Analysis and Evaluation of Antioxidant Flavonoids in Buckwheat Cultivars

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FACULTY OF GRADUATE STUDIES

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OF

DOCTOR OF PHILOSOPHY

TABLE OF CONTENTS

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Abstract

The oxidation of low-density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis. Flavonoids are believed to reduce the risk of cardiovascular disease due to their antioxidant biological property. Quercetin and its glycoside rutin are naturally occurring flavonoids. Buckwheat is generally considered to be a major dietary source of rutin. The difference in the rutin content between species or varieties provides an opportunity to develop buckwheat lines with higher rutin content through hybridization. The objectives of this thesis were: 1) to explore antioxidant effectiveness of quercetin, rutin, α-tocopherol (vitamin E, a lipophilic antioxidant) and ascorbic acid (vitamin C, a hydrophilic antioxidant) in hydrophilic and lipophilic free radical generating systems; 2) to study the effect of quercetin on H_2O_2-induced oxidative stress in cardiomyocytes; 3) to develop a high performance liquid chromatographic assay for rutin in different buckwheat species and quantitatively analyze rutin in buckwheat seeds and leaves; 4) to investigate the protective effects of different buckwheat species or varieties against LDL oxidation and to determine the correlation between rutin content in buckwheat and the antioxidant activity of buckwheat.

The results of this study demonstrated that: 1) Quercetin and its glycoside rutin were more effective in inhibiting lipid oxidation in an aqueous environment surrounding plasma LDL than α-tocopherol or ascorbic acid; 2) Quercetin exhibited an *in vitro* inhibitory effect on the oxidative stress of neonatal cardiomyocytes; 3) A high-performance liquid chromatographic (HPLC) assay for rutin in different buckwheat species which was developed and validated in this project was accurate, rapid, and simple, and suitable for rutin analysis of different buckwheat species in routine application with good reliability and sensitivity. The species *F. tataricum* had the highest rutin content in its seed, followed by *F. homotropicum* , while *F. esculentum,* had the lowest rutin

content in the seed. No correlation in rutin content was found between buckwheat leaves and buckwheat seeds; 4) The protective effect of the three buckwheat species against LDL oxidation was in the order: *F. tataricum* > *F. homotropicum* > *F. esculentum*. The antioxidant activity of the buckwheat cultivars was positively correlated to their rutin content.

The findings in this study demonstrated that quercetin and rutin had the potential to prevent the initiation of lipid oxidation, and that *F. homotropicum* could be used in buckwheat breeding programs. An improved buckwheat line, with high rutin content, has the potential to be used as a functional food for the prevention of cardiovascular diseases.

List of Figures

List of Tables

Chapter I Introduction

1. 1 Lipoprotein peroxidation and antioxidants

1.1.1 Lipid peroxidation

1.1.1.1 Free radicals and reactive oxygen species

Free radicals are defined as any species that exists independently and contains one or more unpaired electrons. They can be formed by the loss or the gain of a single electron from a non-radical. As electrons are relatively stable when paired together in an orbital, radicals are less stable than nonradicals. In the human body, radicals such as superoxide $O_2^{\bullet -}$, hydroxyl $OH^{\bullet}$, peroxyl $RO_2^{\bullet}$, alkoxyl $RO^{\bullet}$, oxides of nitrogen $NO^{\bullet}$ and $NO_2^{\bullet}$, and nonradicals such as hydrogen peroxide H_2O_2, hypochlorous acid HOCL, ozone O_3, and singlet oxygen $^1O_2$ are constantly formed and consist of the reactive oxygen species (ROS) (Aruoma, 1996). Under normal physiological conditions, ROS production is balanced by ROS elimination. However, the excess generation of ROS results in oxidative stress, which is toxic and aggravated by the presence of transition metal ions such as iron and copper (Touyz, 2000). Reactive oxygen species may interact with DNA, lipids, proteins, and carbohydrates, causing damage to these biomolecules, loss of cellular function, and cell death. As a result, oxidative stress has been implicated in the development of various degenerative diseases in aging such as atherosclerosis, cancer, Parkinson's disease, and Alzheimer's disease.

Hydroxyl $OH^{\bullet}$ is one of the most reactive chemical species known. It is so aggressive that it can attack all biological molecules (Halliwell and Gutteridge, 1989). Although $O_2^{\bullet -}$ and H_2O_2 are much less reactive than $OH^{\bullet}$, they are not completely harmless. In a biological system, H_2O_2 can be produced by a dismutation reaction via $O_2^{\bullet -}$ (Equation 1-1), or by several enzymes including glycollate oxidase, D-aminoacid oxidase, and urate oxidase

without $O_2^{\bullet -}$. Hydrogen peroxide can cross cell membranes and react with iron or possibly copper ions to form the hydroxyl radical (Equation 1-2 and Equation 1-3).

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Equation 1-1)}$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^\bullet \quad \text{(Equation 1-2)}$$

$$H_2O_2 + Cu^+ \rightarrow Cu^{2+} + OH^- + OH^\bullet \quad \text{(Equation 1-3)}$$

1.1.1.2 Lipid peroxidation: a free radical chain reaction

Lipids are major constituents of biological membranes. Most lipid molecules found in membranes are long-chain hydrophobic fatty-acids joined by ester bonds to the alcohol glycerol. Some membranes, particularly plasma membranes, contain a significant proportion of the hydrophobic molecule cholesterol. The primary lipids in animal cell membranes are phospholipids, a group of phosphate-containing molecules with structures related to the triglycerides. In the most common phospholipids, known as phosphoglycerides, glycerol forms the backbone of the molecule but only two of its binding sites link to fatty acid residues. The third site links instead to a bridging phosphate group. They are amphipathic molecules, and constitute the lipid bilayer as the basic structure of cell membrane **(Figure 1).**

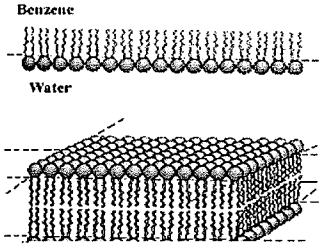


Figure 1. The lipid bilayer as the basic structure of a cell membrane.

The figure is adapted from Wolfe S. L. (1993).

Membrane phospholipid molecules contain a number of unsaturated and polyunsaturated fatty-acid side-chains. Both isolated polyunsaturated fatty acids (PUFAs) and those incorporated into lipids are readily attacked by free radicals that have sufficient reactivity to abstract a hydrogen atom from a methylene ($-CH_2-$) group in the side chain. The greater the number of double bonds in a fatty acid side chain, the easier is the abstraction of a hydrogen atom from a methylene carbon. Therefore, fatty acids with zero, one, or two double bonds are more resistant to free radical attack than polyunsaturated fatty acids. Polyunsaturated fatty acids, once attached by free radicals, are eventually oxidized into toxic lipid peroxides, reducing membrane fluidity, which in turn leads to cell damage.

Lipid peroxidation can be initiated by reactive oxygen species such as superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$) and singlet oxygen ($^1O_2$), which are generated in biological systems (Joseph, *et al.*, 1986). The abstraction of a hydrogen atom by ROS from a methylene group produces a carbon-centred lipid radical, which tends to be stabilized by a molecular rearrangement to form a conjugated diene. In aerobic cells, the conjugated dienes most likely combine with O_2 to give peroxyl radicals. These peroxyl radicals are capable of abstracting hydrogen atoms from another lipid molecule, an adjacent fatty-acid side-chain, then propagating the free radical chain reaction of lipid peroxidation. The combination of the peroxyl radical with the hydrogen that it abstracts produces lipid peroxides and other products (**Figure 2**). Transition metals play an important role in lipid peroxidation. Lipid peroxides are stable at physiological temperatures. However, their decomposition is greatly accelerated in the presence of transition metal ions such as iron or copper. The decomposition of lipid peroxides give both peroxy and alkoxy radicals capable of abstracting hydrogen, thus continuing the chain reaction of lipid peroxidation. The equations (1-4 and 1-5) are as follow:

$$R—OOH + Fe^{2+}\text{-complex} \rightarrow Fe^{3+}\text{-complex} + OH^{-} + R—0\bullet \qquad \text{(Equation 1-4)}$$

$$R—OOH + Fe^{3+}\text{-complex} \rightarrow Fe^{2+}\text{-complex} + H^{+} + R0_2\bullet \qquad \text{(Equation 1-5)}$$

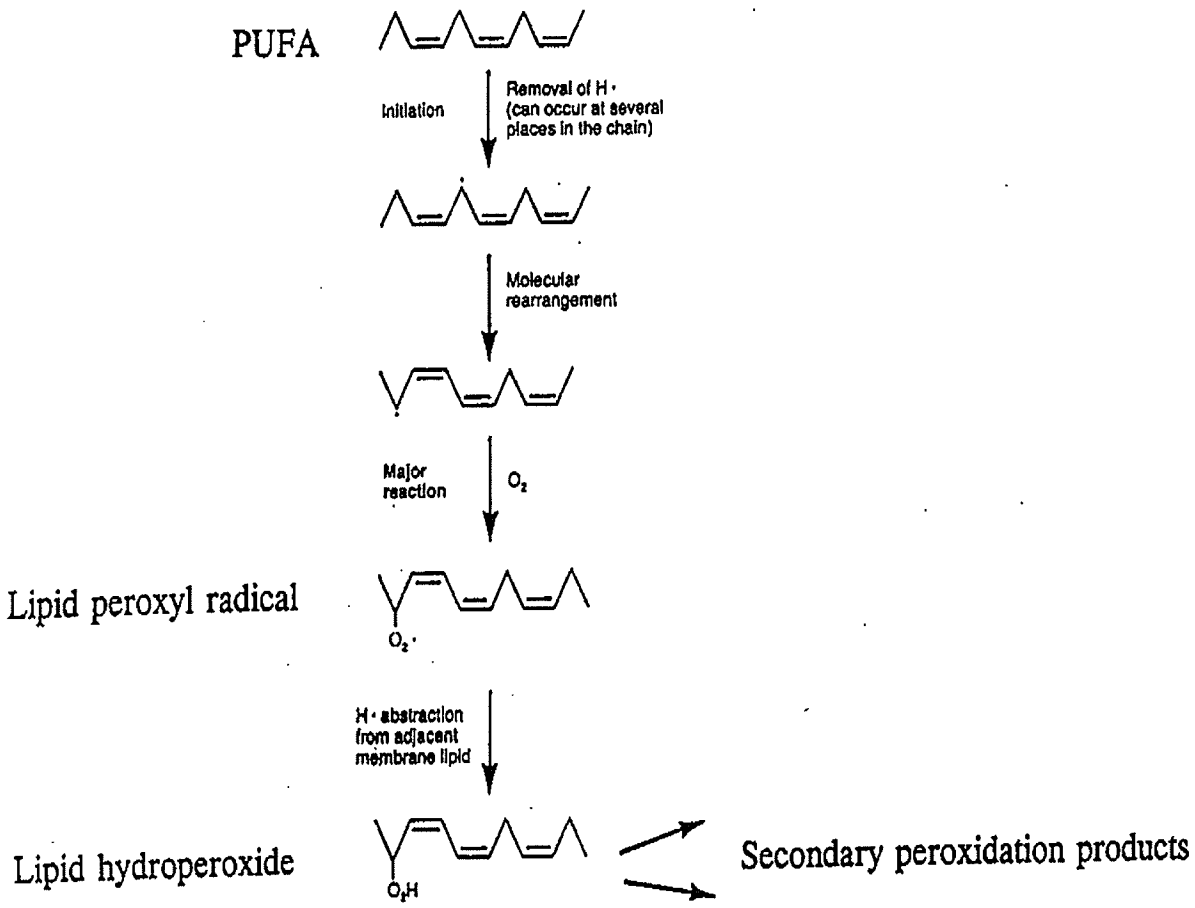


Figure 2. The initiation and propagation reactions of lipid peroxidation

The figure is modified from Barry Halliwell and John M.C. Gutteridge (1999).

1.1.2 The role of oxidized low-density lipoproteins (LDL) in atherosclerosis

Low-density lipoproteins are plasma particles within a density range of 1.019 – 1.063 g/ml with an average diameter of 22 nm. The spherical particles comprise an amphipathic monolayer surrounding a hydrophobic lipid core. The core contains approximately 170 triglyceride and 1600 cholesterol ester molecules and the surface monolayer consists of approximately 700 phospholipid molecules and a single copy of apoB-100 (Esterbauer *et al.*, 1992). Low-density lipoproteins are the vehicles for cholesterols in the circulatory system and their function is to carry cholesterols from the liver to the cells.

Atherosclerotic vascular disease is the leading cause of morbidity and mortality in Western populations. It is well known that elevated plasma concentrations of low-density lipoprotein are associated with accelerated atherosclerosis. The earliest lesion in atherogenesis is a fatty-streak, which results from an accumulation of cells loaded with cholesterol esters (foam cell) just beneath the endothelium. Most foam cells arise from circulating monocytes (macrophages) by their taking up residence beneath the vascular endothelium. The cholesterol accumulating in macrophages originates mainly from the plasma lipoproteins, especially LDL. However, macrophages have a relatively limited number of LDL receptors and these receptors are down regulated in the presence of high plasma LDL concentration (Goldstein and Brown, 1983). The mechanism by which low-density lipoproteins (LDL) exert their effect on atherosclerosis has been investigated extensively. In 1989, Steinberg first proposed the oxidative theory of atherogenesis in which it is not native LDL which is atherogenic but the oxidized form of LDL. Through the action of oxidizing agents, low-density lipoproteins are oxidized to form chemically modified LDL. The oxidized LDL are recognized by specific receptors on the macrophages and are endocytosed at a much higher

rate than native LDL, which increases the accumulation of cholesterol in macrophages and smooth muscle cells. This leads to the formation of foam cells, the cellular hallmark of atherosclerosis. **Figure 3** demonstrates the hypothesis of the atherogenetic pathogenesis. The "oxidation hypothesis" has been supported by indirect evidence including: lipoprotein-like particles with oxidative damage isolated from atherosclerotic lesions (Witzum and Steinberg, 1991), products of lipid peroxidation such as malondialdehyde (MDA) detected in human and animal atherosclerotic lesions (Esterbauer *et al.*, 1992), some structurally unrelated antioxidants retarding lesion formation in hypercholesterolemic animals (Lynch and Frei, 1994), and inhibition of both LDL oxidation and atherosclerosis in animals (Heinecke, 1998; Stocke, 1999). However, the LDL oxidation hypothesis is controversial because of the inconsistent efficacy of antioxidants against atherosclerosis in large-scale intervention studies (Vivekananthan *et al.*, 2003) and the negative effects in some animal experiments (Noguchi, 2002). It has been recognized that atherosclerosis may develop by different mechanisms, LDL oxidation may proceed by either a radical mechanism or a non-radical mechanism, and no antioxidant could be effective in all mechanisms (Niki, 2004). Therefore, the hypothesis that oxidative LDL plays an important role in the pathogenesis of atherosclerosis has been widely accepted.

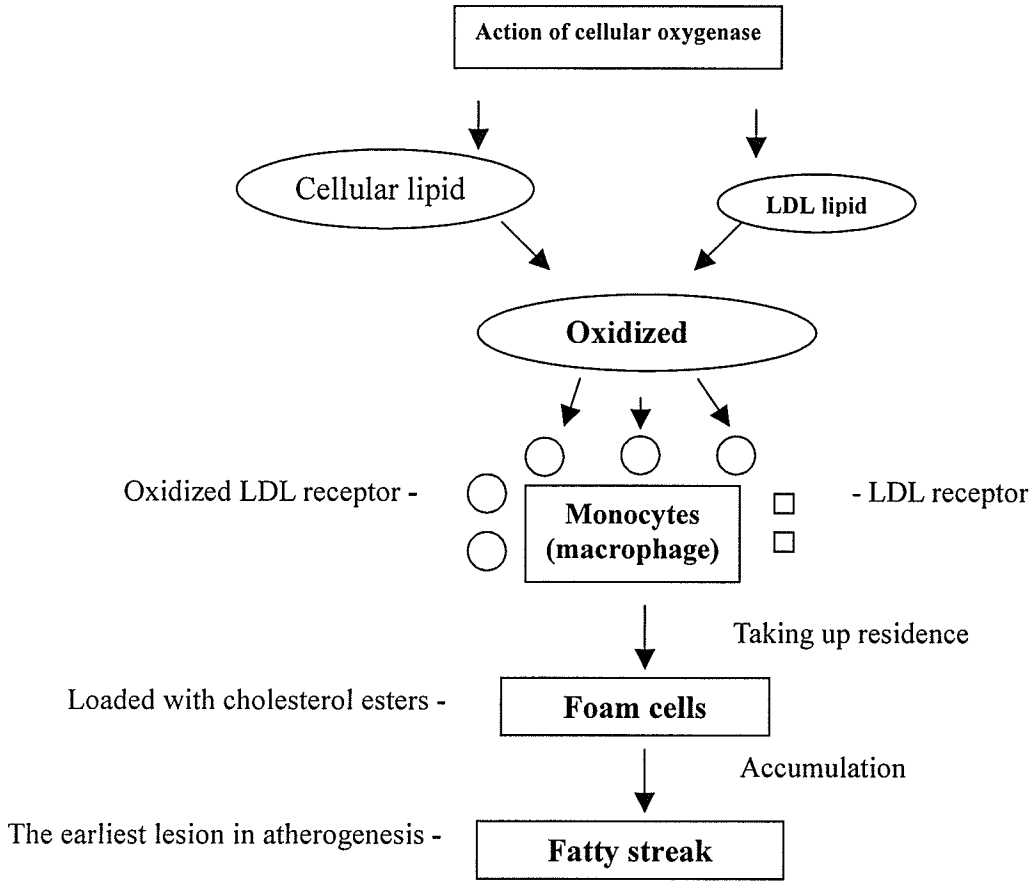


Figure 3. The oxidative theory of atherogenesis.

This figure is modified from Steinberg, D., *et al.* (1989).

1.1.3 Antioxidants and their mechanisms

An antioxidant is defined as any substance that has the ability to delay or inhibit the oxidation of an oxidizable substrate at low concentrations compared to those of the substrate. With lipid peroxidation, antioxidants can act by different mechanisms at different levels in the oxidative sequence. They include (1) scavenging initiating radicals such as $OH^{\bullet}$ to prevent first-chain initiation, (2) binding metal ions (Fe^{2+}, Cu^{+}) in forms that will not generate such initiating species as $OH^{\bullet}$ or will not decompose lipid peroxides to peroxy or alkoxy radicals, (3) scavenging intermediate radicals such as peroxy and alkoxy radicals to prevent propagation of free radical chain reaction, etc. Antioxidants working through the first two mechanisms are called preventative antioxidants, and those which utilize the third mechanism are called chain-breaking antioxidants. It should be noted out that many antioxidants have multiple mechanisms of action. Antioxidants in the body include enzymes, nonenzymatic scavengers, and nutrients. Some are endogenous, whereas others are supplied by food. **Table 1** presents some antioxidants and their functions.

Table 1. Antioxidants and their functions

Antioxidants	Functions
Enzymes	
Catalase	Decomposes hydrogen peroxide to water.
Superoxide dismutase	Converts superoxide to hydrogen peroxide.
Glutathione peroxidase	Catalyzes the detoxifying reduction of hydrogen peroxide.
Nonenzymatic scavengers	
Uric acid	Binds iron and copper.
Glutathione	Regenerates vitamin E
Transferrin, ferritin, ceruloplasmin	Binds reactive forms of copper and iron.
Nutrients	
Vitamin E	Scavenges radicals within membranes.
Carotenoids	Scavenges radicals within membranes.
Ascorbic acid	Regenerates vitamin E, glutathion, and flavonoids.
Flavonoids	Scavenge radicals and chelate metal ions.

This table is modified from Barry Halliwell and John M.C. Gutteridge (1999).

