phenolic acids. Heinonen *et al.* (1998) investigated the antioxidant activity of berry extracts in copper-catalyzed *in vitro* oxidation assays. Miller and Rice-Evans (1997) reported that the antioxidative activity of orange juice was accounted for by hesperidin and narirutin.

2.8.5 Legumes

Tsuda *et al.* (1993b) evaluated methanolic extracts of a number of pulses and found that kidney beans, guar, and tamarind had strong activity. The flours of beans contained only soluble esters, but hydrolysis revealed the presence of trans-ferulic, trans-*p*-coumaric and syringic acids in all species. Drumm *et al.* (1990) indicated the presence of phenolic acids, cinnamic, coumaric, ferulic and sinapic acids in *P. vulgaris.*

Duh *et al.* (1997) investigated the antioxidant activity of methnaolic extracts of hulls of mung bean (*Phaseolus aureus*). Extracts at a concentration of 100 ppm exhibited a stronger activity than 100 ppm of α-tocopherol or 100 ppm of BHA in the prevention of peroxidation of linoleic acid. Moreover, a synergistic effect was observed when 100 ppm of the extract was mixed with 100 ppm of α-tocopherol. The extracts were able to suppress the formation of both the primary and secondary oxidation products in soybean oil.

Carbonava *et al.* (1996) investigated the antioxidative activity of high and low tannin varieties of faba beans (*Vicia faba*) and lentils (*Lens culinaria*). Tannins have

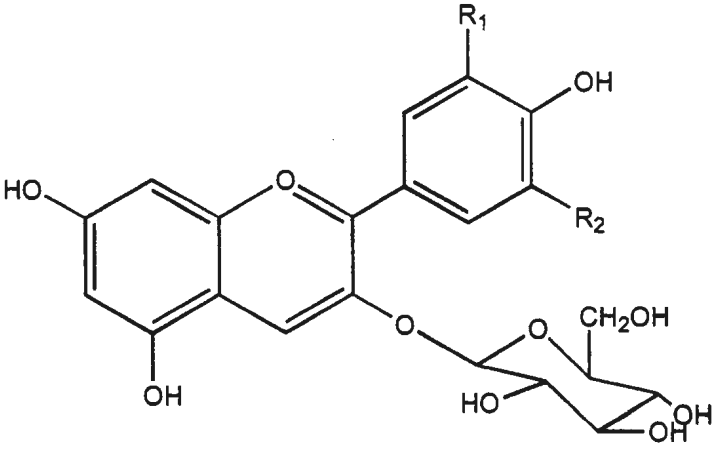
detrimental effects in terms of nutrition as they bind with protein and thereby reduce their digestibility. Tannins also complex with digestion enzymes.

2.8.6 Beans as a source of natural antioxidants

Pea bean (*Phaseolus vulgaris* L) is cultivated throughout the world for its pods and seeds and is consumed in both the western and eastern countries. There are many varieties of seeds of *P. vulgaris* and the size, shape and color in each variety generally differ. The common colours are white, brown, red and black.

The antioxidative activity of extracts of pea beans has been evaluated by different authors (Tsuda *et al.,* 1993 a&b and 1994 a & b; Raab *et al.,* 1997; Tsuda *et al.,* 1997; deMejia *et al.*, 1999). Dry beans contain appreciable quantities of polyphenolic compounds but are typically a poor source of known antioxidants such as ascorbic acid, α-tocopherol and β-carotene (Larson, 1988). Beans contain considerable amounts of phenolic compounds possessing varying degrees of antioxidant activity (Srisuma *et al.,* 1989). The effect of navy bean crude hull extracts on the oxidative stability of edible oils was reported by Onyeneho and Hettiarachchy (1991). The effect of freeze dried navy bean hull extracts were more pronounced than the synergistic effect of BHA, BHT and rosemary extracts in preventing oxidation of vegetable oils. However, it was less effective than TBHQ in inhibiting oxidative rancidity in vegetable oils. Apart from their strong antioxidant

Figure 2.11 Chemical structures of Beans (*Phaseolus vulgaris*)



R_1 = OH, R_2 = H	Cyanidin 3-O-β-D-glucoside
R_1 = H, R_2 = H	Pelargonidin 3-O-β-D-glucoside
R_1 = OH, R_2 =O H	Delphinidin 3-O-β-D-glucoside

activity, bean hull extracts did not impart any visible colour (Onyeneho and Hettiarachchy, 1991). in the oil at the levels (up to 1000 ppm) employed. The extracts were dissolved instantly upon sonication to form an emulsion, which had remained completely dispersed in the oil over a long period of time. Onyeneho and Hettiarachchy (1991) suggested that navy bean hulls can be used commercially to inhibit oxidation of vegetable oils. Tsuda *et al.* (1994a) reported that cyanidin 3-O-β-D-glucoside (C3G), an anthocyanin pigment isolated from red beans, exhibited strong antioxidative activity. C3G, found widely in many seeds, fruits and vegetables plays an important role as a dietary antioxidant after ingestion by preventing lipid peroxidation in cell membranes induced by reactive oxygen radicals.

Depending on the cultivar, bean hulls show a large variation in their flavonoid content and colour. Four colours of bean are most common; black, brown, red and white. These colours are correlated with the colour of the hypocotyl (hull), cotyledon, and leaf veins. The anthocyanin pigmentation in the hypocotyls is determined genetically. The anthocyanin identified in *P. vulgaris* include pelargonidin 3-O-β-D-glucoside, cyanidin 3-O-β-D-glucoside and delphinidin 3-O-β-D-glucoside (Figure 2.11). Tsuda *et al.* (1997) screened 35 species of edible beans for their antioxidant activities and found no antioxidant activity in seed germs; white bean seed coat did not exhibit any antioxidant activity.

Tsuda *et al.* (1997) reported that cyanidin isolated from seed coats of *P. vulgaris* showed marked antioxidant activity in liposomal and rat liver microsomal systems. An

inhibitory effect on malondialdehyde (MDA) levels upon UV irradiation was observed such as radical scavenging effects against hydroxyl radical and superoxide anion radicals.

2.9 Extraction techniques

A number of solvent systems have been used for extraction of antioxidant compounds from plant materials. Ramaratnam *et al.* (1997) extracted antioxidants from vegetables using hot water which involves chopping of vegetables, extracting in hot water for 1h, concentrating under vacuum and freeze-drying. Heinonen *et al.* (1998) used 60% aqueous methanol or 70% acetone to extract antioxidative compounds from berries (black berries, high bush berries, red raspberries, strawberries, sweet cherries) after homogenizing. Grape anthocyanins were extracted with 50% methanol containing 1% trifluoroacetic acid (TFA) by Tamura and Yamagami (1994). Shahidi and coworkers (2001) extracted beach pea phenols (*Lathyrus maritimus* L) using 70% (v/v) aqueous acetone at room temperature.

Antioxidative compounds from natural products may be extracted by employing oils or organic solvents and acetone. In addition, novel methods such as supercritical fluid carbon dioxide can also be employed. Natural materials containing antioxidants such as herbs and spices are mixed with edible oil or fat and the mixture is left at room temperature or slightly elevated temperature over night with or without stirring. The mixture is filtered to separate solid particles and the liquid (oil) part is removed which can be used in food preparation. Antioxidants from powdered materials such as

rosemary, sage, nutmeg, paprika, cocoa have been extracted with edible oils (Pokorny and Korczak, 2001).

The method explained by Bernes *et al.* (1973) involves combining the ground spice with oil, heating to 125°C for 2h with continuous agitation followed by centrifugation and deodourization. This procedure was later improved by Bracco *et al.* (1981) who suggested additional two steps; micronizing spices in edible oil and cleaning the liquid phase by centrifugation the liquid phase and molecular distillation on falling film. The micronization facilitates mechanical transfer of antioxidants to the lipid phase, and molecular distillation allows deodourization or partial cleaning the liquid phase (Pokorny and Koczak, 2001).

Organic solvents such as hexane, acetone and ethyl acetate can be employed to extract antioxidative compounds. The choice of the solvent depends on the materials used. For rosemary and sage the solvents of intermediate polarity are preferable to either non-polar or highly polar solvents (Pokorny *et al.*, 1998). Mixture of organic solvents such as acetone, methanol or ethanol with water have also been tested with lentils; aqueous acetone was found to be the best (Amarowicz *et al.*, 1995). Steam distillation under normal pressure or under vacuum can be employed after extraction with organic solvents (Nakatani and Intani, 1984). Another method for removing volatiles is the use of supercritical extraction with carbon dioxide.

One of the problems encountered in extraction of antioxidants for food preparation is the presence of chlorophyll pigments, which impart a dark colour and pro-oxidant effects in the light. Further fractionation of ethanolic or methanolic extracts and bleaching with activated carbon can be used to minimize the effect of chlorophyll pigments (Nguyen *et al.*, 1993).

Extraction with carbon dioxide under supercritical conditions is selective and a better technique than extracting with organic solvents. A two-stage process of isolating antioxidants from rosemary and sage using supercritical carbon dioxide was developed by Bauman *et al.* (1999). Propane, methanol, ethanol and other substances are used as co-solvents in the extraction procedure. As antioxidants are a more expensive group of food preparations the relatively high price would not play a crucial role if it is compensated by other advantages such as purity and greater efficiency of the process (Pokorny and Korczak, 2001).

Duh *et al.* (1997) extracted antioxidants of mung bean hull using methanol at room temperature. Tsuda *et al.* (1994a) used 0.5% TFA in 80% ethanol to extract antioxidative compounds from bean seeds (*P. vulgaris*). Onyeneho and Hettiarachchy (1991) employed 95% ethanol to extract antioxidative compounds from navy bean hulls. Ganthavorn and Hughes (1997) employed 80% methanol to extract polyphenolic compounds from ground bean (*P. vulgaris*) at 64 °C for 5 min. Tsuda *et al.* (1997) used 80% ethanol containing 0.5% TFA to extract antioxidative compounds from bean seed coats and germs.

2.10 Measuring antioxidative capacity

There are a number of techniques available for measuring plant extracts and their active compounds for antioxidative activity. The choice of the technique depends on the type of extract to be investigated.

2.10.1 Bulk-oil and oil-in-water emulsion systems

Corn oil stripped of its endogenous antioxidants and its oil-in-water emulsions have been employed to evaluate antioxidant activity of different compounds such as L-ascorbic acid and α-tocopherol (Porter, 1993; Frankel *et al.*, 1994). Non-polar antioxidants such as α-tocopherol are relatively ineffective in oil but were strongly effective in oil-in-water emulsions. In contrast, polar antioxidants such as ascorbic acid and or trolox were more effective in oil than in an emulsion system. This phenomenon is known as polar paradox theory (Frankel *et al.*, 1994 and Porter, 1993). Partitioning of antioxidants into phases according to their affinities towards the system was considered responsible for this behaviour (Huang *et al.*, 1996a).

The degree of oxidation may be measured in terms of conjugated dienes (CD) value of the system. Marinova and Yanihleiva (1996) employed triacylglycerols and fatty acid methyl esters from olive oil to evaluate antioxidative capacities of coumaric, ferulic and caffeic acids. Heinonen *et al.* (1998) used lipososme oxidation to measure antioxidative activity of berry phenolics. The liposome prepared with 0.8% lecithin containing 40% phopsphatidylcholamine and oxidation is initiated by adding cupric sulphate in the presence of phenolic substances.

β-Carotene-linoleate model system is another model system widely employed in evaluating antioxidative activities. It is based on minimizing β-carotene loss in the coupled oxidation of linoleic acid and β-carotene using an emulsified aqueous system. The degradation of β-carotene is monitored spectrophotometrically. A number of analytical techniques have been developed to assess the oxidation status of bulk oil or oil emulsion systems. These methods basically measure changes in the concentration of the molecules in the system, which indicates the degree of oxidation.

The peroxide value (PV) is still the most common method of measuring oxidative deterioration of oils. The measurement of PV should normally be combined with a method monitoring secondary oxidation products in order to obtain a correct idea about the oxidation status.

Formation of hydroperoxides from PUFA leads to conjugation of the pentadiene structure causing absorption of UV radiation at 233-234 nm (Gordon, 2001b). Malonaldehyde (MA) can be formed from PUFA with at least 3 double bonds. The concentration of MA may be measured by reacting MA with TBA which yields a red condensation product with MA. The condensation product absorbs light at 532-535 nm range (Gordon, 2001b). Oxidation of bulk oil can be monitored by gravimetric measurements of oxygen uptake or weight gain (Gordon, 2001b). Edible oils increase in weight during early stages of lipid oxidation as fatty acids combine with oxygen during the formation of hydroperoxides. Rapid weight gain occurs after the induction period of oxidation. However, decomposition of hydroperoxides leads to a reduction in weight

(Gordon, 2001b). This method is not suitable for emulsions as they lose water due to evaporation. Zhuang *et al.* (1991) have used an oxygen electrode to monitor oxygen uptake by an emulsion. The active oxygen method (AOM), is a commonly used accelerated method for assessing oxidative stability of oils, which has also been applied to evaluate antioxidants. This methods works on the principle that oxidation of lipids is accelerated by aeration in a tube held at a constant elevated temperature (Gordon, 2001b).

Oil stability instruments (OSI) are the automated versions of AOM. Rancimat manufactured by Omnion Rockland, USA and Oxidograph are some of the examples. These instruments depend on electrical conductivity changes that occur when carboxylic acids generated during oxidation are passed through water (Gordon, 2001b). The oxidograph is an instrument that measures the pressure drop electronically in oxidizing oil sample. The sample of oil or fat is exposed to oxygen at an elevated temperature with stirring to accelerate the oxidation. As the sample absorbs oxygen, the pressure drops and this is measured by oxidograph (Gordon, 2001b). Oxpires is a similar kind of instrument which measures drop in pressure in food systems such as margarine or mayonnaise.

Data from the Rancimat apparatus correlated well with AOM test. Burkow *et al.* (1995) have tested natural tocopherol containing rosemary extracts, sage and lecithin in cod liver oil. The stability of oil has been tested with Rancimat method as well as hypochlorite-activated chemiluminescensce. However, a poor correlation (r=0.339) exists when Rancimat induction times and chemiluminescence data. Wanasundara and

Shahidi (1993) reported that proton nuclear magnetic resonance (H^1NMR) spectroscopy can be employed to evaluate oxidative stability of oils and fat.

2.10.2 Meat model systems

Many of the flavour compounds identified in meat are products of oxidation. These compounds play an important role in the development of the distinctive flavour characteristics of meat. As lipid oxidizes they produce many secondary products such as alcohols, hydrocarbons, ketones and aldehydes among others.

Different meat model systems such as chicken, pork, turkey, beef and fish have been used to evaluate the efficacy of antioxidants and other extracts containing antioxidative compounds. Level of oxidation is monitored by measuring concentration of TBA and headspace gasses. The aldehydes used to monitor lipid oxidation in meat and fish are hexanal and propanal, respectively, as hexanal is the prominent aldehyde formed from oxidation of linoleic acid while propanal being the prominent aldehyde arising from omega-3 fatty acids in marine organisms (Farnkel *et al.*, 1989). Arara *et al.* (1997) explained fluorescence spectroscopy (FS) as a technique assessing antioxidant capacity. FS offers several advantages for evaluation of the efficacy of antioxidants; it is about 1000 times more sensitive than regular spectroscopic methods. It also allows characterization of fluidity and thermotropic phase behaviour that may possible over during lipid oxidation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Red, brown black and white beans were obtained from a local grocery store in Singapore. Sodium carbonate, sodium chloride, methanol, ethanol, hexane, sulphuric acid, hydrochloric acid, glacial acetic acid, chloroform, isooctane, butanol, acetone, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and stripped corn oil were purchased from Fisher Scientific Co (Nepean, ON).

Compounds 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), Trizma base, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), tetrazolium, α-tocopherol, hydrogen peroxide, boric acid, ethidium bromide, sodium hydroxide, butylated hydroxyanisole (BHA), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2-thiobabituric acid (2-TBA), 1,1,3,3-tetramethoxypropane, xanthine oxidase, hypoxanthine, β-carotene, linoleic acid, butylated hydroxytoluene (BHT), catechin, ferrous sulphate, Tween 40 (polyoxyethelene sorbitan monopalmitate), Folin Ciocalteu's phenol reagent, ascorbic acid, copper sulphate, ferric chloride as well as mono- and dibasic sodium and potassium phosphates, vanillin, ethylenediaminetetraacetic acid (EDTA), human low density lipoprotein (hLDL), diethylenetriaminepentaacetic acid, acetontrile, Ferrous ammonium sulphate, deoxyribonucleic acid (DNA) of pBR 322 (*E. coli* strain RRI) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Pentanal, hexanal, heptanal and 2-heptanone were acquired from Aldrich chemical company (St. Louis, MO). Standards of cyanidin and delphinidin chlorides were

obtained from Extrasynthese (Genay, Cedex, France). Standards for procyanidns were a gift from Prof. Jan Oszmianski, Agricultural university, Wroclaw, Poland. Helium, hydrogen, nitrogen and compressed air were obtained from Canadian Liquid Air Ltd. (St. John's, NF).

3.2 Methods

All the experiments were done in triplicates and the results were reported as means ± standard deviation. The methodologies followed are described below.

3.2.1 Determination of proximate composition of beans

3.2.1.1 Moisture content

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

3.2.1.2 Ash content

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

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

3.2.1.3 Crude protein content

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

3.2.1.4 Total lipid content

Total lipid content of the samples was determined using the procedure described by Bligh and Dyer (1959). Approximately 25 g of bean samples were accurately weighed and then extracted over a 3 min period with a mixture of 25 mL of chloroform and 50 mL of methanol (1:2, v/v) using a Polytron homogenizer (Brinkman Instruments,

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

3.2.2 Preparation of bean samples

3.2.2.1 Air Classification

Part of the bean seeds was dehulled using a Seedburo hand grinder (Seedburo equipment company, Chicago, IL). Ground seed fines were first separated using a mesh 30 sieve on a Seedburo portable sieve shaker, the hull were separated on a 757 South Dakota Seed Blower (Seedburo equipment company, Chicago, IL) equipped with a large (4") tube set. Pending on the set up of air pressure, clean hulls and cotyledons were obtained (Figure 3.1). The pressure of air in the tube was controlled by the size of opening at the top.

3.2.2.2 Defatting of samples

The separated hulls and whole bean seeds were ground in an electric grinder (Black and Decker Canada Inc., Brockville, ON) for 15 min and then defatted by

blending with hexane (1:5 w/v, 5 min) in a Waring blender (Model 33BL73, Waring Products Division Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted seeds were vacuum packaged in polythene packets and kept in a freezer at -20°C until used.

3.2.3 Preparation of crude extracts

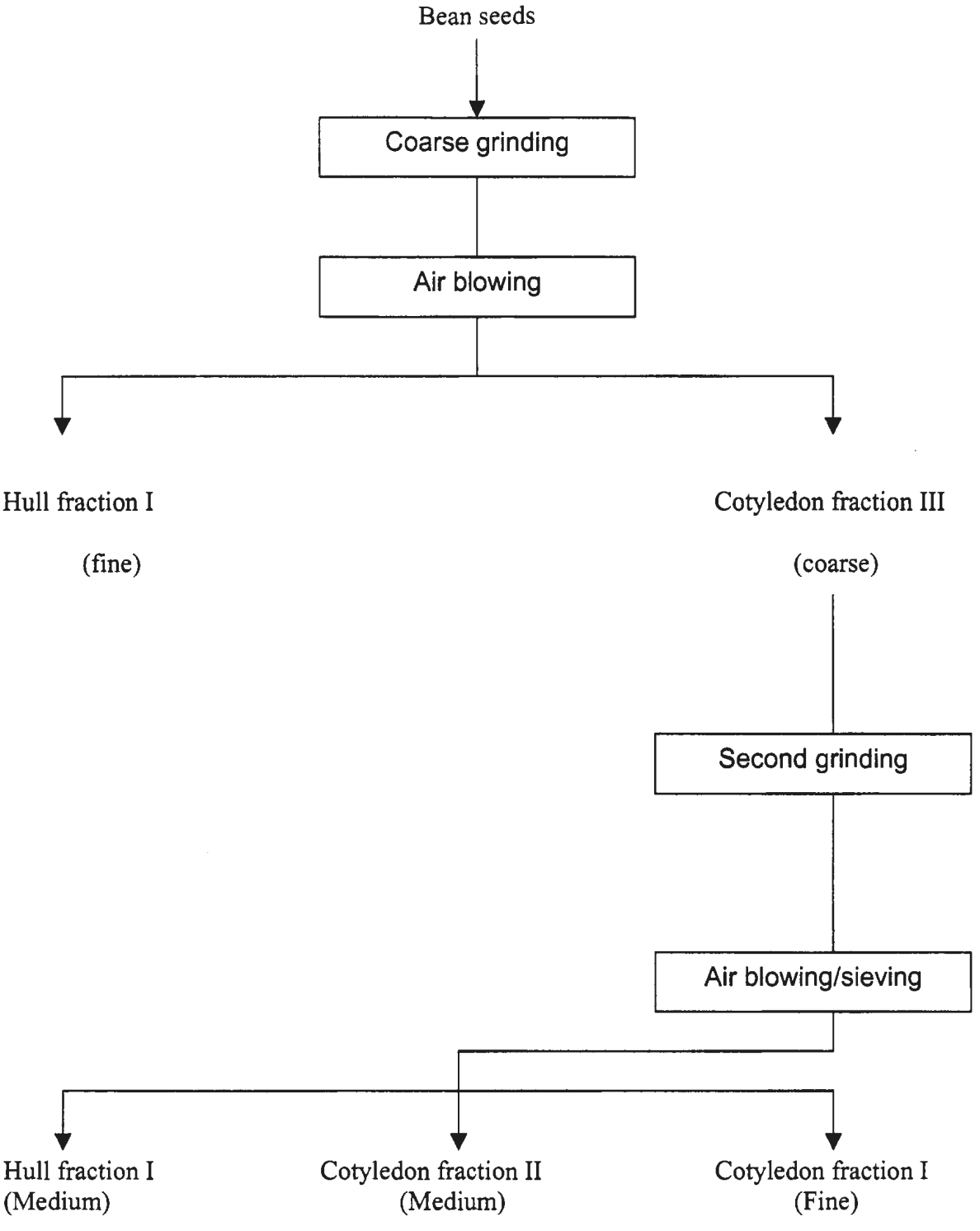
Phenolic compounds present in defatted bean samples (10g) were extracted with 80% acetone (180mL) under reflux in a thermostated water bath at 60°C for 1h. The resulting slurries were centrifuged for 5 min at 4000xg (ICE Centra M5, International Equipment Co., Needham Heights, MA) and supernatants were collected. The residue was reextracted with 80% acetone for 30 min and the supernatants were collected. The combined supernatants were desolventized *in vacuo* at 40°C and the resulting concentrated solutions were lyophilized for 72h at –47°C and $35X10^{-3}$ mbar (Freezone6, Model 77530, Labconco Co., Kansas City, MO).

3.2.4 Determination of total phenolic content

Extracts were dissolved in methanol to obtain a concentration of 0.5mg/mL for whole seed extract and 0.20mg/mL for the hull extracts. The total phenolic content was determined according to an improved version of the procedure explained by Singleton and Rossi (1965).

One millilitre of Folin Ciocalteu's phenol reagent was added to centrifuge tubes containing 1mL of methanolic extracts. Contents were mixed thoroughly and 8.00mL of

Figure 3.1 Flow chart showing the steps of air classification of beans.


Bean seeds
Coarse grinding
Air blowing
Hull fraction I
(fine)
Cotyledon fraction III
(coarse)
Second grinding
Air blowing/sieving
Hull fraction I
(Medium)
Cotyledon fraction II
(Medium)
Cotyledon fraction I
(Fine)

sodium carbonate (75g/L) were added to each tube. To the mixture 10mL of distilled water were added and mixed thoroughly.

Tubes were then allowed to stand for 2h at ambient temperature. Contents were centrifuged for 5min at 4000Xg (ICE Centra M5, International Equipment Co., Needham Heights, MA). Absorbance of the supernatant was read at 765nm. A blank sample for each extract was used for background subtraction. Content of total phenolics in each extract was determined using a standard curve prepared for (+) catechin (Figure A-1). Total extracted phenolics were expressed as mg of (+) catechin equivalents per gram of extract.

3.2.5 Determination of the contents of hydrophilic and hydrophobic phenolic fractions

The crude extract was fractionated into hydrophilic and hydrophobic components by mixing 5g of it with 100mL of water and 100mL of butanol in a 250mL separatory funnel. The mixture was allowed to stand overnight at –4°C. The layers were removed from the funnel separately and desolventised in a rotary evaporator (Büchi, Flawil, Switzerland) under vacuum at 40°C. The resulting solution was lyophilized for 72h at –47°C and $35X10^{-3}$ mbar (Freezone 6, Model 77530, Labconco Co., Kansas City, MO). The weight of each fraction was recorded and the content of phenolics determined as explained in section **3.2.4**.

3.2.6 Qualitative determination of vanillin positive compounds

To 1mL of methanolic solution of crude extract, 5mL of freshly prepared 0.5% vanillin solution in 4% hydrochloric acid were added, mixed vigorously and allowed to stand at 30°C for 20 min. A positive test was indicated by a characteristic pink colour in the solutions (Price and Butler, 1977).

3.2.7 Determination of soluble condensed tannins (SCT)

To 1 mL of methanolic solutions of samples, 5 mL of 0.5% vanillin reagent were added; a 5 mL of 4% concentrated HCl in methanol was used as the reagent blank. The absorbances of sample and blank were read at 500 nm after standing for 20 min in the dark at ambient temperature. The content of condensed tannins in the crude extracts was determined using a standard curve for (+) catechin (Figure A-2) and expressed as g (+) catechin equivalents per 100 g extract (Price *et al.*,1978).

3.2.8 Determination of proanthocyanidin content

The condensed tannins were also assayed colourimetrically by the proanthocyanidin assay explained by Mole and Waterman (1987). One millilitre of methanolic solution of condensed tannin samples was added to 10 mL of the n-butanol-HCl reagent (0.7 g of ferrous heptahydrate in 25 mL of concentrated HCl containing a small volume of n-butanol and made to 1L with n-butanol). The mixture was heated in a sealed ampule for 2 h in a boiling water bath and allowed to cool to ambient temperature. The absorbance of the solution was read at 550 nm against a reagent blank. For

absorbance values of more than 0.75, (A> 0.75) the reaction mixture was diluted with n-butanol. The content of tannins was expressed as A_{550} per gram of extracts.

3.2.9 Determination of protein precipitation capacity (PPC)

The effect of soluble condensed tannins in extracts on formation of soluble condensed tannins (SCT)-protein complex was determined by the method described by Hagerman and Butler (1978) using 1 mg of bovine serum albumin (BSA) per 1 mL concentration. To 1 mL of extract in methanol, 2 mL of a standard BSA solution was added (1 mg of protein/mL in 0.2 M acetate buffer containing 0.17 M sodium chloride, pH 4.0) and mixed well. After 15 min standing at ambient temperature, the solution was centrifuged at 10,000 x g for 15 min. The supernatant was discarded and the surface of the pellet and the tube walls were carefully washed with acetate buffer (pH 4.0) without disturbing the pellet. The pellet was then dissolved in 4 mL of sodium dodecyl sulphate (SDS)-triethanolamine solution [1% SDS and 5% (v/v) triethanolamine in distilled water] and 1 mL of ferric chloride reagent (0.01 M ferric chloride in 0.01 M HCl) was added to it and mixed. After 15 min from the addition of ferric chloride reagent, the absorbance of the solution was read at 510 nm against a reagent blank (4 mL of SDS solution and 1 mL of ferric chloride reagent). The protein precipitation capacity of tannins was expressed as A_{510} per gram of extract.

3.2.10 Evaluation of antioxidative activity of extracts

Different model systems were adopted for evaluating antioxidative properties of crude extracts. The concentrations tested were 50 and 100 ppm phenolics as (+) catechin

equivalents except in the Trolox equivalents antioxidative capacity (TEAC) test. For the convenience of discussion, these concentrations will be referred to, in the text as 50 and 100 ppm.

3.2.10.1 Measurement of total antioxidant capacity by Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay is based on scavenging of 2,2' azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical anion ($ABTS^{\cdot-}$). A solution of $ABTS^{\cdot-}$ was prepared in 0.1M saline phosphate buffer (pH 7.4, 0.15M sodium chloride) (PBS) by mixing 2.5mM 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) with 2.0 mM $ABTS^{2-}$. The solution was heated for 16 min at 60°C, protected from light and stored at ambient temperature until used. The radical solution was used within 2 h as the absorbance of the radical itself depletes with time. Bean extracts were dissolved in PBS at a concentration of 0.17 mg/mL and diluted accordingly to have it fit in the range of values in the standard curve. For measuring antioxidant capacity, 40 μL of the sample were mixed with 1.96 mL of $ABTS^{\cdot-}$ radical solution. Absorbance of the above mixture was monitored at 734 nm over a six min period. The decrease in absorption at 734 nm, 6 min after the addition of a test compound was used for calculating TEAC values. A standard curve was prepared by measuring the reduction in absorbance of $ABTS^{\cdot-}$ solution at different concentrations of Trolox. Appropriate blank measurements (decrease in absorption at 734 nm due to solvent without any compound added) were read and the values recorded (van den Berg et al., 1999).

TEAC values were determined as follows;

$\Delta A_{Trolox} = \{A_{t=0\ Trolox} - A_{t=6\ min\ Trolox}\} - \Delta A_{solvent\ (0-6\ min)}$

$\Delta A_{Trolox} = m_x\ [Trolox]$

$TEAC = \{\Delta A_{extract} / m\}\ x\ d$

Where, ΔA = reduction in absorbance, A= absorbance at a given time, m = slope of the standard curve, [Trolox] = concentration of Trolox, and d = dilution factor.

3.2.10.2 β–Carotene-linoleate model system

β–Carotene-linoleate model system studies were carried out following the spectrophotometric method of Miller (1971) based on the ability of the extracts to decrease the oxidative bleaching of β–carotene in a β–carotene/linoleic acid emulsion. An 8 mg sample of crystalline β – carotene was dissolved in 10 mL of chloroform and 2 mL of the solution were pipetted into a 100 mL round bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40°C, 40 mg of linoleic acid, 400 mg of Tween 40 emulsifier and 100 mL of aerated distilled water were added to the flask with vigorous shaking. Aliquots of 4.8 mL of this emulsion were transferred into a series of tubes containing methanolic extracts so that the final concentration of phenolics was 50 and 100 ppm. The total volume of the system was adjusted to 5.0 mL with methanol. BHA and catechin were used for comparative purposes. Immediately after the addition of emulsion to the tubes, zero time reading was measured at 470 nm using a Hewlett Packard diode array spectrophotometer (Model 8452, Hewlett Packard Co., Mississauga, ON). Subsequent absorbance readings were recorded over a two hour period at 15 min

intervals by keeping the stoppered samples in a thermostated water bath at 50°C. Blank samples devoid of β–carotene were prepared for background subtraction.

3.2.10.3 Bulk stripped corn oil model system

Extracts and standards (BHA, α–tocopherol, catechin) and 5g of stripped corn oil were mixed in 30 mL screw capped glass tubes so that the final concentrations of phenolics were 50 and 100 ppm. Samples were placed in an oven (Thelco, Model 2, Precision Scientifica Co., Chicago, IL) at 60°C for 7 days for Schaal oven studies. Samples for analysis of conjugated dienes (CD), hexanal and 2-thiobarbituric acid reactive substances (TBARS) analysis were drawn on days 0,1,3,5 and 7.