1.1.4. Measurement of lipid peroxidation

A number of techniques are available for measuring lipid peroxidation in biological systems. They can be classified into two categories: 1) determination of the loss of substrates, 2) determination of the production of intermediate products or end products of lipid peroxidation. **Table 2** summarizes some common methods for measuring lipid peroxidation. Additional methods include spin trapping for intermediated radicals, light emission for excited carbonyls and singlet oxygen, gas chromatography measurement of gases formed during lipid peroxide decomposition, and other alternatives. Lipid peroxidation is a complicated process and as each assay measures a different factor, there is no best method to evaluate the complete process. The conjugated diene assay and thiobarbituric acid reactive substances (TBARS) test are two of the most widely used methods to measure the early stage of lipid peroxidation and determine malondialdehyde (MDA) formation during peroxidation, respectively. These techniques are simple and reliable (Halliwell and Chirico, 1993). The conjugated diene assay often has been applied to measure lipid peroxidation in body fluids and tissue extracts of animals, but it can not be used directly on human body fluids because of the presence of many other substances with strong absorbance in the ultraviolet. Analysis of the UV absorbing "diene conjugates" from human body fluids by HPLC techniques revealed that the UV absorbing material consisted almost entirely of a non-oxygen-containing isomer of linoleic acid, octadeca-9 (*cis*) 11 (*trans*)-dienoic acid, which was not found in animal plasma (Dormandy and Wickens, 1987). This method is very useful in studies of pure lipids or isolated lipid fractions. The TBARS test is commonly applied to detect lipid peroxidation. In a TBARS test, malondialdehyde formed during lipid peroxidation reacts with thiobarbituric acid to produce a pink chromogen (allegedly a

[TBA]2 – malondialdehyde adduct) with absorbance at 532 nm (**Figure 4**). This test is applicable to isolated lipid or defined membrane systems such as microsomes and liposomes, but is not ideal for body fluids and tissue extracts as many factors interfere with the test. For

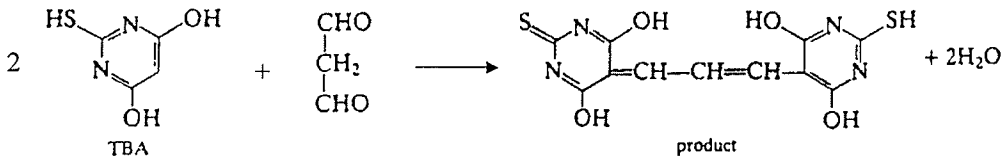


Figure 4. Reaction of two molecules of thiobarbituric acid with one molecule of malondialdehyde

This figure is adapted from Barry Halliwell and John M.C. Gutteridge (1999).

example, aldehydes other than MDA can give products with absorbance around 532 nm. Some chemicals present in human body fluids such as bile pigments and glycoproteins also give positive readings. This problem can be reduced by using HPLC methods to separate the real TBA-MDA adduct from interfering chromogens.

In recent years, some new methods have been developed for detection of lipid peroxidation. Hydroxyoctadecadienoic acid (HODE) and 7-hydroxycholesterol (FCOH) were reported as good oxidative marker *in vivo* to measure lipid peroxidation (Yoshida and Niki, 2004).

Table 2. Methods for measuring lipid peroxidation

Methods	Substances measured
Loss of substrates:	
Uptake of oxygen	Lipid peroxidation is accompanied by the uptake of oxygen in the formation of peroxy radicals and in the peroxide decomposition reactions. The dissolved oxygen concentration can be measured with oxygen electrodes.
Loss of unsaturated fatty acids	Unsaturated fatty acid side-chain is destroyed by lipid peroxidation. Fatty acids can be measured by high performance liquid chromatography or gas liquid chromatography.
Products of peroxidation:	
Conjugated diene	Oxidation of unsaturated fatty acids is accompanied by the formation of a conjugated diene structure that has UV absorbance at 230 – 235 nm. This characteristic can be used to detect lipid peroxidation.
Lipid peroxides	Peroxy radicals abstract hydrogen atoms to form lipid peroxides including lipid hydroperoxide, cyclic peroxide and cyclic endoperoxide. Iodine liberation, glutathione peroxidase, and cyclooxygenase can be applied to measure these lipid peroxides.
Aldehydes	Aldehydes including malondialdehyde (MDA) and polymerization products are from fragmentation of lipid peroxides. They can be detected by gas chromatography – mass spectrometry, fluorescence, and the thiobarbituric acid (TBA) test.

This table is modified from Aruoma O. I. (1996).

1.2 Rutin and flavonoids as antioxidants

1.2.1 Flavonoids and their antioxidant activities

Flavonoids are a group of polyphenolic compounds widely distributed in vegetables, fruit, flowers, seeds, nuts, and bark. The study of flavonoids can be traced back to 1828 when hesperidin, a primary flavanone glycoside in citrus, was first reported by Lebreton. To date, over 5,000 types of flavonoid compounds have been isolated and identified in higher and lower plants. Most flavonoids occur naturally as glycosides (attached to sugars), but occasionally they are also found as aglycones. In general, flowers, leaves, and fruit contain glycosides, woody tissues of plant contain aglycones, and seeds may contain either glycosides or aglycones. Flavonoids are secondary metabolites and arise biogenetically from two main synthetic pathways: the shikimate pathway and the acetate pathway (Bravo, 1998). They contribute to attractive colors in the plant kingdom and function as protectants against harmful UV radiation, bacteria, virus, and oxidative damage (Cook, N. C. and Samman, S., 1996). Medicinal plants rich in flavonoids or concentrated forms of flavonoids have been used for centuries to treat various human conditions such as inflammation, allergy, headache, and common cold (Havsteen, 1983). In 1936, Szent-Gyorgyi proposed the name of vitamin P for a mixture of two citrus flavonoids that were found to be important for proper capillary function and thus began research on the biological properties of flavonoids (Manthey and Buslig, 1998). To date, a wide range of clinical effects has been reported, including antibacterial, antiviral, anti-inflammatory, antiosteoporotic, antithrombogenic, and anticarcinogenic effects. The best-described biological property of flavonoids is their antioxidant capacity, especially their inhibitory effect on lipid peroxidation, because of which they are believed to play a role in preventing the development of atherosclerosis (Nijveldt *et*

al., 2001). Particular interest in flavonoids, for their protective effect against heart disease, came with the report by St. Leger *et al.* (1979) that showed an inverse relationship of coronary heart disease mortality and consumption of wine rich in polyphenolic compounds, based on data of 18 countries. An epidemiological study further indicated that the mortality rate of coronary heart disease in France was much lower than that in other industrialized countries such as the USA and UK, and close to the rates in Japan and China. However, regarding the risk factors for cardiovascular diseases, the French had greater consumption of wine but showed similar average serum cholesterol, blood pressure, and prevalence of smoking to those of USA and UK (Renaud and Lorgeril, 1992). This is the so-called " French Paradox ". The Zutphen Elderly Study was the first epidemiological investigation on the relationship between the dietary flavonoid intake and coronary heart disease mortality. The study authors suggested a protective role of dietary flavonoid intake against coronary heart disease (Hertog *et al.*, 1993). This notion has been supported by other epidemiological studies (Hertog *et al.*, 1995; Knekt *et al.*, 1996).

Flavonoids inhibit lipoprotein peroxidation *in vitro* at the initiation stage by acting as free radical scavengers. Flavonoids can be oxidized by free radicals to produce more stable or less reactive radicals (Equation 2-1):

$$\text{Flavonoid (OH)} + R^{\bullet} \rightarrow \text{Flavonoid } (O^{\bullet}) + RH$$

where $R^{\bullet}$ is either a superoxide anion, a hydroxyl radical, or a peroxynitrite free radical, and $O^{\bullet}$ is an oxygen free radical. Flavonoids can also terminate propagating chain reaction lipid oxidation by donating a hydrogen atom to the peroxyl radical forming a flavonoid radical, which in turn reacts with free radicals resulting in inert products. In addition, some flavonoids inhibit the superoxide-driven Fenton reaction as metal-chelating agents, removing

a causal factor for the development of active oxygen radicals. Quercetin is one such flavonoid known for its iron-chelating or iron-stabilizing properties (Nijveldt *et al.*, 2001; Bors *et al.*, 1990; Bravo, 1998; Bors and Michel, 1999). Although most research on the inhibition of lipoprotein peroxidation by flavonoids and their benefit to cardiovascular system as antioxidants are at *in vitro* level, more *in vivo* studies are being conducted to provide working mechanisms and potentials to support their use in health improvement (van Hoorn *et al.*, 2003).

1.2.2 Structure – activity relationships of flavonoids

Flavonoids are polyphenolic substances based on the flavan nucleus that consists of 15 carbon atoms arranged in three rings (C_6-C_3-C_6), referred to as the A, B, and C rings. They are classified according to the degrees of unsaturation and oxidation of the three rings. The major classes include flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones as shown in **Figure 5**. The structure of flavonoids varies widely within the major classifications, and the principal substitutions are hydroxylation, methylation, and glycosylation. Flavonoids generally occur as *O*–glycosides in which one or more of the flavonoid hydroxyl groups is bound to sugars. Glucose is the most common sugar found in association with flavonoids. Disaccharides or even higher forms are also found to be bound to flavonoids, a common example being rutinose (6-*O*-α-L-rhamnosyl-D-glucose). Flavonoids may also exist in the form of C-glycosides with a direct linkage of the sugar to the benzene nucleus. Glycosylation renders flavonoids more water soluble, making them store readily in the cell vacuole where they are commonly found (Robards and Antolovich, 1997).

The chemical structure of flavonoids has a profound effect on their biochemical and

pharmacological activities in mammalian and other cell systems. The studies on the relationship between chemical structure and protective ability against lipid oxidation have demonstrated that a hydroxyl group at the C-3 position of the C ring, a double bond between carbons two and three (C2-C3) of the C ring, and the carbonyl group at C-4 of the C ring are crucial for flavonoids to exhibit potent antioxidant activities. The number of hydroxyl groups and the pattern of hydroxylation on the A and B rings are also important factors contributing to the inhibition of lipid oxidation. The hydroxyl radical scavenging capacity of flavonoids with four to six hydroxyl substitutions is more effective than that of flavonoids with one to three hydroxyl substitutions, and hydroxyl groups on positions C-5 and C-7 of the A ring and C-3' and C-4' of the B ring increase their antioxidant activities. Flavonoids having both a C-4 carbonyl group and a C-3 or C-5 hydroxyl group, such as quercetin or rutin, can form chelates with iron ions, thus preventing free radical formation in Fenton reactions. Moreover, these flavonoids forming complexes with iron ions still keep their free radical scavenging abilities. In general, the sugar moiety of flavonoid glycosides reduces antioxidant activity of adjacent hydroxyl groups due to steric hindrance. However, flavonoid glycosides, such as rutin and quercetrin, could be hydrolyzed to their corresponding aglycones by human intestinal flora and may also retain their *in vivo* effects (Cook and Samman, 1996).

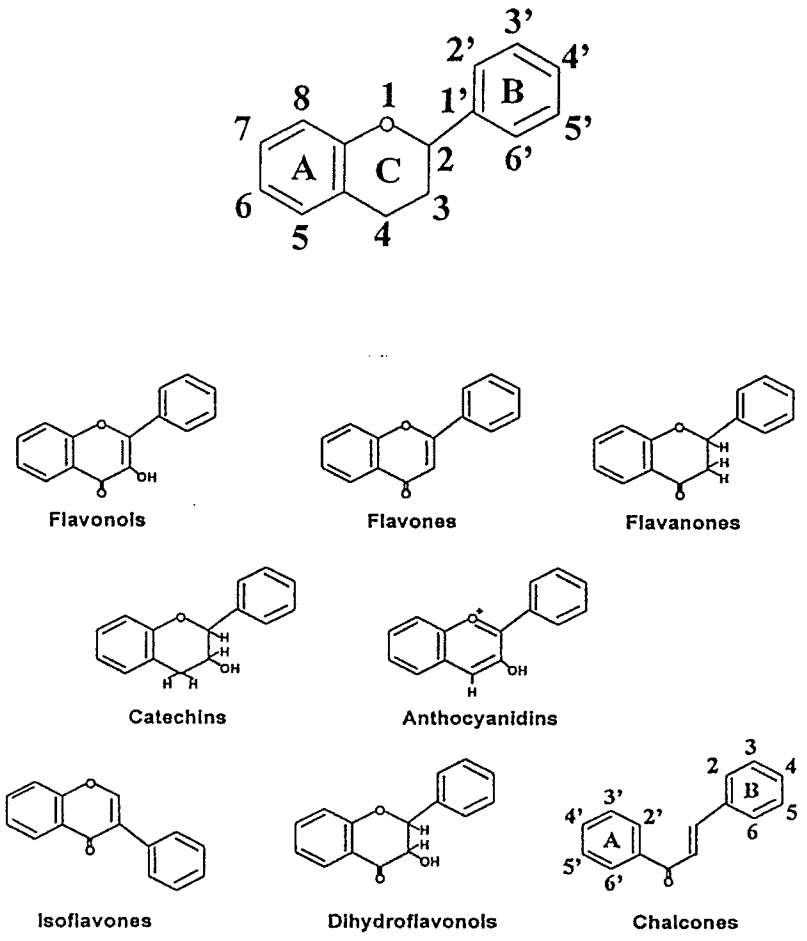


Figure 5. The basic structure of flavonoids and their classification

This figure is adapted from Cook, N. C. and Samman, S. (1996).

1.2.3 Flavonol quercetin and it glycoside rutin

Among the flavonoids, flavonols are of particular interest because of their abundant occurrence in plant foods and their possession of most biological properties of the flavonoids. Flavonols have a double bond between the carbons C_2 and C_3, a keto group in position C_4, and a hydroxyl group in position C_3. Quercetin, 3'. 4'-dihydroxy-flavonol is absorbed in significant amounts from the diet. It has been the most intensely studied flavonoid and exists predominantly in glycosylated forms such as rutin, quercetrin and isoquercetrin. Rutin, quercetin rutinoside, is linked with rutinose. Quercetrin or isoquercetrin, quercetin glucoside, is quercetin linked with glucose. Typical quercetin levels in vegetables are 284 – 486 mg/kg in onions, 110 mg/kg in kale, 30 mg/kg in broccoli, 32 – 45 mg/kg in French beans, 28 – 30 mg/kg in slicing beans, and 21 – 72 mg of quercetin per kg was found in different apple varieties (Hertog *et al.*, 1992). In various red wines, fruit juices, black teas and green teas, quercetin contents were 4.1 – 16 mg/L, 2.5 – 13 mg/L, 10 – 25 mg/L and 13 – 23 mg/L, respectively (Hertog *et al.*, 1993).

Rutin, a 3-*O*-beta-rhamnoglucoside form of quercetin, is the main glycoside naturally occurring in plants (**Figure 6**). It is used medicinally in many countries to reduce capillary fragility associated with some hemorrhagic diseases or hypertension in humans (Yildzogle-Ari *et al.*, 1991). Recent studies have indicated that rutin intake improved rat antioxidant systems in the liver, inhibited the increase in the level of thiobarbituric acid reactive substances (TBARS) in the gastric mucosa caused by ethanol injury, and suppressed oxygen radical overproduction in both rheumatoid arthritis and Fanconi anemia. (Gao *et al.*, 2003; La-Casa *et al.*, 2000; Ostrakhovitch and Afanas'ev, 2001). Dose-dependent effects of rutin were also found in rabbits to inhibit platelet activating factor-induced platelet aggregation, 5-

HT release and the increase of intraplatelet free calcium (Chen *et al.*, 2002). In both *in vitro* and *in vivo* studies, rutin is reported to protect Caco-2 and Hep G2 cells against H_2O_2-induced DNA damage, and reduction in free radical-induced oxidative damage of DNA and proteins was found in rats fed rutin supplemented diet (Aherne and O'Brien, 1999; Funabiki *et al.*, 1999).

Over the past decade, most research on quercetin and rutin has been focused on their capacity as antioxidants. Accumulated evidence has shown their inhibitory effect on lipid peroxidation in biological tissue and subcellular fractions, such as erthrocyte membrane, mitochondria, microsomes, liposomes and low-density lipoprotein (LDL). Using various non-enzymatic and enzymatic lipid peroxidation systems to elucidate the mechanism of antioxidant action of rutin and quercetin, Afanas'ev *et al.* (1989) reported that rutin and quercetin suppressed free radical processes at three stages: the superoxide ion formation, the hydroxyl radical generation from Fenton reaction, and lipid peroxy radical production. It is well known that polyphenols, as reducing agents, work with other reducing agents such as vitamin C, vitamin E and carotenoids to protect the body's tissue against oxidative stress. Many studies have reported the synergistic effect of flavonoids and α - tocopherol or ascorbic acid on lipid peroxidation. Pedrielli and Skibsted (2002) reported the regeneration effect of quercetin on α - tocopherol, and their antioxidant synergy in peroxidating methyl linoleate initiated by α, α'-azoisobutyronitrile. Quercetin and rutin in hawthorn were not only protective to human LDL from Cu^{+2}-mediated lipid oxidation but also effective in preventing the peroxy free radical-induced oxidation of α - tocopherol in human LDL (Zhang *et al.*, 2001). A combination of rutin and α - tocopherol was synergistic compared to rutin or α - tocopherol alone (Pekkarinen *et al.*, 1999). A recent study has also reported the

synergistic effect of rutin combined with a hydrophilic antioxidant ascorbate or a lipophilic antioxidant gamma-terpinene in Cu^{+2}-mediated LDL oxidation (Milds *et al.*, 2004). These activities of quercetin and rutin are relevant to their potential use in nutraceuticals in protecting against coronary heart disease.

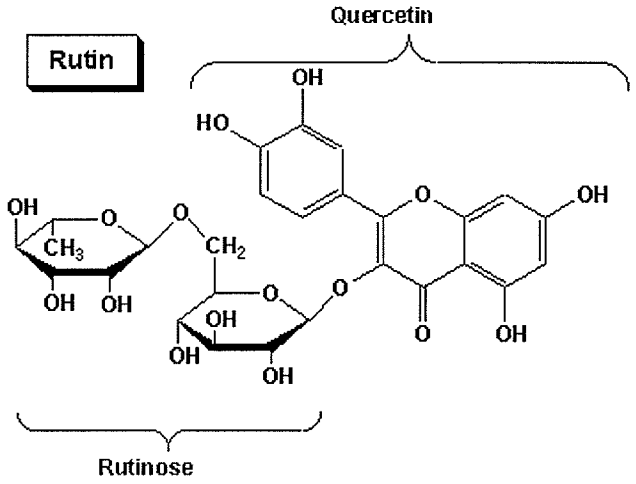


Figure 6. Chemical structures of rutin and quercetin

This figure is adapted from Boyle S.P. (2000).

1.2.4 Flavonoid intake and bioavialability

The early estimation of dietary flavonoid intake in the USA suggested that the average flavonoid intake was approximately 1 g per day (Kuhnau 1976). Later, this value was considered to be overestimated due to inappropriate analytical techniques (Hertog *et al.*, 1992). According to the determination of flavonols and flavones of commonly consumed vegetables, fruits and beverages, and data from the Dutch National Food Consumption Survey 1987-1988, the average flavonoid intake in The Netherlands was estimated to be approximately 23 mg per day, with quercetin representing 16 mg per day. The main dietary sources of flavonoids were tea with 48% of total intake, onions with 29% and apples with 7% of the total (Hertog *et al.*, 1993b). Another rich source of flavonoids was red wine containing 22.5 mg/L (Hertog *et al.*, 1993a). A recent study reported that flavonoid intake varies greatly between countries, with the lowest average intake found to be 2.6 mg/day in Finland and the highest 68.2 mg/day in Japan (Nijveldt *et al.*, 2001).

The beneficial effects of flavonoids in humans are dependent upon their absorption in the small intestine, metabolism in the liver, disposition in tissue and cells, and excretion. As early as in 1970's, a study on the pharmacokinetics of quercetin in humans reported that no measurable plasma concentrations could be detected after oral administration with a dose of 500 mg quercetin and the recovery in faeces was 53%, suggesting extensive degradation by microorganisms in the gut. This result put into question the value of oral administration of flavonoids (Gugler *et al.*, 1975). Recent studies have shown that the absorption and bioavailability of specific flavonoids are much higher than originally believed (Ross and Kasum, 2002). In studies on the bioavailability of quercetin and rutin in rats, Manach *et al.* (1995; 1997) found that dietary flavonols were recovered from rat plasma as conjugated

metabolites at non-negligible concentrations, and high plasma metabolite concentrations were easily maintained with a regular supplementation of quercetin or rutin in the diet because of a low rate of elimination. However, different dietary flavonoids are found to vary considerably in pharmacokinetics. The absorption of flavonoids in the small intestine ranges from 0 to 60% of the dose, and the elimination half-life (T ½) ranges from 2 to 28 hours (Manach and Donovan, 2004). The bound sugar moiety in flavonoids is known to affect their absorption and bioavailabilities. Quercetin aglycone, quercetin-3-glucoside, quercetin rhamnoside, and rutin were reported to have identical quercetin metabolites in rat plasma, but the total metabolite concentrations were quite different. The metabolite levels were higher in quercetin-3-glucoside group than those with the quercetin aglycone, and were very low with the rutin meal and undetectable after the quercetin rhamnoside meal. The peak concentration was also reached much faster after intake of quercetin glucoside than after intake of quercetin rutinoside (Morand *et al.*, 2000; Hollman *et al.*, 1999). These findings were consistent with the human studies on the flavonoid bioavailability from different dietary sources. The absorption of quercetin or quercetin glycosides was found to be greater and faster from onions containing quercetin glucosides than from pure quercetin aglycone, apples containing both glucose and non-glucose quercetin glycosides, buckwheat teas containing quercetin rutinoside, black teas and red wine (Hollman *et al.*, 1995; Hollman *et al.*, 1997; De Vries *et al.*, 1998; De Vries *et al.*, 2001; Graefe *et al.*, 2001). It is suggested that flavonoid aglycone and some glucosides can be absorbed in the small intestine, and that the absorption is enhanced by the attachment of a glucose group. Flavonoids linked to a rhamnose moiety are absorbed from the colon after deglycosylation by microflora, thus their absorption is less rapid and less efficient than those of aglycone and glucosides due to a smaller exchange area

and a lower density of transport systems in the colon as compared to the small intestine. The mechanism of flavonoid glucosides transfer across the small intestine involves the transportation of glucoside into enterocytes by sodium-dependent glucose (SGLT1) where they can be hydrolysed by cytosolic β-glucosidase, or luminal hydrolysis of glucosides by lactase phlorizin hydrolase (LPH), an enzyme at the brush border membrane of the intestinal cells. They are then absorbed through passive diffusion of the released aglycone ((Manach *et al.*, 2004). The position at which the glucose is bound has an effect on the mechanism of flavonoid absorption. Studies in both animal and human models have shown that two pathways of SGLT1 and LPH are involved in the absorption of quercetin-4'-glucoside, and LPH is a major determinant for absorption of quercetin-3-glucoside (Day, *et al.*, 1998; Day, *et al.*, 2000; Day, *et al.*, 2003; Aloys, *et al.*, 2003)

It is believed that flavonoids are in the conjugated form with a glucuronide moiety in intestinal cells, then bound to albumin and transported to the liver, in which the conjugation is extended by adding a sulfate or methyl group (Danny *et al.*, 2003). Morand *et al.*(1998) reported that the circulating metabolites of quercetin in rats were glucurono-sulfo conjugates of isorhamnetin and of quercetin, and glucuronides of quercetin and its methoxylated forms. These metabolites were effective in inhibiting LDL oxidation, and were more potent than the hydrophilic antioxidant trolox. In another human study, the quercetin metabolites in the plasma, 3'-O-methylquercetin and some conjugated derivatives of quercetin, significantly prolonged the lag time of LDL oxidation, but their antioxidant capacity was about one half that of aglycone (Manach *et al.*, 1998). A recent study on quercetin metabolism in rats showed that quercetin is primarily metabolized to glucuronides and sulfoglucuronide and, to a minor extent, to sulfates (Justino *et al.*, 2004). When testing the ability of human plasma

quercetin conjugates to inhibit human LDL oxidation, Janisch *et al.* (2004) reported that intestinal and hepatic metabolism of quercetin had effects on its inhibitory ability against LDL oxidation. The intestinal metabolites (quercetin-7-glucuronide, quercetin-3-glucuronide) were more effective in inhibiting LDL oxidation than hepatic metabolites (isorhamnetin-3-glucuronide, quercetin-3'-sulfate). Quercetin-7-glucuronide was a more efficient antioxidant than quercetin aglycone. It is apparent that flavonoids have great potential for health benefits due to their inherent biological properties as well as the antioxidant activities of their metabolites.

1.3. Buckwheat

1.3.1 Introduction

Buckwheat belongs to the genus *Fagopyum* in the *Polygonaceae* family and is a summer annual plant which thrives at higher altitudes. It is usually treated as a cereal crop but does not resemble the cereals in growth habit. As compared to other grain crops, buckwheat exhibits very strong adaptability to adverse environments with a short growing span. It has coarse, branched stems and large, broadly arrow-shaped leaves. Buckwheat seeds, the fruits of the plant, are pointed, broad at the base, and triangular to nearly round in cross section. The seed size is variable in the different species and varieties. To date, at least 18 species of the genus *Fagopyum* have been identified. Among them, only two species: *Fagopyum tataricum* Gaertn (Tartary buckwheat), named in 1791, and *Fagopyum esculentum* Moench (Common buckwheat), named in 1794, are cultivated in the world. They are widely grown in China, Japan, Korea, India, Nepal, Pakistan, North America, Australia, as well as many European countries such as Russia, Ukraine, Germany, Poland, Slovenia, Hungary,

Yugoslavia, Britain, Italy, Czech Republic, France, Denmark, Sweden, etc. The history of cultivating buckwheat can be dated to ancient times. China is considered to be the original birthplaces of both common buckwheat and tartary buckwheat (Ohnishi and Konishi, 2001), where they have been grown since at least 1000 BC. Around the 13th century, buckwheat was introduced into the European continent from China via the so-called "Silky Route". From then on, it has been adapted in many areas of the world. In the United States, buckwheat is produced extensively in North Dakota as well as in the south central and southwest regions of the United States. It is also produced in South Dakota, Minnesota, Montana, Washington, Pennsylvania, and New York. In Canada, it is mainly produced in Manitoba.

Most of the buckwheat in European countries and the North America is dehulled and milled into flour, making pancakes, muffins, breads, porridge and biscuits. In Asia, buckwheat flour is mainly used to make noodles, dumplings, and buns. Japanese people have enjoyed buckwheat noodles for centuries. In Korea, buckwheat sprouts and germinated buckwheat grains are utilized as functional vegetables or as attractive foods (Lee *et al.*, 2004). In China, buckwheat is occasionally a staple food for people living in harsh climate zones and minority group regions. It also can be used to make buckwheat drinks including vinegar, tea and curative wine. In addition, the buckwheat plant is a good honey source as the blossoms are rich in nectar and blooming continues into the fall. The flowers are fragrant and attractive to bees that use them to produce strongly flavoured, dark honey. Buckwheat hulls are byproducts of buckwheat milling. They are traditionally used as fillings for pillows in Japan and China.

Recently increasing interest has focused on the development of buckwheat as a potential functional food because of its high-quality nutrients and unique components in the seeds.

These components include flavones, buckwheat sterols, d-chiro-inositol and thiamine-binding proteins. They have pharmacological properties and hold potential to benefit human in the treatment of some chronic diseases such as diabetes, hypertension, hypercholesterolemia, as well as other cardiovascular diseases.

1.3.2 Chemical compositions and nutritional properties of buckwheat

Buckwheat contains various kinds of essential nutrients including protein, starch, minerals, vitamins, and some unique components with physiological function and health benefits in human. **Table 3** lists the main chemical compositions of buckwheat seed and groat published by the United States Department of Agriculture, USDA. Buckwheat contains easily digestible and high-quality proteins with a well-balanced amino acid profile. Compared with other crops with lysine as the first limiting amino acid, buckwheat protein has a high lysine content, nearly twice the amounts found in wheat and oats and three times more than that in maize (Thacker *et al.*,1984). From a rat feeding experiment, Kayashita *et al.* (1995) reported that buckwheat protein had the same value as casein and soybean protein, but was more effective in lowering plasma cholesterol level than soybean protein. Lysine/arginine and methionine/glycine ratios are important factors to determine the cholesterol-lowing effects of the plant proteins. Buckwheat is reported to have lower Lys/Arg and Met/Gly ratios in protein than those in most other plant proteins, which gives buckwheat a strong cholesterol-lowing effect (Li and Zhang, 2001). Buckwheat protein extract consumption also increased muscle weight, carcass protein and water, and reduced body fat (Kayashita *et al.* 19999). Starch is the major carbohydrate in buckwheat, making up 51% - 67% of the seed (Pomeranz, 1983). Boiled buckwheat groats have been reported to be rich in resistant starch,

a nutritional variable related to low glycemic index properties, and induce significantly lower postprandial blood glucose and insulin responses compared with white wheat bread (Skrabanja *et al.*, 2001). Kawa *et al.* (2003) reported that buckwheat contained relatively high level of d-chiro-inositol, an insulin mediator with antihyperglycemic property, and buckwheat concentrate reduced serum glucose concentrations in streptozocin-treated rats, suggesting that buckwheat may be useful in the treatment of diabetes. Total lipids in whole buckwheat grain range from 1.5 – 3.7 %. Phospholipids make up 3.6 % of buckwheat flour and 67 % of the conjugated lipids (Pomeranz, 1983). Buckwheat oil recently has been reported to have higher unsaponifiable matter content than those found in rice bran, benne, soybean, and cotton oils, suggesting that buckwheat oil may reduce blood lipid (Wang *et al.*, 2004). The mineral composition of buckwheat is quite complementary to that in cereal crops. It contains important elements such as Fe, Ca, P, Se, Cu, Zn, Mg, I and Pt. Considerable amounts of vitamin B_1 and vitamin B_2 are also found in buckwheat. One hundred grams of buckwheat can supply 40% of the daily adult requirement of vitamin B_1 (Edwardson, 1996). In addition, rheological and chemical assays indicate that buckwheat does not contain gluten, which makes it valuable in the diets for people with celiac disease who are sensitive to wheat or other grains that contain the protein gluten (de Francischi *et al.*, 1994). Phytosterols were reported to inhibit the absorption of cholesterol *in vitro* (Heinemann *et al.*, 1991). Buckwheat seeds contain a certain amount of phytosterols and demonstrated a positive effect in the lowering of blood cholesterol levels (Li and Zhang, 2001). Over the past years, increasing attention has been paid to flavonoids, especially rutin in buckwheat. The high rutin content and its potent antioxidant activity make buckwheat hold a large potential in the prevention and treatment of cardiovascular diseases.

Table 3. Chemical compositions of Buckwheat seed and groats (Robinson, 1980)

Components	Buckwheat seed (%) [1]	Buckwheat groats (%) [2]
Protein	12.3	16.8
Carbohydrate	73.3	67.8
Fat	2.3	3.2
Fiber	10.9	0.6
Ash	2.1	2.2

[1] Buckwheat seed compositions are based on 14% moisture content.

[2] Buckwheat groat compositions are based on 12% moisture content.

1.3.3 *F. esculentum, F. tataricum, and F.homotropicum*

To date, the most popular and extensively consumed buckwheat is common buckwheat (*F. esculentum*). It has been widely planted and utilized all over the world with the advantages of high nutrient content, short growth period and few diseases. Common buckwheat seed is similar in size and weight to barley, and its hull is easily removed. Common buckwheat tastes sweet, so it is also named sweet buckwheat. However, it has never attained the status of the major cereal crops in any region from a production and yield point of view. The causes of low and unstable productivity include self-incompatibility, incompletion of the reproductive organs, failure of fertilization, and seed collapse in the early developmental stage of the embryo (Adachi, 1990).

Tartary buckwheat (*F. tataricum*), also known as bitter buckwheat, is planted and utilized as a traditional food in relative-limited areas including the region of the Himalayan hills from northern Pakistan to eastern Tibet and some regions in the southwest of China. As compared to common buckwheat, Tartary buckwheat is a self-compatible species with higher yield and greater tolerance to environmental stresses such as drought and frost. However, it has some adverse traits such as ease of seed shattering, bitter taste, and adhering seed coat that makes dehulling difficult. In China, Tartary buckwheat used to be considered a "poor quality and not suitable to eat" crop. There has been more attention given to Tartary buckwheat since the ethnic minority people, the Yi tribe in southwest of China, who live in the region where Tartary buckwheat originated and who use Tartary buckwheat as a staple food, were reported to be free from life-style related diseases during the Fifth International Symposium on Buckwheat. The epidemiological investigation on 850 Yi people indicated that Tartary buckwheat intake (100 g/day) was associated with lower serum total cholesterol and low-

density lipoprotein (LDL) cholesterol levels, and a higher ratio of high-density lipoprotein (HDL) to total cholesterol, suggesting a role of Tartary buckwheat consumption in the prevention of both hypertension and hypercholesterolemia (He *et al.*, 1995). Tartary buckwheat tea was also reported to be effective in lowering blood glucose levels both before and after meals in human (Lin *et al.*, 2004). When compared to common buckwheat, Tartary buckwheat has relatively high contents of protein, dietary fiber, vitamin B group, and biologically active components, especially flavonoid compounds (Bonafaccia *et al.*, 2003).

To increase buckwheat yield, breeding programs have been working on the improvement of common buckwheat for more than one hundred years. Although progress on common buckwheat improvement has been slow due to its out-crossing nature as well as the limited within-species variation, new buckwheat varieties have been developed with larger seed size and better groat yield. In recent years, growing attention has been paid to Tartary buckwheat not only because of its self-compatibility characteristic but its unique components that are beneficial to human health. However, crosses between Tartary buckwheat are extremely difficult, and hybridization between common buckwheat and Tartary buckwheat has not developed beyond the vegetative stage. Over the past decade, increasing interest has focused on searching for new species of buckwheat with close relationship to Tartary buckwheat (Campbell, 2004).

The first successful inter-species hybridization of buckwheat was reported by Campbell (1995), in which the self-compatibility trait of homotropicum buckwheat (*F. homotropicum* Ohnishi) was introduced into common buckwheat. *F. homotropicum* is a wild buckwheat species discovered in southwest China (Ohnishi, 1995). Similarly to Tartary buckwheat, it is self-compatible and has better frost and stress tolerance than common buckwheat. However,

it is more closely related to common buckwheat than to Tartary buckwheat based on the analysis of both morphological characteristics and isozyme variability (Ohnishi, 1996). It is believed that *F. homotropicum,* as an intermediate species, has the potential to be a bridge between *F. tataricum* and *F. esculentum* buckwheat in improvement programs.

1.3.4 Rutin and other flavonoids in Buckwheat

Rutin was first discovered in buckwheat by Schunck in 19th century, who stated that 240 g of glucoside was isolated from 30 pounds of fresh leaves (Couch *et al.*, 1946). Among the field crops, buckwheat is the only one that contains rutin. It was cultivated as a source of rutin for herbal drug production in the United States approximately 50 years ago (Ohsawa and Tsutsumi, 1995). Rutin content in buckwheat is quite variable depending on species, cultivars, and environmental conditions such as location and season. It also varies in different organs of the plant.

In mixed varieties of common buckwheat, the rutin content was reported to be higher (2.3 % of dry matter) in the buckwheat leaves and lower (0.02 % of dry matter) in the buckwheat seeds (Holasova *et al.*, 2002). A similar trend was also found in Tartary buckwheat, in which the rutin content was up to 3 % of dry weight in the herb and approximately 0.8-1.7 % of the dry weight in the seed (Fabjan *et al.*, 2003). Kreft *et al.* (1999) reported that on average, 4.6 %, 0.1 % and 0.03 % of rutin were found in the flowers, stems, and leaves of common buckwheat, respectively, with the lowest rutin content being found in the grains (up to 0.01 %). However, Park *et al.* (2004) reported that the rutin content in both common buckwheat and Tartary buckwheat was higher and in the order: flower > leaf > seed > stem > root. This may be explained by different growing conditions that have different influences on rutin

accumulation in different organs of the buckwheat plant (Kreft *et al.*, 2002; Kalinova and Dadakova, 2004). In general, buckwheat herbs have higher rutin contents than do the seeds. The buckwheat seed is an achene, a triangular fruit. The hull, the outer layer of the achene, is a hard fibrous structure. Removing the hulls releases whole groats. The outer part of the groat is called the testa layer. Flavonoids in buckwheat seeds are concentrated in the hull and the testa layer (Salunkhe *et al.*, 1989). In common buckwheat seeds, the rutin content was 0.08 - 0.44 %, 0.07 – 0.08 %, and 0.02 – 0.03 % in the hulls, the bran containing the testa layer and hulls, and the groats, respectively (Steadman *et al.*, 2001). Dietrych-Szostak and Oleszek (1999) reported that buckwheat hulls contained six identified flavonoid compounds, whereas rutin and isovitexin were the only flavonoids in the groats. The total flavonoid contents were 18.8 and 74.0 mg/ 100 g of dry matter in the groats and the hulls, respectively, and the rutin contents was 17.8 and 33.0 mg/ 100 g of dry matter in the groats and the hulls, respectively.

There is a significant difference in rutin content between the two cultivated buckwheat species. Minami *et al.* (1992) reported that Tartary buckwheat seed had much higher rutin content (1523 mg/ 100 g of dry matter) than did common buckwheat seed (15.4 mg/ 100 g of dry matter). This is supported by many studies in which the rutin content was found to be higher in Tartary buckwheat seed than that in common buckwheat seed (Steadman *et al.*, 2001; Fabjan *et al.*, 2003; Chai *et al.*, 2004). Flavonoid compounds in common buckwheat and Tartary buckwheat are different. Fabjan *et al.* (2003) reported that Tartary buckwheat seed contained traces of quercetin and quercetrin, which were not found in common buckwheat seed. This result was consistent with the study by Steadman *et al.*(2001). However, four principal flavonols: rutin, quercetin, kaempferol-3-rutinoside and a flavonol triglycoside were identified in common buckwheat seed (Tian *et al.*, 2002). The inconsistent

results may be due to the different buckwheat varieties or different analytical methods employed in the studies. An obvious variation of rutin content also exists within varieties or strains in the same buckwheat species. Through the investigation of 27 cultivars and strains of common buckwheat originally from Japan, China, Nepal, Russia, Slovenija and France, Kitabayashi *et al.* (1995) reported that the rutin content in the seed exhibited a wide varietal variation ranging from 12.6 to 35.9 mg/ 100 g of dry matter. This is supported by a study on seven common buckwheat varieties in which the rutin content in the seed ranged from 14 to 24 mg/ 100 g of dry matter (Kalinova and Dadakova, 2004). A recent investigation has indicated that significant varietal differences of rutin content were observed in both common and Tartary buckwheat. The range of variation of rutin content was between 0.38 – 0.51 % for twelve common buckwheat varieties grown in different regions in north China and between 0.98 – 1.16 % for ten Tartary buckwheat varieties grown in different regions in both the south and the north of China (Chai *et al.*, 2004).

Environmental conditions are very important factors influencing the rutin level in buckwheat. Flavonoids are aromatic secondary plant metabolite. Their biosynthesis involves the interaction of at least five different pathways, including the glycolytic pathway, the pentose phosphate pathway, the shikimate pathway, the general phenylpropanoid metabolism and the diverse specific flavonoid pathway. Many enzymes are responsible for the different flavonoid class formation and for structure modification, such as hydroxylation, methylation, glycosylation and acylation. The alternative products for a given metabolite may vary in response to environmental stimuli (Robards and Antolovvich, 1997). For example, phenylpropanoid metabolism is stimulated by UV-B radiation. Phenylalanine ammonia-lyase (PAL) catalyses the first step of phenylpropanoid metabolism in higher plants. The positive

relationship between PAL activity and flavone content in the same organ was reported by the study on several organs of buckwheat seedling for both common buckwheat and Tartary buckwheat. Moreover, the proper dosage of radiation increased flavone content as well as PAL activity in buckwheat seedling (Tang and Zhao, 1992). Different levels of UV-B radiation was also reported to affect the rutin content in buckwheat herbs, and the rutin content in leaves and stems depended more on UV radiation than did it in flowers (Kreft *et al.*, 2002). The influence of nitrogen fertilization on the rutin content in buckwheat has been investigated by Kalinova and Dadaova (2004). In their report, nitrogen fertilization decreased rutin content found in achenes, but did not influence those in leaves, flowers and stems.