3.2.10.3.1 Determination of conjugated dienes (CD)

A specified amount of oil (0.02–0.03g) was weighed into a 25 mL volumetric flask, diluted and made up to the mark with isooctane (2,2,4-trimethylpentane). The solution was thoroughly mixed and its absorbance read at 243 nm using a Hewlett Packard diode array spectrophotometer (Model 8452, Hewlett Packard Co., Mississauga, ON). Pure isooctane was used as the reference. Conjugated diene values were calculated using the following equation:

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

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

3.2.10.3.2 Determination of 2-thiobarbituric acid reactive substances (TBARS)

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

3.2.10.3.3 Static headspace gas chromatographic analysis

A Perkin-Elmer 8500 gas chromatograph and an HS-6 headspace sampler (Perkin-Elmer Corp., Montreal, PQ) were used for hexanal analysis of corn oil samples. A high polarity Supelcowax 10 fused silica capillary column (30 m x 0.32 mm internal diameter; 0.10 mm film thickness, Supelco Canada Ltd., Oakville, ON) was used. Helium was used as the carrier gas at an inlet column pressure of 17.5 psig with a split ratio of 7:1. The oven temperature was maintained at 40°C for 5 min and then ramped to 100°C at 20°C/min and held there for 5 min. The injector and flame ionization detector (FID) temperatures were set at 280°C (Wettasinghe and Shahidi, 1996).

For headspace (HS) analysis, 200 mg of oil were transferred into 5 mL glass vials. The vials were then capped with Teflon-lined septa, crimped and stored at –40°C (Ultra Low, Revco Inc., West Columbia, SC) until used. To avoid heat shock after removal from storage, frozen vials were tempered at room temperature for 30 min and then preheated in the HS-6 magazine assembly at 90°C for 45 min equilibrium period. Pressurization time of the vial was 6 s and the volume of the vapour phase drawn was approximately 1.5 mL. Chromatographic peak areas were expressed as integrator count units. Individual volatile compounds were identified by comparing the relative retention times of GC peaks with those of the commercially available standards. Quantitative determination of dominant aldehydes was accomplished using 2-heptanone as an internal standard (Wettasinghe and Shahidi, 1996).

3.2.11 Evaluation of reactive oxygen species (ROS) and DPPH radical scavenging efficacies

3.2.11.1 Hydrogen peroxide scavenging assay

Extracts were dissolved in 3.4 mL of a 0.1M phosphate buffer (pH 7.4) and mixed with 0.6 mL of 53 mM solution of hydrogen peroxide prepared in the same buffer solution. Catechin was used as the reference antioxidant compound. Final concentrations of the extracts were 50 and 100 ppm phenolics as catechin equivalents. Immediately after mixing the zero time absorbance was read at 230 nm and subsequent readings were taken at 10 min intervals over 40 min. For each concentration, a separate blank sample devoid of hydrogen peroxide was used for background subtraction (Ruch *et*

al., 1989). The hydrogen peroxide scavenging capacities were calculated using the following equation.

$$\text{Hydrogen peroxide scavenging capacity, \%} = 100 - \left\{ \frac{[H_2O_2]_{\text{additive}}}{[H_2O_2]_{\text{control}}} \right\} \times 100$$

$[H_2O_2]_{\text{additive}}$ = hydrogen peroxide concentration of medium containing the additive of concern; and $[H_2O_2]_{\text{control}}$ = hydrogen peroxide concentration of the control medium.

3.2.11.2 Superoxide radical scavenging assay

Superoxide radicals were generated via an enzymatic reaction. The reaction mixture contained 1 mL of 3 mM hypoxanthine, 1 mL of 100 milli International Units (mIU) xanthine oxidase, 1 mL of 12 mM diethylenetriaminepentaacetic acid, 1 mL of 186 μM nitro blue tetrazolium and 1 mL of the extracts (final concentration of the phenolics in the assay medium was 50 or 100 ppm phenolics as catechin equivalents). Catechin was used as the reference antioxidant. All solutions were prepared in a 0.1M phosphate buffer (pH 7.4) solution. The absorbance values of the mixtures were read at 560 nm initially at 0 min, and thereafter every 10 min for up to 60 min. Readings at 10 min were used to calculate superoxide radical-scavenging capacities. The following equation was used to calculate superoxide radical scavenging capacity (Nishikimi et al., 1972).

Superoxide radical scavenging capacity,% = 100 – {absorbance of medium containing the additive of concern / absorbance of the control medium} X 100

3.2.11.3 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

One hundred microlitres of a 0.75mM solution of DPPH in methanol were added to a 100 μL of a solution containing the extracts so that the concentration of phenolics in the final assay medium was 50 and 100 ppm catechin equivalents. Contents were mixed well and after 1 min 100 μL of the solution were transferred into a quartz capillary tube. The spectrum was recorded using an electron paramagnetic resonance (EPR) spectrophotometer (Bruker ESP 300 Bruker Analytische Messetechnik) set at 2 x 10^5 receiver gain, 1.0 G modulation amplitude, 200s scan time, 3460 G centre field, 100G sweep width and 0.5 s time constant (Santiago *et al.*, 1992). The reference antioxidant used was catechin. DPPH radical scavenging capacities of the extracts and catechin were calculated using the following equation.

DPPH radical scavenging capacity, % = 100 – { EPR signal intensity for the medium containing the additive / EPR signal intensity for the control medium } X 100

3.2.12 Evaluation of Fe (II) chelation capacity

Solutions of ferrous sulphate [1 mL of 400 μM solution prepared in 10 mM hexamine-HCl buffer containing 10 mM KCl (pH 5.0)] and extracts (1 mL of a solution prepared in the same buffer) were mixed followed by the addition of 0.1 mL of a 1 mM solution of tetramethylmurexide prepared in the same buffer. Catechin was used as the reference antioxidant. The final concentrations of extracts and catechin were 50 and 100 ppm based on phenolics. Absorbance of the reaction mixture was recorded at 460 and 530 nm and the ratio of A_{460} to A_{530nm} calculated. These absorbance ratios were then

converted to the corresponding free Fe (II) concentrations using a standard curve (Terasawa *et al.*, 1991). Iron chelating capacities of the additives were calculated using the following equation.

Fe (II) chelation capacity, % = 100 {Absorbance ratio of medium containing the additive/ absorbance ratio for the control} X 100

3.2.13 Effect of bean extracts on preventing cupric ion induced human low density lipoprotein (hLDL) peroxidation

The method of Hu and Kitts (2000) was used to measure hLDL oxidation. The human LDL (in PBS, pH 7.4 with 0.01% EDTA) was dialyzed against 10 mM PBS (pH 7.4, 0.15M NaCl) at 4°C for 24 h. The EDTA- free hLDL (0.1 mg/ mL) was mixed with 10, 25, 50 and 100 ppm levels of extracts (final concentrations), as catechin equivalents, and catechin dissolved in saline PBS; oxidation was initiated by adding 10 μM $CuSO_4$ at 37°C for 20 h. The mixtures were tested for their content of conjugated dienes at 234 nm. The percentage inhibition of formation of conjugated dienes was calculated using the equation given below.

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

Where, $Abs_{oxidative}$ = absorbance of LDL mixture with $CuCl_2$ only; Abs_{sample} = absorbance of LDL with extract/standard and $CuSO_4$; Abs_{native} = absorbance of LDL without $CuSO_4$

3.2.14 Supercoiled strand DNA scission by peroxyl and hydroxyl radicals

Plasmid supercoiled DNA (pBR 322) was dissolved in 10 mM phosphate buffered saline (PBS) (pH 7.4, 0.15 mM sodium chloride). DNA (20 ng/mL) was mixed with catechin and bean extracts dissolved in the same PBS to obtain final concentrations of 5,10,50 and 100 ppm. Peroxyl radical was generated using AAPH (dissolved in PBS; pH 7.4, 0.15 mM sodium chloride) to attain a final concentration of 1mM and mixed with the DNA and the extract mixture at a total volume of 12 μL. The reactants were incubated at 37°C for 1h in the dark (Hu *et al.*, 2000).

The hydroxyl radical generating system consisted of 100 μM ferric chloride, 100 μM ascorbic acid and 100 μM hydrogen peroxide and 100μM EDTA for non site-specific hydroxyl radical generation (Halliwell *et al.*,1987). For site-specific hydroxyl radical generation EDTA was replaced with PBS (Hu and Kitts, 2001). The total volume was adjusted to 12 μL with PBS and the reaction mixture was incubated at 37°C for 1 h in the dark. Upon completion of incubation, the loading dye (3 μL) (consisting 0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose in distilled water) was added to the sample and loaded to a 0.7% (w/v) agarose gel prepared in Tris-acetic acid-EDTA buffer (40 mM Tris acetate, 2mM EDTA, pH 8.5). Horizontal gel electrophoresis was performed at 32v for 8h. DNA strands were stained with 0.5 μg/mL ethidium bromide and visualized under ultraviolet light. Images were analyzed using AlphaEase ^{TM} stand alone software (Alpha Innotech Co., San Leandro, CA). The protective effect of extracts and catechin was calculated based on the following equation.

$$\text{DNA retention, \%} = \left\{ \frac{\text{Supercoiled DNA content in sample}}{\text{Supercoiled DNA content in control}} \right\} \text{x } 100\%$$

3.2.15 Identification of active compounds in bean extracts

3.2.15.1 Analysis of anthocyanidins

Ten milligrams of sample were accurately weighed in 8 mL capacity thick-walled screw-top glass tubes, sealed with a teflon-lined screw cap and 3 mL of acidified butanol (950 mL of n-butanol mixed with 50 mL of concentrated HCl) and 0.1 mL of iron reagent (0.5 g of $FeNH_4(SO_4)_2$ dissolved in 25 mL of 2M HCl) were added and vortexed. The samples so prepared were kept in a boiling water bath for 50 min. The samples were then cooled, transferred to 10 mL volumetric flasks and adjusted the volume to 10 mL with acidified butanol (Porter *et al.,* 1986). The samples were passed through a 0.45 μM filter, filtrates were collected and mixed with 250 μL methanol. Samples (20 μL) were injected onto Shimadzu HPLC system (Mandel Scientific Co., Guelph, ON) (two LC 10AD pump, SPD M10A diode array detector, SCL-10A system controller, CTO 10AS column oven). The conditions for separation were as follows: column LUNA (5μ C 18; 4.6 X 250 mm) Phenomenex; mobile phase; 4% aqueous phosphoric acid/acetonitrile (80:20; v/v); flow rate 1 mL/min; oven temperature 25°C; detection at 525 nm (Hong and Wrolstad, 1990).

3.2.15.2 Analysis of procyanidins

Analysis of procyanidins was carried out according to the procedure explained by Oszmianski and Moutounet, (1996). Twenty milligrams of sample were dissolved in 10 mL of methanol and 20 μL of samples so obtained were injected onto a Shimadzu HPLC system. The HPLC system and the column used for proanthocyanidin analysis was the

same as that used in section **3.2.15.1** for anthocyanidin analysis. The samples were separated in a gradient system having the following parameters: A, 5% (v/v) acetonitrile in 5% (v/v) of acetic acid; B, 40% acetontrile in 5% acetic acid (v/v); gradient B, 0% to B= 100% in 50 min.

3.2.15.3 Analysis of phenolic acids

Approximately 50 mg of each bean extract were dissolved in 10 mL of 2M NaOH and hydrolyzed for 4 h at room temperature under nitrogen atmosphere. After acidification to pH 2, using 6M HCl, free phenolic acids were extracted 5 times into 15 mL diethyl ether using a separatory funnel. The ether extract was then evaporated to dryness under vacuum at 30°C. The dry residue was then dissolved in 2 mL of methanol and filtered through a 0.45 μM nylon filter (Amarowicz and Weidner, 2001). The sample so obtained was injected onto a Shimadzu HPLC system described in section **3.2.15.1.** The conditions for separation were as follows: pre-packed LiChrospher 100 RP-18 column (5μM, 4.0 X 259 mm, Merck); reverse phase: water-acetontrile-acetic acid (88:10:2; v/v/v); flow rate 1 mL/min; oven temperature 25°C; at injection volume 20 μL, detector was set at 260 nm (Amarowicz and Shahidi, 1994).

3.2.16 Statistical analysis

All experiments were carried out in triplicates and results were reported as mean ± standard deviation. The significance of differences among the values was determined at $p < 0.05$ using analysis of variance (ANOVA) followed by Tukey's multiple range test (Snedecor and Cochran, 1980).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Proximate composition of beans

The contents of moisture, ash, crude protein and total lipids of red, brown, black and white beans are listed in the Table 4.1. These values are in agreements with the data reported by Berrios *et al.* (1999).

4.2 Yield and phenolic contents of crude extracts

Different solvents have been employed to extract phenolic compounds from plant materials. Naczk *et al.* (2001) used 70% acetone to extract phenolic compounds from canola and rapeseed hulls. Heinonen *et al.* (1998) used 60% aqueous methanol or 70% acetone to extract antioxidative compounds from homogenized berries (black berries, high bush berries, red raspberries, strawberries, sweet cherries). Grape anthocyanins have been extracted with 50% methanol containing 1% trifluoroacetic acid (TFA) as reported by Tamura and Yamagami (1994). Shahidi and coworkers (2001) extracted beach pea phenols (*Lathyrus maritimus* L) using 70% (v/v) aqueous acetone at room temperature. Methanol and methanol-water (50:50 v/v) were used to extract phenolics of beans; methanol was found to yield the highest total phenolic content of beans (Meja *et al.,* 1997). Phenolic compounds from beans were also extracted with 70% methanol (Raab *et al.,*1997), 80% methanol (Ganthavorun and Hughes, 1997), 80% ethanol containing 0.5% trifluoroacetic acid (Tsuda *et al.,* 1997) and 95% ethanol (Onyeneho and Hettiarachchy, 1991). Hussein *et al.* (1990) reported that 70% acetone extracted the highest total phenolic content from beans among several solvents used, namely water,

Table 4.1 Proximate composition (%) of red, brown, black and white bean Seeds[1].

Bean Type	Moisture	Ash	Crude protein	Total fat
Red	13.14±0.10[b]	4.43±0.12[a]	26.10±0.74[a]	1.47±0.90[a]
Brown	11.30±0.11[a]	4.30±0.21[a]	25.70±0.60[a]	1.49±0.84[a]
Black	11.24±0.20[a]	5.03±0.31[a]	25.90±0.26[a]	1.58±0.61[a]
White	11.15±0.25[a]	4.40±0.20[a]	25.30±0.81[a]	1.49±0.72[a]

[1]Data expressed on a dry weight basis.
Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.

10% acetone, methanol containing 1% HCl, 10% tartaric acid in methanol and 70% aqueous acetone.

In this study, preliminary experiments revealed that 70% acetone was the best solvent for the extraction of phenolics from beans at 60°C for 60 min as it afforded a maximal yield of phenolics. The yields of whole bean extracts ranged from 12.2 to 20.6% (w/w), being highest for whole black beans and lowest for white beans; values were in between for red and brown beans. The same pattern of yield was observed for hulls but the values were comparatively higher (19.0 - 26.6%) than those of whole seeds (Table 4.2).

All bean extracts showed a positive result for vanillin test indicating the presence of condensed tannins; however, results for white bean extracts were inconclusive. Literature data and the preliminary studies indicated the presence of catechin or related compounds in beans. Therefore, the total phenolic contents were reported as catechin equivalents. In addition, catechin was used as a reference antioxidant in other experiments.

Out of the four bean varieties tested, red whole beans had the highest content of phenolics. The total phenolic content of brown whole beans was comparable to that of red whole bean extract while the black whole bean extract had a moderate amount and the white whole bean extract had the lowest amount.

Table 4.2 Yield and phenolic content of bean extracts[1].

Sample	Yield of extract (g/100g of defatted meal)	Phenolic content (mg catechin equivalents per gram extract)		
		Total	Hydrophilic	Hydrophobic
RWE	16.7±2.8[a]	93.6±2.1[c]	30.2±1.8[c]	63.0±2.8[c]
BWE	17.1±2.1[a]	91.4±1.6[c]	29.6±0.3[c]	61.4±1.2[c]
LWE	20.6±1.2[b]	44.0±2.5[b]	12.9±1.6[b]	31.0±0.6[b]
WWE	12.2±0.9[a]	04.9±0.8[a]	01.6±0.5[a]	03.1±0.2[a]
RHE	19.0±1.3[b]	223.5±1.9[d]	74.8±2.2[d]	148.1±1.7[d]
BHE	18.1±0.8[b]	253.2±2.3[e]	82.5±0.9[e]	171.0±0.6[e]
LHE	26.6±1.6[c]	270.0±1.6[f]	83.0±1.7[e]	187.9±3.1[f]
WHE	14.1±3.1[a]	06.7±0.9[a]	04.7±2.2[a]	02.1±0.3[a]

[1]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; WWE, white whole bean extract; RHE, red bean hull extract; BHE, brown bean hull extract; LHE, black bean hull extract; and WHE, white bean hull extract.

In all four bean types, the hulls contained a higher amount of total phenolics when compared to that of whole seeds. Therefore, it can be concluded that phenolic compounds are mainly concentrated in the seed coats. Total phenolic content of black bean extract (LHE) was the highest among all hull extracts while white hull extract (WHE) had the lowest. These results are in agreement with those of Drumm *et al.* (1990) and Tsuda *et al.* (1994) who reported that white beans had a low polyphenolic content compared to coloured beans. The order of total phenolic content in the whole bean extracts was red = brown > black > white. However, in the case of hull extracts the order was black > brown > red > white. The reason for this difference may be attributed to the bean size and hence the proportional difference in hull content in different beans (Table 4.2).

The antioxidant activity varies with the nature of solvent used for extraction of phenolics. Hu and Kitts (2000) reported that chloroform extracts of *Echinacea* root extract did not show any $ABTS^{\cdot-}$ scavenging activity, whereas methanolic fraction exhibited antioxidant activity. Methanol with a high polarity compared to chloroform was effective in extracting polar antioxidative compounds from *Echinacea* roots. The ratio of hydrophilic to hydrophobic phenolics of both whole seed and hull extracts was approximately 1:2 (red, brown and white) and 1:3 (black). In all four bean types the hydrophobic phenolic fraction is more than that of its hydrophilic counterpart. Polarity and solubility of antioxidants determine their concentrations in different locations in various multiphase food and model systems. Their antioxidant activity is affected by their diffusion rates, stability, and degree of dissociation, which may change with their

location in these systems (Huang *et al.*, 1996). The hydrophobic and hydrophilic counterparts present in a mixture are responsible for the overall antioxidant activity (Huang *et al.*, 1994). Plant extracts contain a mixture of compounds with different polarities and hence, their activity depends on the model system used for evaluation. In addition, the activity of some of the antioxidant compounds depend on the pH of the medium. Tsuda *et al.* (1994) reported that the antioxidant activities of anthocyanin pigments isolated from beans depend on the pH of the lionleic acid system used. Therefore, various models systems, free radical scavenging assays, human LDL oxidation model systems and supercoiled DNA scission studies were carried out to a gain a comprehensive understanding of the nature and activities of bean extracts.

4.3 Tannin content

Tannins are complex phenolic compounds widely distributed in plants. The molecular weights of tannins are in the range of 500-3000 Da. Tannins can be classified as either condensed or hydrolysable on the basis of their structural type and their reactions towards hydrolytic agents (Naczk *et al.*, 1994). Tannins may be considered as anti-nutritional compounds due to their ability to bind with proteins and enzymes, thus making them unavailable for absorption in the human digestive system. A number of colorimetric assays have been proposed for quantitation of tannins but only a few of them are specific towards condensed tannins. These include the modified vanillin method of Price *et al.* (1978) and proanthocyanidin assay of Mole and Waterman (1987). The proanthocyanidin assay is carried out in butanol and depends on acid hydrolysis of the interflavanol bonds of condensed tannins to produce anthocyanidins.

The content of tannins in beans, as determined by vanillin, proanthocyanidin and protein precipitation assays is summarized in the Table 4.3. The hull extracts contained a high amount of tannins compared to whole seed extracts. The reason for the low tannin contents in whole beans may be attributed to the dilution effect of the tannins in hulls with cotyledons. The highest tannin content was found in brown bean hulls (2.67 mg/100g extract) followed by red bean hulls (1.63 mg/100g extract) and the lowest in hulls of black beans. White beans did not contain any detectable amount of tannins. The content of tannins in beans as determined by the proanthocyanidin assay showed a good correlation ($r = 0.94$) with the results obtained using the vanillin assay.

The condensation of anthocyanin, which is a major group of flavonoid compounds in beans, with other flavonoids through C-4 position results in the formation of proanthocyanidin polymers e.g. condensed tannins (Foo and Porter, 1980). The antioxidant activity of these compounds would be expected to be similar to their parent flavonoid compounds and dependent on the presence or absence of C-3` and C-4` hydroxyl groups. The condensed tannins composed of gallic acid and epicatechin are effective antioxidants (Salah *et al.*,1995); the antioxidant activity was increased with the number of hydroxyl groups in the molecules.

Table 4.3 Tannin content of bean extracts[1].

Sample	Catechin equivalents[2] (mg/100 g extract)	Absorbance/g[3]	Slope[4]
RWE	0.384±0.001[b]	717±2[c]	0.696±0.017[c]
BWE	0.381±0.006[b]	626±3[b]	0.459±0.008[b]
LWE	0.049±0.002[a]	108±4[a]	0.210±0.008[a]
WWE	ND	ND	ND
RHE	1.634±0.019[d]	2640±15[d]	4.200±0.032[e]
BHE	2.677±0.026[e]	3830±25[f]	4.376±0.150[e]
LHE	1.372±0.058[e]	3000±16[e]	3.303±0.068[d]
WHE	ND	ND	ND

[1]Results reported are mean values of three determinations ± standard deviation.
[2]Determined by vanillin assay.
[3]Determined by proanthocyanidin assay.
[4]Determined by protein precipitation assay.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: ND, not detectable; RWE, red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; WWE, white whole bean extract; RHE, red bean hull extract; BHE, brown bean hull extract; LHE, black bean hull extract; and WHE, white bean hull extract.

4.4 Trolox equivalent antioxidant capacity (TEAC) of bean extracts

TEAC assay is based on the scavenging of 2,2'-azinobis-(3-ethylbenzothiaoline-6-sulphonate) radical anion ($ABTS^{\cdot-}$) which is generated by heating of $ABTS^{2-}$ with 2,2'-azobis-(2-amidinopropane) (ABAP). TEAC value of a compound represents the concentration of a Trolox solution that has the same antioxidant capacity as the unknown compound or mixture of compounds. The value of TEAC can be considered as a stoichiometric number because TEAC for Trolox is set at 1. Usually the mixture of extract under investigation is diluted to have it fit in the concentration range of the standards. The Trolox standards (0 to 10 μM) resulted in a decrease in the absorbance at 734 nm in the range of 0-0.4 at 734 nm over 6 min.

The TEAC values of extracts are determined by the measurement of the absorbance of $ABTS^{\cdot-}$ at 734 nm in the presence of the extract, and compared with that of Trolox. TEAC depends on the time point used to read the absorbance value. Trolox reacts instantly with $ABTS^{\cdot-}$ and the reaction is completed within one minute. In contrast, the extracts take 6 min to complete the reaction indicating a biphasic pattern of reaction. Reactions of Trolox, whole bean extracts and hull extracts with $ABTS^{\cdot-}$ are illustrated in Figures 4.1 and 4.2, respectively.

The extracts were prepared at a concentration of 0.20 mg/mL and diluted accordingly to fit in the desired range. Whole seed extracts were used at the same concentration while the hull extracts were diluted to a final concentration of 0.05 mg/mL. Table 4.4 lists the TEAC values of whole seed and hull extracts of beans. van den Berg *et al.*

(1999) reported that a biphasic behaviour can be observed to a lesser degree in compounds such as vitamin C and to a greater extent in flavonoids and other mixtures of extracts. Two time points (10 s and 6 min) which can be used for measuring absorbance have been tested. Due to the biphasic nature of most antioxidative compounds, 6 min which includes a greater part of the slow-biphasic reaction (van den Berg *et al.*, 1999) has shown good results: Therefore, 6 min was used as the time point in the present study. Whole bean extracts showed low TEAC values ranging from 4.64 to 8.84 (whole bean extracts are 4.64 to 8.84 times effective as Trolox) whereas TEAC values of hull extracts varied from 40.74 to 46.68 (hull extracts were 40.74 to 46.68 times effective as Trolox). In both cases the red bean extract showed the highest TEAC value followed by extracts of brown and black beans. TEAC value can be used to rank the unknown antioxidants. In both whole and hull extracts at the same concentration, TEAC value for beans was in the descending order of red > brown > black. TEAC of red and brown bean hulls were about 5.5 times higher than that of the corresponding whole seed extracts while black hulls were about 9 times more effective than their corresponding whole seed extracts. TEAC method is useful in screening antioxidants but antioxidant effectiveness must also be studied by other methods because their activity in foods is dependent on a variety of factors, including polarity, solubility and metal-chelating capacity.

As depicted in Figure 4.3, the total phenolic contents of bean extracts are well correlated with TEAC values ($r = 0.934$). However, this is contrary to the findings of Ganthavorn and Hughes (1997) who reported that the concentration of bean polyphenolic compounds was not consistent with antioxidant capacity. This may be due to different

Figure 4.1 Reaction of Trolox (3μM) and whole bean extracts at a final concentration of 0.2 mg/mL of the assay medium with 2,2'-azinobis(3-ethylbenzthaizoline-6-sulphonic acid) radical anion ($ABTS^{\cdot-}$).
Error bars are very small and therefore have been omitted.
Abbreviations used are: RWE, red bean whole extract; BWE, brown bean whole extract; and LWE, black bean whole extract.

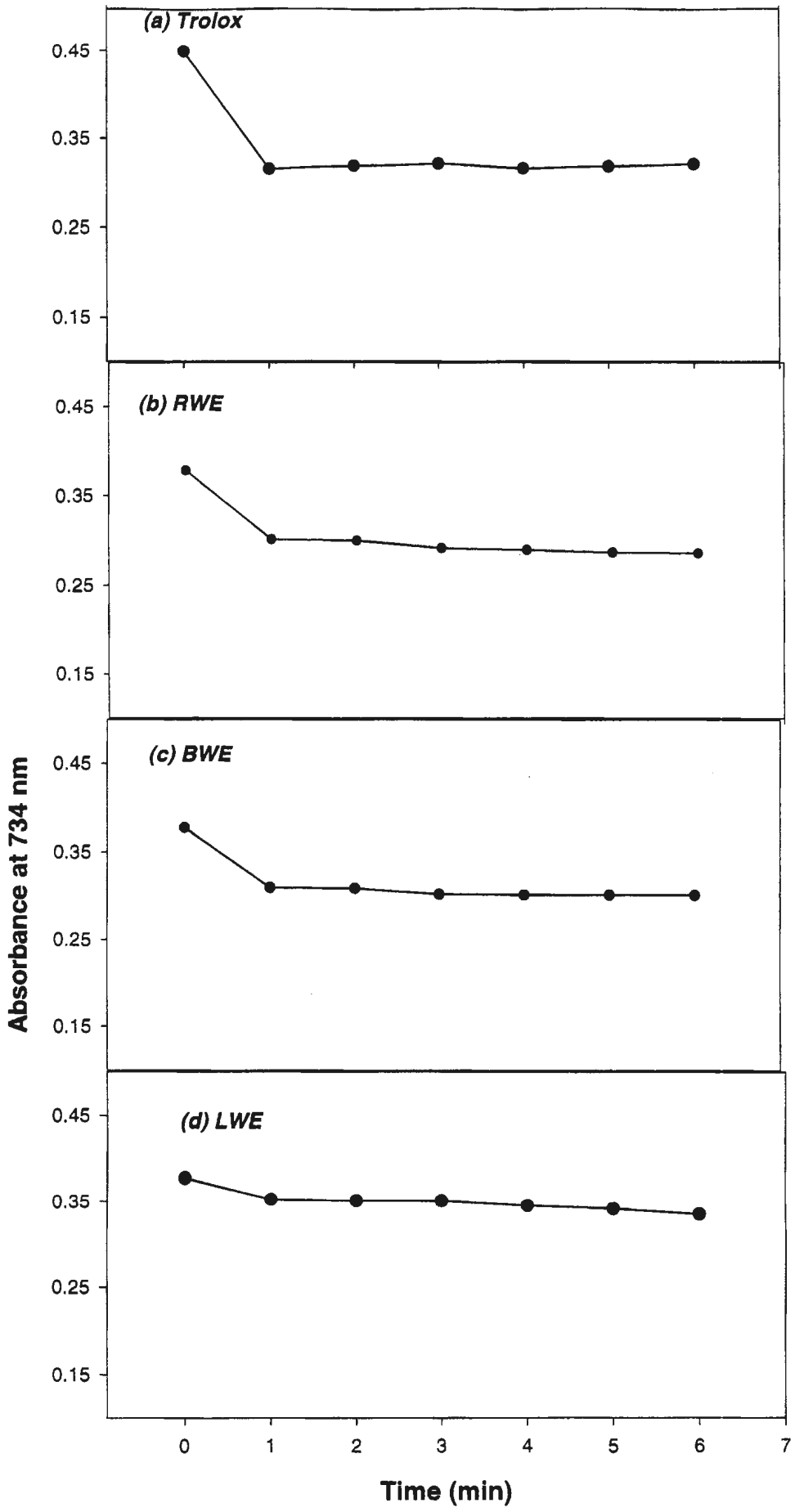

(a) Trolox
(b) RWE
(c) BWE
(d) LWE
0.45
0.35
0.25
0.15
Absorbance at 734 nm
0
1
2
3
4
5
6
7
Time (min)

Figure 4.2 Reaction of Trolox (3μM) and bean hull extracts at a final concentration of 0.05 mg/mL of the assay medium with 2,2'-Azinobis (3-ethylbenzthaizoline -6-sulphonic acid) radical anion ($ABTS^{\cdot\cdot}$).
Error bars are very small and therefore have been omitted.
Abbreviations used are: RHE, red bean hull extract; BHE, brown bean hull extract; and LHE, black bean hull extract.