An animal study (mouse) showed that Tartary buckwheat leaf demonstrated antioxidant properties by increasing the activities of superoxide dismutase (SOD) and GSH-peroxidase (GSH-Px) in blood, liver and heart, and by decreasing the amount of malondialdehyde (MDA) in these organs (Wang *et al.*, 1992). Another clinical study also reported that common buckwheat herb tea had a favorable influence on patients with chronic venous insufficiency (CVI) to prevent further oedema development (Ihme *et al.*, 1996). Do the flavonoids contribute to the biological properties of buckwheat beneficial in human health? Besides rutin, buckwheat also contains flavonoids such as quercetin, kaempferol, morin, catechins, vitexin, orientin, hyperin, kaempferol-3-rutinoside etc. In a study on the antioxidant capacities and total phenolic contents in the honeys from seven different floral sources, a linear correlation was observed between antioxidant capacity and total phenolic content. Buckwheat honey, with a high phenolic content, was effective in inhibiting *in vitro* human serum lipoprotein oxidation (Gheldof and Engeseth, 2002). A significant relationship between total phenolic content as well as rutin content and antioxidant activity of mixed

varieties of common buckwheat was found in the evaluation of antioxidant activities of buckwheat seed, dehulled seed, hull, straw and leaf (Holasova *et al.*, 2003). In a comparison of total phenolics, flavanol, proanthocyanidin, and flavonoid in a French common buckwheat variety, 'La Harpe', with an antioxidant capacity of scavenging hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hypochlorous acid (HOCl), Quettier-Deleua *et al.*(2000) reported that the higher efficiency of buckwheat flour should be attributed mainly to flavanols, with a complementary contribution of the flavonoids, rather than solely to rutin. Nevertheless, rutin is the major flavonoid in buckwheat. It is believed to have an important role in the pharmacological activity of buckwheat. With respect to this area, more *in vivo* studies on animals and clinical research in humans are needed.

Increasing rutin content in buckwheat has been an important objective in buckwheat breeding programs for many years. A wide variation of rutin content within varieties or the significant difference in the rutin content between two cultivated species provides a great potential to develop buckwheat lines with higher rutin content through inter-varietal or inter-specific hybridizations. The discovery of the wild species, *F. homotropicum* has brought a new tool for breeding work. *F. homotropicum*, with a self-compatibility trait similar to Tartary buckwheat, and morphological characteristics and isozyme variability similar to common buckwheat, could serve as an intermediate species, contributing to the hybridization between Tartary buckwheat and common buckwheat.

Chapter II Hypothesis and Objectives

Quercetin has been the most intensively studied flavonoid due to its effective antioxidant activity and significant absorption from the diet. It exists predominantly in glycosylated form such as rutin, rather than in its aglycone form. Buckwheat is generally considered to be a major dietary source of rutin. The difference in the rutin content between species or varieties provides an opportunity to develop a buckwheat line with high rutin content through hybridization. Kade Research Ltd has a very active buckwheat breeding program in North America, which has been working on the development of buckwheat lines from crosses between *F. tataricum, F. esculentum and F. homotropicum* for many years. Our project was to develop a rapid and accurate assay for rutin in buckwheat and to study the antioxidant activity of buckwheat lines in collaboration with this breeding program. In this thesis, we tested the hypothesis that the rutin content in improved buckwheat lines was higher than in their parents and that the buckwheat lines with higher rutin content had more effectively antioxidant activity than those with lower rutin content.

The objectives of the project were (1) to develop a high performance liquid chromatographic assay for rutin in different buckwheat species and quantitatively analyze rutin in buckwheat seeds and leaves. A large number of samples require quantitative analysis for rutin content in the breeding work. The objective was to provide an efficient HPLC assay to screen the rutin content in buckwheat seeds and leaves, and to validate this analytical method for routine application; (2) to compare the antioxidant effectiveness of quercetin, rutin, α-tocopherol (vitamin E, a lipophilic antioxidant) and ascorbic acid (vitamin C, a hydrophilic antioxidant) in hydrophilic and lipophilic free radical generating systems; It is well known that quercetin and its glycoside rutin have antioxidant activity. However, their

relative antioxidant capacity has been controversial due to differences in study methodology (Hopia and Heinonen, 1999). The present objective was to elucidate the differences in antioxidant activities of these antioxidant compounds in aqueous and lipid environments; (3) to investigate the protective effects of different buckwheat species or different buckwheat varieties against low-density lipoprotein (LDL) oxidation and to determine the correlation between the rutin content and the antioxidant activity in buckwheat. It has been suggested that LDL oxidation is most likely initiated in the aqueous environment surrounding the lipoprotein particle (Frei, 1995). The objective of the present study was to reveal the potential for developing buckwheat lines with increased rutin content to prevent the initiation of lipid peroxidation for the benefit of the cardiovascular system; (4) to study the effect of quercetin on H_2O_2-induced oxidative stress in cardiomyocytes using dichlorofluorescein (DCFH) as a cellular fluorescent marker. In order to more completely understand the roles of oxidative stress in various diseases, including atherosclerosis, and to implement treatment or prevention, a technical method to evaluate the oxidative stress in living cell models is very important. A number of researchers have reported on the application of a DCFH method for the evaluation of natural antioxidants at the cell level (Lin *et al.*, 2000; Bestwick and Miline, 2001; Takamatsu *et al.*, 2003). However, these studies almost always used HL-60 cells. When considering the pharmacological relevance to cardiovascular system, the objective of the present study was to employ cardiomyocytes to feedback *in vitro* results into *in vivo* evaluation of antioxidants.

Chapter III Materials and Methodology

3.1 Materials

3.1.1 Chemicals

1. Rutin: Sigma Chemical Co., St. Louis, MO, U.S.A.
2. Quercetin: Sigma Chemical Co., St. Louis, MO, U.S.A.
3. Ascorbic acid: Sigma Chemical Co., St. Louis, MO, U.S.A.
4. α-Tocopherol: Sigma Chemical Co., St. Louis, MO, U.S.A.
5. 2-Aminoethyl-diphenylborate: Sigma Chemical Co., St. Louis, MO, U.S.A.
6. 1,1,3,3-Tetra-methoxypropane: Sigma Chemical Co., St. Louis, MO, U.S.A.
7. Dithiobisnitrobenzoic acid: Sigma Chemical Co., St. Louis, MO, U.S.A.
8. Phenylmethylsulfonyl fluoride: Sigma Chemical Co., St. Louis, MO, U.S.A.
9. Ethylenediaminetetraacetic acid (EDTA): Sigma Chemical Co., St. Louis, MO, U.S.A.
10. Thimerosal: Sigma Chemical Co., St. Louis, MO, U.S.A.
11. Thiobarbituric acid: Sigma Chemical Co., St. Louis, MO, U.S.A.
12. Trichloroacetic acid: Sigma Chemical Co., St. Louis, MO, U.S.A.
13. Sodium chloride (NaCl): Sigma Chemical Co., St. Louis, MO, U.S.A.
14. Sodium phosphate (monobasic): Sigma Chemical Co., St. Louis, MO, U.S.A.
15. Sodium phosphate (dibasic): Sigma Chemical Co., St. Louis, MO, U.S.A.
16. Potassium chloride (KCl): Sigma Chemical Co., St. Louis, MO, U.S.A.
17. Potassium phosphate (monobasic): Sigma Chemical Co., St. Louis, MO, U.S.A.
18. Potassium phosphate (dibasic): Sigma Chemical Co., St. Louis, MO, U.S.A.
19. Aluminium chloride ($AlCl_3 \cdot 6H_2O$): Sigma Chemical Co., St. Louis, MO, U.S.A.
20. Copper sulfate ($CuSO_4$): Fisher Scientific, Fair lawn, NJ, U.S.A.

21. Magnesium sulfate ($MgSO_4$): Fisher Scientific, Fair Lawn, NJ, U.S.A.

22. Calcium chloride (CaC_{l2}): Anachemia Ltd., Montreal, QB, Canada

23. Diacetate ester of 2' 7' –dichlorofluorescin (DCFH-DA): Sigma Chemical Co., St. Louis, MO, U.S.A.

24. Dulbecco's modified eagle medium/F 12 (DEME/F-12): Gibco-BRL Life Technologies Inc., Burlington, ON, Canada

25. Fetal calf serum: Gibco-BRL Life Technologies Inc., Burlington, ON, Canada

26. Hank's buffered salt solution: Gibco-BRL Life Technologies Inc., Burlington, ON, Canada

27. Penicillin-streptomycin: Gibco-BRL Life Technologies Inc., Burlington, ON, Canada

28. 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH): Wako Chemical USA, Inc., Richmond, VA. U.S.A.

29. 2,2'-Azobis(2,4-dimethylvaleronitrile) (AMVN): Wako Chemical USA, Inc., Richmond, VA. U.S.A.

30. Hydrochloric acid: Fisher Scientific, Nepean, Ontario, Canada

31. Hydrogen peroxide (30%): Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

32. Acetic acid (glacial): Stanchem, Etobicoke, Ontario, Canada

33. Cholesterol INFINITY assay kit: Sigma Chemical Co., St. Louis, MO, U.S.A.

34. DC Protein assay kit: BIO-RAD. Mississauga, ON, Canada.

3.1.2 Solvents

1. Ethanol: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.
2. Methanol: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

3. Acetonitrile: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

4. Iso-amyl alcohol: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

3.1.3 Plant Samples

All buckwheat seed and leaf samples of different species were provided by Kade Research, Morden, Manitoba, Canada.

3.1.4 Supplies

1. 15 ml SC polypropylene tube: Sarstedt Inc., Newton, NC, U.S.A.

2. 50 ml polypropylene centrifuge tube: Fisher Scientific, Pittsburgh, PA, U.S.A.

3. Glass test tube: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

4. 250 ml separatory funnel: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

5. 100 ml volumetric flask: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

6. 250 ml Erlenmyer flask: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

7. 25 ml glass bottle: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

8. 10 ml serologische pipette: SARSTEDT, Aktiengesellschaft & Co., France

9. Microcentrifuge polypropylene tube: Sarstedt Inc., Newton, NC, U.S.A.

10. Tissue culture plate (24 – well): Corning Inc., Corning, NY, U.S.A.

11. PD10 desalting columns: Amershampharmacia Biotech, Baie d'Urfe', QC. Canada

12. 1.5 ml disposable cuvette: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

16. 3.0 ml disposable cuvette: Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

17. Glass vial for HPLC: Waters Corporation, Milford, MA, U.S.A.

18 Plastic spring (insert) for HPLC: Waters Corporation, Milford, MA, U.S.A.

3.1.5 Equipments

1. UV-visible spectrophotometer (UV-160): Shimadzu Corporation, Kyoto Japan.
2. Cary 1 UV-visible spectrophotometer: Varian Australia Pty Ltd., Mulgrave, Victoria, Australia
3. Gyrotory water bath shaker (model G76D): New Brunswick Scientific Co. Inc., Edison, N.J. U.S.A.
4. Greenway water bath: Lab – line Instruments Inc., Melrose Park, Illinois, U.S.A.
5. Greenway Super-mixer: Lab – line Instruments Inc., Melrose Park, Illinois, U.S.A.
6. PH meter: Fisher Accumet Meter 15, Denver Instrument Company, U.S.A.
7. Scale: 1) Mettler – Toledo AG, Type BB 244, Switzerland.
   2) Mettler AG 50, Swizerland.
8. Beckman PC Centrifuge: U.S.A.
9. Beckman Optima LE – 80K: Beckman, Canada.
10. Nikon Diaphot TME: Nikon Corporation, Japan.
11. Lamda DG-4 illumination system:

    175 watt Xenon arc lamp; Sutter Instrument Company, CA, U.S.A.

    12 bit, 12.5 MHz sensiCam CCD camera: Cooke Corporation, NY, U.S.A.
12. High performance liquid chromatography (HPLC):

The HPLC system was comprised of a Waters 2690 (alliance) pump, a Xterra MS reversed-phase C18 column (4.6 x 250 mm; particle size 5 μM) and a Waters 996 Photodiode Array Detector. All of above instruments were purchased from Waters Corporation, Milford, MA, U.S.A.

3.1.6 Software

1. Millennium ^{32} (version 3.2) Chromatography: Waters Corporation, Milford, MA, U.S.A.

2. Millennium ^{32} Photodiode Array Chromatography: Waters Corporation, Milford, MA, U.S.A.

3. Cary WinUV software: Varian Australia Pty Ltd., Mulgrave, Victoria, Australia

4. Imaging Workbench 2.1: Axon Instruments, Inc., Foster City, CA. U.S.

5. Statistical Analysis System (version 8, 2002): SAS Institute Inc., Cary, NC, U.S.A.

3.2 Methodology

3.2.1 Quantitative analysis of total flavonoids and rutin in buckwheat seed and leaf

3.2.1.1 Buckwheat sample preparation

1. Buckwheat seed

Buckwheat seeds were manually dehulled and ground to a fine powder with a mortar and pestle immediately before an experiment.

1. Buckwheat leaf

Buckwheat leaves were pulverized with a mortar and pestle immediately before an experiment.

3.2.1.2 Extraction procedure of total flavonoids for spectrophotometric assay

The method used for extracting total flavonoids from buckwheat seed was as described by Oomah and Mazza (1996) with slight modifications.

Finely ground buckwheat seed samples (0.02 – 0.2 g) were transferred into a 25ml glass bottle and 8 ml of 80% methanol added. The sample weight was dependent on the buckwheat

species: 0.02 g for *F. tataricum*, 0.1 g for *F. homotropicum* and 0.2 g for *F. esculentum.* The bottle was placed on a Gyrotory water bath shaker, shaking (200 rpm) under reflux for 2 hours at 70 ^{o} C. The sample bottle was put into a cold-water bath. Once the extract had cooled to room temperature, it was transferred to a 15 ml polypropylene tube and centrifuged at 1000 rpm for 10 minutes on a Beckman PC Centrifuge. After removing the supernatant, 1 ml of 80% methanol was added to the residue, which was then mixed using a vortex mixer for a few seconds, and again centrifuged under the same conditions. This process was repeated one more time. The three supernatants were combined and made up to a total of 10 ml with 80% methanol. This buckwheat extract was then ready for quantification of the total flavonoids.

3.2.1.3 Quantitative analysis of total flavonoids in buckwheat seed by spectrophotometric assay

The method used for determining total flavonoid content in buckwheat seed was performed as described by Oomah and Mazza (1996).

Buckwheat extract (0.1 – 1.0 ml) was diluted with deionized water to 2 ml of final volume. 1% 2-aminoethyl-diphenylborate methanol solution (0.1 ml) was added to the extract solution and mixed using a vortex mixer. The absorption of the extract solution was measured at 404 nm on a UV-visible spectrophotometer (UV-160) and compared to that of a standard rutin curve.

3.2.1.4 Extraction procedure of rutin for spectrophotometric assay

The method used for extracting rutin from buckwheat seed and leaf was as described in the procedure provided by Kade research with modification.

Samples analysed were 0.2 g for buckwheat leaf and 0.2 – 2.0 g for buckwheat seed depending on species (0.2 g for *F. tataricum*, 1.0 g for *F. homotropicum* and 2.0 g for *F. esculentum*). The buckwheat sample was transferred into a 250 ml Erlenmyer flask and 25 ml of 95% ethanol added. The flask was then placed into a water bath, shaking (50 rpm) under reflux for 3 hours at 70 $^{\circ}$ C. The sample flask was put into a cold-water bath. Once the extract cooled to room temperature, it was transferred to a 50 ml polypropylene tube and was centrifuged at 1000 rpm for 10 minutes on Beckman PC Centrifuge. After removing the supernatant, 10 ml 95% ethanol was added to the residue, mixing on a vortex mixer for a few seconds, and re-centrifuged at the same condition. This step was repeated one more time. The three supernatants were combined and made up to a total of 50 ml with 95% ethanol. The buckwheat extract was then ready for quantification of rutin.

3.2.1.5 Quantitative analysis of rutin in buckwheat seed and leaf by spectrophotometric assay

The method used for determining rutin content in buckwheat seed or leaf was described in the procedure provided by Kade Research with modification.

Ten ml of buckwheat extract were pipetted into a 250 ml separatory funnel, and added 10 ml of iso-amyl alcohol and 25 ml of 0.1 M $AlCl_3$ solution. The funnel was gently shaken and solvents were allowed to separate. The bottom layer, an aqueous yellow solution, was drained off into a 100 ml volumetric flask. This step was repeated two more times. The

yellow solution was diluted to 100 ml with distilled water in the volumetric flask. The absorption of yellow solution was measured at 416 nm on the UV-visible spectrophotometer (UV 160). Ther rutin content in the buckwheat seed or leaf was calculated from absorbance measurement.

3.2.1.6 Extraction procedure of rutin for high performance liquid chromatographic assay

This method used for extracting rutin from buckwheat seed was developed in our laboratory.

Extraction kinetics of rutin from buckwheat seed

A *F. tataricum* seed sample (0.02 g) was added to 8 ml of 80% methanol in each of five 25ml glass bottles, labeled 1#, 2#, 3#, 4# and 5#, respectively. The bottles were placed in a Gyrotory water bath shaker, shaking (200 rpm) under reflux at 70 $^{\circ}$ C for different time periods: 5 minutes for 1#, 10 minutes for 2#, 30 minutes for 3#, 60 minutes for 4# and 120 minutes for 5#. The sample glass bottles were then placed into a cold-water bath. Once the extract cooled to room temperature, it was transferred to a 15 ml polypropylene tube and centrifuged at 1000 rpm for 10 minutes on a Beckman PC Centrifuge. After removing the supernatant, 1 ml of 80% methanol was added to the residue, vortexed for a few seconds, and centrifuged as stated above. This step was repeated one more time. The three supernatants were combined, made up to total 10 ml with 80% methanol and then mixed on the vortex. One ml of the solution was added to a 1.5 ml microcentrifuge tube and centrifuged at 14,000

rpm for 10 minutes on the centrifuge. The supernatant was loaded into a HPLC vial. The buckwheat extract was ready for the quantification of rutin.

Extraction of rutin from different buckwheat species

Buckwheat seed sample (0.02 – 0.2 g) was added to 8 ml of 80% methanol in a 25ml glass bottle. The amount of sample was dependent on the buckwheat species: 0.02 g for *F. tataricum*, 0.1 g for *F. homotropicum* and 0.2 g for *F. esculentum.* The bottle was placed on Gyrotory water bath shaker, shaking (200 rpm) under reflux at 70 ° C for the optimal time period that was chosen based upon the extraction kinetic study. The buckwheat extract was ready for the quantification of rutin following the procedure described above.

3.2.1.7 Chromatographic separation and quantitation of rutin

This method used for quantifying rutin in buckwheat seed was developed and validated in our laboratory.

The HPLC system was controlled by Millennium ^{32} Chromatography software and Millennium ^{32} Photodiode Array Chromatography software. The mobile phase consisted of 2.5% acetic acid and acetonitrile with the ratio of 80:20. The mobile phase was filtered through a 0.45 μm membrane and degassed prior to use. The separation was performed by isocratic elution at a flow rate of 0.8 ml/min. The injection volume was 10 μl, and the column temperature was 22.5 ° C. Eluates from the column were detected at 360 nm with the retention time being 6.9 minutes for rutin. The peak area was used for the quantification based on the rutin standard calibration curve.

For validation of the analytical method, linearity, accuracy, precision, and sensitivity were determined. The linearity was investigated over a range of 5 – 200 μg/ml from 7 concentrations. Each solution was analyzed in duplicate and average absorbance was calculated for each concentration level. The slope, the y-intercept, the residual sum of squares, and the coefficient of determination (R^2) for the line of best fit were reported. The accuracy was measured by the percentage recovery for the whole procedure or for the HPLC system, and the precision was measured by the repeatability for the whole procedure or for the HPLC system. The sensitivity was expressed by the limit of detection (LOD) and the limit of quantification (LOQ).

Sensitivity: The limit of detection and the limit of quantification were estimated based on the linearity data over the low concentration range of 0 - 50 μg/ml from 6 concentrations. Each solution was analyzed in duplicate and average absorbance was calculated for each concentration level. The uncertainty of responses (S_y) was determined using the following equation:

$$S_y = \sqrt{RSS / (n - 2)}$$

Where: RSS = the residual sum of squares

n = number of linearity points

After S_y was determined, calculated the standard deviation of the y – intercept (σ) by the equation:

$$\sigma = S_y \sqrt{1 / [n - (\sum Xi)^2 / \sum Xi^2]}$$

Where: n = number of linearity points

x_i = individual concentration value

The limit of detection and the limit of quantification were calculated by:

LOD = 3.3 σ / m

LOQ = 10 σ / m

Where σ = the standard deviation of the y – intercept

m = slope of the best fit line

Accuracy: To determine accuracy, recoveries of the whole procedure and HPLC system for rutin analysis were tested by spiking known amounts of standard rutin into the buckwheat seed at the start of extraction and into the buckwheat seed extract prior to the HPLC injection, respectively. Three buckwheat cultivars/lines, representing the three species, respectively, were used. For each buckwheat sample, standard rutin at three different concentration levels were added to buckwheat seed or to buckwheat seed extract. Each buckwheat sample was performed three replicate determinations corresponding to each concentration level. Recovery of rutin for the whole procedure or for the HPLC system was calculated from the equation below:

$$\textbf{\% Recovery of rutin} = \frac{Cm \cdot 100}{Cc}$$

Where Cm = Measured value of rutin concentration

Cc = Calculated value of rutin concentration

Precision (Repeatability)**:** To determine precision, three buckwheat cultivars/lines, representing the three species, respectively, were used. Each buckwheat sample was performed six replicate determinations. Each sample preparation (buckwheat extract) was injected into HPLC once. The relative standard deviation (RSD) was calculated from the six replicate determinations.

3.2.2 Protective effect of flavonoids or buckwheat against lipid peroxidation in low-density lipoprotein of rabbits

3.2.2.1 Low-density lipoprotein isolation

This method was adapted from the laboratory at St. Boniface Hospital Research Centre. Blood from male New Zealand White rabbits (2.5 – 3.0 kg) were fed with 0.5% cholesterol-supplemented diet for 8 weeks up to 16 weeks. Rabbits were anesthetized with constant flow 2% isoflurane and the blood was collected by cardiac puncture in a 10 ml vascutainer containing 17.55 mg ethylenediaminetetraacetic acid (EDTA) that prevents LDL from oxidation. The blood was spun down in a clinical centrifuge at 3000 rpm for 15 minutes at 4^{0} C to collect the plasma. To inhibit lecithin: cholesterol acyltransferase, proteolysis, and bactericide, the following solutions: 1.5 mM DTNB (dithiobisnitrobenzoic acid), 2 mM PMSF (phenylmethylsulfonyl fluoride), and 0.08 mg/ml thimerosal were required, respectively. Thus 2 ml of 3% DTNB, 1 ml of 3.5% PMSF, and 1 ml of 0.8% thimerosal were added to every 10 ml of plasma. Plasma density was adjusted to 1.006 and 1.063 g/ml to isolate VLDL and LDL respectively by sequential ultra centrifugation overnight using Ti50.2 rotor run in Beckman Optima LE – 80K at 4^{0} C. The LDL fraction was then dialyzed in a PD10 column against 0.15 M NaCl and 1 mM EDTA (pH 7.4). Determination of the protein content was performed by Lowry's protein assay using the DC Protein Kit and the cholesterol content was measured enzymatically using a Cholesterol INFINITY assay (Liu, K. *et al.*, 1993; Liu, K and Pierce G.N., 1993). The LDL fraction was kept in a 1.5 ml cryovial wrapped with aluminium foil at 4^{0} C for one week.

3.2.2.2 Buckwheat extract preparation

A. A buckwheat seed sample (2 g) was transferred into a 250 ml Erlenmyer flask and 30 ml of 95% ethanol was added. The flask was then placed into a water bath, shaking (50 rpm) under reflux for 3 hours at 70 $^{\circ}$ C. The sample flask was placed into a cold-water bath. Once the extract had cooled to room temperature, it was transferred to a 50 ml polypropylene tube and then centrifuged at 1000 rpm for 10 minutes on a Beckman PC Centrifuge. After removing the supernatant, 10 ml 95% ethanol was added to the residue, mixing on a vortex mixer, and re-centrifuged. This step was repeated one more time. The three supernatants were combined and made up to a total of 50 ml with 95% ethanol. The buckwheat extract was ready for the measurement of antioxidant activity using AAPH or AMVN and LDL model with TBARS test and conjugated diene assay.

B. A buckwheat seed sample (1 g) was added to 8 ml of 80% methanol in a 25ml glass bottle. The bottle was then placed into Gyrotory water bath shaker, shaking (200 rpm) under reflux for 30 minutes at 70 $^{\circ}$ C. The sample glass bottles were immediately placed into an ice-water bath. Once the extract had cooled to room temperature, it was transferred to a 15 ml polypropylene tube and centrifuged at 1000 rpm for 10 minutes on a Beckman PC Centrifuge. After removing the supernatant, 1 ml of 80% was added to the residue, and then mixed on a vortex mixer, and re-centrifuged. This step was repeated one more time. The three supernatants were combined and made up to a total of 10 ml with 80% methanol. The buckwheat extract was ready for the measurement of antioxidant activity of three buckwheat species using the Cu^{2+} and LDL model with conjugated diene assay.

3.2.2.3 Thiobarbituric acid-reactive substances test for measurement of lipid peroxidation

The method was a modification of the report by Massaeli *et al.* (1999).

The thiobarbituric acid-reactive substances (TBARS) assay is commonly used to measure malondialdehyde (MDA) produced in peroxidizing lipid systems. Heated with TBARS reagent at low pH, MDA forms pink chromogens having an absorbance at 535 nm. In this study, the azo-compounds 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) were used to generate free radicals in the aqueous phase and within the lipid region, respectively. In order to compare the effectiveness of antioxidants in both lipophilic and hydrophilic free radical generating systems, the concentration of AAPH or AMVN that produces similar amounts of MDA in LDL need to be used. Therefore, MDA production in LDL was examined at various concentrations of each azo-compound, and the specific concentrations of AAPH and AMVN that induced similar amounts of MDA production were chosen for the subsequent work.

A stock solution of (AAPH) or a stock solution of (AMVN) was freshly prepared by weighing a predetermined amount of the chemical to be dissolved in phosphate buffer solution (PBS, pH 7.4) or 95% ethanol, respectively. In the absence or presence of a test compound or buckwheat extract, 1 ml of lipoprotein (0.5 mg protein/ml in phosphate buffer solution, pH 7.4) in a 15 ml screw cap polystyrene centrifuge tube was incubated in a hydrophilic free radical generating system AAPH or a lipophilic free radical generating system AMVN for 60 minutes at 37 ^{0}C in water bath. For the test compounds, except for ascorbic acid that was dissolved in phosphate buffer solution, the other test compounds were dissolved in methanol. The volume of the all test compound solution added to 0.99 ml of

lipoprotein solution was 10 µl. For buckwheat extract, 10 µl of extract was added to the lipoprotein solution. 95% ethanol or methanol (1%, final), which did not influence the assay, was also added in the control. The extent of lipid peroxidation was determined using thiobarbituric acid-reactive substances. TBARS reagent, 2 ml aliquot of 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N HCL, was added to the lipoprotein and heated for 15 minutes at 100 ^{0} C in boiling water. After cooling to room temperature, the sample was centrifuged at 2000 rpm for 10 minutes on Beckman CP Centrifuge. The absorbance of the supernatant was measured at 535 nm using a UV-visible spectrophotometer (UV 160). Using 1,1,3,3-tetra-methoxypropane as a reference standard, the TBARS results were expressed as Malondialdehyde (MDA) equivalents and the results were expressed as nmole MDA/mg protein.

3.2.2.4 Conjugated diene assay for measurement of lipid peroxidation

The method was as described by (Regnstrom *et al.*, 1993) with modification.

The peroxidation of unsaturated fatty acids (PUFA) is accompanied by formation of conjugated dienes with absorption in the UV range at 234 nm. This characteristic absorption can be used to measure an early stage in the lipid peroxidation process. AAPH was used to induce the lipid peroxidation. Fresh stock solution of 60 mM AAPH was prepared by dissolving the chemical in phosphate buffer solution (PBS, pH 7.4). For the test compounds, ascorbic acid was dissolved in a phosphate buffer solution, and other test compounds were dissolved in methanol. For the buckwheat extract, 10 µl of extract was added to the lipoprotein solution. In the absence or presence of a test compound or buckwheat extract, 100 µl of 60 mM AAPH was added to 4.9 ml of lipoprotein (0.1mg protein/ml in PBS, pH 7.4) in

a glass test tube. The concentration of AAPH in the reaction system was 1.2 mM. After mixing the sample by shaking the test tube very gently, 2.8 ml of sample solution was added to a 3 ml quartz cuvette. The sample was incubated in the hydrophilic free radical generating system AAPH at 37 ^{0} C by placing the cuvette in the Cary 1 UV-visible spectrophotometer. The control contained 0.2 % methanol (final) that did not affect the assay. Diene formation in lipoprotein was measured by reading absorbance at 234 nm. The time course of oxidation was followed by the absorbance reading every 30 min for 8 hours. When cupric ion (Cu^{2+}) was used to induce lipid peroxidation, $Cu^{2+}SO_4$ solution was added to lipoprotein to make a 12 μM $Cu^{2+}SO_4$ lipoprotein solution. In the determination of antioxidant activity of buckwheat extract, the procedure was as above, except that the time course of oxidation was measured for 6 hours.

The lag time to diene formation in LDL, the slope of the propagation phase and the area under the oxidation curve (AUC) were used to describe the oxidation curves. The lag time was determined graphically by the intersection of the tangents of the propagation curve with the initial oxidation curve. The slope and the AUC were calculated.

3.2.3 Effect of quercetin on oxidative stress in cardiomyocytes

Neonatal rat cardiomyocytes were provided by St.Boniface Hospital Research Centre.

The fluorescent microscopic method used to measure the oxidative stress in cardiac myocytes was previously described by Swift and Sarvazyan (2000) with a slight modification in our laboratory. 2' 7' –dichlorofluorescein (DCF) was used as a fluorescent marker to detect cellular oxidative stress. The cells were seeded onto two sterile 24-well plates containing 1 ml of culturing medium (DF-10) and placed in an incubator with an atmosphere

of 5% (v/v) CO_2 at 37 ° C for 24 hours after being delivered to our laboratory. The wells of each plate were divided into three groups: 8 wells for the negative control, 8 wells for the positive control and 8 wells for the quercetin treatment. After changing the medium, 5 μl of quercetin methanol solution was added to each well of the treatment group, making the concentration of quercetin in the well 5 μM. Five μl of methanol was added to the wells of the negative or the positive control group (0.5% v/v). The plates were placed into the incubator at the same conditions as above described for 24 hours. A 10 mM stock solution of DCFH-DA was freshly prepared in 100% ethanol on a daily basis, wrapped in aluminum foil and kept at – 20 ° C. It was diluted in Ca^{2+}/Mg^{2+}-containing Hank's buffer (pH 7.4) to 10 μM just before experiments. The cells were transferred into Ca^{2+}/Mg^{2+} -containing Hank's buffer and were loaded with DCFH-DA probe by incubating the cells at room temperature for 20 minutes in the dark. Then the cells were washed three times with Ca^{2+}/Mg^{2+} -containing Hank's buffer to remove the extracellular probe and exposed to 1 mM hydrogen peroxide (H_2O_2) by incubating the cells for 8 minutes at 37 ° C. The cells were washed three times with Ca^{2+}/Mg^{2+} -containing Hank's Buffer, and then ready for image.

Instrumentation used for image included an inverted Nikon Diaphot TME with epifluorescence optics, Lamda DG-4 illumination system equipped with a 175 watt Xenon arc lamp and a 12 bit, 12.5 MHz sensiCam CCD camera. Images were conducted immediately using 488 nm excitation wavelength/515 nm emission wavelength and recorded images were analyzed using an Axon Integrated Imaging System (Axon Instruments). The degree of the fluorescence brightness correlated directly to the extent of oxidative stress in the cell. The area fluorescent intensity of individual cells was determined using Axon Imaging Workbench Software. Mean fluorescence intensity in each well was calculated by

averaging area intensities from 15-20 cells (randomly selected) per field. The results were obtained from six repeated experiments.

3.3 Statistical analysis

All collected data were subjected to analysis of variance (ANOVA) using the general linear models of statistical analysis system software (version 8). The significant differences among multiple groups were determined by the Duncan's multiple-range test ($P < 0.05$). The paired individual comparisons were made using *t* – test, and significance was determined when $P < 0.05$.

Chapter IV Results

4.1 Quantitative analysis of flavonoids in different buckwheat species

4.1.1 Total flavonoid and rutin contents in three buckwheat species

The seed samples of three buckwheat species (*Fagopyrum esculentum, Fagopyrum homotropicum, and Fagopyrum tataricum*) were harvested in 1998, and provided by Kade Research (Morden, Manitoba, Canada). In this study, 11 buckwheat cultivars/lines were involved to represent the three different species, respectively.

F.esculentum: Koto, Mancan, Manizoba, Koban

F.homotropicum: K980856, K980855, K980854

F.tataricum: B930586, B930550, B930554, B880276

4.1.1.1 Quantification of rutin in different buckwheat species by high performance liquid chromatographic assay

A. Validation of HPLC assay for rutin

A representative chromatogram of standard rutin using the HPLC method developed in our laboratory is shown in **Figure 7**. The retention time of rutin was 6.9 minutes. The calibration curve was constructed by plotting the peak areas versus concentrations of rutin, ranging from 5 μg – 200 μg /ml over which linearity was indicated by the correlation coefficient of 0.9995. The slope, the y-intercept, and the residual sum of squares for the line of best fit were 1.84×10^4, 15.6, and 5.30×10^6, respectively (**Figure 8**).

In order to obtain the optimum time for extracting rutin from the buckwheat seed, a study of extraction kinetics was done by checking five individual time points: 5 minutes, 10 minutes, 30 minutes, 60 minutes and 120 minutes. The *F. tataricum* line B930550 was used

in this study, and the results from three repeat experiments are as shown in **Table 4** and **Figure 9**. The rutin content at the extraction times of 30 minutes, 60 minutes and 120 minutes were significantly higher than those with the extraction time of 5 minutes and 10 minutes. Five-minute extraction yielded the lowest rutin content with a mean value of 1528 mg per 100 g of seeds. No significant differences in rutin content were found from 30 minutes to 120 minutes of extraction time, which were 1662, 1664 and 1658 mg per 100 g of seeds, respectively. Therefore, 30-minute extraction time was used in our study.

The limit of detection (LOD) and the limit of quantification (LOQ) were established by the construction of a standard calibration curve in the low concentration range from 0 - 50 μg/ml. The calibration curve and the regression equation were shown in **Figure 10.** The residual sum of squares for the line of best fit was 6.90×10^{7}. The values of LOD and LOQ calculated from the equations described in Chapter III (**3.2.1.7)** were 0.6 μg/ml and 1.8 μg/ml, respectively. Mancan, B930550, and B980854 representing *F. esculentum, F. tataricum and F. homotropicum,* respectively, were used to determine the accuracy of the whole procedure and of the HPLC system for three species. The results are presented in **Table 5 and Table 6.** The recoveries of rutin for the procedure were 94.3% for *F. esculentum*, 93.8% for *F. homotropicum* and 94.0% for *F.tataricum*. No significant differences in recovery were found between the three buckwheat species. For the HPLC system, the recoveries of rutin in *F.esculentum* and *F. homotropicum* were 100.8% and 100.4%, respectively, higher than that in *F. tataricum* (94.4%). The same three buckwheat cultivar/lines were also used for the determination of repeatability. The results are presented in **Table 7**. The relative standard deviations from six determinations were 2.53% for *F. esculentum*, 4.85% for *F. homotropicum* and 1.88% for *F. tataricum.*

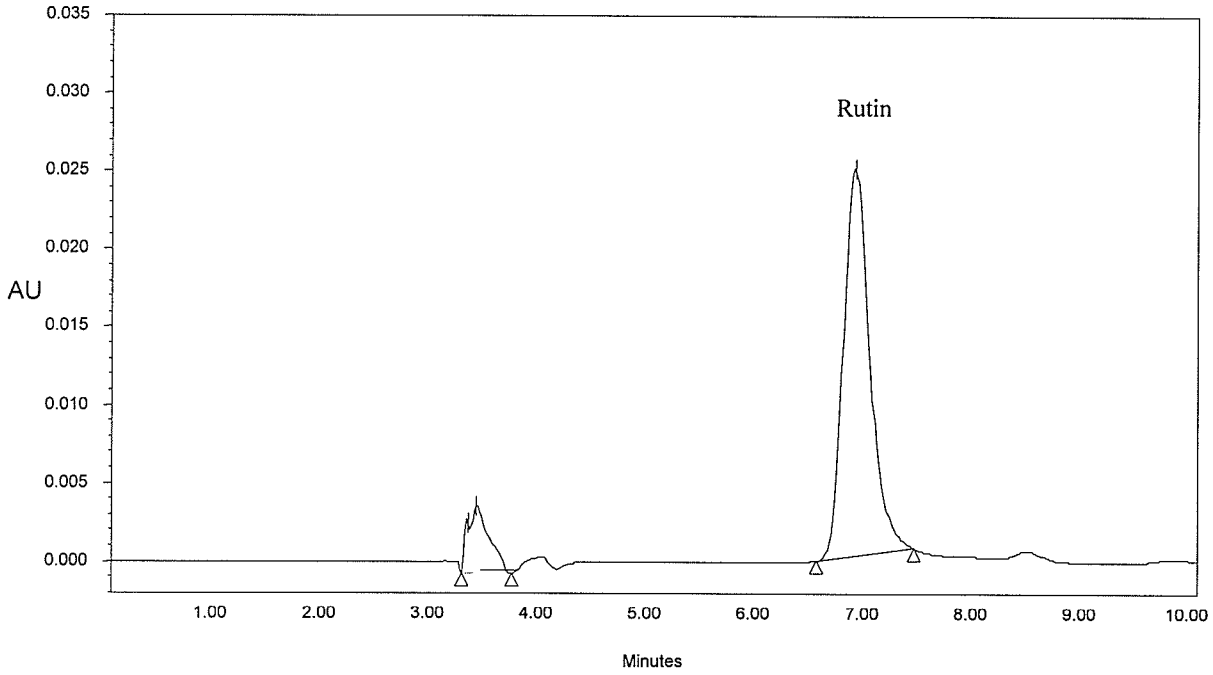


Figure 7. The HPLC chromatogram of rutin (retention time is 6.9 minute)

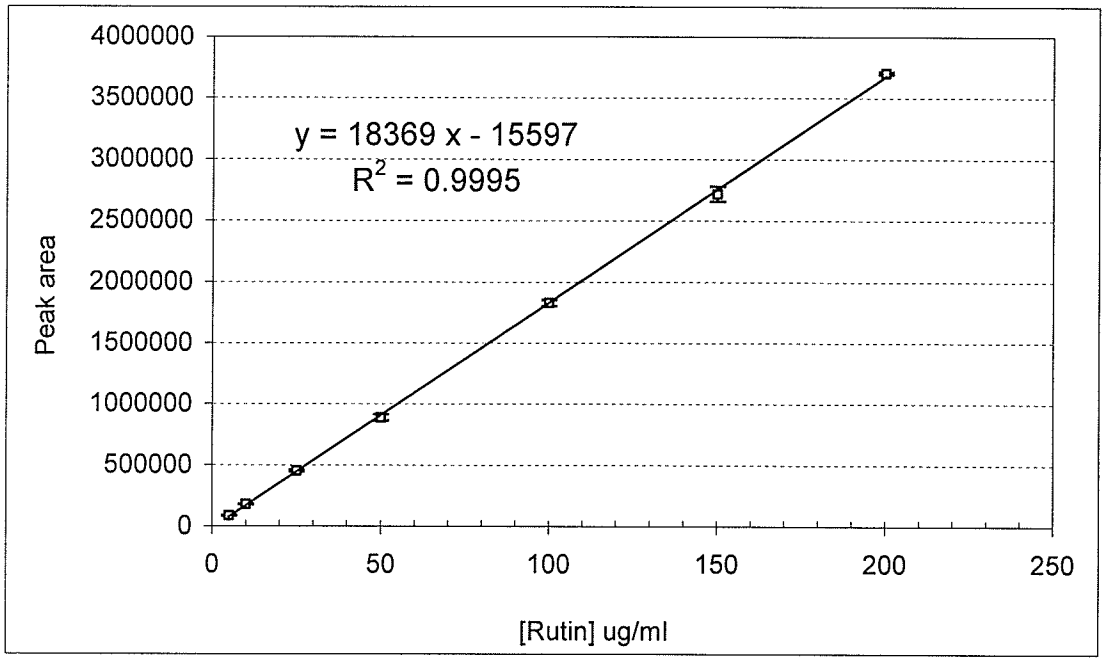


Figure 8. The HPLC calibration curve for rutin in the working concentration range (5 – 200 µg/ml). Each point on the curve represents the mean ± standard deviation (n = 2).