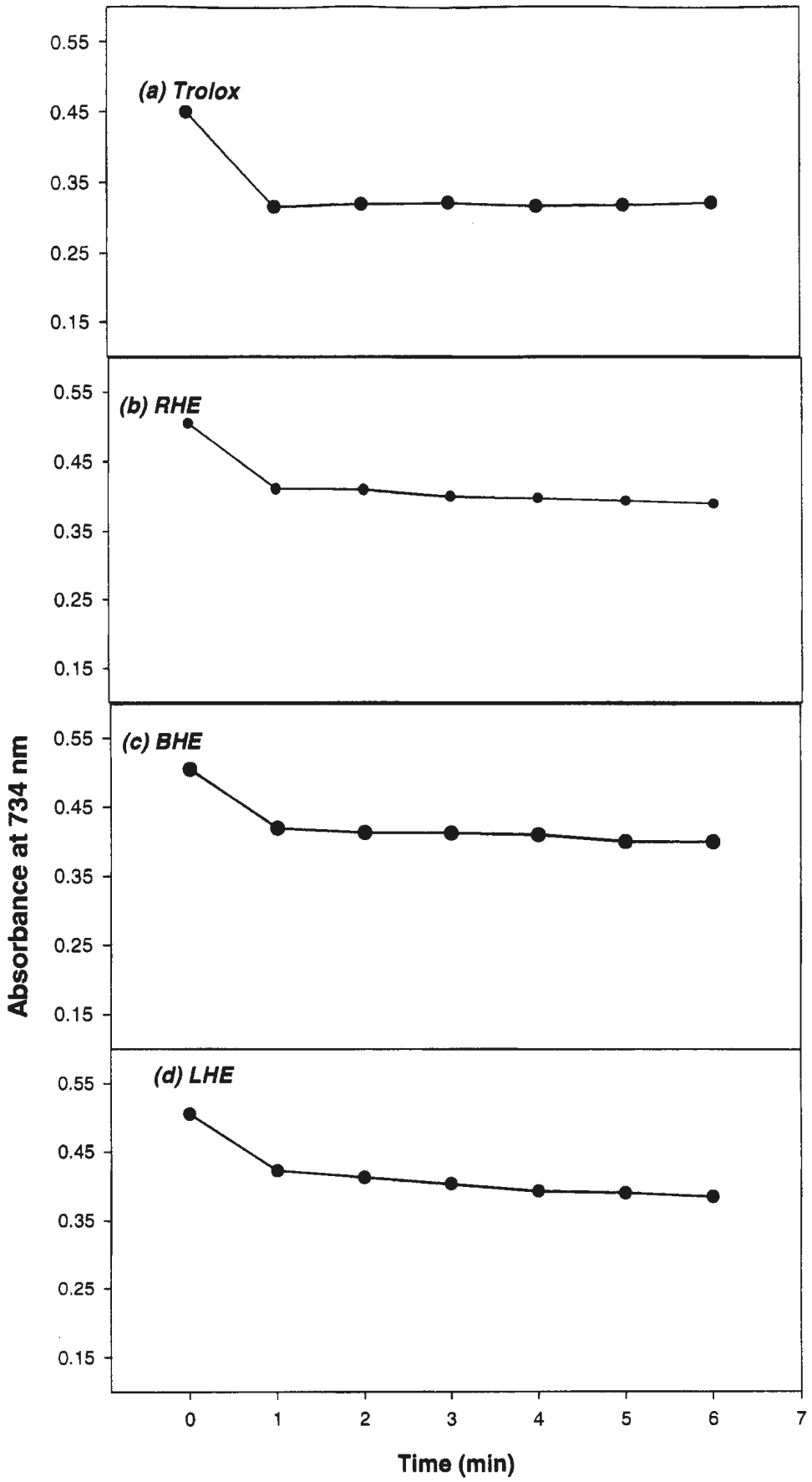

(a) Trolox
(b) RHE
(c) BHE
(d) LHE
0.55
0.45
0.35
0.25
0.15
Absorbance at 734 nm
0
1
2
3
4
5
6
7
Time (min)

Table 4.4 Trolox equivalent antioxidant capacity (TEAC) values of whole seed and hull extracts of beans[1].

Extract	Original concentration (mg/mL)	Dilution factor	Final concentration (mg/mL)	TEAC
RWE	0.2	1	0.2	8.84±0.31[c]
BWE	0.2	1	0.2	7.46±0.20[b]
LWE	0.2	1	0.2	4.64±0.61[a]
RHE	0.2	4	0.05	46.68±0.12[f]
BHE	0.2	4	0.05	42.99±0.68[e]
LHE	0.2	4	0.05	40.74±0.23[d]

[1]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red bean whole extract; BWE, brown bean whole extract; LWE, black bean whole extract; WWE, white bean whole extract; RHE, red bean hull extract; BHE, brown bean hull extract; LHE, black bean hull extract; and WHE, white bean hull extract.

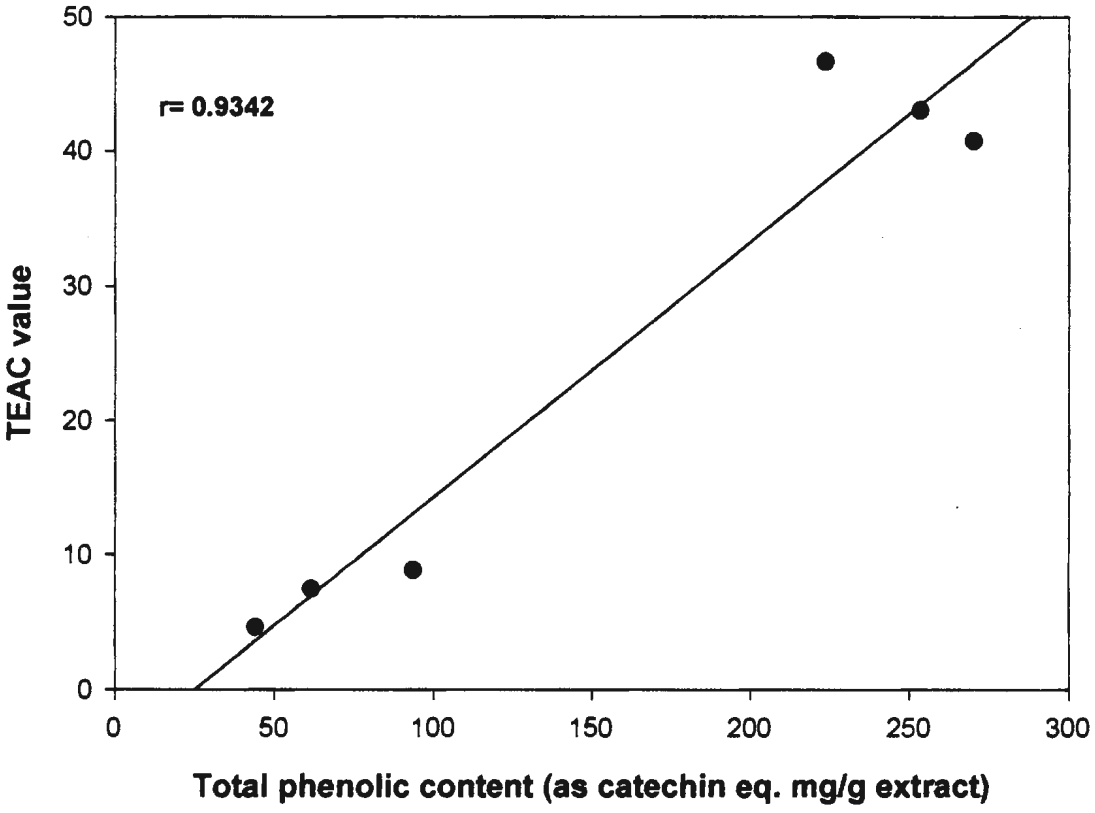
individual phenolics present in different mixtures. However, the results of the present study indicated that phenolic compounds in bean extracts significantly contribute to their total antioxidant capacity as measured by TEAC.

The bean hull extracts contain a high concentration of anthocyanins which impart a strong colour to the resultant extract solution. The extracts when dissolved in usual solvents yield a very intense colour which interferes with most colourimetric methods of estimation. Therefore, only the whole bean extracts were tested in most of the tests explained in the following sections.

4.5 Antioxidant efficacy of beans

Different model systems have been employed to evaluate the antioxidant efficacy of various plant materials. Model systems such as β-carotene-linoleate (Miller, 1971), bulk and oil-in-water corn oil (Frankel *et al.*, 1994), liposome (Tsuda *et al.*, 1993), rabbit erythrocyte membrane systems (Tsuda *et al.*, 1994) are among the methods used for estimation of antioxidant activity. Wettasinghe and Shahidi (1999) used a β-carotene-linoleate model system to evaluate the antioxidant activity of borage and evening primrose extracts. Tsuda *et al.* (1993) used a linoleic acid system to evaluate the antioxidants of beans.

Figure 4.3 Relationship between the total phenolic content of bean extracts and TEAC values


r= 0.9342
TEAC value
Total phenolic content (as catechin eq. mg/g extract)
0
10
20
30
40
50
0
50
100
150
200
250
300

Onyeneho and Hettiarachchy (1991) employed soybean and sunflower oil storage studies to investigate the antioxidative effects of navy bean hull extracts. Corn oil stripped of its endogenous antioxidants has been used to evaluate antioxidant activity of Trolox, L-ascorbic acid, α-tocopherol and L-ascorbyl 6-palmitate (Frankel *et al.,* 1994). The β-carotene (Miller, 1971) and the bulk corn oil model systems (Frankel *et al.*, 1994) have been employed to assess the antioxidant activity of the whole seed extracts of beans.

4.5.1 β-Carotene-linoleate model system

β-Carotene has a natural orange colour which degrades upon oxidation. The linoleic acid radical formed upon abstraction of a hydrogen atom may attack the highly conjugated β-carotene, thus leading to the loss of its natural orange colour. The presence of phenolic antioxidants prevents the loss of colour by neutralizing linoleic acid free radical. The greater the potential of the antioxidant, the lesser the depletion of colour. Thus, this system can be employed to evaluate the efficacy of unknown antioxidative compounds (Wettasinghe and Shahidi, 1999). In β-carotene-linoleate model system the amount of β-carotene remaining in the assay medium after 120 min was used as an indicator for the efficacy of antioxidants. The initial content of β-carotene was 76±5 μg. Table 4.5 lists the amount of β-carotene in μg left after 120 min of assay with different antioxidative extracts at two concentration levels (50 and 100 ppm as catechin equivalents).

The control sample, devoid of antioxidant extracts, lost almost 85% of its initial β-carotene content after 120 min assay. As there is no protection for β-carotene, the

Table 4.5 Retention capacity of β-carotene after 2h assay in a β-carotene-linoleate model system at 50 and 100 ppm levels[1]

Additive	Amount of β-carotene (μg) retained after 2h of assay (Initial content is 76±4μg)	
	50 ppm	100 ppm
RWE	45.68±0.68[c]	51.51±0.25[c]
BWE	45.32±0.25[c]	50.06±0.33[c]
LWE	28.59±0.08[b]	32.89±0.65[b]
Catechin	55.56±0.12[d]	61.02±0.15[d]
BHA	62.24±0.45[e]	69.65±0.14[e]
Control	1.76±0.09[a]	1.76±0.18[a]

[1]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; and LWE, black whole bean extract.

linoleic acid free radical attacks the highly conjugated β-carotene molecule, thus reducing the β-carotene content. Therefore, as shown in Figures 4.4 and 4.5, the amount of β-carotene present is decreased a fast rate. On the other hand, the presence of extracts changes the pattern of β-carotene loss. In the presence of additives, the loss of β-carotene occurred according to a second order polynomial function. As expected, BHA, a reference antioxidative compound, exerted the strongest antioxidative effect at both 50 and 100 ppm levels.

Out of the three bean extracts tested, red and brown whole bean extracts showed a retention of 50-52% of the original β-carotene content at 100 ppm level while black whole bean extract showed a retention of 33% which is significantly ($p<0.05$) lower compared to the effect of red and brown whole bean extracts. At 50 and 100 μM concentrations, cyanidin-3-O-β-D-glucoside and cyanidin extracted from beans have shown a stronger antioxidant activity than α-tocopherol in a linoleic acid system (Tsuda *et al.*, 1994). The β-carotene-linoleate system is comparable to an oil-in-water emulsion. Therefore, it can be speculated that hydrophobic antioxidants can be more effective in oil-in-water type emulsion. In the assay, the hydrophobic fraction of antioxidants might have performed effectively in protecting β-carotene. It is evident that the protective effect of the extracts is attributed to their phenolic content although structural effects are also very important. Red and brown bean extracts with a high total phenolic content exerted a great effect in β-carotene retention while the black bean extract had a relatively low effect.

Figure 4.4 Effect of bean extracts at 50 ppm, as catechin equivalents, on the retention of β-carotene in a β-carotene-linoleate model system.

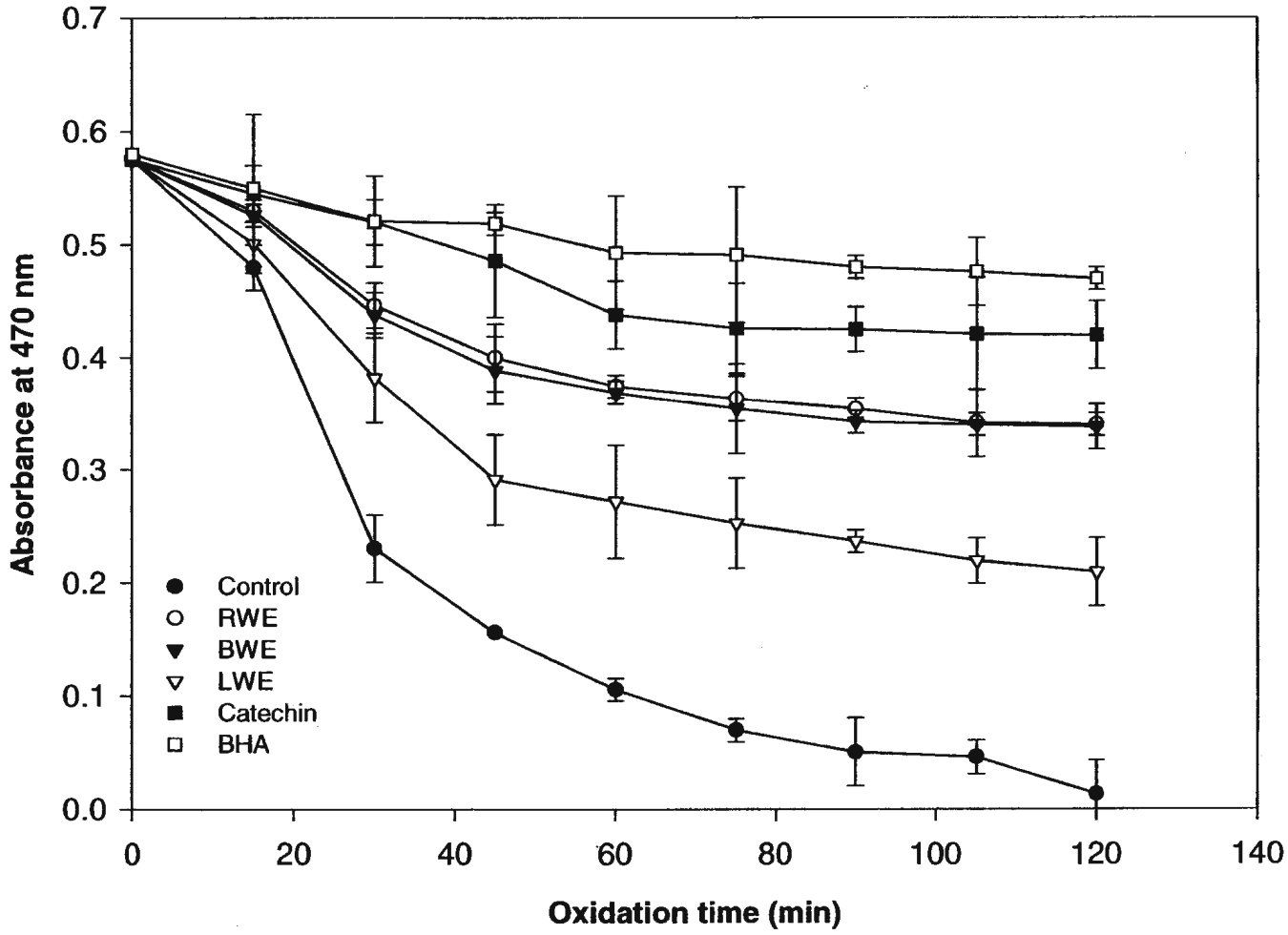
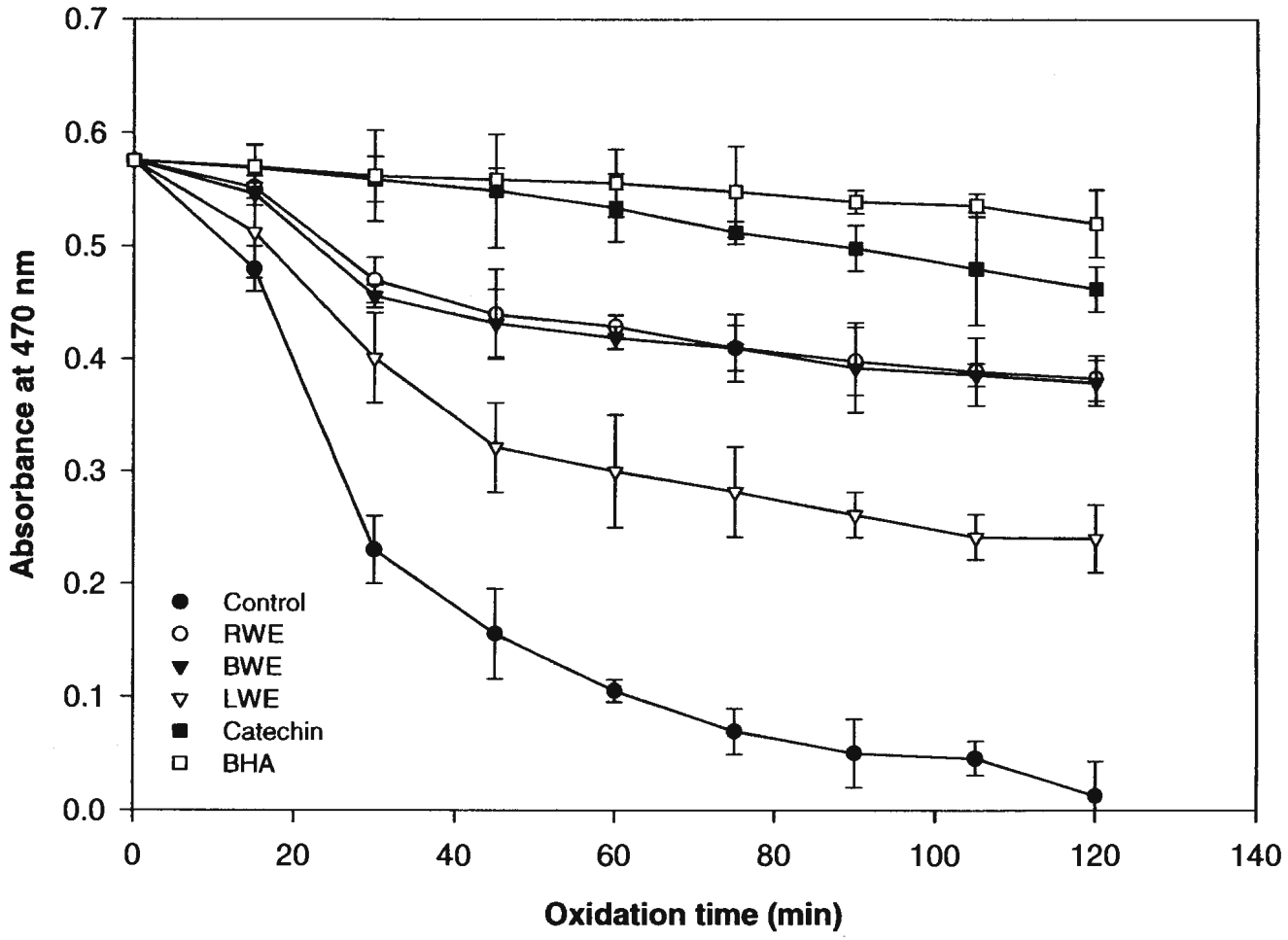

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 470 nm
0
20
40
60
80
100
120
140
Oxidation time (min)
Control
RWE
BWE
LWE
Catechin
BHA

Figure 4.5 Effect of bean extracts at 100 ppm, as catechin equivalents, on the retention of β-carotene in a β-carotene-linoleate model system.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 470 nm
0
20
40
60
80
100
120
140
Oxidation time (min)
Control
RWE
BWE
LWE
Catechin
BHA

Tsuda *et al.* (1993) reported that the effect of crude bean extracts in inhibiting oxidation in a linoleic acid system measured by the thiocyanate method was comparable to that of BHA while α-tocopherol was not as effective as the extracts in controlling the oxidation. Tsuda *et al.* (1994) indicated that seed coat extracts of red and black beans exhibited strong antioxidant activity which was comparable to that of BHA while white bean seed coat did not show any antioxidant activity in a linoleic acid system as measured by the 2-thiobarbituric acid reactive substances (TBARS).

Tsuda *et al.* (1994) observed that antioxidant activity of two pigments isolated from red bean seed coat; cyanidin (Cy) and cyanidin-3-*O*-β-D-glucoside (C3G) in a linoleic acid system. C3G showed a stronger antioxidant activity than α-tocopherol at 100 μM concentration while Cy showed the strongest activity and Cy showed a marked inhibition of lipid peroxidation even at 5 μM concentration. Meanwhile C3G and α-tocopherol were not effective at this concentration.

4.5.2 Bulk corn oil model system

Commercially available corn oil, stripped of its endogenous antioxidants, was used to evaluate the efficacy of different extracts in preventing its oxidative deterioration. Conjugated dienes (CD), hexanal and TBARS in the treated oil were used as indicators of oxidation. CD measures the formation of primary oxidation products, whereas hexanal, which is the prominent volatile formed in corn oil arising from oxidation of linoleic acid, and TBARS measure secondary oxidation products.

Stripped corn oil containing two levels of antioxidant extracts (50 and 100 ppm) and reference antioxidant was used. The oils were kept at 60°C for seven days. CD, hexanal and TBARS values of the oil samples collected on days 0,1,3,5 and 7 are listed in Tables A-1 through A-6 in the appendix. CD values of treated oils increased by about 3-6 fold at the end of a 7-day storage period whereas the control showed about 6 fold increase. BHA exerted the greatest inhibition on the formation of CD both at 50 and 100 ppm concentrations. Figures 4.6 and 4.7 depict the percentage inhibition of formation of CD, hexanal and TBARS of stripped corn oil at 50 and 100 ppm levels on the 7^{th} day of storage, respectively. Regardless of the concentration used BHA and catechin reduced CD formation by 30-50% as compared to the control. At 100 ppm level both red and brown bean extracts were able to reduce formation of CD by 32%.

At 50 and 100 ppm concentrations, the formation of CD was reduced up to 44% and up to 47%, respectively. The order of effectiveness was catechin > BHA > RWE = BWE = LWE > α-tocopherol. The results show that bean extracts are more effective than α-tocopherol in inhibiting the formation of CD in corn oil.

Formation of TBARS was inhibited by 2-51 and 14-56% at 50 and 100 ppm levels, respectively. The order of activity followed a similar trend to that observed for both CD and hexanal. In all cases, α-tocopehrol was ineffective in controlling oxidation of corn oil. The inhibition of hexanal formation decreased in the order of catechin > RWE = BWE = BHA > LWE > α-tocopehrol. The hexanal concentration showed a 160-

fold increase in untreated oil samples whereas the increase was 16-126 fold in treated samples at the end of the 7-day storage period.

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

4.6 Efficacy of bean extracts in scavenging of reactive oxygen species (ROS)

Tsuda *et al.* (1994) reported that anthocyanin pigments from beans play a role in prevention of lipid peroxidation of cell membranes induced by active oxygen radicals in living systems. The efficacy of bean extracts in scavenging ROS is discussed in the following sections.

4.6.1 Hydrogen peroxide scavenging capacity

The hydrogen peroxide scavenging capacity of bean extracts (after 10 min of the assay) is shown in Table 4.6. Between 58 and 70% of hydrogen peroxide present in the assay medium was scavenged by additives used at 50 ppm level within the first 10 min. When used at 100 ppm level, scavenging of hydrogen peroxide by the additives was 65–92%.

Figure 4.6 Effect of bean extracts at 50 ppm as catechin equivalents, on the formation of conjugated dienes (CD), hexanal and 2-thiobarbutirc acid reactive substances (TBARS) at 60°C on day 7 in a corn oil model system.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; and LWE, black whole bean extract.

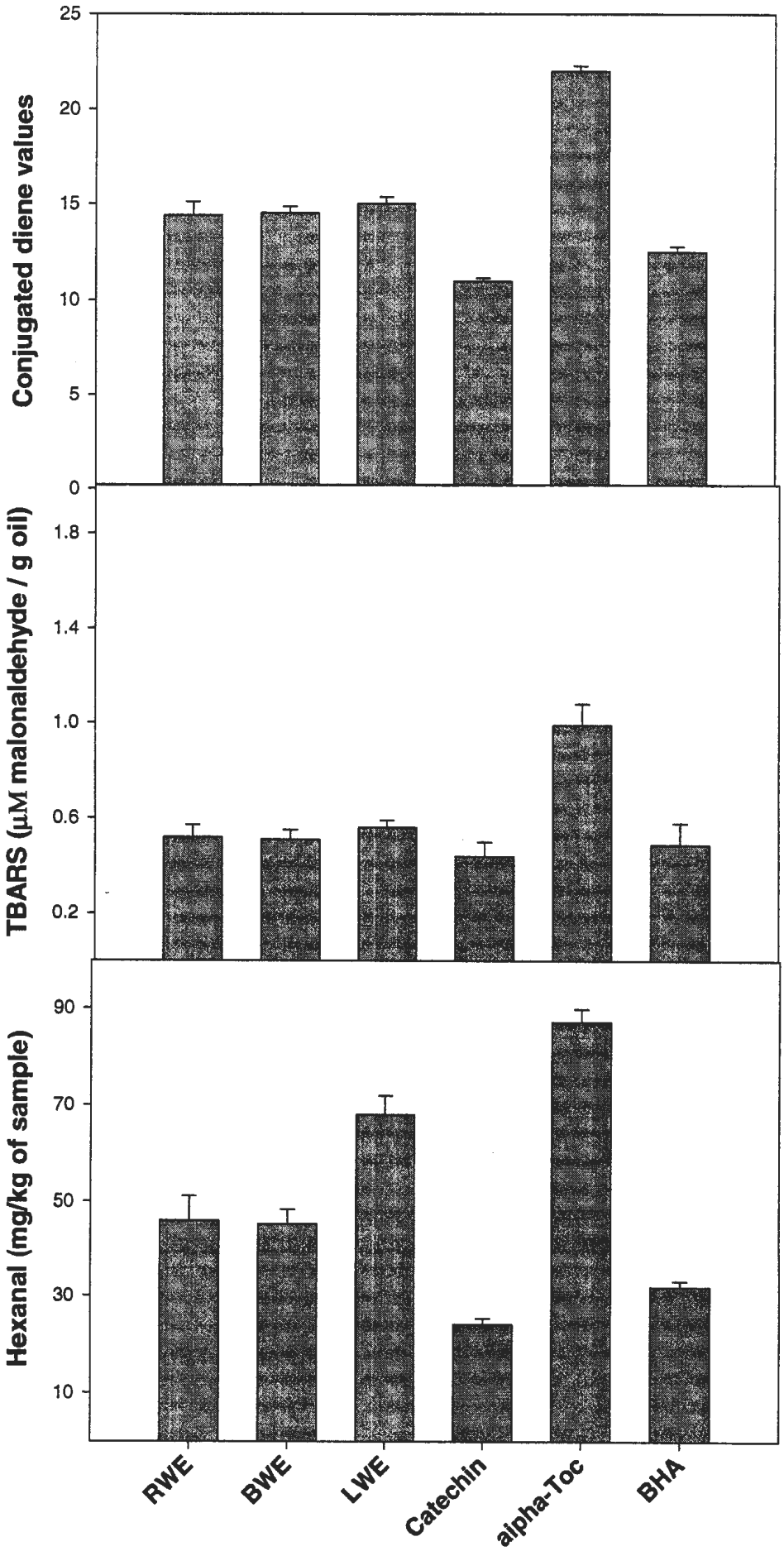

Conjugated diene values
25
20
15
10
5
0
TBARS (μM malonaldehyde / g oil)
1.8
1.4
1.0
0.6
0.2
Hexanal (mg/kg of sample)
90
70
50
30
10
RWE
BWE
LWE
Catechin
alpha-Toc
BHA
Additive

Figure 4.7 Effect of bean extracts at 100 ppm as catechin equivalents, on the formation of conjugated dienes (CD), hexanal and 2-thiobarbutirc acid reactive substances (TBARS) at 60°C on day 7 in a corn oil model system.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; and LWE, black whole bean extract.

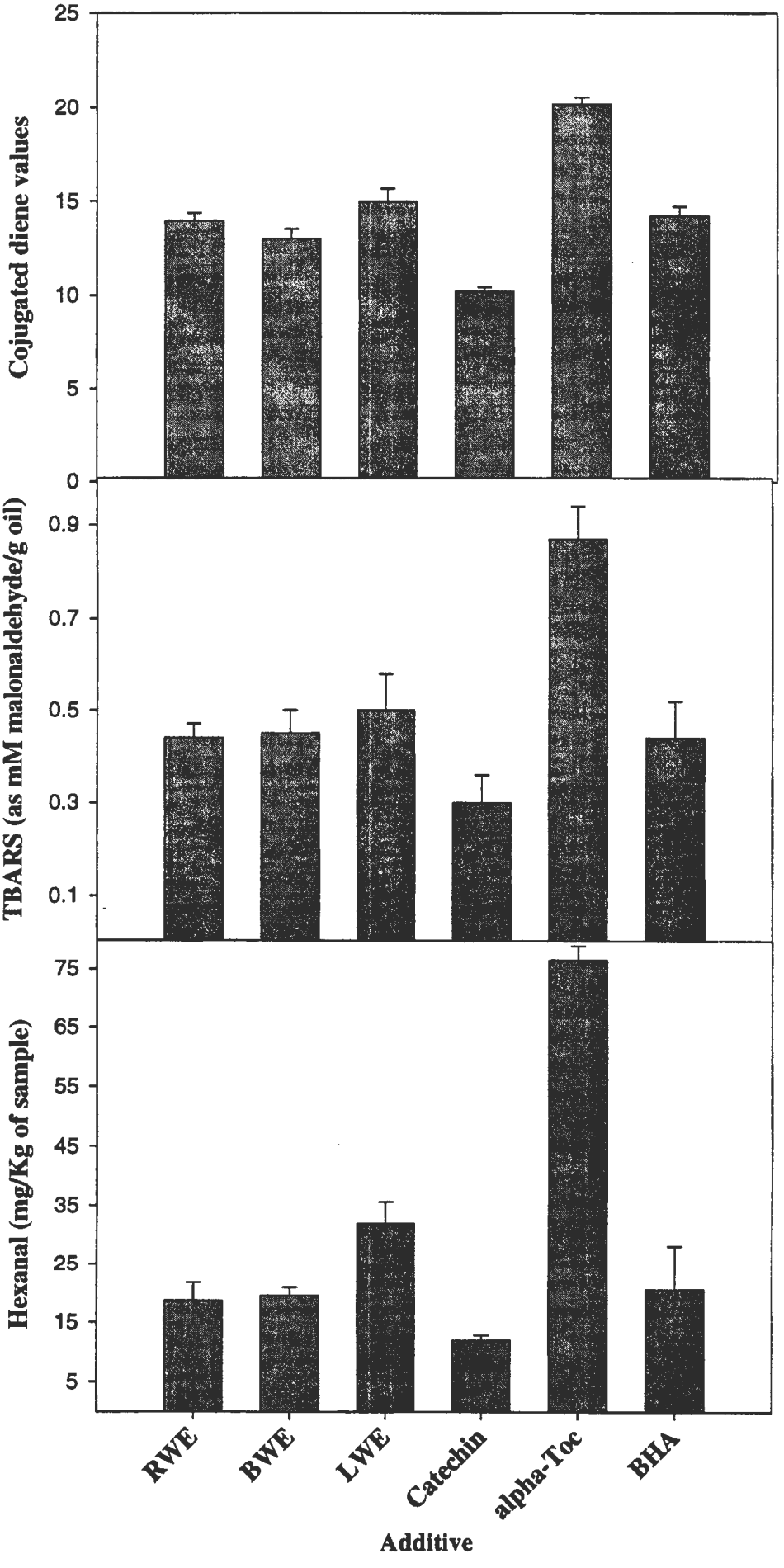

Cojugated diene values
25
20
15
10
5
0
TBARS (as mM malonaldehyde/g oil)
0.9
0.7
0.5
0.3
0.1
Hexanal (mg/Kg of sample)
75
65
55
45
35
25
15
5
RWE
BWE
LWE
Catechin
alpha-Toc
BHA
Additive

Table 4.6 Hydrogen peroxide scavenging capacity of bean extracts at 50 and 100 ppm levels as catechin equivalents[1].