Table 4. Extract kinetics of rutin in Tartary buckwheat (mg/ 100 g seeds)

	Time (minutes)				
	5	10	30	60	120
	1511 1529 1544	1585 1625 1569	1630 1666 1689	1670 1630 1691	1620 1668 1685
Mean ± SD [1, 2]	1528 ± 17 [c]	1593 ± 28 [b]	1662 ± 30 [a]	1664 ± 31 [a]	1658 ± 34 [a]
RSD % [3]	1.1	1.8	1.8	1.9	2.0

[1] The data are expressed as means ± standard deviation (SD, n = 3). [2. a, b, c] Values sharing the same letter within a row are not significantly different by Duncan's multiple-range test ($P < 0.05$). [3] RSD: relative standard deviation.

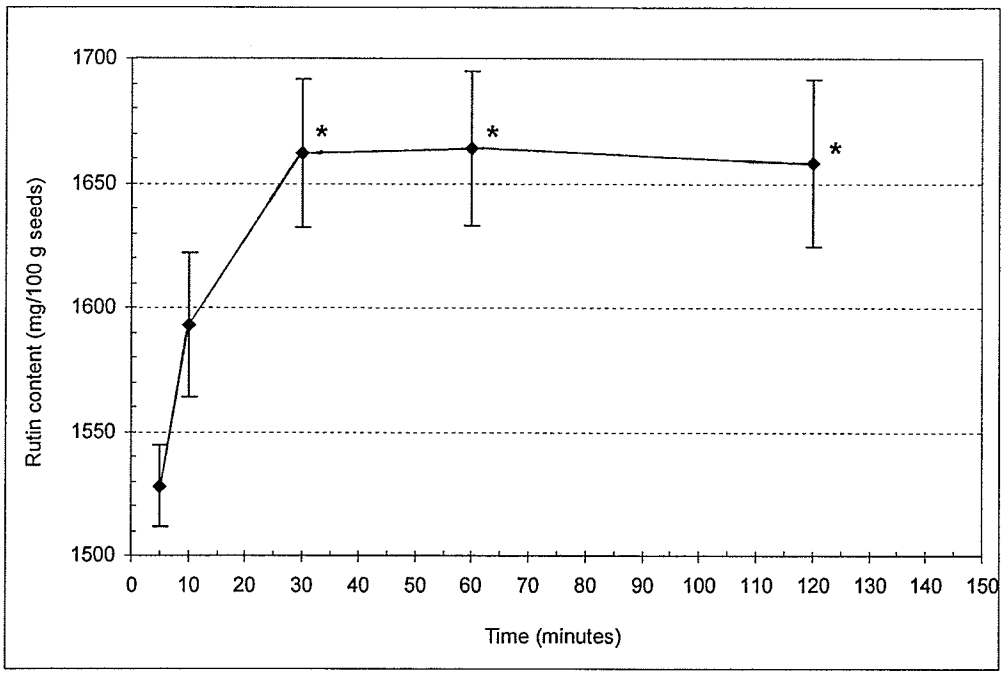


Fig. 9. Extract kinetics of rutin from buckwheat seeds. Each point on the curve represents the mean ± standard deviation (n = 3). * $P < 0.05$.

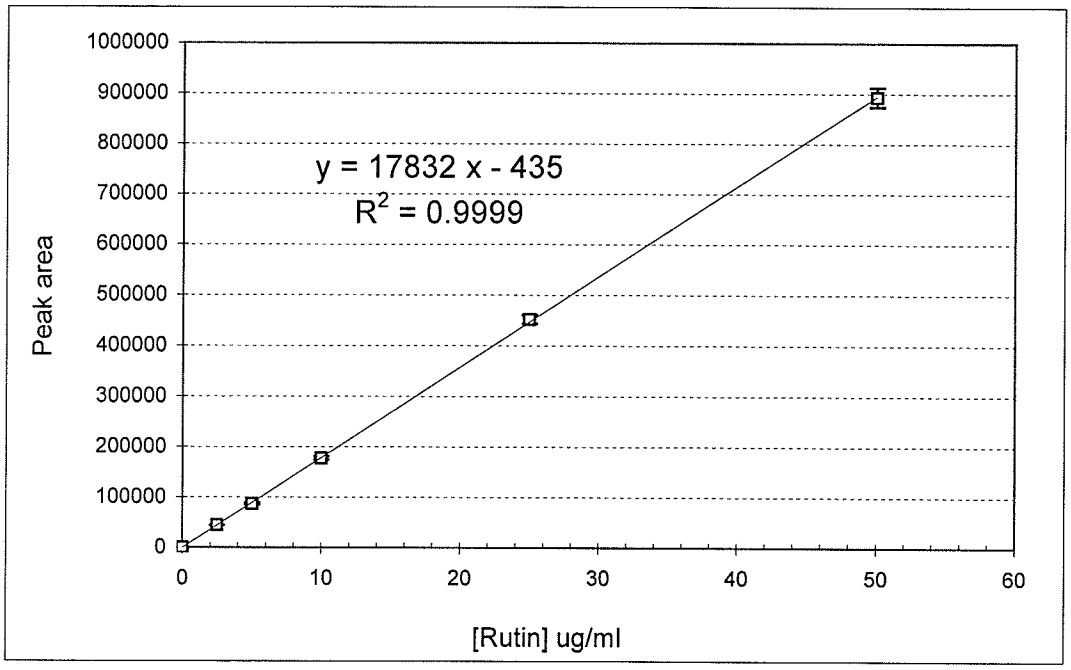


Figure 10. HPLC calibration curve of rutin in the low concentration range (0 – 50 µg/ml). Each point on the curve represents the mean ± standard deviation (n = 2).

Table 5. Accuracy of the procedure for analysis of rutin in different buckwheat species

Species	Rutin added in buckwheat extract (mg/ml)	Rutin in extract (mg/ml)		Recovery (%)	
		Measured *	Calculated	Individual	Mean
F. esculentum		$0.0050\pm1\times10^{-4}$			94.3
	0.0025	$0.0072\pm2\times10^{-4}$	0.0075	96.0	
	0.0050	$0.0095\pm2\times10^{-4}$	0.0100	95.0	
	0.0100	$0.0139\pm4\times10^{-4}$	0.0150	92.0	
F. homotropicum		$0.0074\pm4\times10^{-4}$			93.8
	0.0100	$0.0164\pm8\times10^{-4}$	0.0174	94.2	
	0.0250	$0.0309\pm14\times10^{-4}$	0.0324	95.4	
	0.0500	$0.0527\pm25\times10^{-4}$	0.0574	91.8	
F. tataricum		$0.0333\pm6\times10^{-4}$			94.0
	0.0250	$0.0553\pm10\times10^{-4}$	0.0580	95.4	
	0.0500	$0.0785\pm14\times10^{-4}$	0.0830	94.6	
	0.1000	$0.1224\pm24\times10^{-4}$	0.1330	92.0	

* The data are expressed as means ± standard deviation from three replicate determinations.

Table 6. Accuracy of HPLC system for analysis of rutin in different buckwheat species

Species	Rutin added in buckwheat extract (mg/ml)	Rutin in extract (mg/ml)		Recovery (%)	
		Measured [1]	Calculated	Individual	Mean [2]
F. esculentum		0.0053			100.8 [a]
	0.0025	$0.0081\pm1\times10^{-4}$	0.0078	103.8	
	0.0050	$0.0103\pm1\times10^{-4}$	0.0103	100.0	
	0.0100	$0.0151\pm2\times10^{-4}$	0.0153	98.7	
F. homotropicum		0.0076			100.4 [a]
	0.0100	$0.0176\pm2\times10^{-4}$	0.0176	100.0	
	0.0250	$0.0333\pm4\times10^{-4}$	0.0326	102.2	
	0.0500	$0.0570\pm7\times10^{-4}$	0.0576	99.0	
F. tataricum		0.0330			94.4 [b]
	0.0250	$0.0559\pm7\times10^{-4}$	0.0580	96.3	
	0.0500	$0.0772\pm11\times10^{-4}$	0.0830	93.0	
	0.1000	$0.1248\pm16\times10^{-4}$	0.1330	93.8	

[1]The data are expressed as means ± standard deviation from three replicate determinations.

[2 a, b] Values sharing the same letter within a column are not significantly different by Duncan's multiple-range test ($P < 0.05$).

Table 7. Repeatability of the analytical method for rutin by HPLC

Species	Rutin (mg/100 g seed)	Mean ± SD [1]	RSD (%) [2]
F. esculentum	19.4 19.7 20.7 20.1 19.6 19.2	19.8 ± 0.5	2.53
F. homotropicum	75.0 77.9 70.8 78.4 74.3 69.5	74.3 ± 3.6	4.85
F. tataricum	1675.7 1653.3 1630.0 1692.5 1621.0 1614.4	1647.8 ± 31.0	1.88

[1] The data are expressed as means ± standard deviation of six replicate determinations.

[2] RSD: relative standard deviation.

B. Rutin content in different buckwheat species

The representative HPLC chromatograms for rutin in buckwheat extracts of the three species are shown in **Figure 11, Figure 12,** and **Figure 13.** The retention times of rutin in the three different buckwheat species meet the retention time of the rutin standard with a value of 6.9 minutes. There were no interfering peaks with any of the buckwheat species.

Quantitative analysis of rutin in 11 buckwheat cultivars/lines was performed in triplicate and the results are presented in **Table 8** and **Table 9**. The rutin content in the three buckwheat species were: 17.9-25.2 mg/100 g seed in *F. esculentum*, 74.3-142.6 mg/100 g seed in *F. homotropicum*, and 1569.9-1794.8 mg/100 g seed in *F. tataricum*, respectively. The average rutin contents differed significantly among the three buckwheat species, 1669.2 mg/100 g seed in *F. tataricum*, 101.4 mg/100 g seed in *F. homotropicum*, and 20.0 mg/100 g seed in *F. esculentum*.

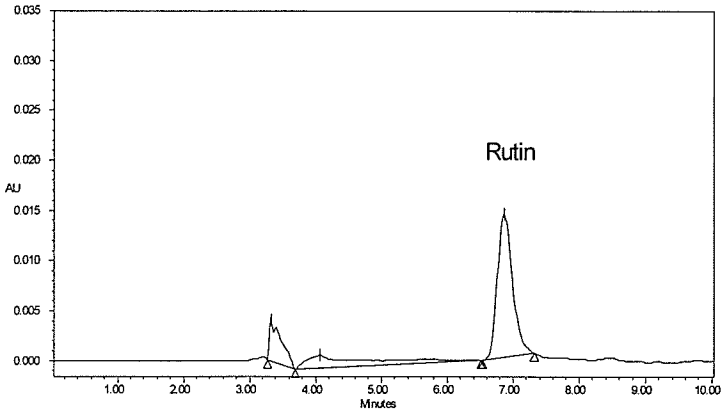


Figure 11. The HPLC chromatogram of *F. esculentum* extract at 360 nm (retention time is 6.87 minute)

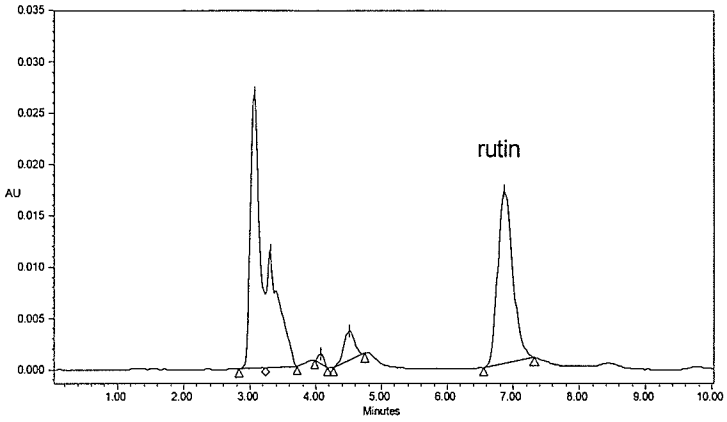


Figure 12. The HPLC chromatogram of *F. homotropicum* extract at 360 nm (retention time is 6.87 minute)

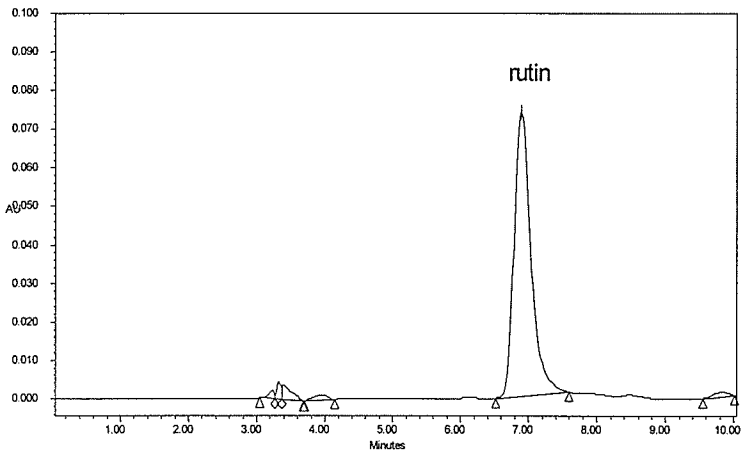


Figure 13. The HPLC chromatogram of *F. tataricum* extract at 360 nm (retention time is 6.90 minute)

Table 8. Rutin content in different buckwheat cultivars/lines (mg/100 g seed) [1, 2]

F.esculentum		*F. homotropicum*		*F.tataricum*	
Line	Mean ± SD	Line	Mean ± SD	Line	Mean ± SD
Koban	19.8 ± 0.5 [b]	K980855	87.5 ± 0.6 [b]	B930550	1647.8 ± 30.3 [b]
Koto	16.9 ± 0.7 [c]	K980856	142.6 ± 3.3 [a]	B930554	1569.9 ± 21.3 [c]
Mancan	25.2 ± 0.7 [a]	K980854	74.3 ± 3.5 [c]	B930586	1794.8 ± 25.5 [a]
Manizoba	17.9 ± 1.0 [c]			B880276	1664.5 ± 15.8 [b]

[1] The data are expressed as means ± standard deviation (n = 3). [2. a, b, c] Values sharing the same letter within a row are not significantly different by Duncan's multiple-range test ($P < 0.05$).

Table 9. Rutin content in different buckwheat species (mg/100 g seeds)

Species	Mean ± SD [1, 2]
F.esculentum	20.0 ± 3.7 [c]
F. homotropicum	101.4 ± 36.2 [b]
F. tataricum	1669.2 ± 93.3 [a]

[1] The rutin content for each species are expressed as means ± standard deviation from the cultivars/lines within the species. [2. a, b] Values sharing the same letter within a column are not significantly different by Duncan's multiple range test ($P < 0.05$).

4.1.1.2 Total flavonoids in three buckwheat species

Quantitative analysis of total flavonoids in 11 buckwheat culivars/lines was performed in triplicate and results were presented in **Table 10**. The flavonoid contents of buckwheat were significantly different depending on the buckwheat species, ranging from 28.4 to 44.4 mg/100 g seed for *F. esculentum*, 288.1 to 409.9 mg/100 g seed for *F. homotropicum*, and 1954.0 to 2152.9 mg/100 g seed for *F. tataricum*.

Table 10. Total flavonoids in different buckwheat species (mg/100 g seed)

	F.esculentum		*F. homotropicum*		*F.tataricum*	
	Line	Flavonoids	Line	Flavonoids	Liner	Flavonoids
	Koban	32.7	B980855	353.6	B930550	2017.3
	Koto	42.2	B980856	409.9	B930554	1954.0
	Mancan	44.4	B980854	288.1	B930586	2152.9
	Manizoba	28.4			B880276	2027.5
Mean ± SD [1,2]	36.9 ± 7.6 [c]		350.5 ± 60.9 [b]		2037.9 ± 83.3 [a]	

[1] The data are expressed as means ± standard deviation. [2 a, b, c] Values sharing the same letter within a row are not significantly different by Duncan's multiple-range test ($P < 0.05$).

4.1.2 Rutin content in *F. tataricum*

In this study, four *F. tataricum* lines (B830222, K970848, B930555 and B770198) were used to investigate the rutin content in the seeds and leaves of the species *F. tataricum* using a spectrophotometric assay. The results of the rutin content in the leaves and seeds of individual plants for each line are presented in **Table 11** and **Table 12,** respectively. B930555 had the highest rutin content in the leaves with an average of 6.59 % of dry matter, followed by B770198 (5.89 %). B830222 and K970848 showed a significantly ($P < 0.05$) lower rutin content in the leaves of 5.41 % and 5.68 %, respectively, than that found in B930555. However, the data from the seed samples of individual plants indicated that the line B830222 had the highest rutin content with an average of 1.98 %, followed by the K970848 (1.81 %) and the B770198 (1.88 %). The lowest rutin content in the seeds was found in B930555 with an average of 1.76 %. The rutin content in different buckwheat line seeds, which were obtained from the mixture of individual plants in each line were determined six times. B830222 also showed the highest rutin content (2.05 %) followed by K970848 (1.89 %) and B770198 (1.87 %), and the lowest rutin content was still found in B930555 seeds with an average of 1.71 %(**Table 13**).

Table 11. Rutin content in the leaves of individual plants (%)

Plant number	B830222	K970848	B930555	B770198
1	4.41	5.51	8.08	5.78
2	4.19	5.71	6.03	5.75
3	5.33	5.29	8.51	7.36
4	5.53	6.69	5.60	5.97
5	6.19	6.07	6.49	6.63
6	5.77	6.39	6.36	6.15
7	5.88	5.38	5.88	4.64
8	5.36	4.74	5.75	4.44
9	6.69	5.30		6.63
10	5.39			5.07
11	4.49			6.13
12	6.01			6.12
13	5.06			
Number of plants	13	9	8	12
Mean ± SD [1,2]	5.41 ± 0.73 [b]	5.68 ± 0.61 [b]	6.59 ± 1.10 [a]	5.89 ± 0.84 [a b]

[1] The data are expressed as means ± standard deviation. [2 a, b, c] Values sharing the same letter within a row are not significantly different by Duncan's multiple-range test ($P < 0.05$).

Table 12. Rutin content in the seeds of individual plants (%)

Plant number	B830222	K970848	B930555	B770198
1	1.99	2.06	1.64	1.71
2	1.83	2.03	1.78	1.86
3	2.06	1.84	1.74	1.71
4	1.92	1.90	1.84	1.88
5	1.95	1.68	1.83	1.92
6	2.06	1.89	1.77	1.90
7	2.13	1.70	1.72	1.84
8	1.86	1.83	1.74	1.80
9	1.97	1.80	1.77	20.1
10	2.04	1.95	1.72	1.88
11		1.71		1.94
12		1.77		1.78
13		1.89		1.86
Number of plants	10	13	10	13
Mean ± SD [1,2]	1.98 ± 0.09 [a]	1.81 ± 0.10 [b]	1.76 ± 0.06 [c]	1.88 ± 0.07 [b]

[1] The data are expressed as means ± standard deviation. [2 a, b, c] Values sharing the same letter within a row are not significantly different by Duncan's multiple-range test ($P < 0.05$).

Table 13. Rutin content in the seed of different lines (%)

	B830222	K970848	B930555	B770198
	2.08	1.83	1.80	1.94
	2.06	1.86	1.76	1.87
	1.95	1.95	1.74	1.82
	1.99	1.82	1.72	1.80
	2.12	1.90	1.64	1.98
	2.10	1.96	1.62	1.83
Mean ± SD [1,2]	2.05 ± 0.06 [a]	1.89 ± 0.06 [b]	1.71 ± 0.07 [c]	1.87 ± 0.07 [b]

[1] The data are expressed as means ± standard deviation (n = 6). [2 a, b, c] Values sharing the same letter within a row are not significantly different by Duncan's multiple-range test ($P < 0.05$).

4.1.3 Rutin content in *F. homotropicum*

4.1.3.1 Selection of a *F. homotropicum* for the breeding with *F.esculentum*

To investigate the rutin content in *F. homotropicum* leaves and seed, individual leaf samples within the lines 990049 and 990050, and individual seed samples within the line 990051 were analyzed using the spectrophotometric assay. The results are presented in **Table 14**. To select an *F. homotropicum* with a high rutin content for developing high rutin varieties, six lines were analysed: B930290, K950818, K970851, K980853, K980855, and K980856 (**Table 15**).

Table 14. Rutin content in the individual *F. homotropicum* seed and leaf samples (% of dry matter)

Plant number	*F. homotropicum* seed 990051	*F. homotropicum* leaf 990049	*F. homotropicum* leaf 990050
1	0.037	1.28	0.84
2	0.051	1.29	1.05
3	0.046	1.68	1.34
4	0.031	2.57	1.44
5	0.038	1.32	0.66
6	0.029	1.00	3.22
7	0.043	2.59	2.46
8	0.052	2.56	1.98
9	0.035	3.32	2.52
10	0.078	3.36	1.62
11		3.49	0.71
12		2.07	
13		1.42	
14		0.92	
15		1.87	
16		1.63	
17		0.85	
18		1.39	
19		0.80	
Number of plants	10	19	11
Mean ± SD [1, 2]	0.044 ± 0.014 [b]	1.86 ± 0.88 [a]	1.90 ± 1.24 [a]

[1] The data are expressed as means ± standard deviation. [2 a, b, c] Values sharing the same letter within a row are not significantly different by Duncan's multiple-range test ($P < 0.05$).

Table 15. Rutin content in the different *F. homotropicum* lines (% of dry matter)

Cultivar	Rutin content [1, 2]
B930290	0.055 ± 0.002 [f]
K950818	0.082 ± 0.003 [e]
K970851	0.266 ± 0.006 [a]
K980853	0.192 ± 0.004 [b]
K980855	0.170 ± 0.002 [c]
K980856	0.154 ± 0.004 [d]

[1] The data are expressed as means ± standard deviation (n = 3). [2] [a, b, c, d, e, f] Values sharing the same letter within a column are not significantly different by Duncan's multiple range test ($P < 0.05$).

4.1.3.2 New cross Q010001 between *F. homotropicum* and *F. esculentum*

The rutin content of the cross Q010001 and its parents *F. homotropicum* K970851 and T990036, a cross between *F. homotropicum* and *F. esculentum* are presented in **Table 16.** To examine the distribution of rutin content in the second generation of Q010001, 35 individual leaf samples and 117 individual seed samples were analyzed using the HPLC assay. The results are presented in **Table 17** and **Table 18,** respectively. The distributions of the rutin content in the leaf and seed are shown in **Figure 14** and **Figure 15**. Plant samples of the second generation were used to further determine the correlation of the rutin content between buckwheat seed and leaf and the results indicated that there was no correlation of rutin content between buckwheat leaves and seeds with a correlation coefficient value of 0.1866 **(Figure 16).**

Table 16. Rutin contents in the cross Q010001 and its parents K970851 and T990036

Buckwheat	Rutin content (mg/100 g seed) *
T990036	77 ± 3
K970851	266 ± 6
Q010001	121 ± 4

* The data are expressed as means ± standard deviation (n = 3).

Table 17. Rutin content in buckwheat leaves of Cross Q-F2 populations

Plant number	Rutin content (% of dry matter)	Plant number	Rutin content (% of dry matter)
1	1.75	21	2.70
2	1.51	22	2.19
3	2.05	23	3.09
4	2.13	24	1.95
5	1.54	25	2.57
6	1.01	26	1.85
7	1.58	27	2.23
8	2.11	28	0.89
9	2.47	29	1.86
10	1.89	30	2.55
11	2.61	31	3.35
12	3.58	32	3.92
13	1.88	33	2.54
14	2.51	34	5.39
15	1.89	35	4.05
16	1.79		
17	2.00		
18	2.78		
19	2.00		
20	1.88		

Table 18. Rutin content in buckwheat seeds of Cross Q-F2 populations

Plant number	Rutin content (mg/100 g seed)	Plant number	Rutin content (mg/100 g seed)	Plant number	Rutin content (mg/100 g seed)
1	79	41	12	81	29
2	180	42	16	82	32
3	61	43	8	83	11
4	32	44	107	84	44
5	11	45	4	85	47
6	12	46	24	86	22
7	13	47	83	87	10
8	5	48	27	88	72
9	ND	49	37	89	7
10	ND	50	22	90	4
11	12	51	30	91	10
12	26	52	37	92	18
13	41	53	48	93	17
14	12	54	24	94	11
15	4	55	36	95	14
16	30	56	11	96	53
17	6	57	42	97	55
18	73	58	18	98	36
19	31	59	84	99	19
20	32	60	23	100	43
21	22	61	46	101	39
22	47	62	31	102	19
23	27	63	23	103	29
24	13	64	16	104	40
25	66	65	55	105	10
26	15	66	58	106	10
27	8	67	32	107	68
28	62	68	8	108	15
29	12	69	14	109	17
30	14	70	15	110	37
31	258	71	39	111	15
32	4	72	62	112	21
33	26	73	6	113	15
34	23	74	65	114	98
35	48	75	13	115	11
36	76	76	24	116	44
37	84	77	17	117	3
38	40	78	24		
39	6	79	26		
40	17	80	12		

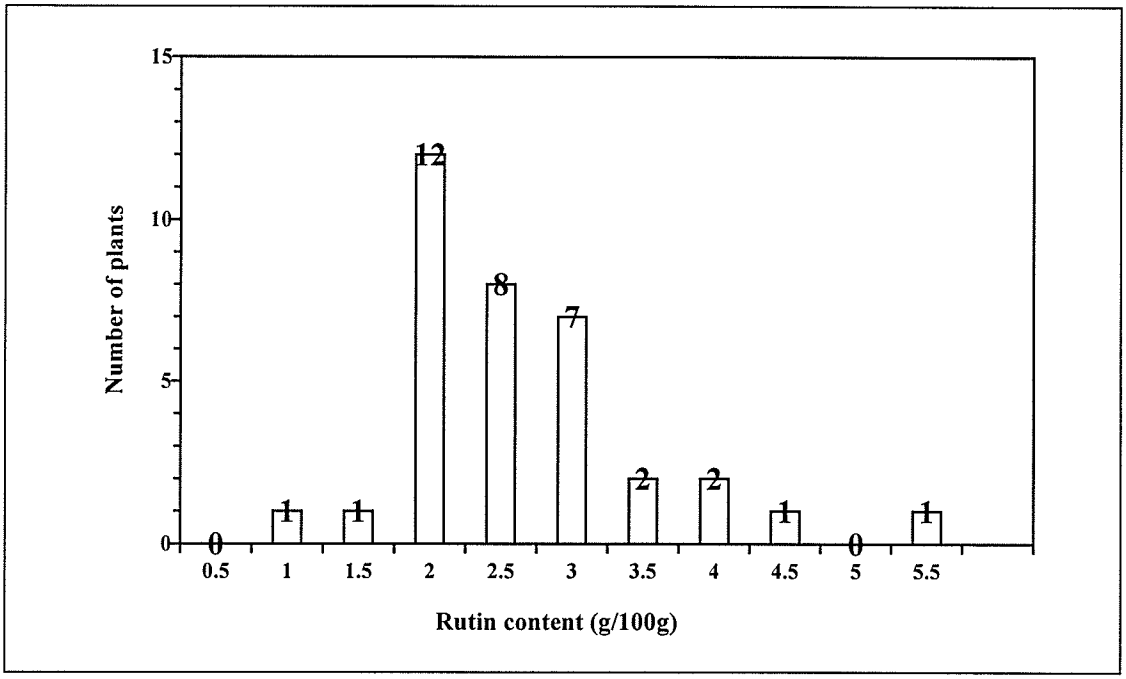


Figure 14. Distribution of rutin content in the leaf of the second generation of the cross Q010001 (N=35)

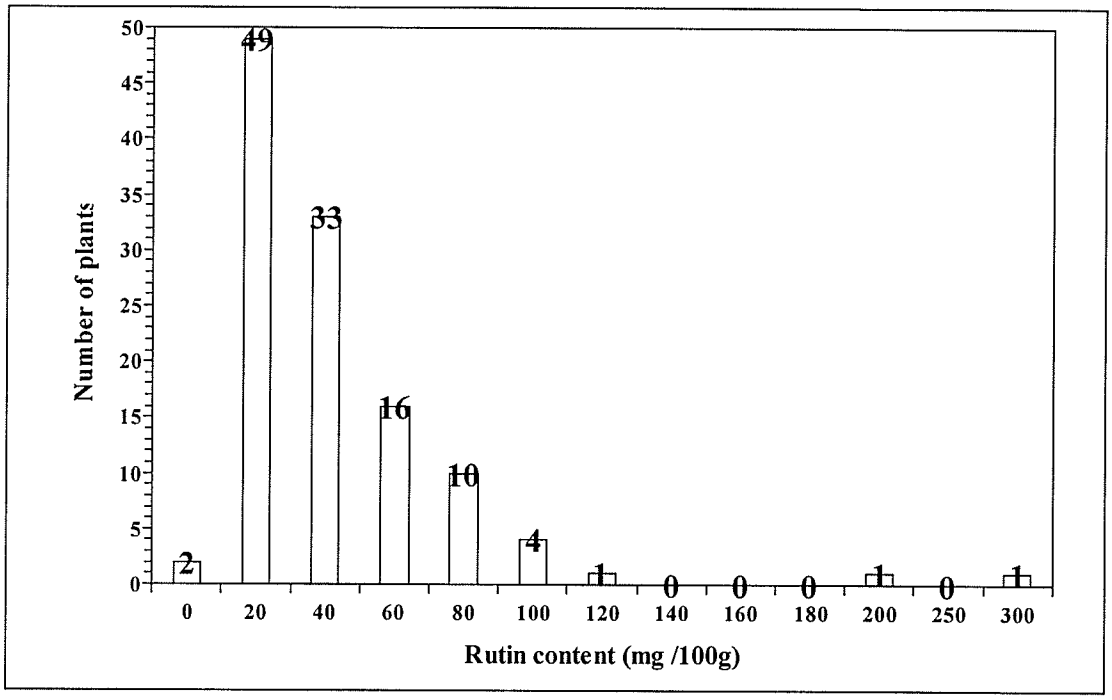


Figure 15. Distribution of rutin content in the seed of the second generation of the cross Q010001 (N=118)

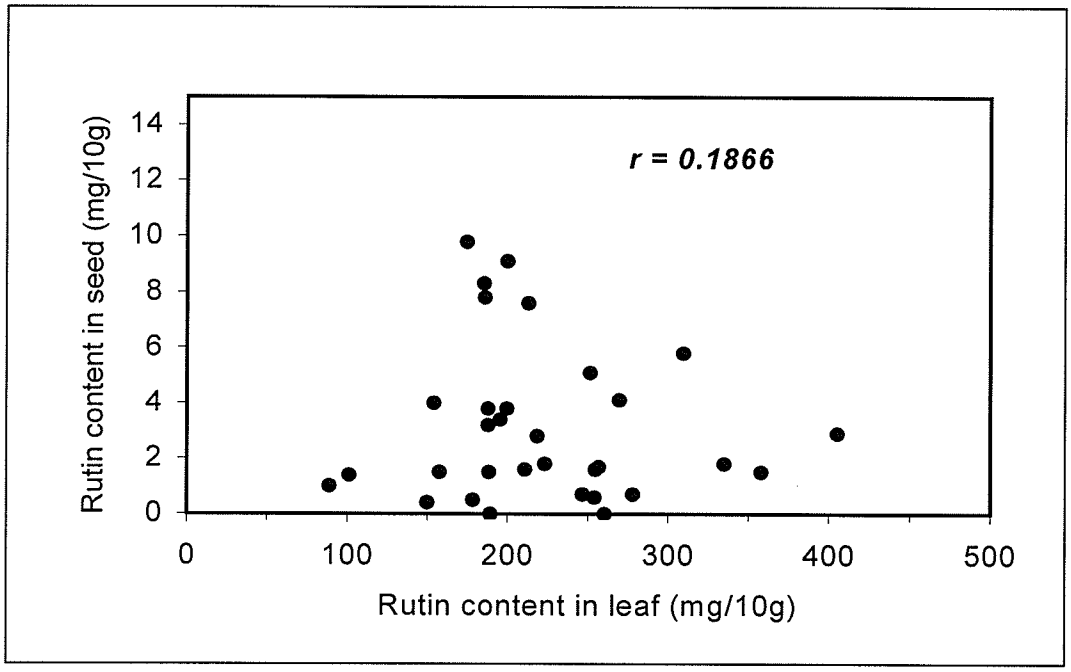


Figure 16. Correlation of rutin contents between leaf and seed in the second generation of the cross Q010001 (N=35)

4.2 Protective effects of flavonoids and buckwheat against lipid peroxidation in low-density lipoprotein of rabbits

4.2.1 Effects of rutin and quercetin on low-density lipoprotein peroxidation in hydrophilic and lipophilic free radical generating systems

Applying both the thiobarbituric acid-reactive substances (TBARS) test and conjugated diene measurement to isolated LDL from the plasma of rabbits, we compared the antioxidant effectiveness of quercetin, rutin, α-tocopherol (vitamin E), and ascorbic acid (vitamin C) in hydrophilic and lipophilic free radical generating systems. The objective was to elucidate the difference in antioxidant activities of these compounds in both aqueous and lipid environments. The azo-compounds 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) were used as a hydrophilic and lipophilic free radical generator, respectively.

4.2.1.1 Antioxidant activities of the test compounds with low-density lipoprotein in aqueous or lipid environment using TBARS assay

To compare the effectiveness of antioxidants in both lipophilic and hydrophilic free radical generating systems, the concentrations of AAPH and AMVN producing a similar amount of MDA in LDL were used. MDA production in LDL was examined at various concentrations of each azo-compound, and the specific concentrations of AAPH and AMVN inducing a similar amount of MDA production were chosen for the subsequent study. The results, shown in **Figure 17,** indicated that as a free radical initiator, AMVN was more effective than AAPH in inducing LDL peroxidation. AMVN (4 mM) and AAPH (18 mM) generating a similar quantitie of MDA were selected for comparative work.

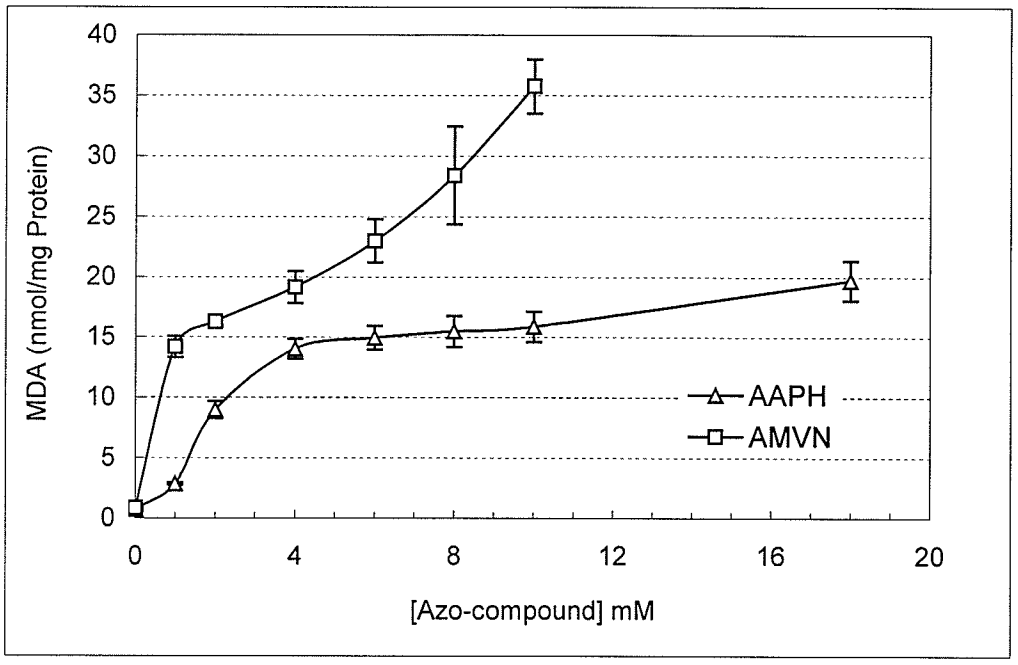


Figure 17. MDA formation in LDL in a hydrophilic or a lipophilic free radical generating system. LDL (0.5 mg protein / ml) was incubated with varying concentrations of AAPH or AMVN for 1 hour at 37 ^{0} C. Each point on the curves represents the mean ± standard deviation of six independent experiments. No significant difference in MDA production of LDL was found between 18 mM AAPH system (19.5 ± 1.8 nmol/mg protein) and 4 mM AMVN system (19.1 ± 1.6 nmol/mg protein) ($P < 0.05$).

The protective effects of quercetin, rutin, α-tocopherol and ascorbic acid against LDL peroxidation in a hydrophilic free radical generating system are shown in **Figure 18, Figure 19, Figure 20** and **Figure 21**, respectively. The inhibitory capacities of all test compounds increased with the concentration. At concentrations from 1 to 15 μM, quercetin significantly reduced MDA production with 29% inhibition at 2.5 μM and 78% inhibition at 15 μM. Thereafter its effect remained stable (**Figure 18**). At 2.5 μM, rutin produced a 20% inhibition of MDA production, rising to 77% inhibition when its concentration was increased to 30 μM **(Figure 19)**. A concentration of 1.56 μM α-tocopherol provided a 25 % protection against lipid peroxidation. A 79% reduction in MDA formation was obtained when the concentration of α-tocopherol was increased to 50 μM **(Figure 20)**. Ascorbic acid was far less effective in inhibiting lipid peroxidation. It significantly inhibited 13% MDA production at a concentration of 50 μM, with its protective capacity against lipid peroxidation increasing linearly until the concentration reached 300 μM **(Fig. 21)**. The IC_{50} values, the concentrations inhibiting 50% of the control, were the criterion used to compare the efficacy of antioxidants and calculated graphically from dose-response curves. In this study, the IC_{50} values against AAPH – induced LDL peroxidation were 4.1 μM for quercetin, 5.8 μM for α-tocopherol, 7.1 μM for rutin and 157.3 μM for ascorbic acid **(Table 19).** With the lipophilic free radical generating system, no compounds except for α-tocopherol exhibited a significantly protective effect against lipid peroxidation in the 0 – 30 μM concentration range. The antioxidant activity of α-tocopherol was lower in the AMVN system than with AAPH. A concentration of 1.56 μM α-tocopherol did not significantly inhibit lipid peroxidation, while a 25% reduction in MDA production was obtained when the concentration increased to 6.25

μM. The IC_{50} value of α-tocopherol against AMVN - induced LDL peroxidation was 16 μM **(Table 19)**.

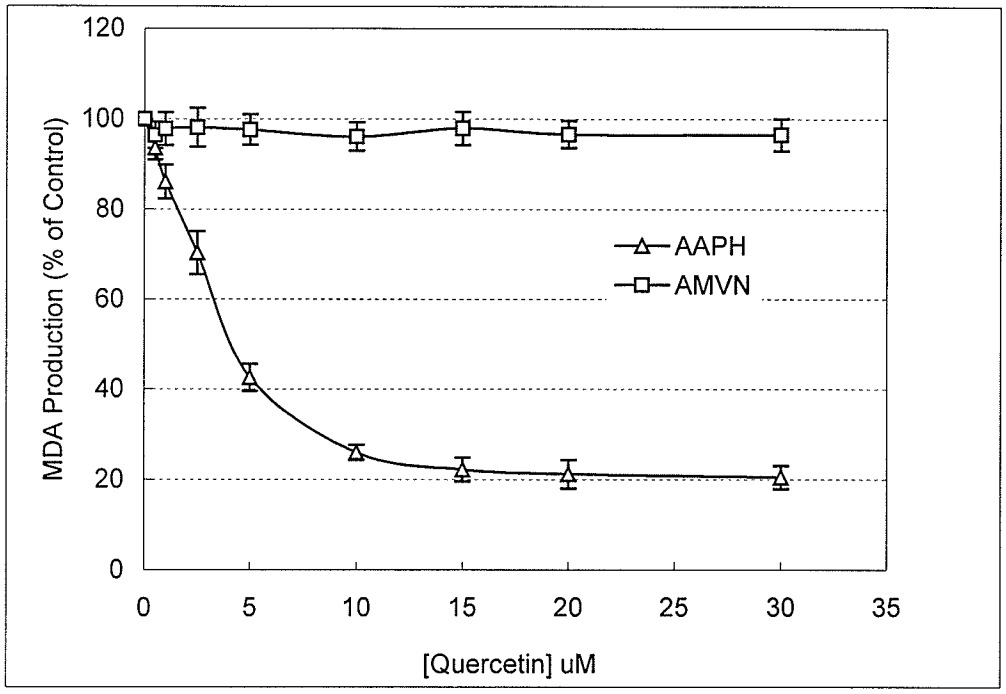


Figure 18. A comparison of the effect of quercetin on AAPH and AMVN induced MDA production in LDL. LDL (0.5 mg protein / ml) was co-incubated with quercetin at different concentrations and 18 mM AAPH or 4 mM AMVN for 1 hour at 37 ^{0} C. Each point on the curves represents the mean ± standard deviation of six independent experiments. Quercetin, at the concentration > 1.0 μM, significantly reduced MDA production in AAPH system but no inhibitory effect on MDA production was observed in AMVN system ($P < 0.05$).