Additive	Hydrogen peroxide scavenging capacity, %	
	50 ppm	**100 ppm**
Catechin	70.6±3[c]	92.3±1[c]
RWE	67.0±1[c]	76.1±3[b]
BWE	57.6±2[a]	73.4±2[b]
LWE	61.8±2[b]	65.2±1[a]

[1]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; and LWE, black whole bean extract.

At 50 ppm concentration catechin showed the strongest effect by scavenging 70.6% of the hydrogen peroxide present in the assay medium. RWE was in the same order of magnitude in scavenging hydrogen peroxide. This was followed by LWE which showed 61.8% scavenging. The least effective additive was BWE. When used at 100 ppm level, again catechin showed the strongest effect by scavenging 92.3% hydrogen peroxide present in the assay medium. The effectiveness of RWE and BWE was not significantly ($p>0.05$) different while LWE exhibited the least scavenging effect (65.2%). These results show that all three bean extracts are quite effective in scavenging hydrogen peroxide. Of the three extracts, RWE and BWE were more effective than LWE in scavenging hydrogen peroxide in the model system. Figures 4.8 and 4.9 depict the decrease of absorbance in the assay medium which reflects the depletion of hydrogen peroxide in the presence of antioxidative extracts and catechin at 50 and 100 ppm levels as catechin equivalents, respectively.

Hydrogen peroxide is a weak oxidizing agent that can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. But the ability of hydrogen peroxide producing other ROS such as hydroxyl radical cannot be ignored. Hydrogen peroxide is found to be toxic to cells at 10–100 μm levels and can cross biological membranes rapidly to form hydroxyl radicals (Halliwell and Gutteridge, 1999). Furthermore, DNA is an important target to be damaged when hydrogen peroxide is added to mammalian cells (Halliwell and Gutteridge, 1989). Any biological system generating superoxide radical can produce hydrogen peroxide by dismutation reactions, unless of course all the superoxide radicals are intercepted by other molecules such as

Figure 4.8 Hydrogen peroxide scavenging activity of bean extracts (at 50 ppm level as catechin equivalents) as exhibited by the reduction of absorbance of hydrogen peroxide at 230 nm

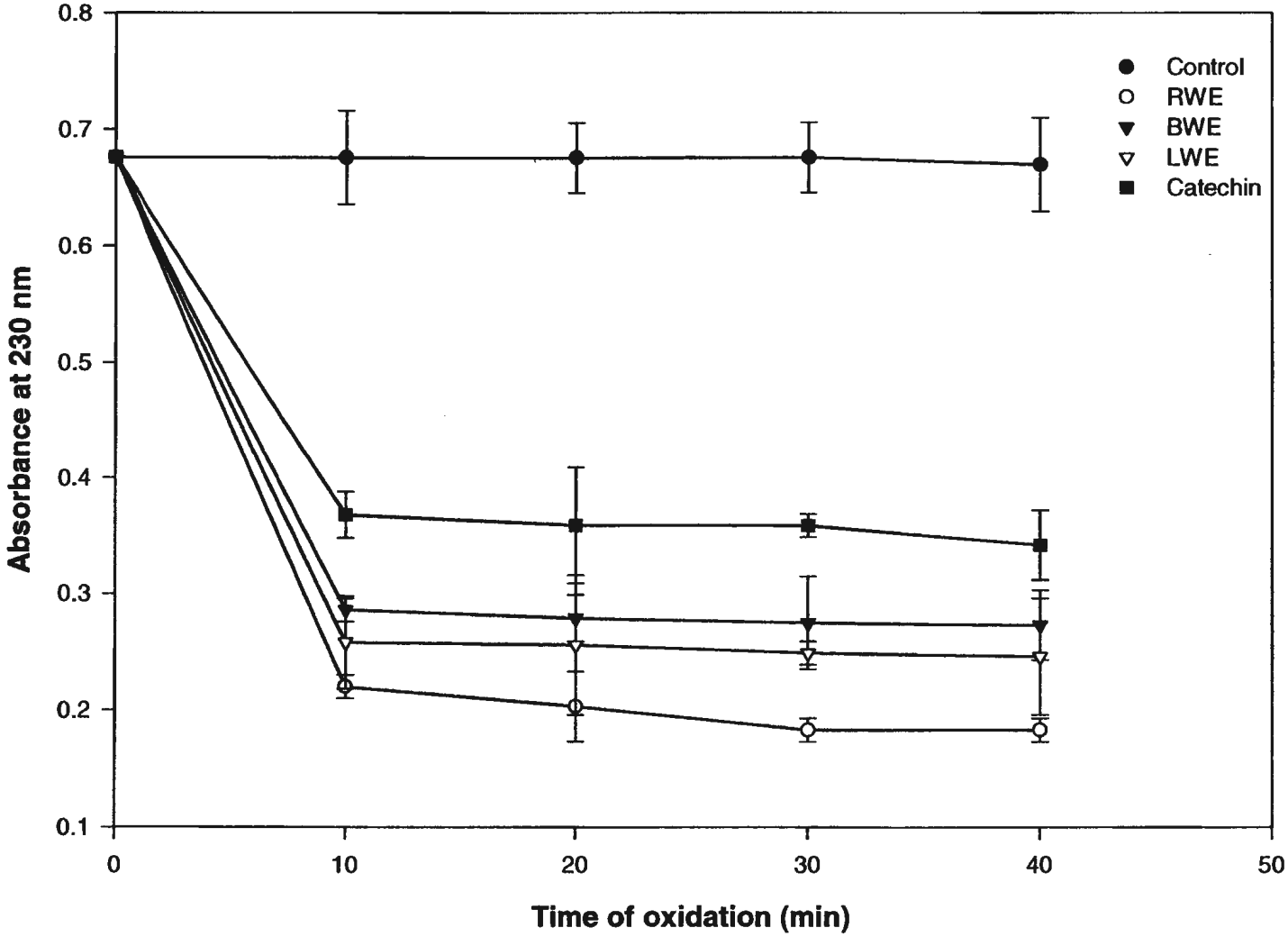
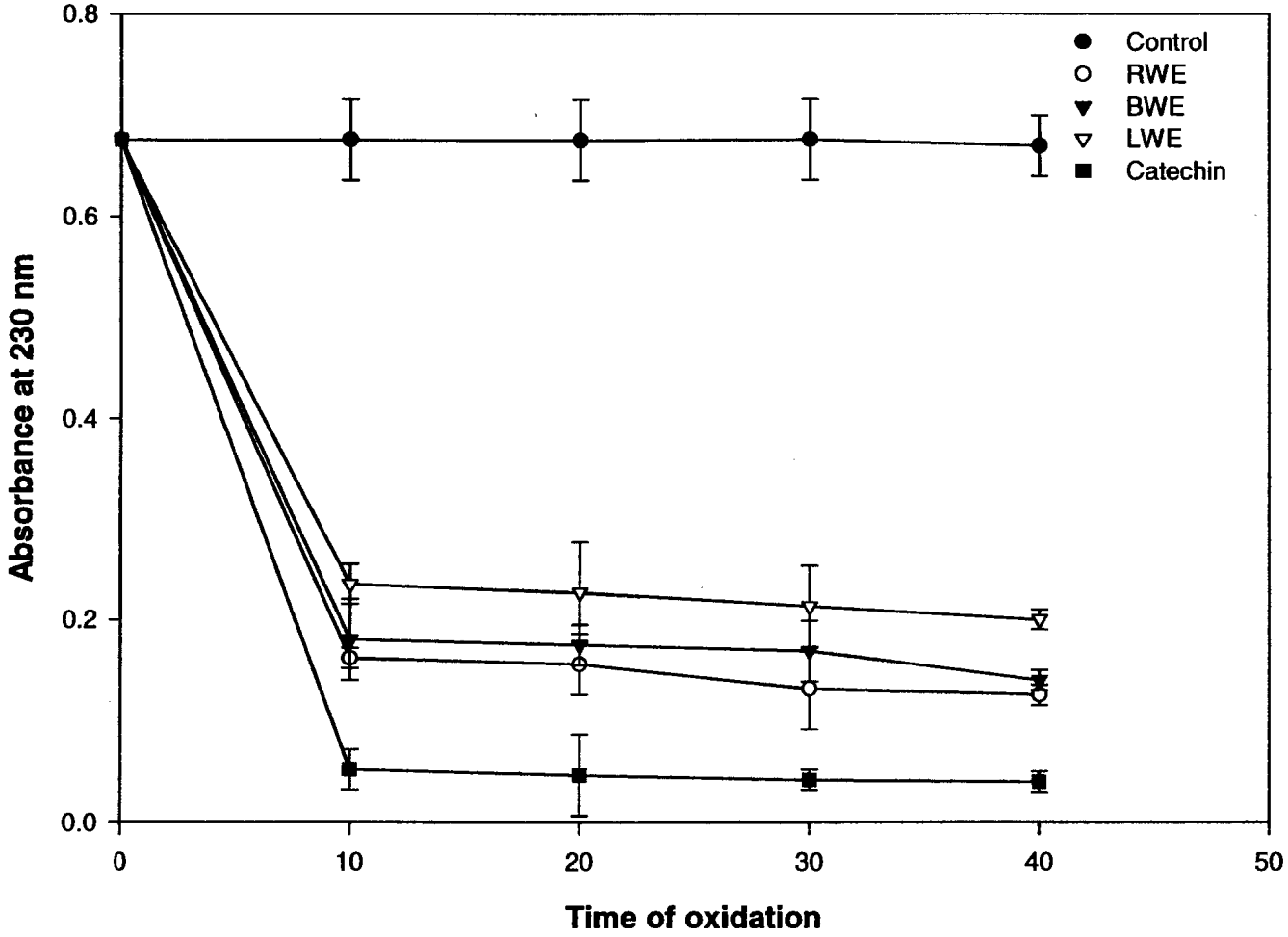

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance at 230 nm
0
10
20
30
40
50
Time of oxidation (min)
Control
RWE
BWE
LWE
Catechin

Figure 4.9 Hydrogen peroxide scavenging activity of bean extracts (at 100 ppm level as catechin equivalents) as exhibited by the reduction of absorbance of hydrogen peroxide at 230 nm


0.8
0.6
0.4
0.2
0.0
Absorbance at 230 nm
0
10
20
30
40
50
Time of oxidation
Control
RWE
BWE
LWE
Catechin

cytochrome c. Hydrogen peroxide formation has been frequently derived from mitochondria and microsomes. There are also several enzymes that produce hydrogen peroxide without intermediacy of free superoxide radical which include glycollate oxidase, D-amino acid oxidase and urate oxidase (Halliwell and Gutteridge, 1989).

4.6.2 Superoxide radical scavenging activity

Various methods are available for producing superoxide radical in the laboratory. There are generally two superoxide anion sources used; enzymatic and non-enzymatic sources such as the phenazine methosulphate-NADH system or potassium superoxide. In the present study, superoxide radical was enzymatically produced using hypoxanthine /xanthine oxidase (X/XO) system. The generation of superoxide radical was characterized by the development of an ink-blue colour in the assay medium due to the reduced nitro blue tetrazolium (Nishikimi *et al.*, 1972). The production of blue colour was used to monitor the production of superoxide radical in the assay medium.

In the control (devoid of any extract), the blue colour developed continuously and leveled off after 1 h. All the three bean extracts showed a dose-dependent scavenging activity. Figures 4.10 and 4.11 depict the absorbance increase in the assay media in the presence of 50 and 100 ppm of extracts, respectively. When used at 100 ppm level, LWE showed the highest scavenging capacity of 60% next to catechin. The scavenging capacities of RWE and BWE were 54 and 53%, respectively, and they were not significantly ($p>0.05$) different from each other (Table 4.7).

Table 4.7 Superoxide radical scavenging capacity of bean extracts at 50 and 100 ppm levels as catechin equivalents[1].

Additive	Superoxide radical scavenging capacity, %	
	50 ppm	**100 ppm**
Catechin	68±2[b]	77±1[c]
RWE	25±1[a]	54±2[a]
BWE	24±1[a]	53±2[a]
LWE	29±2[a]	60±3[b]

[1]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; and LWE, black whole bean extract.

Figure 4.10 Superoxide radical scavenging of bean extracts (at 50 ppm as catechin equivalents) as exhibited by the reduction of the intensity of the reduced nitro blue tetrazolium indicator at 560 nm

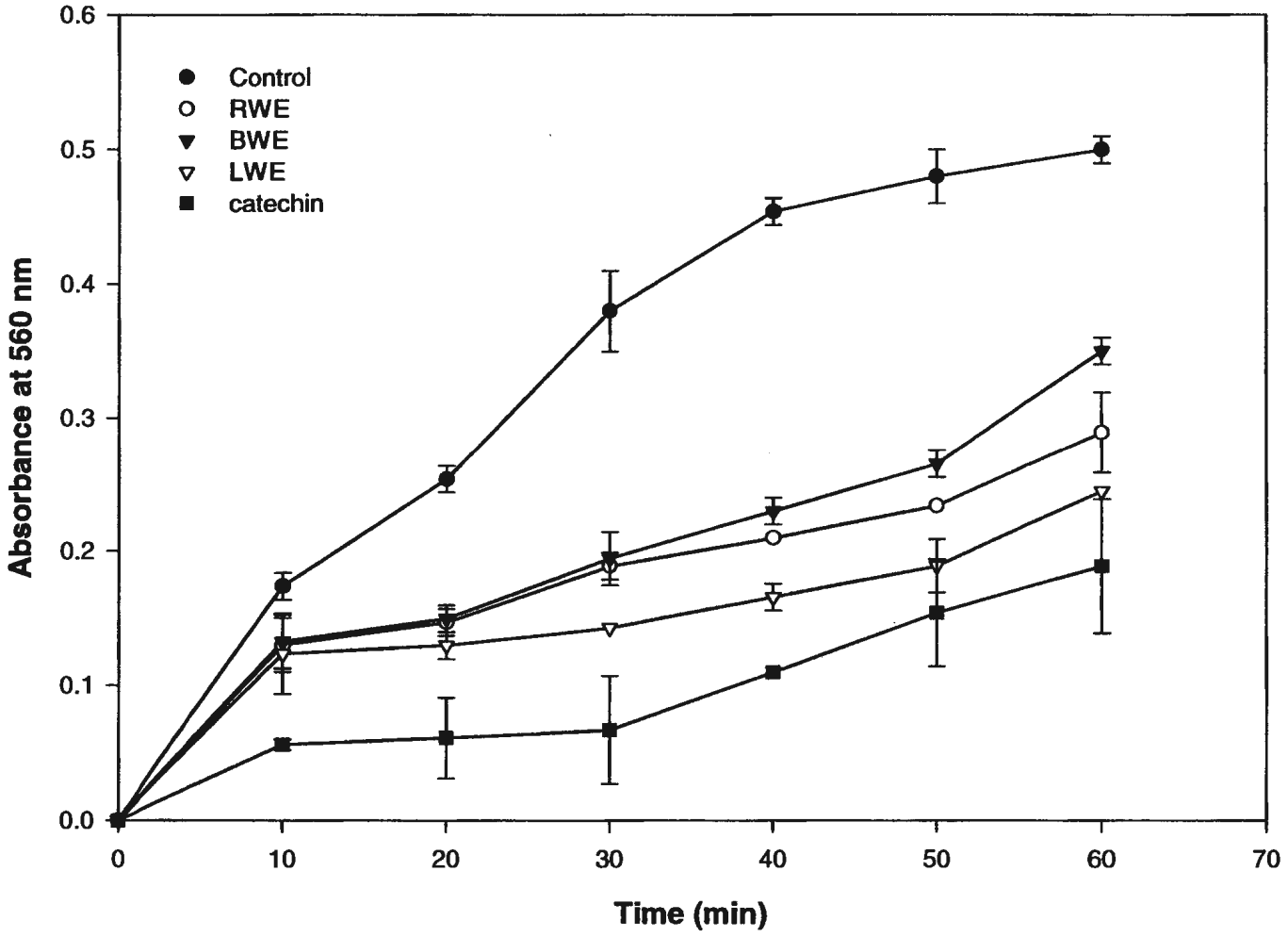
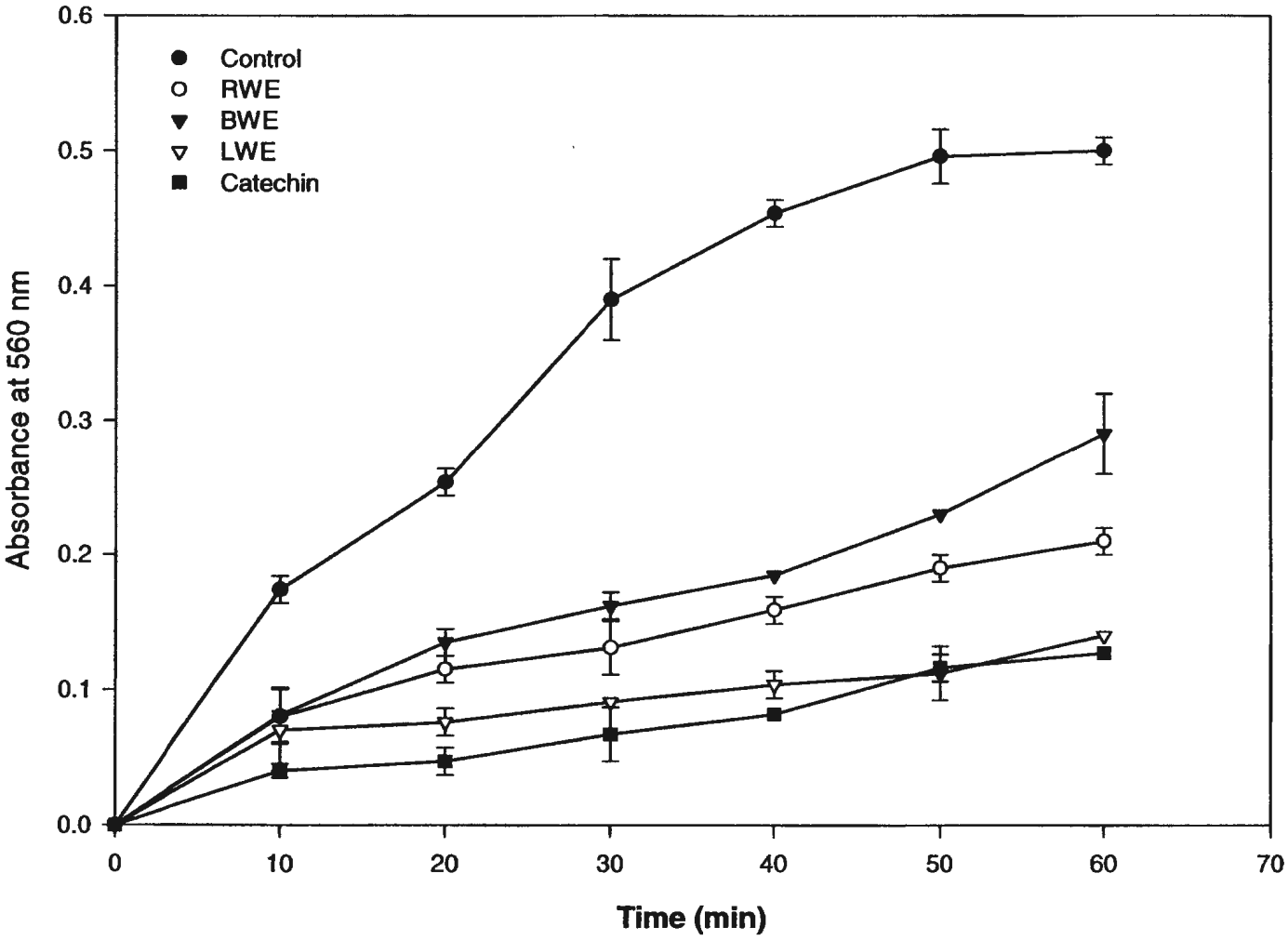

Control
RWE
BWE
LWE
catechin
Absorbance at 560 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
Time (min)

Figure 4.11 Superoxide radical scavenging of bean extracts (at 100 ppm as catechin equivalents) as exhibited by the reduction of the intensity of the reduced nitro blue tetrazolium indicator at 560 nm


Control
RWE
BWE
LWE
Catechin
Absorbance at 560 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
Time (min)

Tsuda *et al.* (1997) have investigated the superoxide radical scavenging activity of two pigments isolated from beans; cyanidin (Cy) and cyanidin-3-*O*-β-D-glucoside (C3G). The pigments have shown superoxide radical scavenging activity, however, the extent of the effect has not been greater than that of catechol and pyrogarol. The concentration for 50% inhibition (IC_{50}) values for Cy and C3G were 13.4 and 12.4, respectively (Tsuda *et al.*, 1997).

Scavenging ability depends on the chemical structures of flavonoids (Hanasaki *et al.*, 1994). In enzymatic (X/XO) systems, the scavenging effect may come directly from the radical-quenching effect or/and the enzyme-inhibiting effect (Shi and Noriko, 2001). The structure-activity relationship of flavonoids as inhibitors of xanthine oxidase and as scavengers of the superoxide radical was recently reported (Cos *et al.*, 1998). The presence of hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 are essential for a high inhibiting activity on xanthine oxidase. On the other hand, for a high superoxide scavenging activity, presence of a hydroxyl group at C-3` in the B ring and at C-3 is essential (Shi and Noriko, 2001). Based on the effect on xanthine oxidase and superoxide radical, flavonoids are classified into six groups; superoxide radical scavengers without inhibiting activity on xanthine oxidase, xanthine oxidase inhibitors without any additional superoxide scavenging, xanthine oxidase inhibitors with an additional superoxide scavenging activity, xanthine oxidase inhibitors with an additional prooxidant activity, flavonoids with marginal effect on xanthine oxidase but with prooxidant effect on the production of superoxide radical.

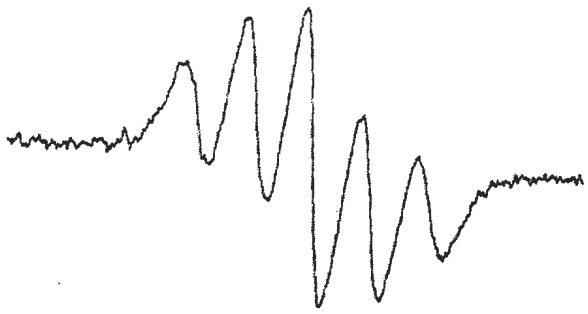
Hanasaki *et al.* (1994) reported that out of 11 flavonoids tested all, except (-)-epicatechin and fisetin, had an inhibitory effect on xanthine oxidase with more than 20% at the dose of their half inhibition concentration (IC_{50}), in X/XO system, thus a great part of superoxide scavenging effect observed with the flavonoids was due to the inhibitory effect on xanthine oxidase. The effect of bean extracts may be due to their activity on xanthine oxidase and also or their effect on superoxide radical itself, thus extracts used fall into one of the first three categories.

4.6.3 DPPH radical scavenging capacity

EPR spectrum of DPPH radical solution devoid of any antioxidant (control) and spectra after adding whole bean and bean hull extracts are depicted in Figures 4.12 and 4.13, respectively. Table 4.8 summarizes the results of DPPH radical scavenging capacity of bean extracts.

Catechin, RWE and BWE showed a 100% DPPH radical scavenging at both 50 and 100 ppm levels. At 50 ppm level, LWE showed a comparatively poor DPPH radical scavenging capacity of 22%. However, when used at a 100 ppm concentration, LWE also showed 100% scavenging of DPPH radical. The results show that all three bean extracts are powerful organic free radical scavengers when used at 100 ppm concentration. At lower concentrations, catechin, RWE, and BWE are better than LWE in scavenging organic free radicals.

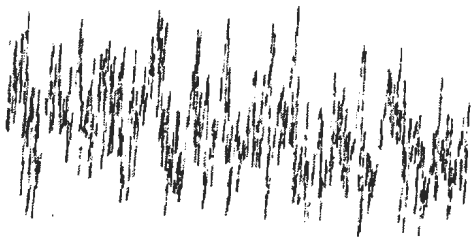
Figure 4.12 Electron paramagnetic resonance (EPR) spectra of 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) as affected by whole bean extracts. Columns 1 and 2 represent the effects of 50 ppm and 100 ppm extracts, respectively.



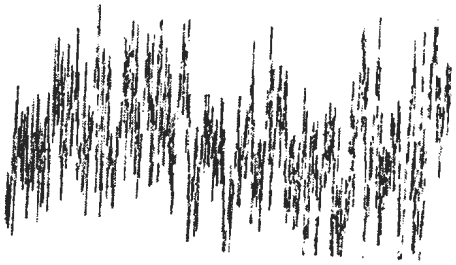
1

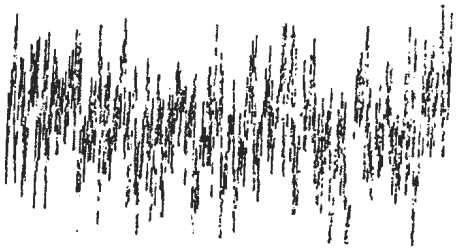
2

Control

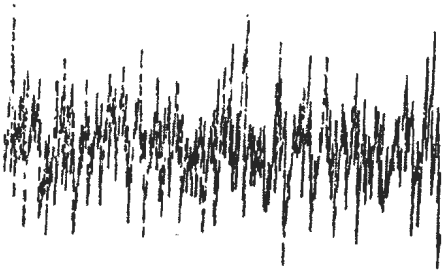


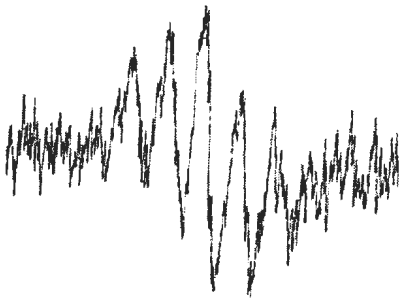
RWE



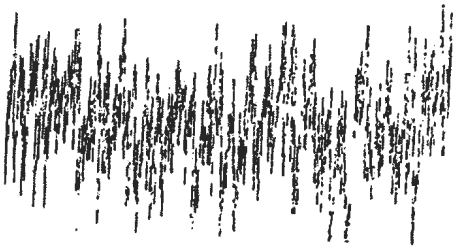


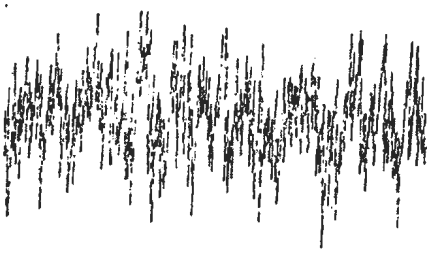
BWE





LWE





Catechin

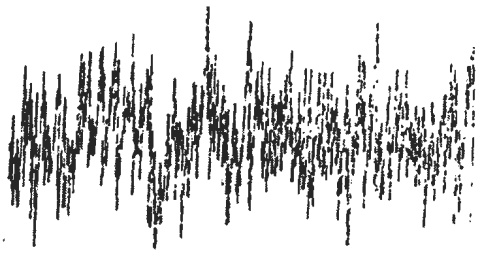
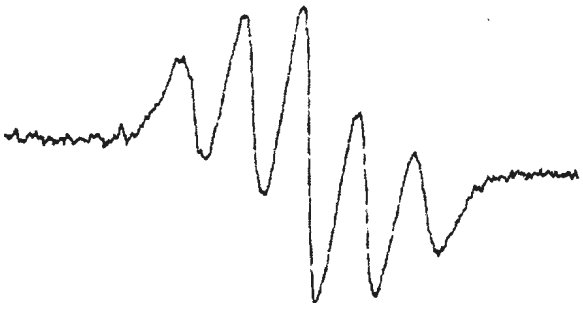


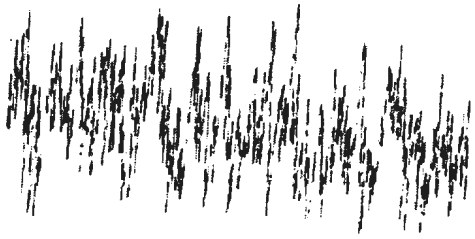
Figure 4.13 Electron paramagnetic resonance (EPR) spectra of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) radical as affected by bean hull extracts. Columns 1 and 2 represent the effects of 50 ppm and 100 ppm extracts, respectively.



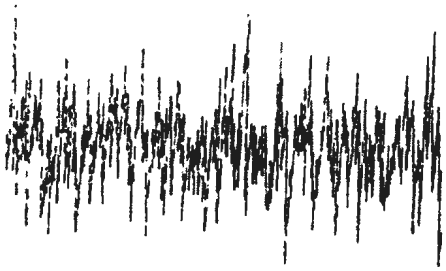
1

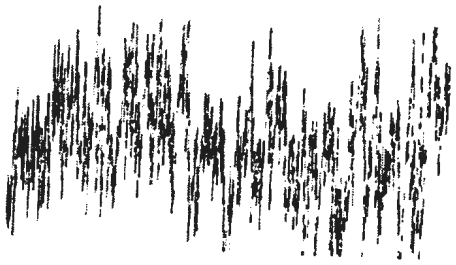
2

Control

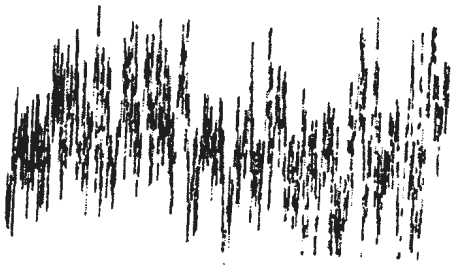


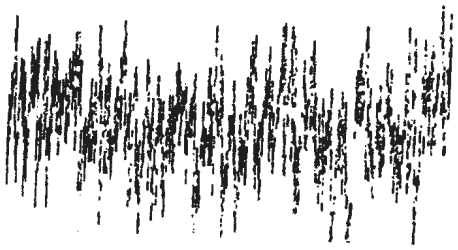
RHE



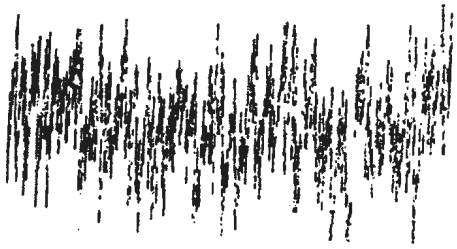


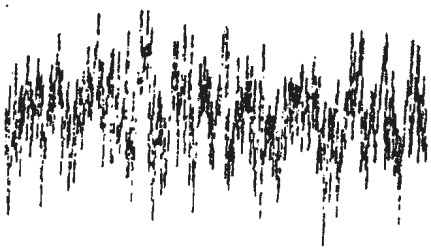
BHE





LHE





Catechin

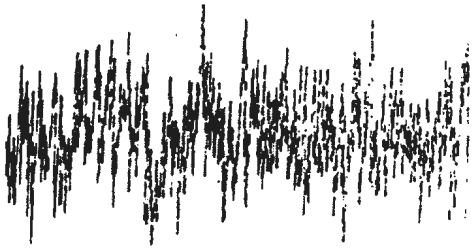


Table 4.8 DPPH radical scavenging capacity of the bean extracts at 50 and 100 ppm concentrations as catechin equivalents

Additive	DPPH radical scavenging capacity, %	
	50 ppm	**100 ppm**
Catechin	100±0[b]	100±0[a]
RWE	100±3[b]	100±1[a]
BWE	100±1[b]	100±2[a]
LWE	22±1[a]	100±3[a]
RHE	100±2[b]	100±2[a]
BHE	100±1[b]	100±1[a]
LHE	100±3[b]	100±1[a]

[1]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; RHE, red bean hull extract; BHE, brown bean hull extract; and LHE, black bean hull extract.

As expected, all three bean hull extracts showed 100% scavenging at both 50 and 100 ppm concentrations. The high total phenolic contents of bean hulls examined may be responsible for their strong radical scavenging activity.

Scavenging effect of various antioxidants on the DPPH radical was strongly concentration dependent (Lai *et al.*, 2001). In general, the scavenging effect on DPPH radical increases with increasing antioxidant concentration to a certain extent and then levels off with further increase (Lai *et al.,* 2001). The scavenging percentage on the DPPH radical was found to be 68.6% for crude Hsian-tsao plant extract at 1.25 mg/ mL and 71.7% for α-tocopherol and BHT at a dose level of 0.31 mg/mL (Lai *et al.*, 2001). Hu and Kitts (2000) have evaluated three *Echinacea* species for their DPPH radical scavenging effects. All three species showed strong radical sequestering capacities at 600-1000 μg/mL. The DPPH$^{\cdot}$, a stable free organic radical, was used to evaluate the free radical scavenging properties of natural antioxidants. DPPH is a stable radical which has been used to evaluate the antioxidant activity of plant and microbial extracts (Ko *et al.*, 1998). DPPH radical scavenging assay has been used to evaluate antioxidant activity of *Echinacea* root extracts (Hu and Kitts, 2000); Hsian-tsao leaf gum (Lai *et al.,* 2001).

The reduction of DPPH$^{\cdot}$ in the presence of additives was monitored by measuring the intensity of the electron paramagnetic resonance (EPR) signal. Hydrogen donated by antioxidants can neutralize DPPH$^{\cdot}$ into a non-radical (DPPH)H, as follows (Blois, 1958).

$$DPPH^{\cdot} + AH \longrightarrow (DPPH)H + A^{\cdot}$$

4.7 Fe^{2+} chelating capacity of bean extracts

Among primary catalysts that initiate oxidation *in vivo* and *in vitro*, transition metal ions and complexes containing metals such as haemoproteins have been identified. Transition metal ions such as iron, copper, manganese, magnesium and zinc participate in direct and indirect initiation of lipid oxidation (Schaich, 1980). Metals such as iron, copper, manganese, nickel and cobalt at their higher valence state are known to participate in direct initiation of lipid oxidation via electron transfer and lipid alkyl radical formation while lower valence metals can directly initiate lipid oxidation via the formation of reactive oxygen species (Kanner, 1986).

In the determination of Fe^{2+} chelating capacity of bean extracts, tetramethylmurexide (TMM) indicator was used to quantify the free Fe^{2+} concentration. Tetramethylmurexide is a metal chelating agent that shows an absorbance maximum at 530 nm. However, shifting of the absorbance maximum to 460 nm occurs when metal ions chelate with tetramethylmurexide. The ratio of absorbance at 460 nm to 530 nm is linearly correlated with the metal ion concentration (Asakura *et al.*, 1990).

Upon addition of a known concentration of Fe^{2+} to a buffered solution of antioxidant extracts, some of the metal ions chelate with the extract leaving the rest in the buffered solution as free metal ions. Fe^{2+} left behind in the solution as free ions reacts with tetramethylmurexide which in turn results in shifting the absorbance maximum from 530 nm to 460 nm.

The unreacted Fe^{2+} concentration was determined from the calibration line (Figure A-7 in appendix) and the concentration of ions chelated by extracts was calculated by subtraction (Asakura *et al.,* 1990). Table 4.9 shows the Fe^{2+} chelation capacities of bean extracts at 50 and 100 ppm levels as catechin equivalents.

At 50 ppm level, catechin showed the highest chelation capacity of 60% of the initial Fe^{2+} content while RWE and BWE showed moderate chelation capacities of 58 and 56%, respectively. Chelation capacity of LWE was significantly ($p<0.05$) lower than that of RWE and BWE at 50 ppm level. When used at 100 ppm level, catechin exhibited almost 100 % chelation while RWE and BWE showed 72 and 69% chelation, respectively. LWE showed fairly good chelation capacity at 100 ppm level (60%), but this was significantly ($p<0.05$) lower than that of other extracts used in this study at the same concentration.

Wettasinghe and Shahidi (2002) have reported that borage crude extracts chelated 43 and 91% of the original Fe^{2+} concentration when used at 100 and 200 ppm as catechin equivalents, respectively. Evening primrose crude extracts showed metal chelation capacity of 63 and 100% at 100 and 200 ppm levels, respectively (Wettasinghe and Shahidi, 2002). It is evident that bean crude extracts show better Fe^{2+} chelation efficacy compared to borage and evening primrose. The phenolic compounds are the major fractions that chelate metal ions although non-phenolic constituents in the crude extracts may also participate in sequestering of metal ions (Wettasinghe and Shahidi, 2002).

Table 4.9 Fe^{2+} concentration (μM) and percentages of chelated ferrous ion[1] by bean extracts at 50 and 100 ppm concentrations as catechin equivalents[2]

Additive	50 ppm		100 ppm	
	μM	%	μM	%
Catechin	240±4[d]	60	395±8[d]	99
RWE	232±6[c]	58	289±4[c]	72
BWE	224±2[b]	56	276±7[b]	69
LWE	156±5[a]	39	240±5[a]	60

[1]The initial concentration of ferrous is 400μM.
[2]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; and LWE, black whole bean extract.

Metal chelation is an example of secondary antioxidant mechanism by which antioxidants can influence the oxidation process (Hall, 2001). As in other flavonoid antioxidants, the position and number of hydroxyl groups play an important role in radical scavenging and metal chelation mechanisms (Rice-Evans, 1995). Unlike other flavonoids, anthocyanins and anthocyanidins are lacking the C-4 carbonyl group suggesting that metal-chelation activity is due to the presence of ortho hydroxyl groups at C-3` and C-4` positions in the B ring.

4.8 Effect of bean extracts in preventing copper-mediated human low density lipoprotein (hLDL) oxidation

Oxidation of LDL *in vivo* may contribute to the pathology of atherosclerosis (Steinberg *et al.*, 1989; Steinbrecher, 1987). Thus, this has led to increased interest in investigating the role of natural antioxidants in preventing oxidation of LDL and membrane lipids. It has been reported that anthocyanins derived from grape juices (Frankel *et al.*, 1998), wine (Tesissere *et al.*, 1996) and berries (Abuja *et al.*, 1998) are the major compounds contributing to the *in vitro* antioxidant activity, thus preventing oxidation of LDL. The copper-induced LDL oxidation method has served as a useful model for evaluating natural antioxidants (Yan *et al.*, 1995; Heinonen *et al.*, 1998). Cupric ion and AAPH have been widely employed as oxidizing agents to induce LDL oxidation in studies involving *in vitro* models of evaluating antioxidative extracts.

Conjugated dienes can be used as an indicator of level of peroxidation of LDL in antioxidant evaluating studies. Recent studies have shown that Cu^{2+}-mediated oxidation of the LDL can exhibit different kinetics depending on Cu^{2+} concentration. Propagation can proceed when antioxidants are depleted, at high Cu^{2+} concentrations or when they are present in low concentrations (Ziouzenkova *et al.,* 1998).

At high mole ratios of at least 10 Cu^{2+} ions per one LDL molecule, oxidation continues to propagate after all the available antioxidants are consumed (Ziouzenkova *et al.,* 1998). The increase in CD during the propagation phase was reported to be mainly due to the formation of cholesteryl linoleate hydroperoxides and substantial amounts of cholesteryl linoleate hydroxides (Esterbauer *et al.,* 1992)

In the present study, EDTA-free hLDL was used to initiate the oxidation process in the presence of different concentrations of bean extracts and catechin (2-50 ppm) (Table 4.10). The dose-dependent pattern of inhibition of oxidation exerted by bean extracts is depicted in Figure 4.14.

All extracts tested and catechin showed almost 100% inhibition of oxidation at 50 ppm concentration (as catechin equivalents). At 2, 5 and 10 ppm concentrations the level of protection by RWE and BWE was not significantly ($p<0.05$) different from one another, but the protection by LWE was significantly ($p< 0.05$) lower than that of RWE and BWE. All three bean extracts showed a significantly ($p<0.05$) higher activity against LDL oxidation, at both 2 and 5 ppm levels, than catechin. This behaviour may be

attributed to the synergistic effect in a mixture of natural compounds present in bean extracts.

The inhibition of copper-induced LDL oxidation by flavonoids has been reported by various authors (Dewhalley *et al.*, 1990; Mangiopane *et al.*, 1992; NegreSalvayre and Salvayre, 1992). The protective effect of antioxidants from *Echinacea* root extracts towards LDL oxidation was attributed to the protective affinity reported by chelating Cu^{2+}. In interpreting the findings, it is important to note the fact that there was no evidence of prooxidant activity of extracts toward hLDL oxidation within the range of concentrations tested although the extracts were added before the initiation of oxidation. Similar results have been reported by Hu and Kitts (2000) for *Echinacea* root extracts. On the other hand, Abuja *et al.* (1998) have reported that eldberry shows prooxidant activity towards hLDL in a similar *in vitro* model system when the extracts were added after 20 min from the start of oxidation.

Abuja *et al.* (1998) reported that anthocyanins and other phenolic compounds from eldberry reduce Cu^{2+} to Cu^{+} upon binding to LDL, thus leading to enhanced oxidation when antioxidant extracts were added after the initiation of oxidation. The binding of redox-active cupric ion to LDL at both low and high-affinity binding sites is critical for the initiation of LDL oxidation (Ziouzenkova *et al.*, 1998). However, when the antioxidant compounds are present from the beginning, no prooxidant activity occurred. Whenever the constituents of the extracts are present before the start of oxidation, they prevent oxidation. Therefore, at the end of the lag phase of oxidation,

Table 4.10 Inhibition percentages of conjugated diene (CD) formation by bean extracts at different concentrations in copper-mediated hLDL oxidation[1]

Additive	Concentration of additives in ppm (as catechin equivalents)				
	2	5	10	25	50
RWE	81.2±3.2^{c}	89.5±2.5^{c}	97.6±1.9^{c}	99.1±3.6^{a}	99.9±2.9^{a}
BWE	83.3±1.6^{c}	87.1±3.2^{c}	98.2±2.9^{c}	99.6±3.5^{a}	99.9±1.8^{a}
LWE	61.4±0.9^{b}	66.0±1.7^{b}	80.0±2.9^{a}	95.7±3.1^{a}	98.2±2.4^{a}
Catechin	13.5±0.8^{a}	33.0±1.9^{a}	89.0±2.5^{b}	97.2±0.6^{a}	99.9±2.6^{a}

[1]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; and LWE, black whole bean extract

they are already consumed, thus they cannot exert a prooxidant effect towards LDL oxidation (Abuja *et al.*, 1998). However, the consequences of the prooxidant potential of antioxidant compounds from eldberry is probably marginal *in vivo* (Abuja *et al.*, 1998). Furthermore, glycosylated flavonoids and anthocyanidins have recently been found to be absorbed without deglycosylation in the human gut (Paganga and Rice-Evans, 1997). Supplementation with a high dose of these compounds may contribute markedly to the antioxidant capacity of body plasma (Abuja *et al.,* 1998).

Antioxidant extracts may exert different effects towards prevention of LDL oxidation depending on their chemical nature. Lipophilic substances such as vitamin E (α- tocopherol, TocOH), and carotenoids tend to accumulate in plasma lipoprotein (Esterbauer *et al.*, 1991). On the other hand, antioxidants which cannot enter the lipid moiety of LDL would be less efficient as they are unable to encounter lipophilic radicals which can possibly attack LDL. These compounds may act synergistically with lipophilic antioxidants by regenerating them (Abuja *et al.*, 1998). This kind of behaviour has been observed for vitamin C and also for phenolic substances (Hayakawa *et al.*, 1997) by reducing α-tocopherol radical (TocO$^{\cdot}$) to TocOH (Kangan *et al.*, 1992).

4.9 Supercoiled DNA scission study

Oxidants produced as by-products of mitochondrial electron transport and products from lipid peroxidation that escape the numerous antioxidant defense systems can cause damage to cellular macromolecules including DNA and such damage can lead to mutations and cancer (Ames and Shigenaga, 1993). Oxidation damage of DNA results in a wide range of scission products which include strand breaks and sister chromatid exchange, DNA-DNA, and DNA-protein cross-links as well as base modifications (Ames and Shigenaga, 1993). DNA damage is often measured as single strand-breaks, double strand-breaks or chromosomal aberrations (Breimer, 1990). In the present study, the bean extracts were evaluated on their capacity of inhibiting peroxyl and hydroxyl radical induced-DNA supercoiled (Form 1) strands scission. Figure A-8 through A-12) show the activity of three bean extracts and the reference antioxidant, catechin. Table 4.11 summarizes the percentage DNA retention capacities calculated from the band intensities in Figures A-8 to A-12.

The DNA retention capacities of RWE, BWE and catechin were not significantly ($p>0.05$) different from each other against AAPH-derived peroxyl radical damage at a 5 ppm level while that of LWE was low. At all other concentrations (10, 50 and 100 ppm as catechin equivalents) all three extracts and catechin showed very high DNA retention which was not significantly ($p>0.05$) different from one another. Radicals cleave supercoiled plasmid DNA (form I) to nicked circular DNA (form II) or at higher concentrations of radicals to linear DNA (form III). The presence of peroxyl radical resulted in a dramatic scission of supercoiled DNA. This was clearly seen in the wells

Table 4.11 Retention % of supercoiled DNA (form I) by bean extracts and catechin at different concentration (as catechin equivalents) in peroxyl radical-induced DNA strand scission studies[1]

Radical	Extract	5 ppm	10 ppm	50 ppm	100 ppm
Peroxyl	RWE	89.5±4.5^{a,b}	96.6±2.5^{a}	97.5±3.9^{a}	100±0.4^{a}
	BWE	92.6±3.9^{b}	97.2±0.8^{b}	97.3±4.8^{a}	100±0.6^{a}
	LWE	86.4±2.1^{a}	94.0±3.6^{a}	98.5±5.1^{a}	100±0.5^{a}
	Catechin	94.5±2.9^{b}	98.2±0.7^{b}	99.0±2.6^{a}	100±0.1^{a}

[1]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: RWE, red whole bean extract; BWE, brown whole bean extract; and LWE, black whole bean extract

where the reaction mixture did not contain any antioxidant. The radical concentration used in the present study was not sufficient enough to destroy the nicked circular DNA which is more difficult to be destroyed as compared to DNA of form I. This is clearly seen in lane 2 (Figures A-8 through A-13). As the concentration of antioxidative extract was increased the protective effect against nicking of supercoiled DNA also increased. Peroxyl radicals are more stable than other oxygen radicals (Ross and Bielski, 1990) and have the ability to diffuse relatively far from the site of their generation before they react with a target molecule (Morrero and Marnett, 1993). It is well documented that the frequently associated oxidative stress occurring in biological systems attributed to peroxyl radicals. Furthermore, unlike other oxygen-derived free radicals they have the ability to insert oxygen atoms onto non-aromatic carbon double bonds to produce epoxides (Ingold, 1969) which leads to the induction of mutation that can contribute to the multistage process of carcinogenesis (Sims and Grover, 1974). In the absence of any antioxidant, it may be expected that the peroxyl radical abstract a hydrogen atom from the nearby DNA to generate new radicals, which in turn evokes a free radical chain reaction resulting in the destruction of the DNA molecule. However, in the presence of antioxidants, this chain reaction is terminated by abstracting a hydrogen atom from the antioxidant molecule (Hu and Kitts, 2000). Hu and Kitts (2000) reported that bamboo leaf extracts (BLE) prevented the breakage of superoxide DNA strand to a similar extent to that of Trolox, a water soluble analogue of tocopherol, with free radical chain breaking activity in peroxyl radical induced-DNA scission studies. BLE showed 37.5 and 82.1% protection against DNA breakage at 10 and 20 μg, respectively.

In the present study, neither bean extracts nor catechin exhibited protection of hydroxyl radical induced-DNA nicking in both site-specific and non site-specific models. However, no prooxidant effects were observed in the range of concentrations used in the study. The concept of site-specific effect of hydroxyl radical was described by Gutteridge (1984). In the absence of EDTA, iron ions bind to deoxyribose molecules and bring about a site-specific reaction in the molecule. However, in the presence of EDTA, iron ion is removed from binding site to form EDTA metal complex and produce hydroxyl radical that can be removed by hydroxyl radical scavengers. The most hydroxyl radical scavengers show poor inhibitory effects for site-specific hydroxyl reactions (Gutteridge, 1984). A similar trend was observed in the present study with bean extracts.

Barbara *et al.* (1997) have evaluated antioxidant and antigenotoxic properties of flavonoids from yellow and green beans using single strand breaks (SSB) in DNA with the help of alkaline version of Comet assay. Of the six flavonoids tested in the study (quercetin, kaempferol, quercetin-3-*O*-rutinoside, kaempfeorl-3-*O*-rutinoside, quercetin-3-*O*-glucuronide and kaempferol-3-*O*-glucuronide) only quercetin was able to decrease SSB induced by hydrogen peroxide, tert-butylhydroperoxide (t-BuOOH) and linoleic acid hydroperoxide (LAOOH). Furthermore, despite the antioxidant activity of aglycone kaempferol enhanced the level of SSBs caused by hydrogen peroxide and (t-BuOOH) (Barbara *et al.*, 1997). This might have been attributed to prooxidant effects of the flavonoids towards DNA.

Shun-Huali *et al.* (2000) reported that all four phenolic compounds tested, namely 1,2,4-benzenetriol, gallic acid, gossypol and caffeic acid tested enhanced cleavage of superociled plasmid DNA to nicked circular DNA; this system was similar to that employed in the present study. The damage percentage observed ranged from 20-100 of DNA molecules based on densitometric analysis at a 0.02mM concentration of phenolic compounds (Shun-Hulai *et al.,* 2000). Aruoma *et al.* (1998) have reported that hydroxytyrosol present in extra virgin olive oil strongly inhibited LDL oxidation, but exhibited extensive cleavage of DNA in hydroxyl radical induced-DNA model system. When tested in a human lymphocyte, using Comet assay, ascorbic acid was found to induce a marked dose-dependent increase in DNA breakage (Anderson and Phillips, 1999). Furthermore, high plasma ascorbic acid concentrations increased percentage of cell aberrations in vivo studies (Anderson and Phillips, 1999).

Okada and Okada (2000) reported that water soluble proteins from broad beans (*Vicia faba*) positively affected the suppression of fibroblast senescence in a cellular aging study. It has been speculated that water soluble proteins from beans can improve cellular protection against oxidative damage. This study demonstrated that non-phenolic compounds present in bean extracts examined may also provide cellular protection.

4.10 Active compounds in bean extracts

High performance liquid chromatographic (HPLC) analysis of the bean extracts showed the presence of different anthocyanidins, procyanidins and phenolic acids. Each compound was tentatively identified by its retention time and comparing with standards

under the same conditions. The chromatograms obtained for HPLC analysis are illustrated in Figures A-13 through A-18

4.10.1 Anthocyanidins

The anthocyanidins identified in the bean hull extracts were delphinidin and cyanidin (Table 4.12). Red and brown bean extracts contained both delphinidin and cyanidin while black contained only cyanidin. White bean extracts did not contain any of the two anthocyanidins. Black bean extracts contained the highest amount of cyanidin (122 mg/g extract) followed by brown (85 mg/g extract) while red bean extracts contained the lowest (41 mg/g extract). Red bean extracts contained significantly higher ($p>0.05$) amounts of delphinidin (9.5 mg/g extract) compared to brown bean extract (5.6 mg/g). The anthocyanidins are the natural pigments responsible for the red, blue and purple colour of many plants. A number of workers have reported HPLC method for separation of both anthocyanin (Wolf and Nagel, 1978; Strack *et al.,* 1980) and their aglycons (Wilkinson *et al.,* 1997). Variation of absolute peak retention times from one study to another and lack of availability of pure standards have made the anthocyanin analysis difficult by conventional methods. Hong and Wrolstad (1990) explained a systematic HPLC method to analyze anthocyanidins with good reproducibility using on-line photodiode array detection.

Anthocyanin and anthocyanidins which are metabolic products of flavanones (Hall, 2001) constitute a major portion of bean antioxidants (Tsuda *et al.,* 1994). Most of the naturally occurring anthocayanidins and anthocyanins carry hydroxyl groups at C-4`.

Table 4.12 Anthocyanidin and procyanidin contents of bean extracts[1]

Compound[2]	RHE	BHE	LHE	WHE
Delphinidin	9.5±1.2[b]	5.6±0.5[a]	ND	ND
Cyanidin	41.0±2.1[a]	85.0±2.1[b]	122.3±4.2[c]	ND
Procyanidin X	45.1±1.4[a]	120.0±1.1[b]	ND	ND
Procyanidin B_1	2.1±0.4[a]	ND	9.2±0.6[b]	ND
Procyanidin C_1	14.0±0.9[a]	ND	14.3±0.3[a]	ND
Procaynidin C_2	51.0±2.1[b]	42±2.1[a]	192.0±4.0[c]	ND

[1]Results reported are mean values of three determinations ± standard deviation.
[2]Expressed as mg/g extract.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: ND, not detectable; RHE, red bean hull extract; BHE, brown bean hull extract; LHE, black bean hull extract; and WHE white bean hull extract.

Anthocyanins such as cyanidin and delphinidin, found in beans, contain a hydroxyl group at the C-3` position while pelargonidin does not (Mazza and Miniati, 2000) Figure 4.14A.

Compared with flavones, anthocyanidins are less active, and this may be attributed to the lack of C-4 carbonyl that, in conjugation with the C-2: C-3 double bond, plays an important role in the efficiency of antioxidants (Wang, 1997). Presence of a hydroxyl group at the 5` position does not enhance antioxidant activity, contrary to other flavonoids (Wang, 1997). The sugar moiety attached to the flavylium cation seems to influence antioxidant activity, in general, which may account for the difference in their radical scavenging activity (Wang, 1997; Rice-Evans *et al.*, 1996). The addition of glucose enhanced activity to a greater degree than rhamnoglucose and galactose did (Wang, 1997). The difference in molecular structure of sugars may cause changes in the structural configurations of the anthocyanins that enhance or diminish their ability to form a stable radical (Kandaswami and Middleton, 1994).

The major antioxidative activity of anthocyanin pigments in beans can be ascribed to the reducing power of the *O*-di hydroxy structure in the B ring in cyanidin and delphinidin with higher TEAC values. Dehydroxylation to a monophenol in the B ring as in pelargonidin (1.3) gives much the same value as kaempferol of flavon-3-ol (Rice-Evans *et al.*, 1996).

Rice-Evans *et al.,* (1996) reported that the presence of a third hydroxyl group in the B ring does not enhance the effectiveness against aqueous phase radicals in delphinidin compared with cyanidin. Delphinidin which carries 6 free hydroxyl substituents at 3,5,7,3`,4 and5` positions has a TEAC value of 4.44 while cyanidin with 5 free hydroxyl substituents at 3,5,7,3` and 4` positions has a TEAC value of 4.0 (Rice-Evans *et al.,* 1996). It is noteworthy to mention that the TEAC values of delphinidin and cyanidin are comparable with strong antioxidative compounds such as epicatechin gallate (TEAC, 4.9), epigalactocatechin gallate (TEAC, 4.8) and quercetin (TEAC, 4.7) (Rice-Evans *et al.,* 1996). Other anthocyanidins have moderate TEAC values i.e. pelargonidin (TEAC, 1.3), peonidins (TEAC, 2.2) malvidin (TEAC,2.1) (Rice-Evans *et al.,* 1996).

Glycosyslation of anthocyanin in the 3-position diminishes the antioxidative activity (Rice-Evans *et al.,* 1996). Tsuda *et al.* (1994b) reported that cyanidin exhibited a high antioxidant activity compared to cyanadin-3-*O*-β-D-glucoside (C3G) in a rabbit erythrocyte membrane ghost system. Furthermore, the extent of antioxidant activity of cyanidin was similar to the same as tocopherol. Tsuda *et al.* (1994a) measured the antioxidative activity of pelargonidin 3-*O*-β-D-gluceride, delphinidin 3-*O*-β-D-gucoside and C3G in a linoleic acid system at pH 7. C3G exhibited strong antioxidative activity while the other two pigments have not shown considerable antioxidant activity. When tested at pH 3 and 5, they also exhibited strong antioxidative activity suggesting that antioxidant activity of anthocyanidins depends on the pH of the medium.

The daily intake of anthocyanins in humans has been estimated to be as much as 125-180 mg/ day in the USA (Kuhnau, 1976) due to their widespread distribution and occurrence in fruits and vegetables (Wang *et al.,* 1997). Anthocyanins have shown to have some positive therapeutic effects as in diabetic retiontherapy (Scharrer and Ober, 1981). A commercial preparation of bilberry anthocyanins containing largely glycosides of delphinidin and cyanidin (Baj *et al.*, 1977) has been used to treat various microcirculation diseases (Mian *et al.*, 1977). Anthocyanins are also known to possess antineoplastic (Kameil *et al.,* 1995), radiation-protecting (Minkova *et al.,* 1990), vasotonic (Colantuoni *et al.,* 1991), vasoprotective and and anti-inflammatory (Lietti *et al.*, 1976), chemoprotective (Karaivanova *et al.,* 1990) and hepatoprotective effects (Mitcheva *et al.*, 1993).

4.10.2 Procyanidins

Procyanidin X is expressed as equivalents of procyanidin B_3 (based on their close retention times and similar UV spectra). Procyanidin C_2 is the sum of two compounds C_2 and another compound which collectively yielded a broad peak. Red bean extract contained all the procyanidins X, B_2, C_1 and C_2 while brown bean extract contained only X and C_2. Black bean extracts contained B_2, C_1 and C_2 while white bean contained none of the above procyanidins (Figure 4.14C).

da Silva *et al.* (1991) evaluated procyanidins extracted from grape seeds in different aqueous model systems. When compared to Trolox, all procyanidins tested showed a better superoxide radical scavenging capacity at both pH 7.5 and 9. Hydroxyl

radical scavenging capacities of procyanidins were comparable to those of ethanol and mannitol (da Silva *et al.,* 1991); the dimer procyanidin B_2 was more active than the monomers [(+) catechin and (-)-epicatechin]. The same relationship had been observed with DPPH (Ariga *et al.*, 1988) and with peroxy radical (Ariga and Hamano, 1990).

4.10.3 Phenolic acids

The phenolic acids of bean extracts were extracted and quantified (Table 4.13). Figure 4.14B depicts the chemical structures of the phenolic acids identified in the bean extracts. Drumm *et al.* (1990) determined ferulic, *p*-coumaric, sinapic and cinnamic acids in four varieties of beans (navy, dark red kidney, pimanto and black tutrle soup). The overall results of the present study compare well with that of Drumm *et al.* (1990).

Of the four types of beans examined, only black beans contained vanillic acid (254 μg/ g extract) while it did not contain any caffeic acid. Black beans contained a substantial amount of *p*-coumaric acid and high amount of ferulic and sinapic acids. Red beans contained the highest amount of *p*-coumaric acid compared to the other beans. None of the other bean types contained caffeic acid. Interestingly, white bean contained significantly ($p<0.05$) high amount of sinapic and ferulic acids compared to the other beans with substantial amount of *p*-coumaric acid. Among the most widely distributed phenylpropanoids in plant tissues are the hydroxycinnamic acids which includes coumaric, caffeic and ferulic acids produced from

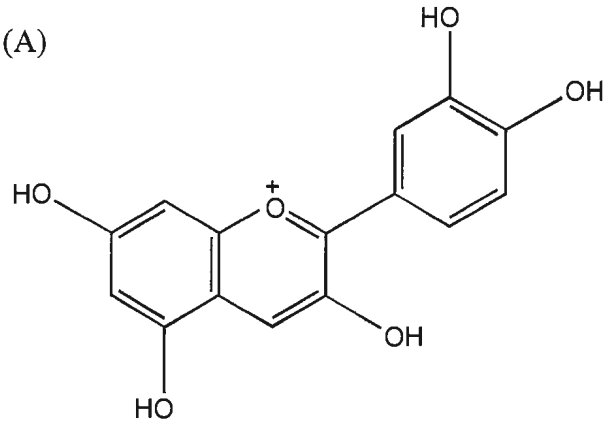
Table 4.13 Phenolic acids content[1] of bean extract[2]

Extract	Vanillic	Caffeic	*p*-Coumaric	Ferulic	Sinapic
RHE	ND	155.1±3.2[a]	1206.0±11[d]	139.8±4.3[b]	283.0±3.1[c]
BHE	ND	ND	209.2±4.2[c]	119.9±1.2[a]	87.2±2.8[a]
LHE	254.2±2.1[a]	ND	96.2±3.6[a]	146.3±2.3[c]	220.6±3.3[b]
WHE	ND	ND	152.4±4.2[b]	1217.0±8.9[d]	603.4±2.1[d]

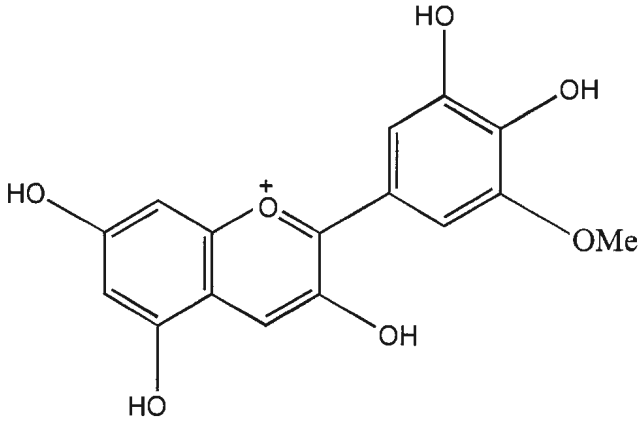
[1]Expressed as μg/g extract.
[2]Results reported are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are: ND, not detectable; RHE, red bean hull extract; BHE, brown bean hull extract; LHE, black bean hull extract; and WHE white bean hull extract.

Figure 4.14 Chemical structures of phenolic acids identified and quantified in bean Extracts: Anthocyanidins (A) phenolic acids (B) and procyanidin (C).

(A)

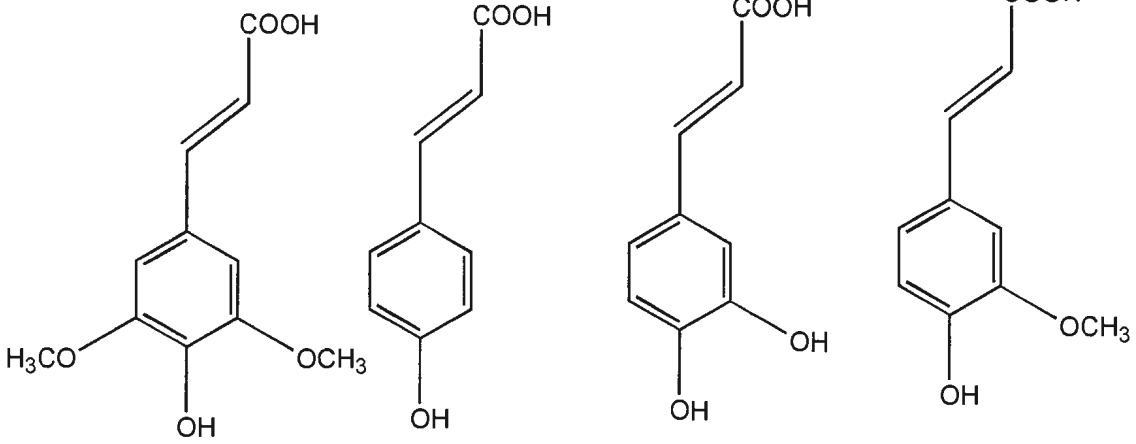


Cyanidin



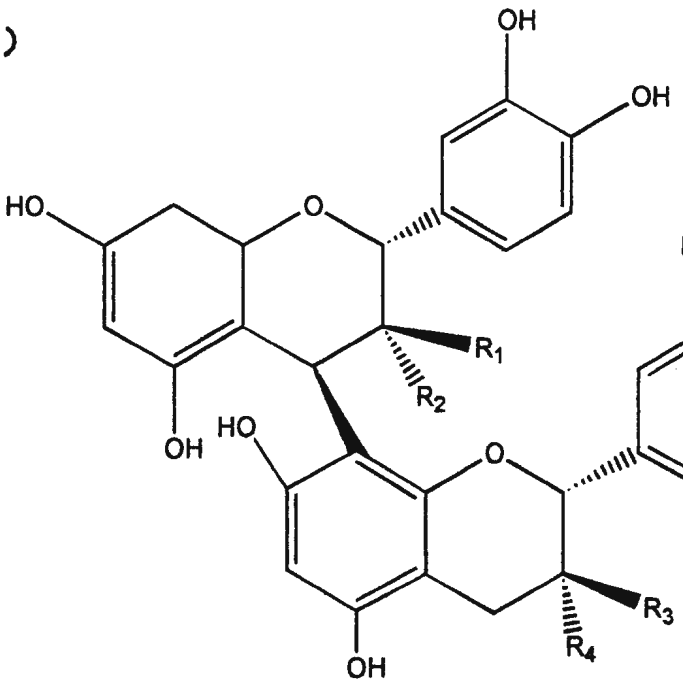
Delphinidin

(B)

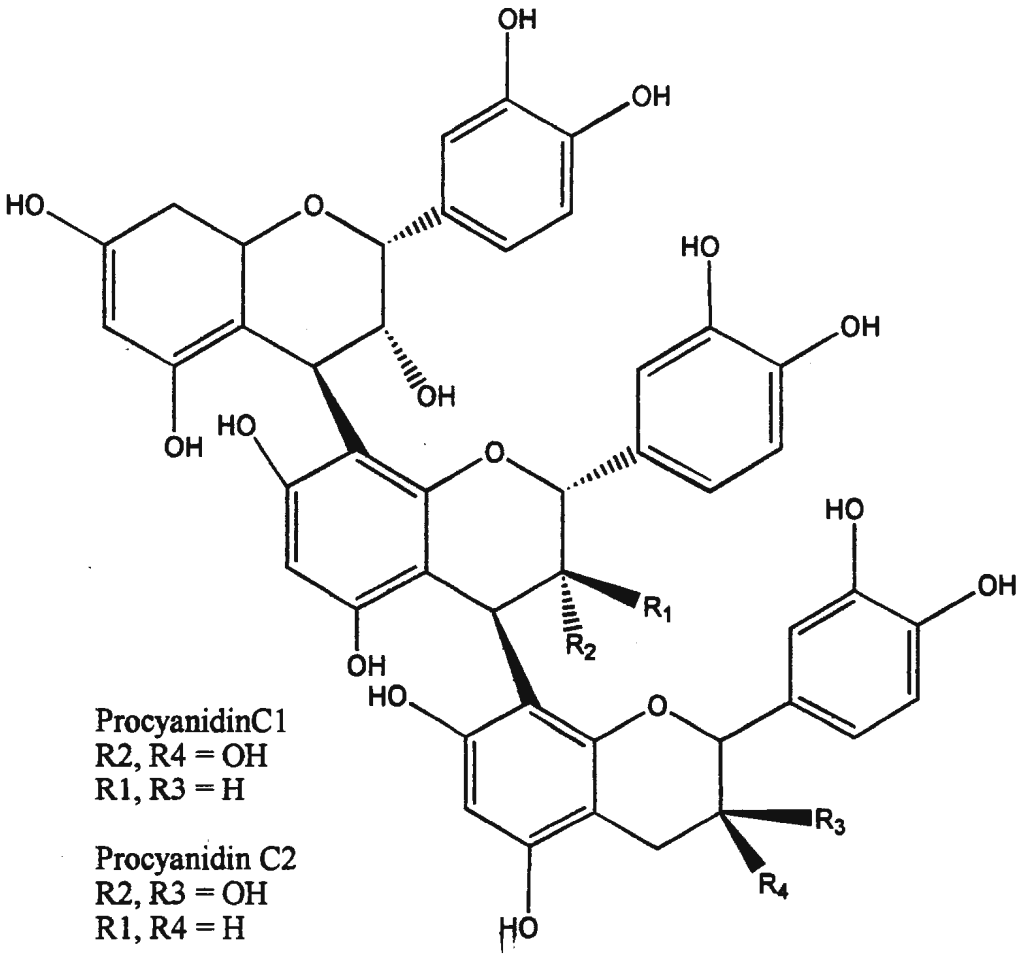


Sinapic acid p-coumaric acid Caffeic acid Ferulic acid

(c)



Procyanidin B2
R2, R4 = OH
R1, R3 = H



ProcyanidinC1
R2, R4 = OH
R1, R3 = H

Procyanidin C2
R2, R3 = OH
R1, R4 = H

shiklimate pathway from L-phenylalanine or L-tyrosine (Rice-Evans et al., 1996). These workers determined the total antioxidant capacity of different phenolic acids. Based on TEAC values, *p*-coumaric acid exhibited the highest antioxidative activity (TEAC, 2.22) followed by ferulic (TEAC 1.9) vanillic (1.43) and caffeic (TEAC, 1.26) acids.

The substitution of 3-hydroxyl group of caffeic acid by a methoxy group (ferluic acid) considerably enhanced the antioxidative effect of the compound. Cuvelier *et al.* (1992) reported that sinapic acid was the strongest phenolic acid in preventing accelerated autoxidation of methyl linoleate indicating methoxy substitution enhances antioxidant activity as might be expected for a better electron-donating effect of methoxy as compared to hydroxyl group.

CONCLUSIONS AND RECOMMENDATIONS

Total phenolic content of bean hull extracts ranged from 223-270 mg/g extract as catechin equivalents. The total phenolic contents of the whole seed extracts were low compared to that of hulls due to the dilution effect of cotyledons. Trolox equivalent antioxidative capacity of (TEAC) of the hull extracts ranged from 41 to 47 while the TEAC values of the whole bean seed extracts varied from 4 to 9. Based on the total phenolic and condensed tannin contents as well as total antioxidant capacity, as measured by TEAC value, it can be deduced that coloured beans possess superior antioxidant activity compared to white beans. Analysis of active compounds in the hulls lend further support to this observation as red, brown bean hulls contained the anthocyanidins; delphinidin and cyanidin while black bean hull contained only cyanidin; These compounds are responsible for the colour of bean hulls. The bean extracts contained phenolic acids such as ferulic, vanillic, *p*-coumaric and sinapic acids. Thus, antioxidative compounds were concentrated in the hulls of beans. Despite their low antioxidant activity, white beans contained high amounts of ferulic and sinapic acids along with a substantial amount of *p*-coumaric acid. Thus, the high antioxidaive capacities of red, brown and black bean hulls are mainly due to their anthocyanin pigments. Furthermore, fractionation of bean extracts revealed the presence of more hydrophobic constituents compared to hydrophilic compounds.

Evaluation of the activity in a β-carotene model system revealed that red, brown and black whole bean extracts were capable of inhibiting oxidation (33-52%) of β-carotene as compared to the control (2%) when used at 100 ppm level as catechin

equivalents. Similar results were obtained in a bulk corn oil model system. When used at 100 ppm level as catechin equivalents, red, brown and black whole bean extracts were efficient in scavenging hydrogen peroxide (65-76%), superoxide (53-60%) and DPPH (100%) radicals. Furthermore, red, brown and black whole bean extracts exhibited strong Fe^{2+} chelation capacity (60-72%) when used at 100 ppm level as catechin equivalents.

Red, brown and black whole extracts exhibited total inhibition of oxidation of Cu^{2+}-mediated human LDL oxidation as measured by conjugated dienes when used at 25 and 50 ppm as catechin equivalents. Red, brown and black whole seed extracts were capable of inhibiting peroxyl radical-induced supercoiled DNA nicking while they did not show any protection against hydroxyl radical-induced DNA nicking. Based on the results of the present study, it can be concluded that beans, especially those with coloured coats possess good antioxidant properties.

Antioxidative compounds in beans, especially anthocyanins, may play an important role as a source of plant-based dietary antioxidant in the human body. Apart from high protein and crude fibre contents, beans can also be considered as a potent source of antioxidants. Thus, beans may play an important role in the daily diet by providing protection against oxidative stress caused by various free radicals. However, the *in vivo* antioxidative effects of the extracts were not evaluated. Furthermore, some phenolic antioxidants are reported to have prooxidant effects, especially *in vivo,* and these should also be evaluated in different systems and under varied conditions.

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APPENDIX

Figure A-1 Dependence of the abosrobance of catechin-metal complex at 765 nm on The content of catechin in the medium.

Correlation coefficient (r) = 0.9977
Equation of the line is Y=aX where,
Y= absorbance at 765 nm (A_{765nm})
X=Concentration of catechin in the assay medium in μg/10 mL (C)
A=0.0107
Therefore, C=93.45 X A_{765nm}

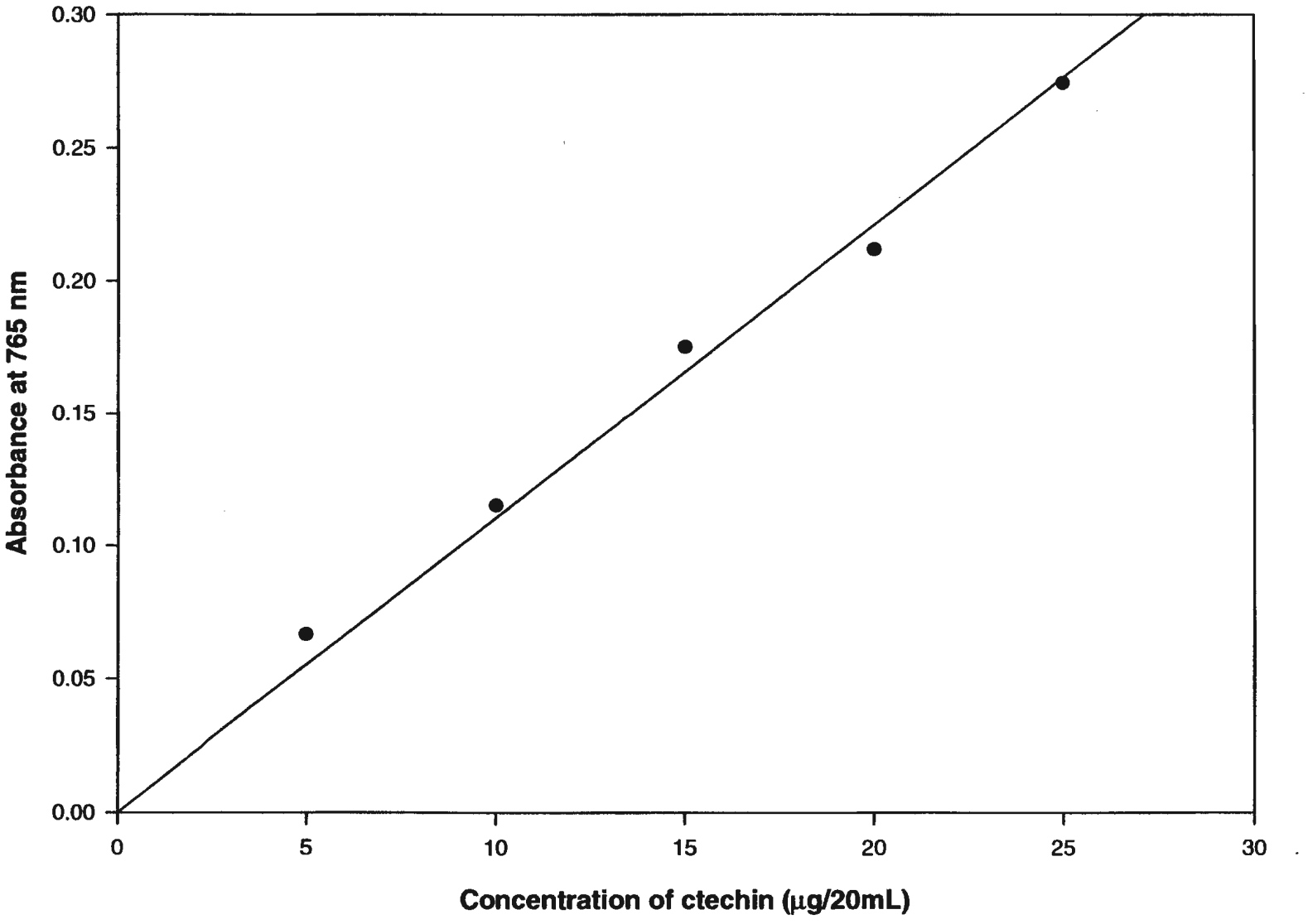

0.30
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance at 765 nm
0
5
10
15
20
25
30
Concentration of ctechin (µg/20mL)

Figure A-2 Dependence of absorbance of catechin-vanillin complex at 500 nm on the concentration of catechin

Correlation coefficient (r) = 0.9969
Equation of the line is Y=aX where,
Y= absorbance at 500 nm (A_{500nm})
X=Concentration of catechin in the assay medium in mg/mL (C)
A=0.4873
Therefore, C=2.052 X A_{500nm}

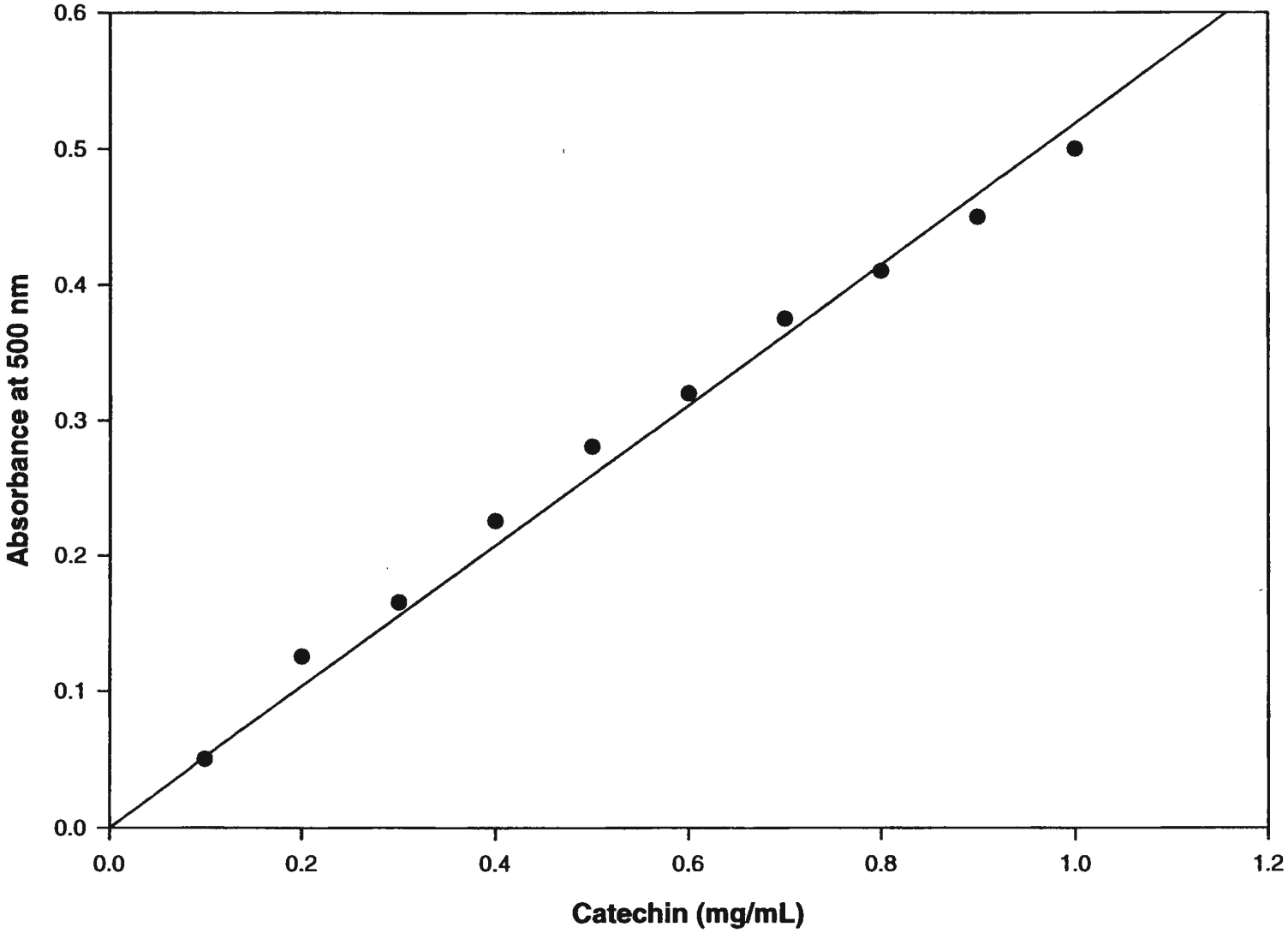

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 500 nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Catechin (mg/mL)

Figure A-3 Dependence of the absorbance of malonadehyde (MA)-thiobarbituric acid (TBA) complex at 532 nm on the content of MA and related compounds

Correlation coefficient (r) = 0.9828
Equation of the line is Y=aX where,
Y= absorbance at 532 nm (A_{532nm})
X=Concentration of malonaldehyde (μg/10 mL) in the assay media (C)
a=0.02648
Therefore, C=37.76 X A_{532nm}

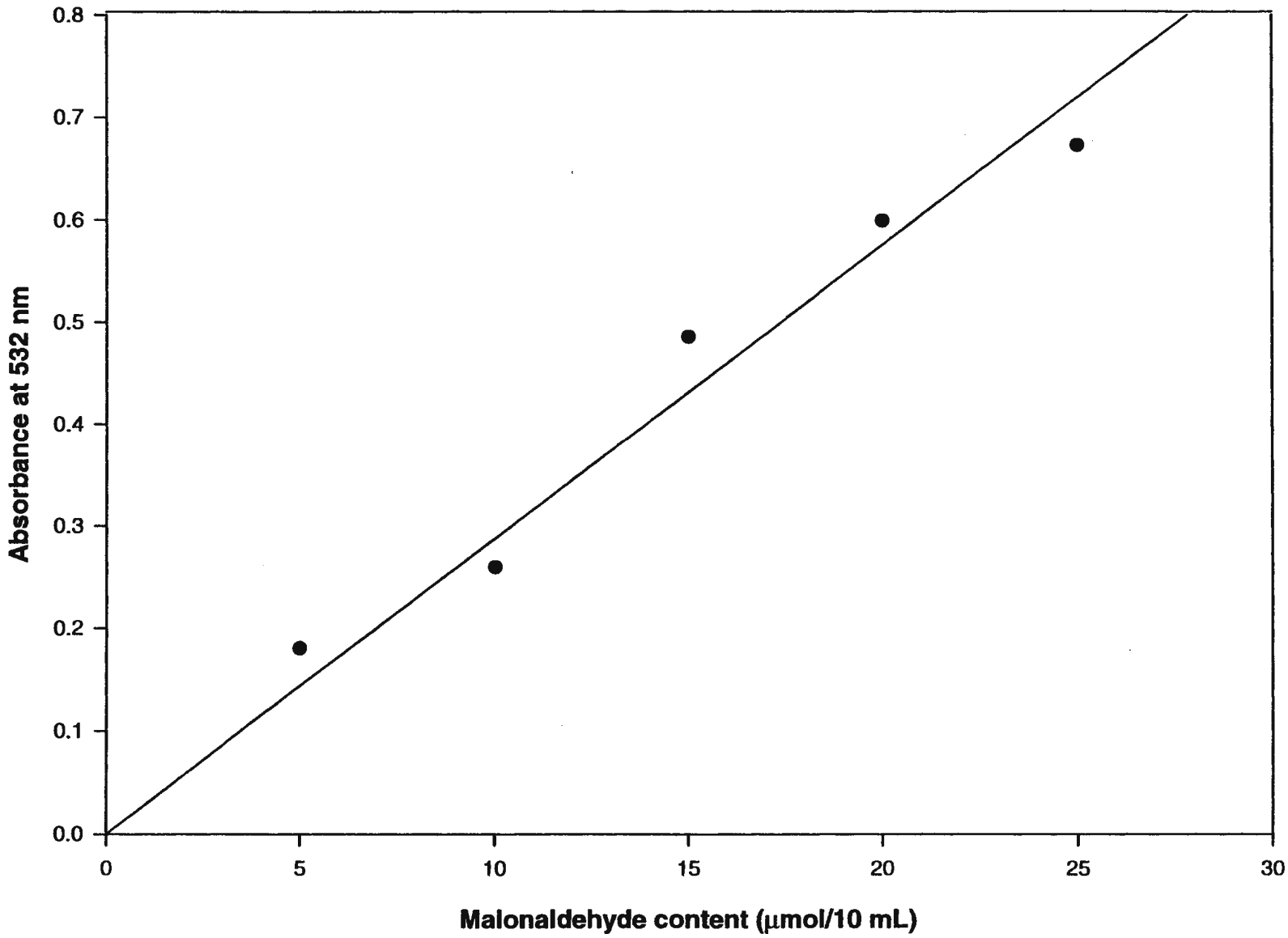

Absorbance at 532 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
5
10
15
20
25
30
Malonaldehyde content (μmol/10 mL)

Figure A-4 Dependence of the drop in absorbance of 2,2'-azinobis-(3-ethylbenzthioline-6-sulphonate) at 734 nm radical cation ($ABTS^{\cdot}$) solution on the concentration of Trolox.

Correlation coefficient (r) = 0.9983
Equation of the line is Y=aX where,
Y= absorbance at 734 nm (A_{734nm})
X=Concentration of Trolox (μM) in the assay media (C)
a=0.03297
Therefore, C=30.33 X A_{734nm}

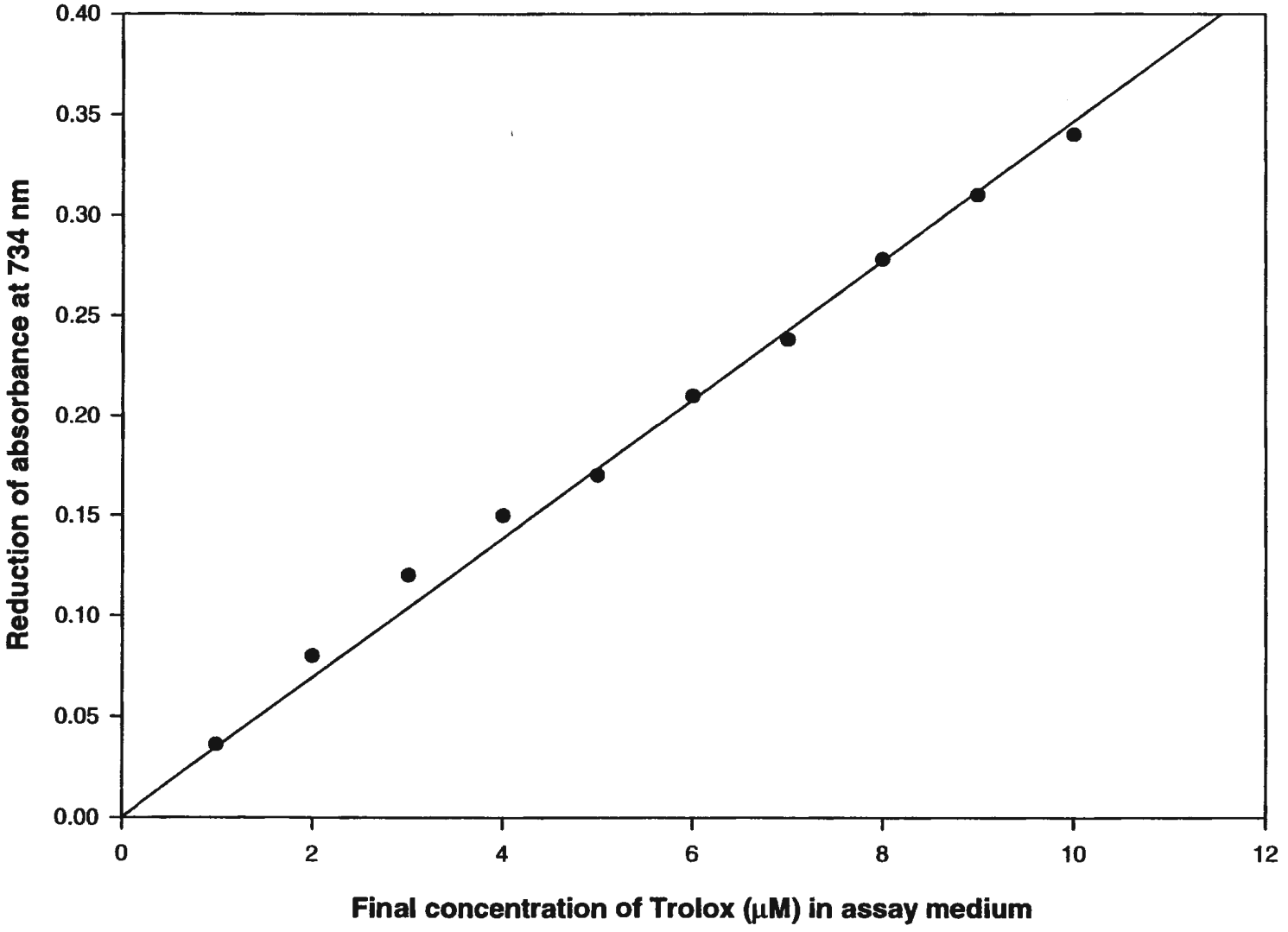

0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Reduction of absorbance at 734 nm
0
2
4
6
8
10
12
Final concentration of Trolox (μM) in assay medium

Figure A-5 Dependence of the absorbance of β-carotene at 470 nm on the concentration of β-carotene.

Correlation coefficient (r) = 0.9989
Equation of the line is Y=aX where,
Y= absorbance at 470 nm (A_{470nm})
X=Concentration of β-carotene (μg/10 mL) in the assay media (C)
a=0.00682
Therefore, C=146.62 X A_{470nm}

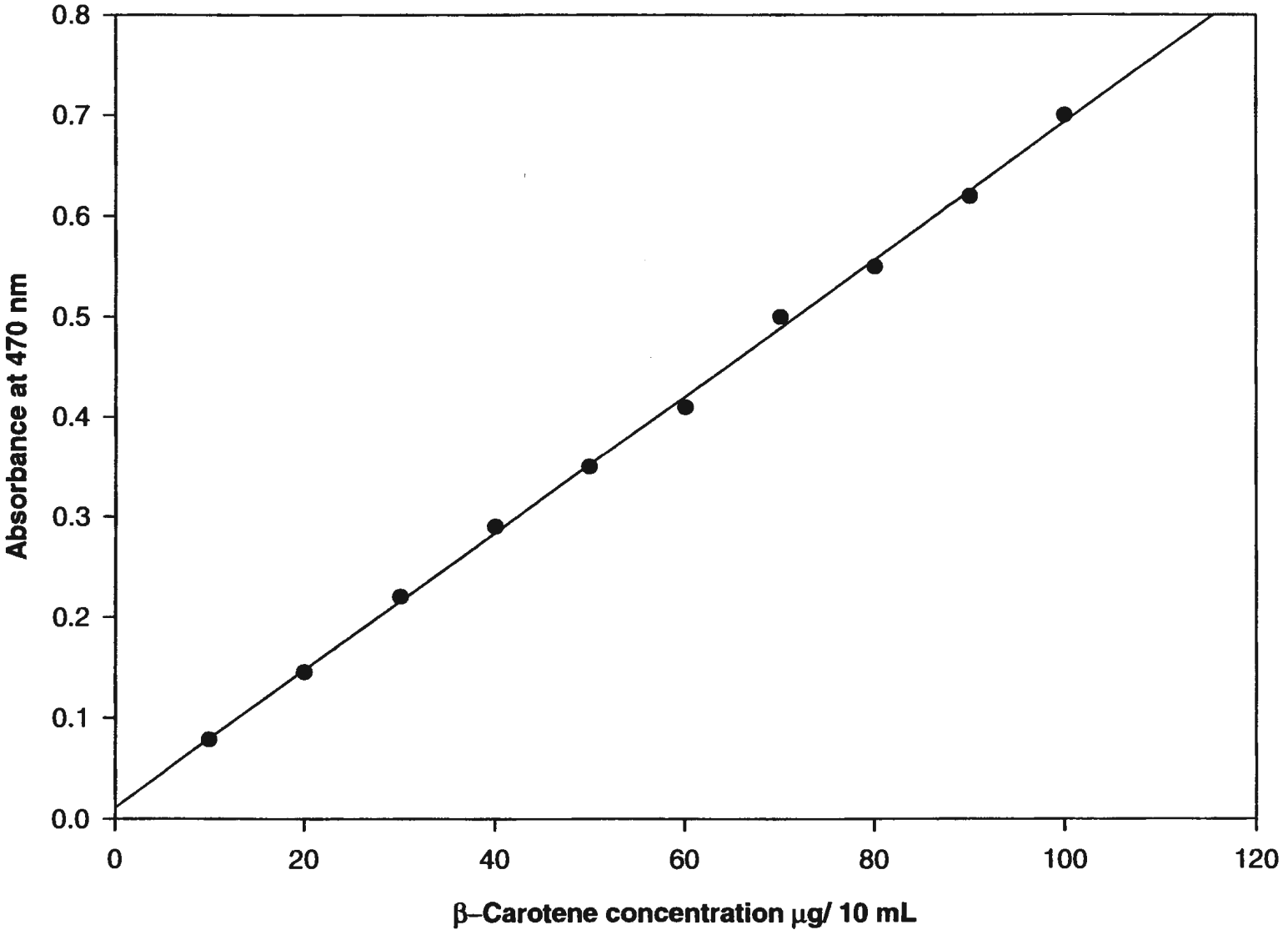

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 470 nm
0
20
40
60
80
100
120
β–Carotene concentration µg/ 10 mL

Figure A-6 Dependence of the absorbance of hydrogen peroxide at 230 nm on the concentration of hydrogen peroxide.

Correlation coefficient (r) = 0.99949
Equation of the line is Y=aX where,
Y= absorbance at 230 nm (A_{230nm})
X=Concentration of hydrogen peroxide (mM) in the assay media (C)
a=0.0680
Therefore, C=14.70 X A_{230nm}

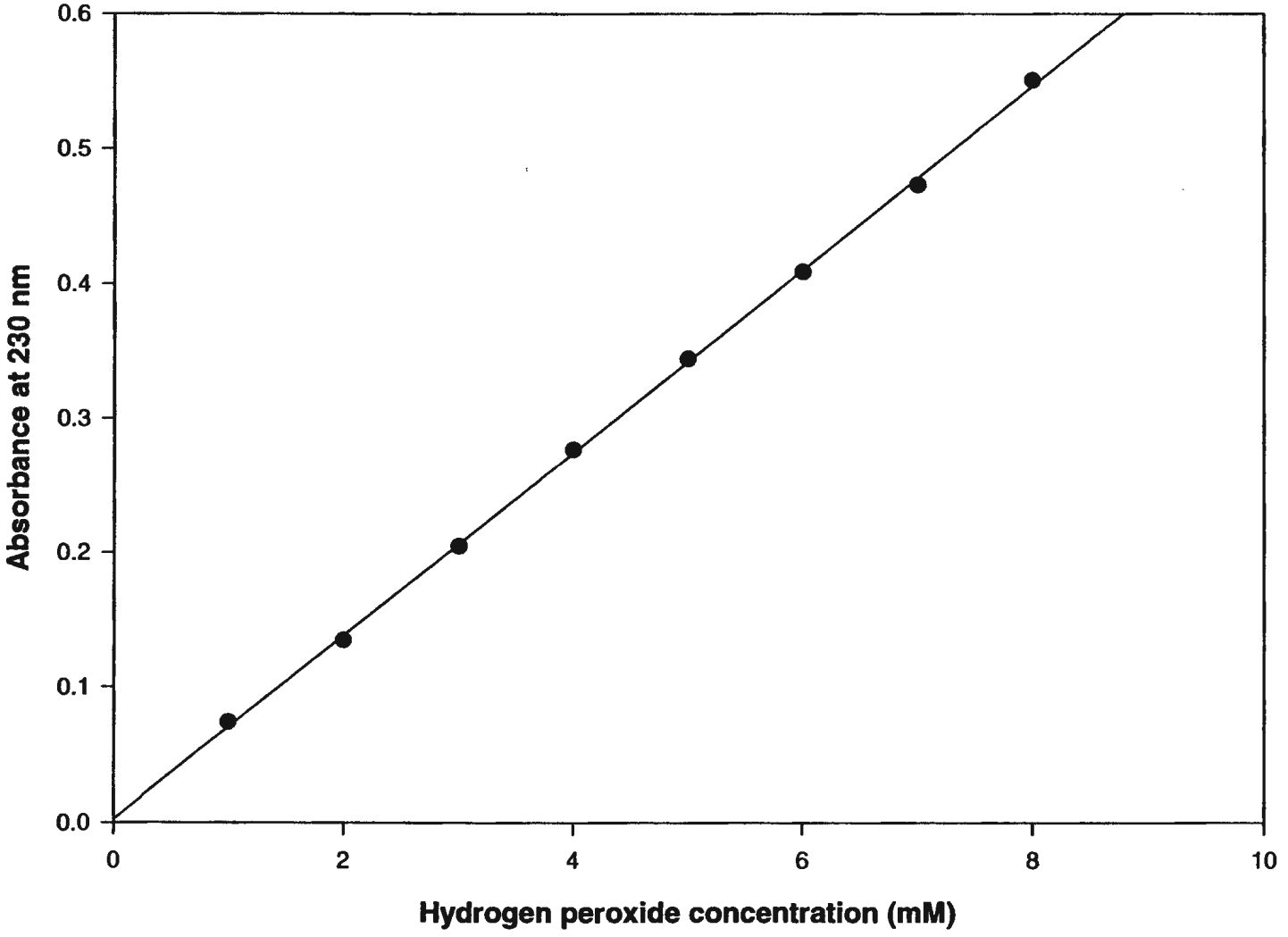

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 230 nm
0
2
4
6
8
10
Hydrogen peroxide concentration (mM)

Figure A-7 Dependence of the absorbance ratio (460 nm/ 530 nm) on the concentration of free ferrous ions in μM

Correlation coefficient (r) = 0.9980
Equation of the line is Y=aX where,
Y= absorbance ratio ($A_{460\ nm/530\ nm}$)
X=Concentration of free ferrous ions (μM) in the assay media (C)
a=0.000617
Therefore, C=1620.74 X $A_{460\ nm/\ 530\ nm}$

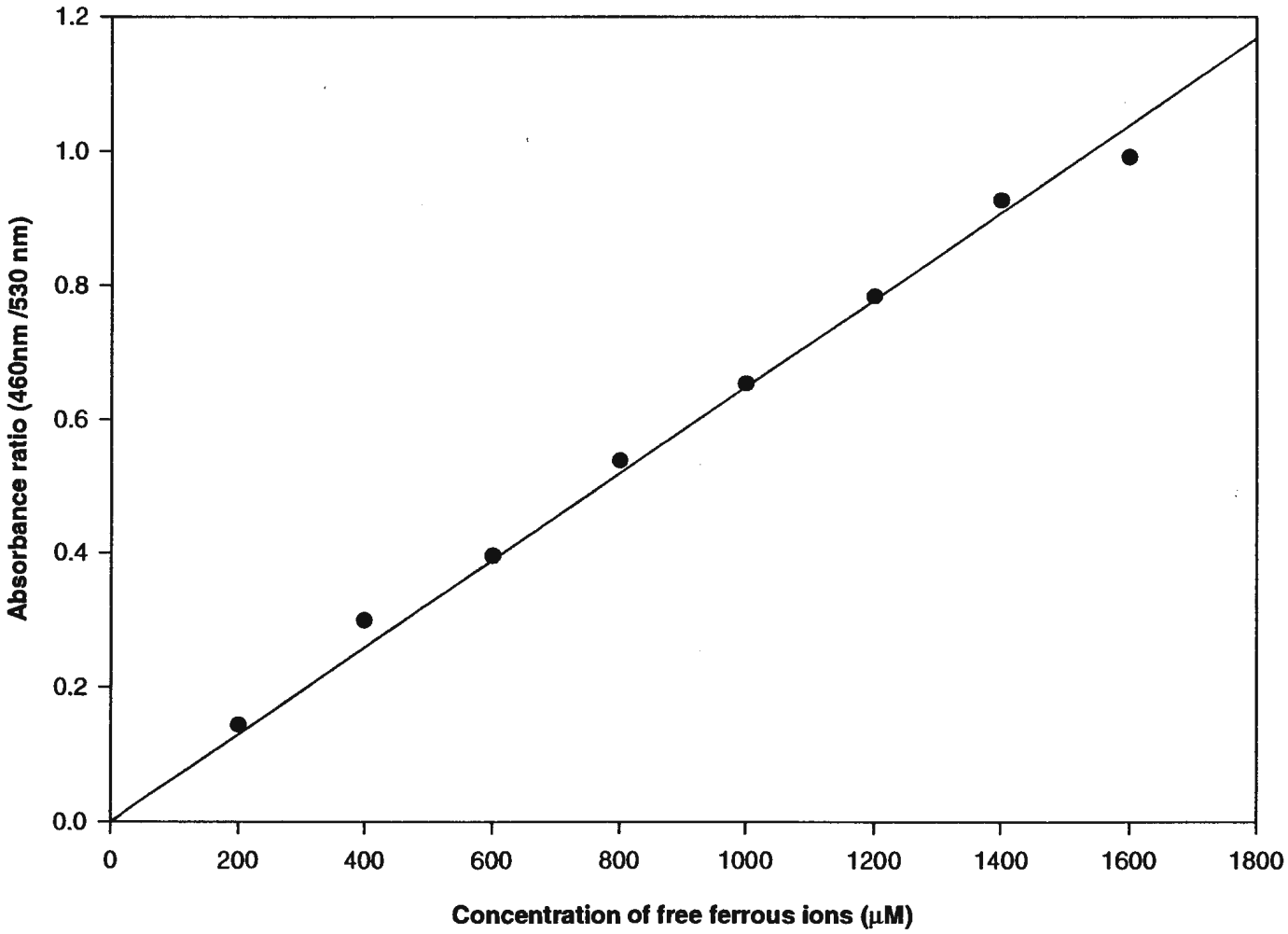

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance ratio (460nm /530 nm)
0
200
400
600
800
1000
1200
1400
1600
1800
Concentration of free ferrous ions (μM)

Figure A-8 Effect of catechin in preventing peroxyl radical induced DNA scission.

Lane 1 = DNA + PBS; Lane 2 = DNA + 1mM AAPH ; Lane 3 = DNA + 1mM AAPH + catechin 5 ppm; Lane 4 = DNA + catechin 10 ppm + 1mM AAPH; Lane 5 = DNA + 1mM AAPH + catechin 50 ppm; Lane 6 = DNA + 1 mM AAPH + catechin 100 ppm.

Abbreviations used: AAPH, 2,2`-azobis-(2-methylpropionamidine) dihydrochloride; S, supercoiled DNA strand; N, Nicked DNA strand.

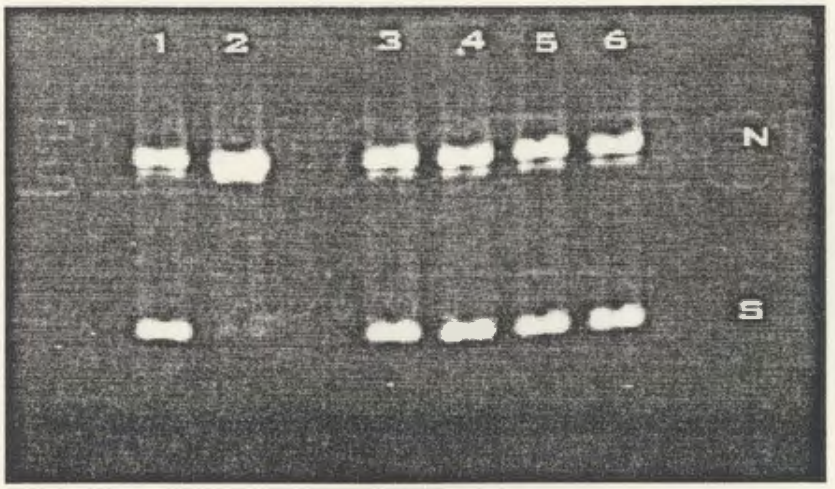

1
2
3
4
5
6
N
S

Figure A-9 Effect of RWE in preventing peroxyl radical induced DNA scission.

Lane 1 = DNA + PBS; Lane 2 = DNA + 1mM AAPH ; Lane 3 = DNA + 1mM AAPH + RWE 5 ppm; Lane 4 = DNA + mM AAPH +RWE 10 ppm + ; Lane 5 = DNA + 1mM AAPH + RWE 50 ppm; Lane 6 = DNA + 1 mM AAPH + RWE 100 ppm.

Abbreviations used: RWE, Red whole bean extract; AAPH, 2,2`-azobis-(2-methylpropionamidine) dihydrochloride; S, supercoiled DNA strand; N, Nicked DNA strand.

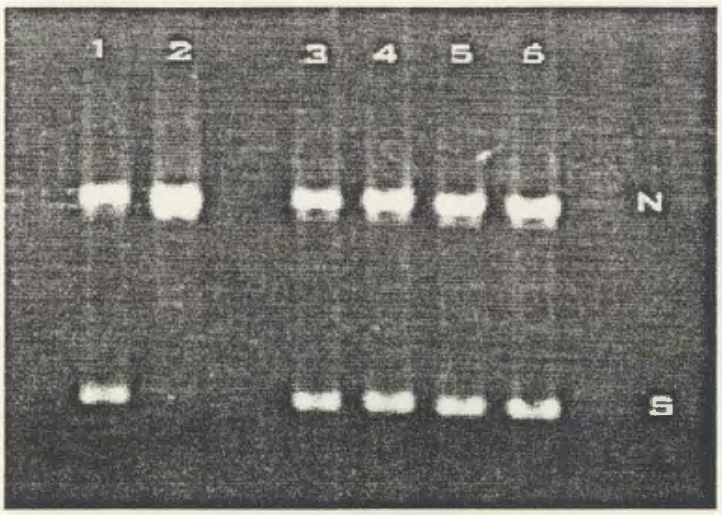

1
2
3
4
5
6
N
S

Figure A-10 Effect of BWE in preventing peroxyl radical induced DNA scission.

Lane 1 = DNA + PBS; Lane 2 = DNA + 1mM AAPH ; Lane 3 = DNA + 1mM AAPH + BWE 5 ppm; Lane 4 = DNA + BWE 10 ppm + 1mM AAPH; Lane 5 = DNA + 1mM AAPH + BWE 50 ppm; Lane 6 = DNA + 1 mM AAPH + BWE 100 ppm.

Abbreviations used: BWE, Brown whole bean extract; AAPH, 2,2`-azobis-(2-methylpropionamidine) dihydrochloride; S, supercoiled DNA strand; N, Nicked DNA strand.

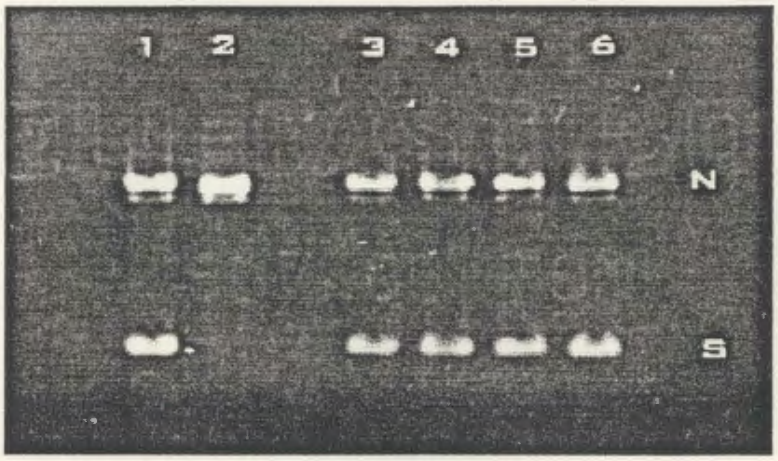

1 2 3 4 5 6
N
S

Figure A-11 Effect of LWE in preventing peroxyl radical induced DNA scission.

Lane 1 = DNA + PBS; Lane 2 = DNA + 1mM AAPH ; Lane 3 = DNA + 1mM AAPH + LWE 5 ppm; Lane 4 = DNA + LWE 10 ppm + 1mM AAPH; Lane 5 = DNA + 1mM AAPH + LWE 50 ppm; Lane 6 = DNA + 1 mM AAPH + LWE 100 ppm.

Abbreviations used: LWE, Brown whole bean extract; AAPH, 2,2`-azobis-(2-methylpropionamidine) dihydrochloride; S, supercoiled DNA strand; N, Nicked DNA strand.

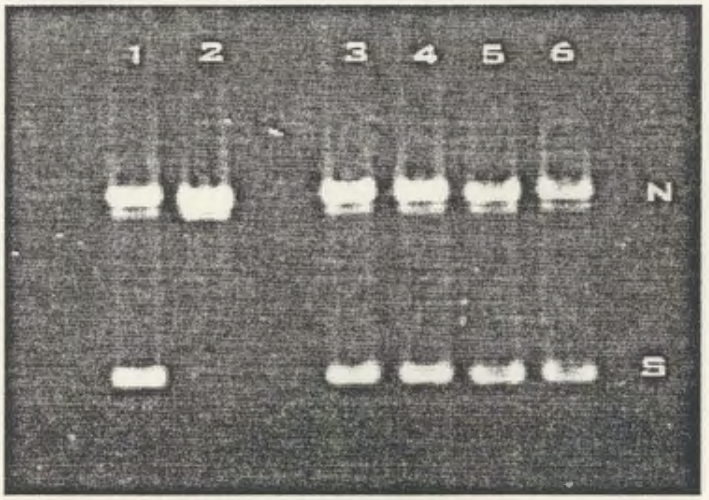

1
2
3
4
5
6
N
S

Figure A-12 A representative figure to illustrate the effect of whole bean extracts in preventing hydroxyl radical induced DNA scission.

Lane 1 = DNA + PBS; Lane 2 = DNA + hydroxyl radical; Lane 3 = DNA + Hydroxyl radical + RWE 5 ppm; Lane 4 = DNA + RWE 10 ppm + hydroxyl radical; Lane 5 = DNA + hydroxyl radical + RWE 50 ppm; Lane 6 = DNA + hydroxyl radical + RWE 100 ppm.

Abbreviations used: RWE, Brown whole bean extract; S, supercoiled DNA strand; N, Nicked DNA strand.

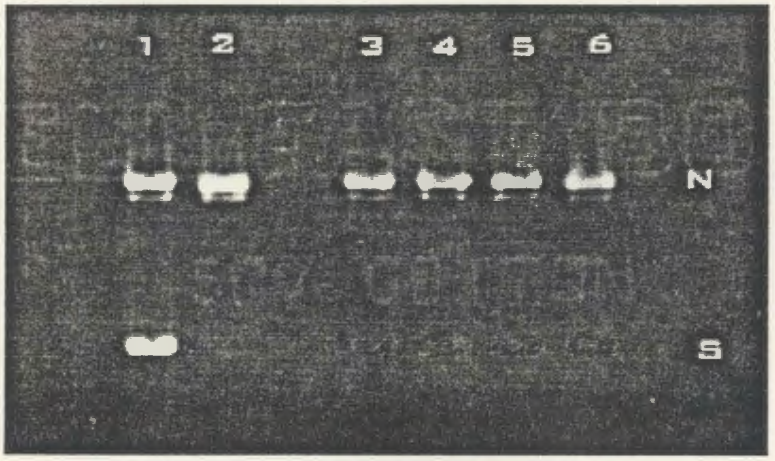
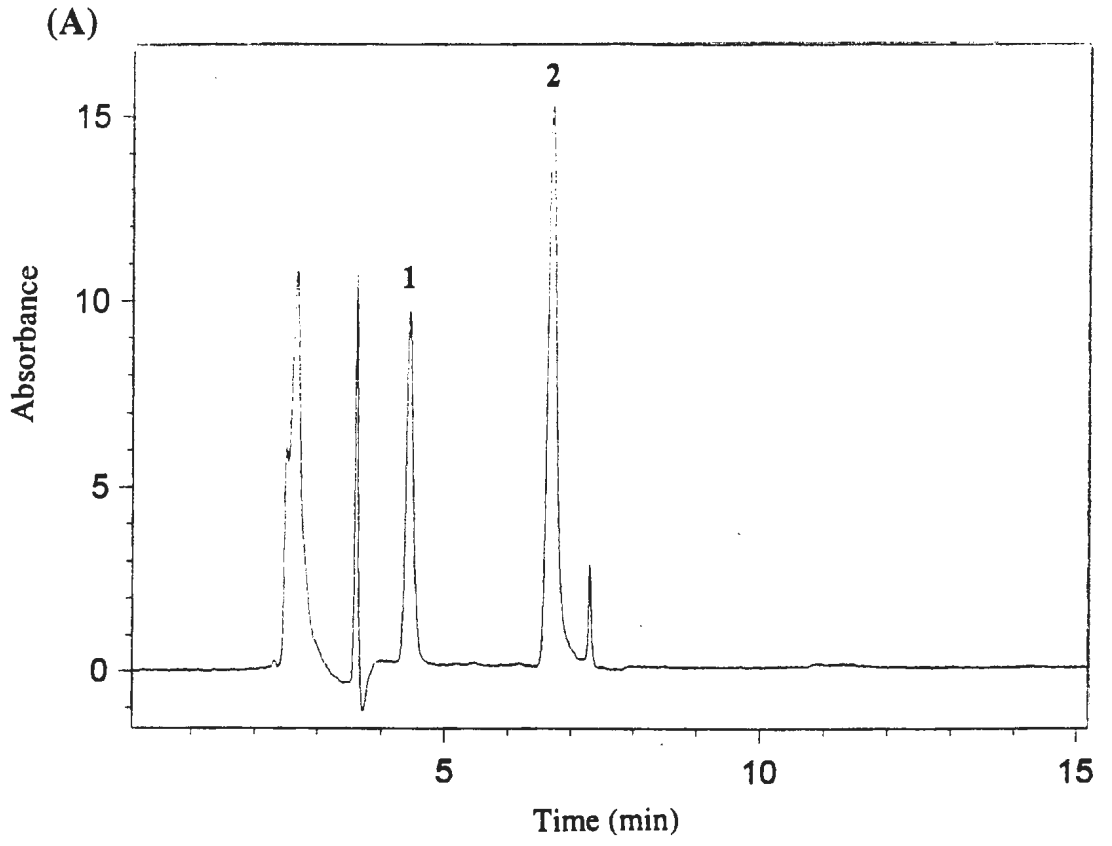

1
2
3
4
5
6
N
S

Figure A-13 High performance liquid chromatographic profiles of hydrolyzed (A) red and (B) brown bean hull extracts at 525 nm. Peaks: 1, Delphinidin, 2, Cyanidin.


(A)
Absorbance
15
10
5
0
1
2
5
10
15
Time (min)

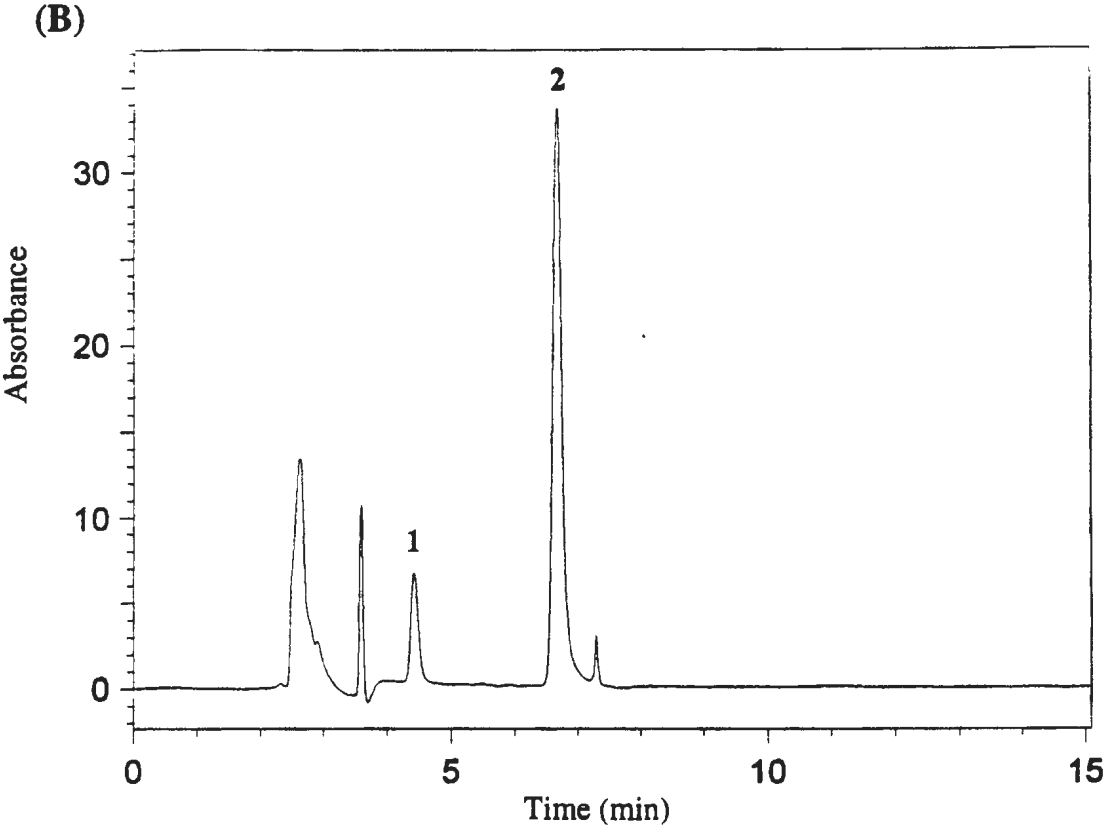
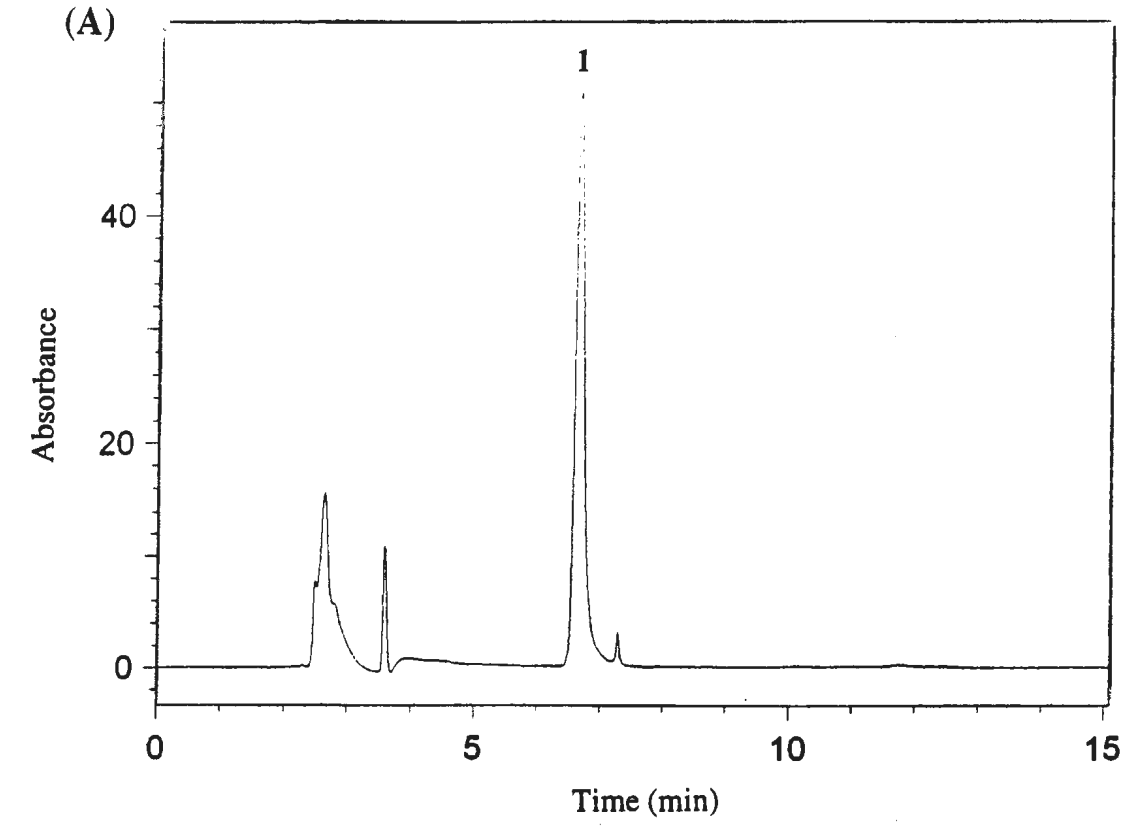

(B)
Absorbance
30
20
10
0
1
2
0
5
10
15
Time (min)

Figure A-14 High performance liquid chromatographic profiles of hydrolyzed (A) black and (B) white bean hull extract at 525 nm. Peaks: 1, Cyanidin.


(A)
1
40
20
0
Absorbance
0
5
10
15
Time (min)

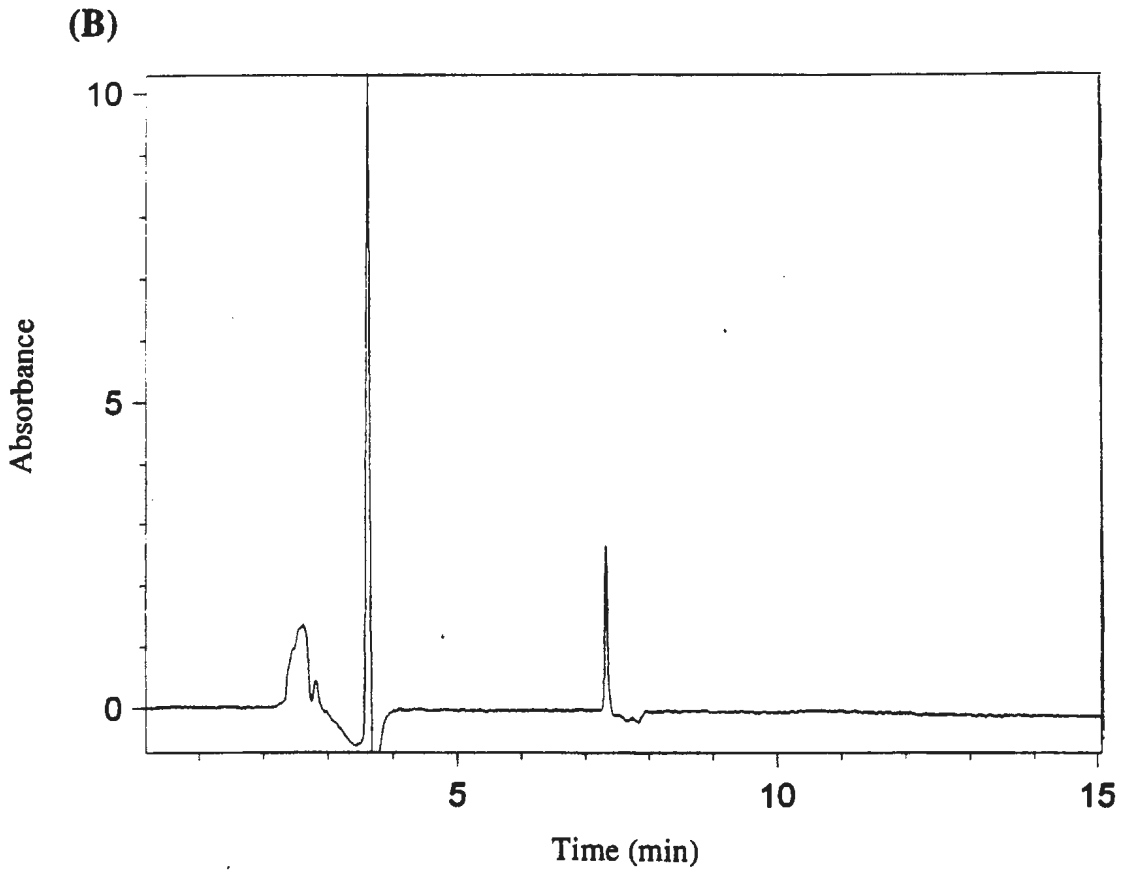
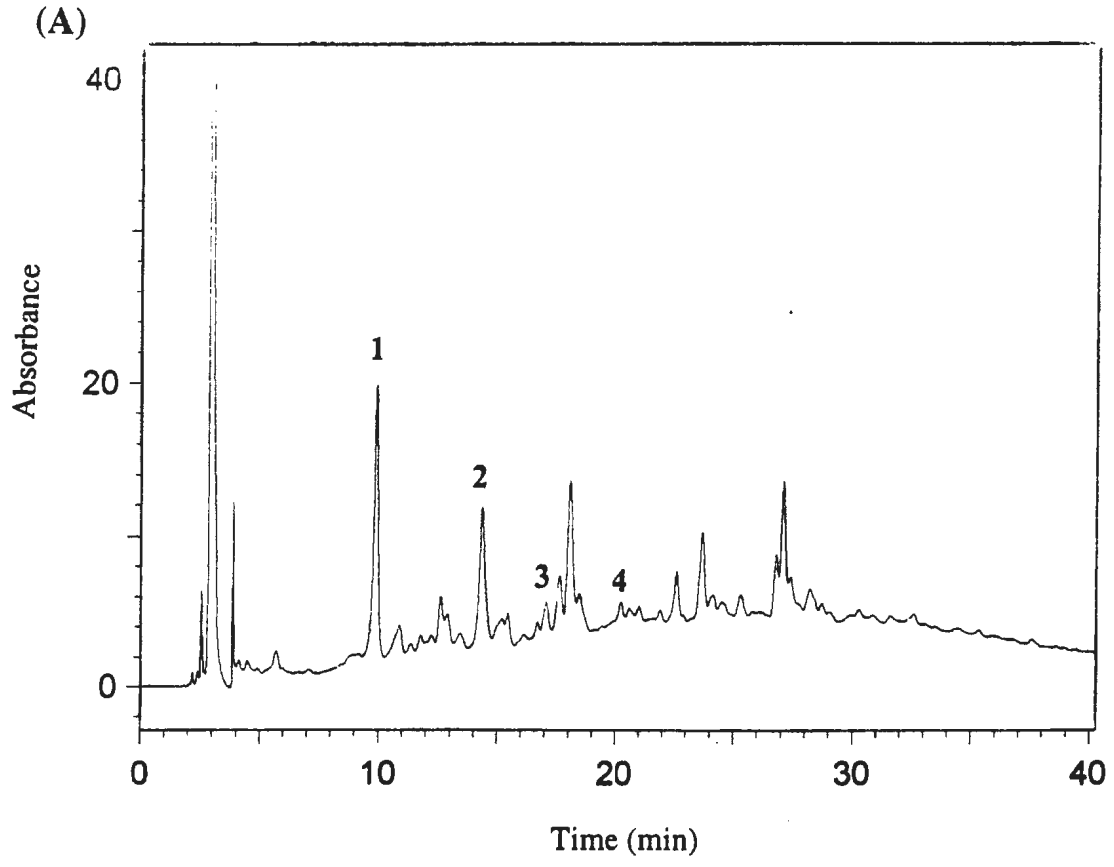

(B)
10
5
0
Absorbance
5
10
15
Time (min)

Figure A-15 High performance liquid chromatographic profiles of hydrolyzed (A) red and (B) brown bean hull extract at 525 nm. Peaks: 1, Procyanidn X; 2, Procyanidin C_2; 3, Procyanidin B_2; 4, Procyanidin C_1


(A)
40
20
0
Absorbance
1
2
3
4
0
10
20
30
40
Time (min)

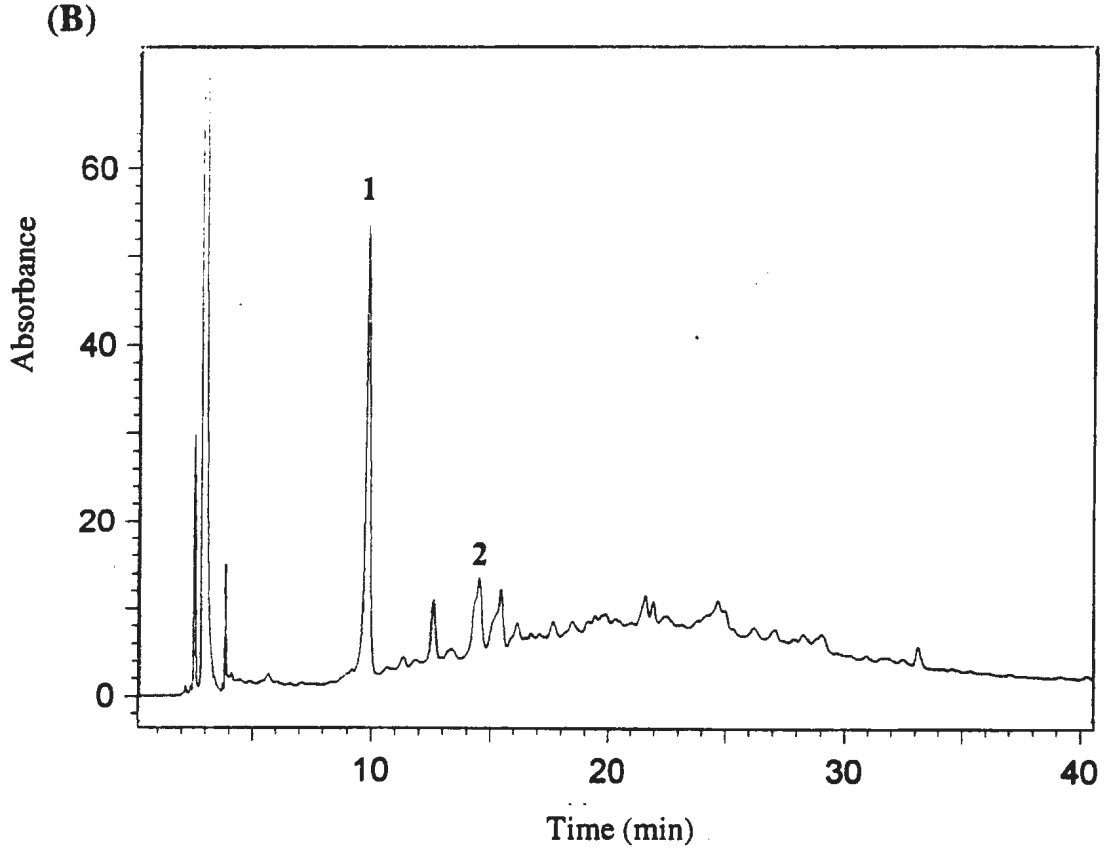
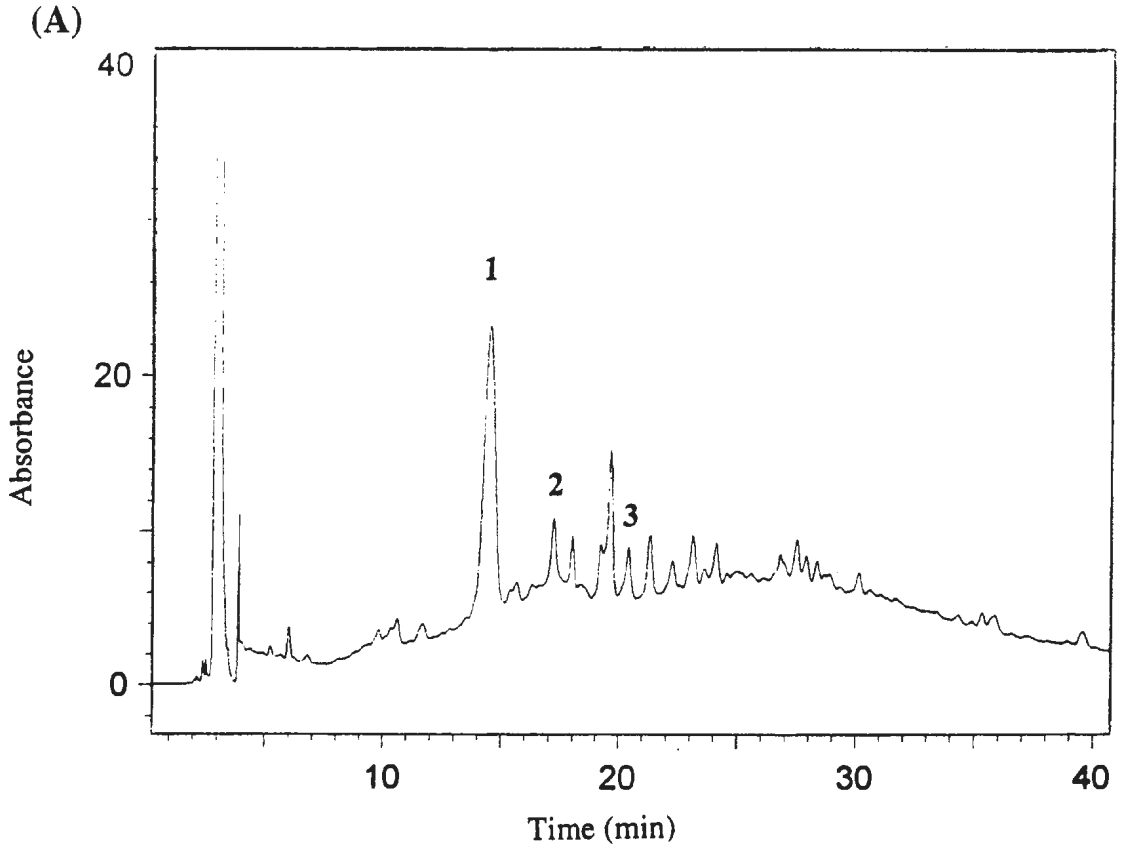

(B)
60
40
20
0
Absorbance
1
2
10
20
30
40
Time (min)

Figure A-16 High performance liquid chromatographic profiles of hydrolyzed (A) black and (B) white bean hull extract at 525 nm. Peaks: 1, Procyanidin C_2; 2, Procyanidin B_2; 3, Procyanidin C_1


(A)
40
20
0
Absorbance
1
2
3
10
20
30
40
Time (min)

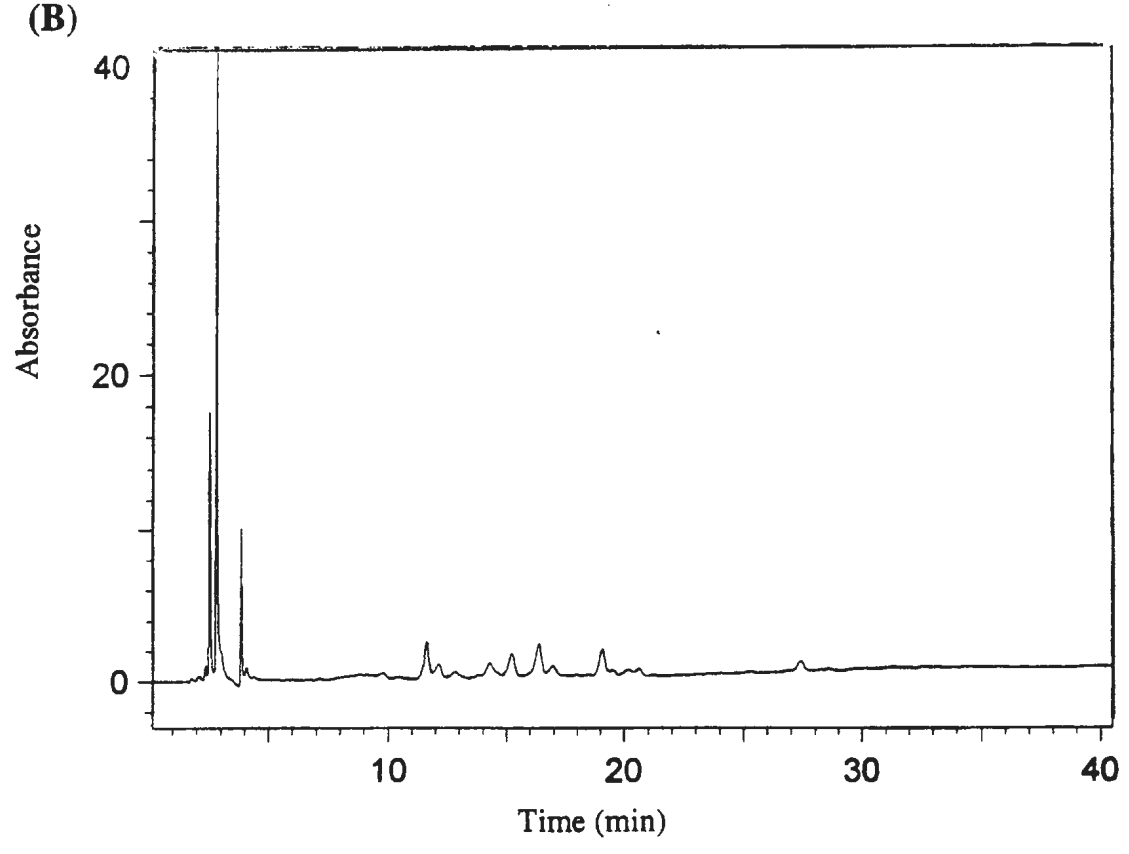
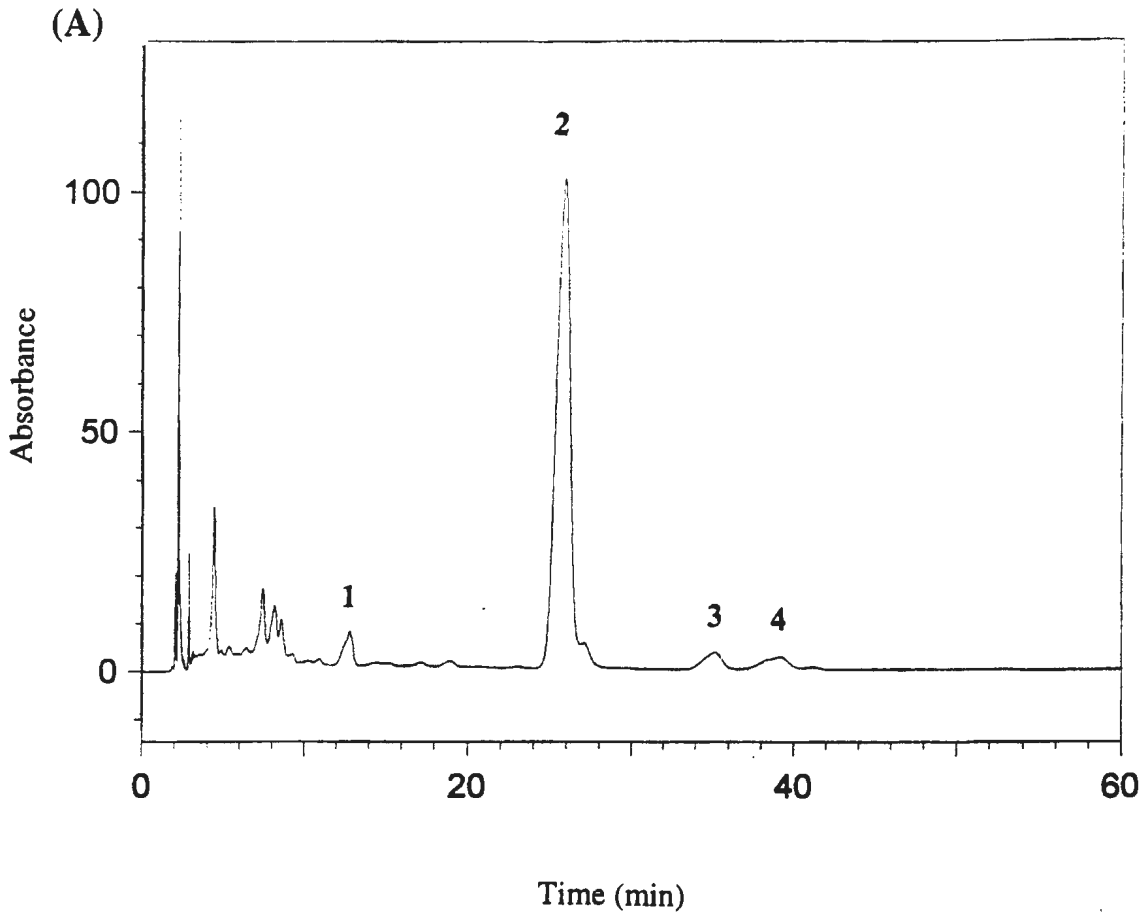

(B)
40
20
0
Absorbance
10
20
30
40
Time (min)

Figure A-17 High performance liquid chromatographic profiles of phenolics acids in (A) red and (B) brown bean hull extract at 330 nm. Peaks: 1, caffeic acid; 2, *p*-coumaric acid; 3, ferulic acid; 4, sinapic acid; 5, vanillic acid.


(A)
Absorbance
100
50
0
1
2
3
4
0
20
40
60
Time (min)

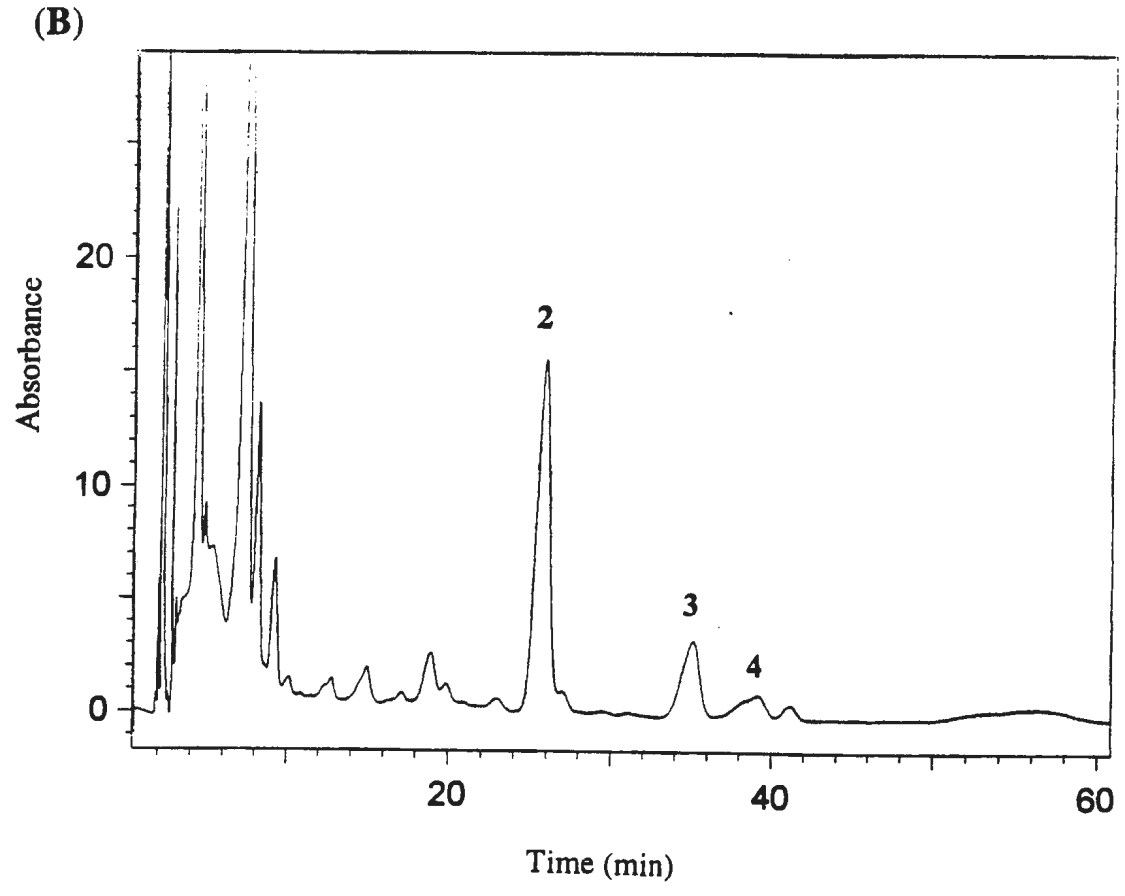
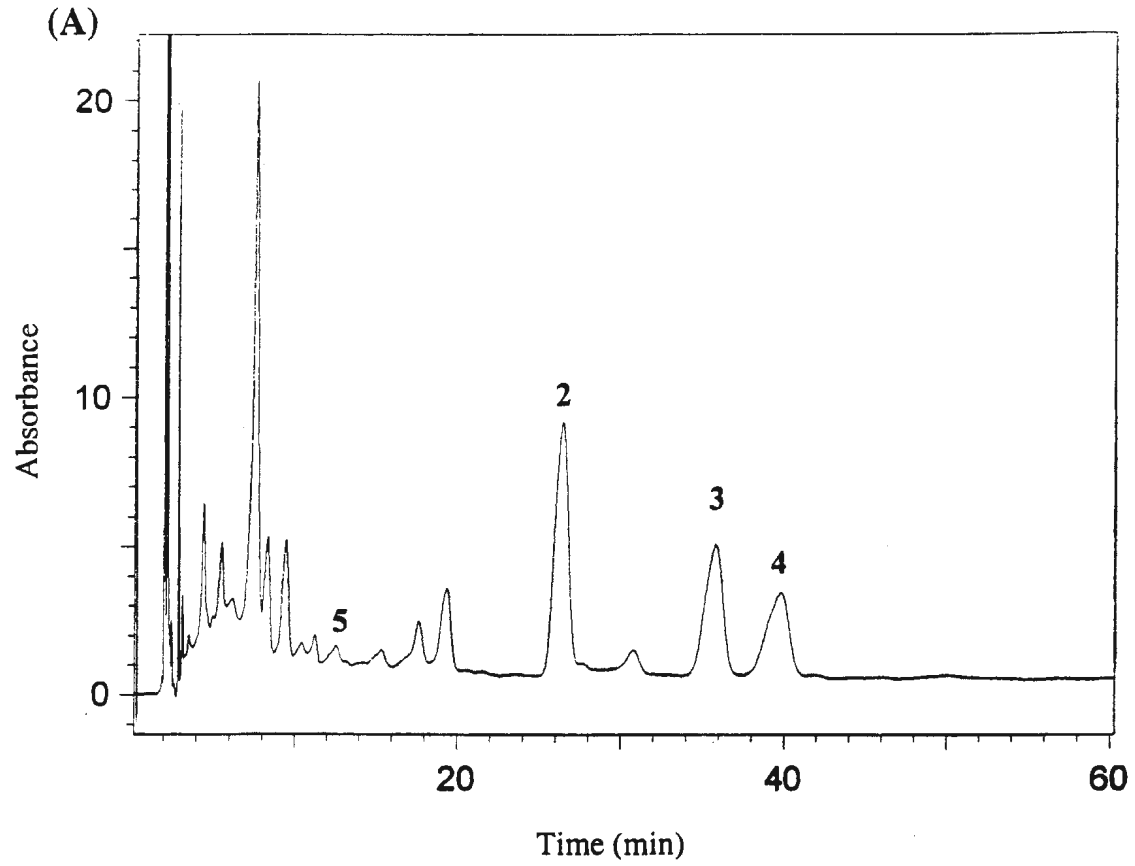

(B)
Absorbance
20
10
0
2
3
4
20
40
60
Time (min)

Figure A-18 High performance liquid chromatographic profiles of phenolics acids in (A) black and (B) white bean hull extract at 330 nm. Peaks: 1, caffeic acid; 2, *p*-coumaric acid; 3, ferulic acid; 4, sinapic acid; 5, vanillic acid.


(A)
20
10
0
Absorbance
2
3
4
5
20
40
60
Time (min)

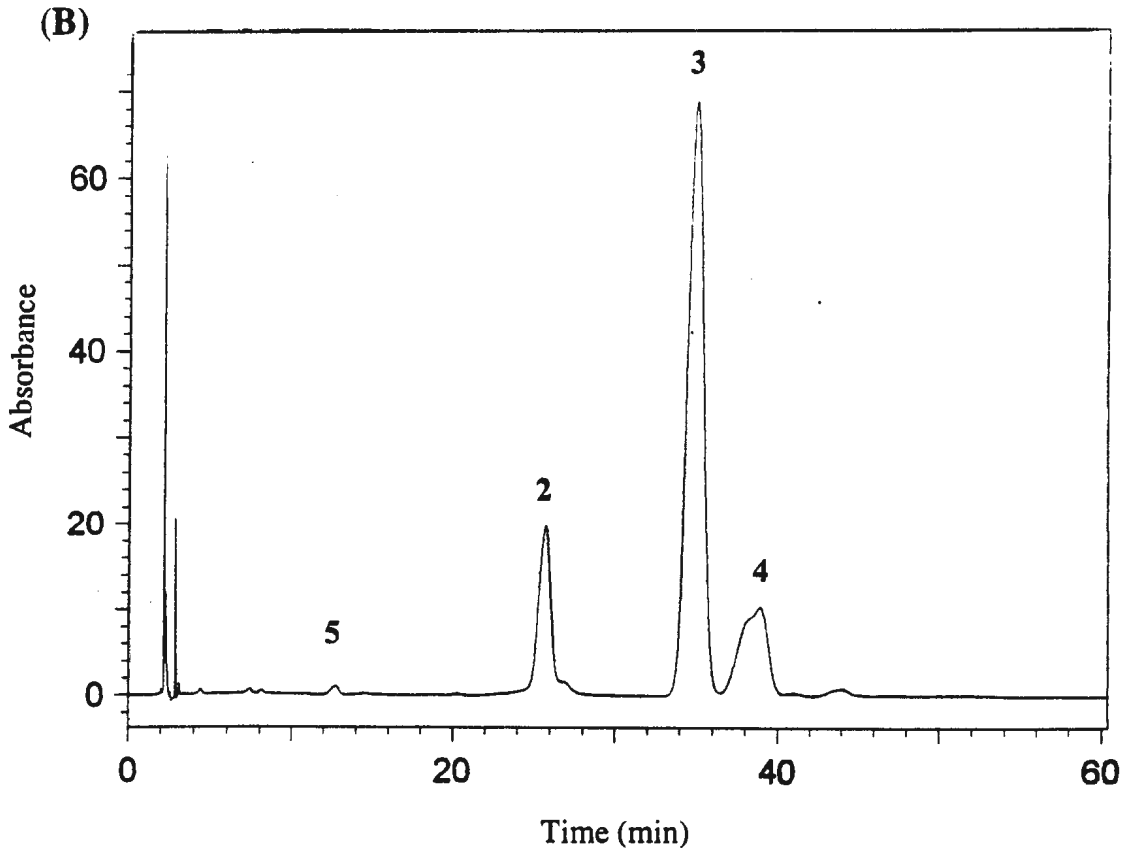

(B)
60
40
20
0
Absorbance
3
2
4
5
0
20
40
60
Time (min)

Table A-1 Conjugated diene (CD) values of bean extracts at 50 ppm concentration as catechin equivalents and reference antioxidants at 50 ppm in stripped bulk corn oil system during a 7-day storage period at 60°C[1].

Additive	Storage time in days				
	0	1	3	5	7
Control	3.39±.43^{a}	4.17±.06^{a}	6.16±.17^{b}	12.56±.32^{c}	19.43±.65^{d}
RWE	3.31±.01^{a}	3.46±.05^{a}	5.90±.34^{a,b}	9.61±.39^{b}	14.40±.70^{c}
BWE	3.38±.51^{a}	3.41±.35^{a}	6.15±.41^{b}	10.31±.47^{b}	14.50±.36^{c}
LWE	3.46±.09^{a}	3.44±.09^{a}	6.14±.36^{a,b}	10.63±.91^{b}	15.00±.35^{c}
Catechin	3.39±.21^{a}	3.39±.39^{a}	5.21±.27^{a}	7.00±.61^{a}	11.00±.15^{a}
A-Tocopherol	3.41±.42^{a}	3.91±.42^{a}	6.25±.51^{b}	13.14±.24^{c}	22.01±.29^{c}
BHA	3.37±.01^{a}	3.61±.36^{a}	5.20±.27^{a}	8.10±.43^{a}	12.51±.28^{b}

[1]Results are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are : RWE, Red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; BHA, butylated hydroxyanisole.

Table A-2 Conjugated diene (CD) values of bean extracts at 100 ppm concentration as catechin equivalents and reference antioxidants at 100 ppm in stripped bulk corn oil system during a 7-day storage period at 60°C[1].

Sample	Storage time in days				
	0	1	3	5	7
Control	3.39±.09[a]	4.17±.15[b]	6.16±.36[b]	12.5±.72[d]	19.42±.68[d]
RWE	3.32±.15[a]	3.44±.27[a]	5.50±.24[a,b]	9.09±.36[b]	13.96±.39[b,c]
BWE	3.36±.36[a]	3.45±.30[a]	5.87±.43[b]	9.93±.76[b,c]	13.00±.51[b]
LWE	3.41±.46[a]	3.49±.51[a]	5.37±.52[a,b]	10.00±.69[b,c]	14.97±.70[c]
Catechin	3.37±.09[a]	3.41±.60[a]	4.91±.32[a]	6.81±.47[a]	10.21±.21[a]
A-Tocopherol	3.30±.13[a]	3.91±.46[b]	6.01±.23[b]	11.30±.32[c,d]	20.16±.39[d]
BHA	3.41±.07[a]	3.90±.27[a,b]	5.50±.31[a,b]	9.10±.46[b]	14.21±.49[b,c]

[1]Results are mean values of three determinations ± standard deviation..

Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.

Abbreviations used are : RWE, Red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; BHA, butylated hydroxyanisole.

Table A-3 TBARS (as μmoles malonladehyde equivalents)of bean extracts at 50 ppm concentration as catechin equivalents and Reference antioxidants at 50 ppm in stripped bulk corn oil system during a 7-day storage period at 60°C[1].

Additive	Storage time in days				
	0	1	3	5	7
Control	0.089±.01[a]	0.17±.03[a]	0.30±.06[c]	0.65±.09[d]	1.01±1.3[d]
RWE	0.09±.03[a]	0.13±.02[a]	0.26±.02[a,b]	0.41±.01[b,c]	0.52±.05[b,c]
BWE	0.10±.02[a]	0.15±.01[a]	0.27±.03[b]	0.45±.04[b,c]	0.51±.04[b,c]
LWE	0.09±.01[a]	0.21±.03[a]	0.29±.02[b]	0.49±.03[b,c]	0.56±.03[c]
Catechin	0.12±.02[a]	0.18±.03[a]	0.21±.02[a]	0.31±.08[a]	0.44±.06[a]
A-Tocopherol	0.11±.07[a]	0.21±.03[a]	0.29±.01[b]	0.510±.02[c]	0.99±.09[d]
BHA	0.09±.05[a]	0.12±.06[a]	0.24±.03[a,b]	0.40±.07[a,b]	0.49±.09[a,b]

[1]Results are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are : RWE, Red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; BHA, butylated hydroxyanisole.

Table A-4 TBARS (as μmoles malonadehyde equivalents) of bean extracts at 100 ppm concentration as catechin equivalents and reference antioxidants at 100 ppm in stripped bulk corn oil system during a 7-day storage period at 60°C[1].

Additive	Storage time in days				
	0	1	3	5	7
Control	0.089±.01[a]	0.17±.03[a]	0.30±.02[b]	0.65±.03[d]	1.01±.07[d]
RWE	0.12±.03[a]	0.16±.02[a]	0.22±.03[a,b]	0.31±.04[a,b]	0.44±.03[b]
BWE	0.10±.01[a]	0.15±.03[a]	0.20±.02[a,b]	0.39±.01[b,c]	0.45±.05[b]
LWE	0.09±.02[a]	0.20±.02[a]	0.24±.06[a,b]	0.32±.03[a,b,c]	0.50±.08[b]
Catechin	0.12±.03[a]	0.13±.02[a]	0.16±.03[a]	0.24±.07[a]	0.30±.06[a]
A-Tocopherol	0.09±.04[a]	0.19±.05[a]	0.28±.06[b]	0.410±.05[c]	0.87±.07[c]
BHA	0.09±.03[a]	0.10±.06[a]	0.20±.07[a,b]	0.36±.08[b,c]	0.44±.08[b]

[1]Results are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are : RWE, Red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; BHA, butylated hydroxyanisole.

Table A-5 Hexanal content (mg/Kg of sample) of bean extracts and reference antioxidants at 50 ppm (as catechin equivalents) in a bulk corn oil model system at 60°C[1]

Additive	Storage time in days				
	0	1	3	5	7
Control	0.68±.03[a]	12.60±.20[c]	27.30±2.1[d]	78.60±3.91[d]	109.60±6.91[c]
RWE	0.69±.06[a]	2.20±.30[a]	5.90±.96[a]	21.36±.91[a]	46.02±5.12[b]
BWE	0.69±.07[a]	2.10±.13[a]	5.44±.97[a]	23.16±1.32[a]	45.32±3.11[b]
LWE	0.68±.02[a]	2.03±.91[a]	11.68±.39[b]	38.78±4.10[b]	67.69±3.96[c]
Catechin	0.73±.06[a]	2.18±.09[a]	5.96±.41[a]	17.62±.99[a]	23.98±1.30[a]
α-Tocopherol	0.69±.05[a]	9.65±.17[b]	20.21±1.3[c]	64.12±4.61[c]	87.14±2.71[d]
BHA	0.72±.02[a]	1.33±.02[a]	5.67±.91[a]	22.65±3.60[a]	31.90±1.24[a]

[1]Results are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are : RWE, Red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; BHA, butylated hydroxyanisole.

Table A-6 Hexanal content (mg/Kg of sample) of bean extracts and reference antioxidants at 100 ppm (as catechin equivalents) in a bulk corn oil model system at 60°C[1]

Additive	Storage time in days				
	0	1	3	5	7
Control	0.68±.07[a]	12.66±1.31[c]	27.36±.96[c]	78.61±3.2[e]	109.6±6.65[e]
RWE	071±.02[a]	1.40±.46[a]	4.98±.91[a]	12.65±1.31[a]	18.64±3.15[a,b]
BWE	0.69±.10[a]	1.32±.17[a]	4.78±.10[a]	13.47±.92[a]	19.54±1.35[b]
LWE	0.68±.12[a]	1.58±.35[a]	5.36±.96[a]	24.68±2.13[c]	31.87±3.71[c]
Catechin	0.69±.09[a]	1.12±.27[a]	3.74±.35[a]	9.66±1.8[a]	11.74±.84[a]
α-Tocopherol	0.71±.12[a]	9.17±.03[b]	19.65±1.10[b]	60.00±3.59[d]	76.56±2.46[d]
BHA	0.73±.17[a]	1.44±.03[a]	4.00±.37[a]	15.31±.98[b]	20.63±.76[b]

[1]Results are mean values of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.
Abbreviations used are : RWE, Red whole bean extract; BWE, brown whole bean extract; LWE, black whole bean extract; BHA, butylated hydroxyanisole.