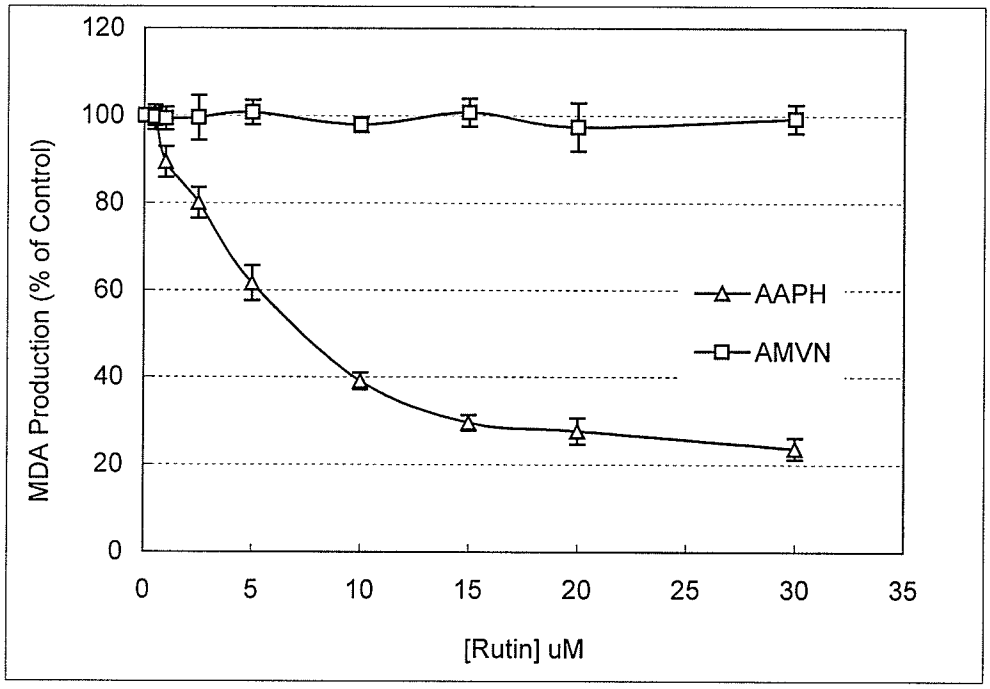


Figure 19. A comparison of the effect of rutin on AAPH and AMVN induced MDA production in LDL. LDL (0.5 mg protein / ml) was co-incubated with rutin at different concentrations and 18 mM AAPH or 4 mM AMVN for 1 hour at 37 ^{0} C. Each point on the curves represents the mean ± standard deviation of six independent experiments. Rutin, at the concentration > 1.0 μM, significantly reduced MDA production in AAPH system but no inhibitory effect on MDA production was observed in AMVN system ($P < 0.05$).

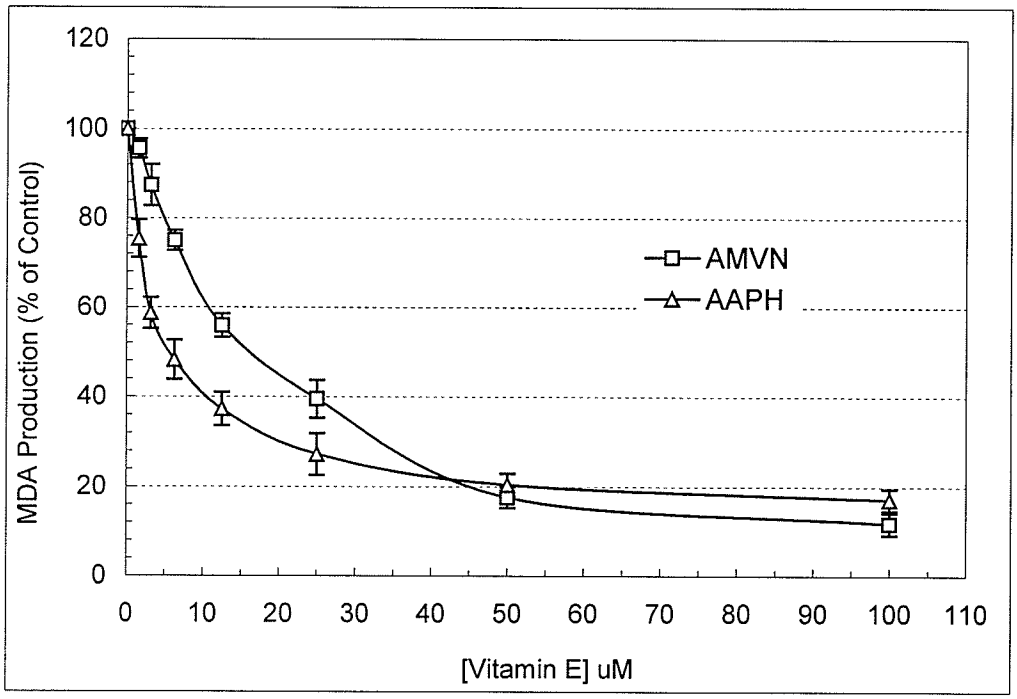


Figure 20. A comparison of the effect of α-tocopherol on AAPH and AMVN induced MDA production in LDL. LDL (0.5 mg protein / ml) was co-incubated with vitamin E at different concentrations and 18 mM AAPH or 4 mM AMVN for 1 hour at 37 ^{0} C. Each point on the curves represents the mean ± standard deviation of six independent experiments. Vitamin, at the concentration > 1.6 μM, significantly reduced MDA production in AAPH system, and inhibitory effect on MDA production was also observed in AMVN system when the concentration > 3.1 μM ($P < 0.05$)

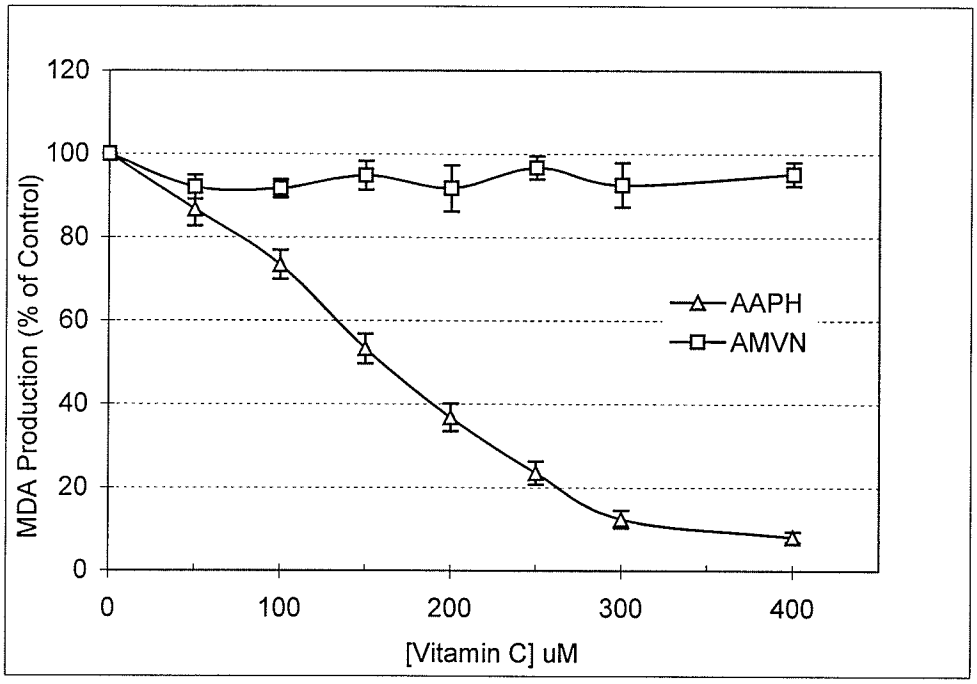


Figure 21. A comparison of the effect of ascorbic acid on AAPH and AMVN induced MDA production in LDL. LDL (0.5 mg protein / ml) was co-incubated with vitamin C at different concentrations and 18 mM AAPH or 4 mM AMVN for 1 hour at 37 ^{0} C. Each point on the curves represents the mean ± standard deviation of six independent experiments. Vitamin C, at the concentration > 50 μM, significantly reduced MDA production in AAPH system but no inhibitory effect on MDA production was observed in AMVN system ($P < 0.05$).

Table 19. IC_{50} values of antioxidants against LDL peroxidation [1, 2]

Antioxidants	IC_{50} (μM) in AAPH System	IC_{50} (μM) in AMVN System
Quercetin	4.1 ± 0.4^{d}	UD [3]
Rutin	7.1 ± 0.5^{b}	UD
α-tocopherol	5.8 ± 1.1^{c}	16.0 ± 1.6
Ascorbic acid	157.3 ± 8.8^{a}	UD [3]

[1] The IC_{50} values, the concentrations inhibiting 50% of the control, were calculated graphically from dose-response curves. [2] The data are expressed as means ± standard deviation (n = 6) and the values sharing the same letter are not significantly different by Duncan ($P < 0.05$). [3] UD = undetectable.

4. 2.1.2 Effects of test antioxidants on the early stage of lipid peroxidation in an aqueous area surrounding LDL by conjugated diene assay

The time plots of conjugated diene formation in LDL in the hydrophilic free radical generating system in the absence or presence of selected antioxidants at various concentrations are shown in **Figure 22 – Figure 25**. The concentration – response effects of all test compounds on the lag time of LDL peroxidation are shown in **Figure 26 – Figure 29**. To compare the effects of the test compounds on lipid peroxidation, AUC, lag time and slope relative to the control for each test concentration were calculated and are presented in **Table 20**. Compared with the control, quercetin, rutin, α-tocopherol and ascorbic acid produced a significant reduction in AUC at all test concentrations except for α-tocopherol at 0.63 μM. Linear regression analysis revealed an inverse linear correlation between concentration and relative AUC value for quercetin ($R^2 = 0.9928$, $\alpha = 0.01$), rutin ($R^2 = 0.9949$, $\alpha = 0.01$), α-tocopherol ($R^2 = 0.9622$, $\alpha = 0.01$) and ascorbic acid ($R^2 = 0.8681$, $\alpha = 0.05$). All compounds also demonstrated a concentration – response effect on the lag time of LDL peroxidation. The relative lag time increased linearly for all compounds: quercetin ($R^2 = 0.9991$, $\alpha = 0.01$), rutin ($R^2 = 0.9940$, $\alpha = 0.01$), α-tocopherol ($R^2 = 0.9768$, $\alpha = 0.01$) and ascorbic acid ($R^2 = 0.8774$, $\alpha = 0.05$). The concentration increasing the lag time to 50% greater than that of the control (CLT_{50}) was the criterion used to compare the efficacy of antioxidants. The CLT_{50} values were determined graphically from the dose-response curves. Quercetin and rutin with CLT_{50} values of 0.50 μM and 0.81 μM, respectively, produced a greater increase in lag time than α-tocopherol (3.39 μM). Ascorbic acid while having the highest CLT_{50} value of 15.00 μM showed the weakest ability to delay the lag time. No significant difference in CLT_{50} value was found between quercetin and rutin. The minimum concentrations of the test

compounds to produce a significant decrease in slope relative to the control were as follows: quercetin at 0.5 μM with a 7.2% decrease; rutin at 2 μM with a 15.2% decrease; and α-tocopherol at 5 μM with 21.1% decrease. In comparison with the control, ascorbic acid significantly increased the slope at all test concentrations ($P < 0.05$). No linear correlation between relative slope and concentration was observed with any test compound.

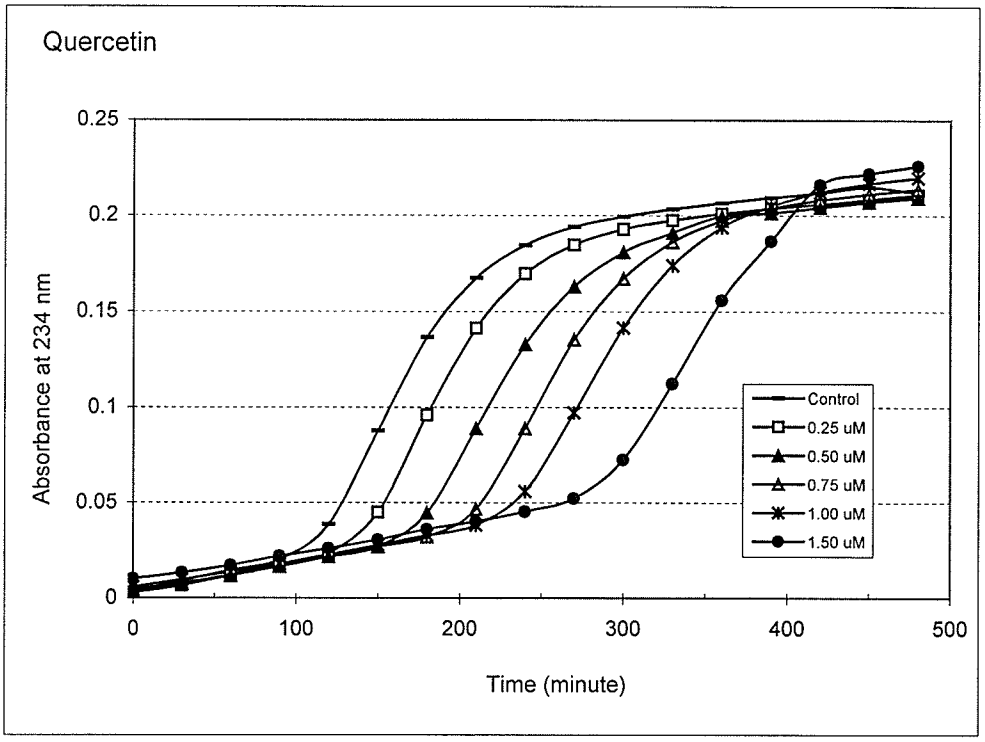


Figure 22. Oxidation – time curves of LDL in a hydrophilic free radical (AAPH) generating system. LDL (0.1 mg protein /ml) was co-incubated with 1.2 mM AAPH and quercetin at different concentrations for 8 hours at 37 ^{0}C. Each point on the curves represents the mean ± standard deviation of three independent experiments.

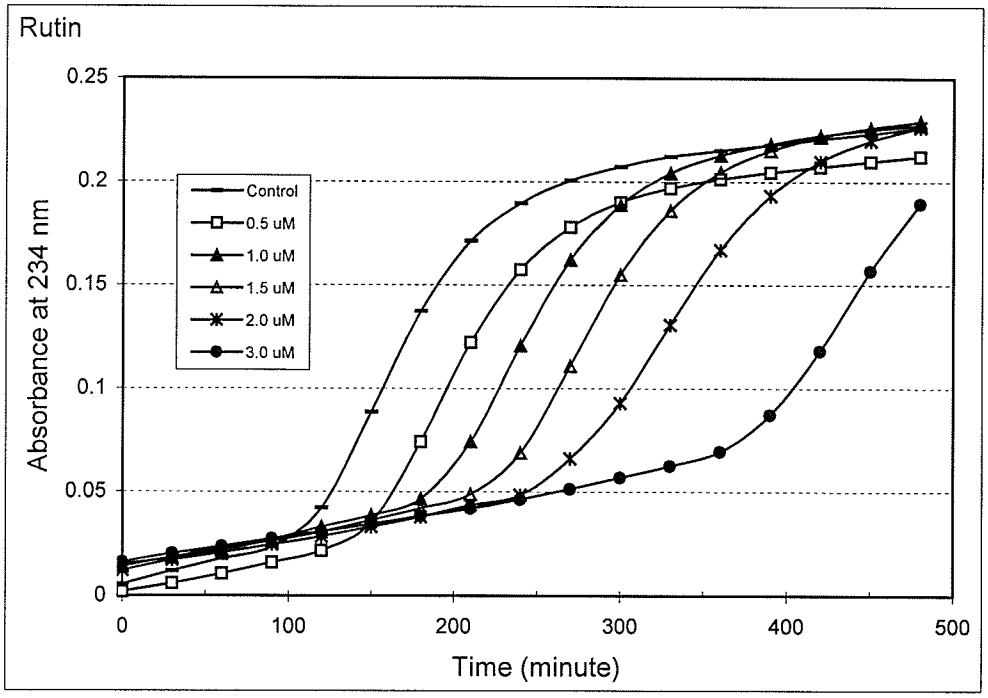


Figure 23. Oxidation – time curves of LDL in the hydrophilic free radical (AAPH) generating system. LDL (0.1 mg protein /ml) was co-incubated with 1.2 mM AAPH and rutin at different concentrations for 8 hours at 37 ^{0}C. Each point on the curves represents the mean ± standard deviation of three independent experiments.

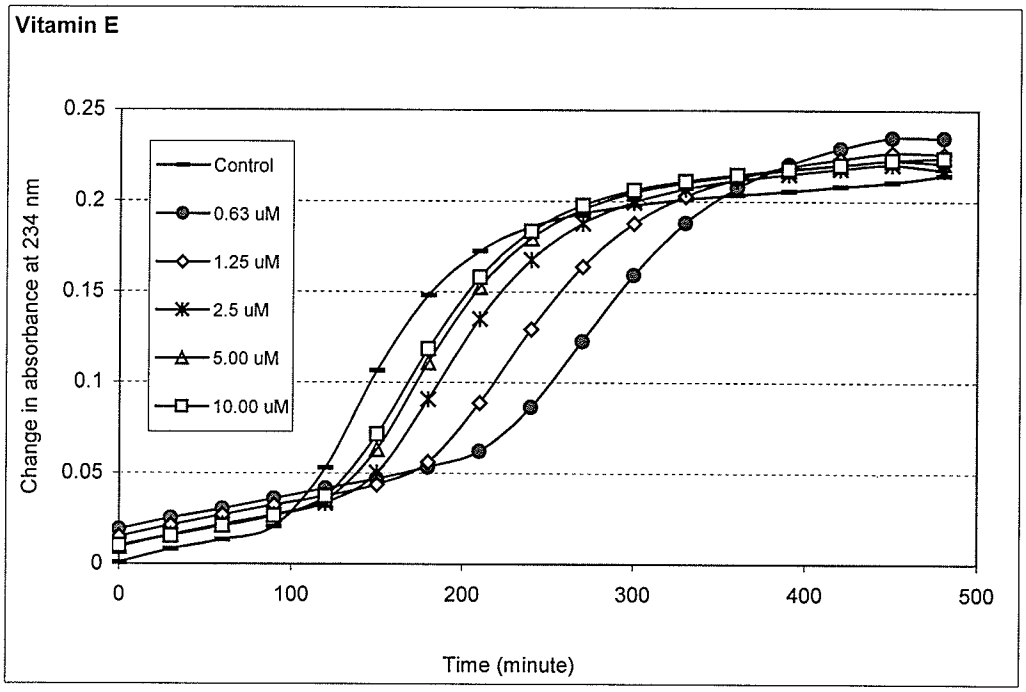


Figure 24. Oxidation – time curves of LDL in the hydrophilic free radical generating system. LDL (0.1 mg protein /ml) was co-incubated with 1.2 mM AAPH and Vitamin E at different concentrations for 8 hours at 37 ^{0}C. Each point on the curves represents the mean ± standard deviation of three independent experiments.

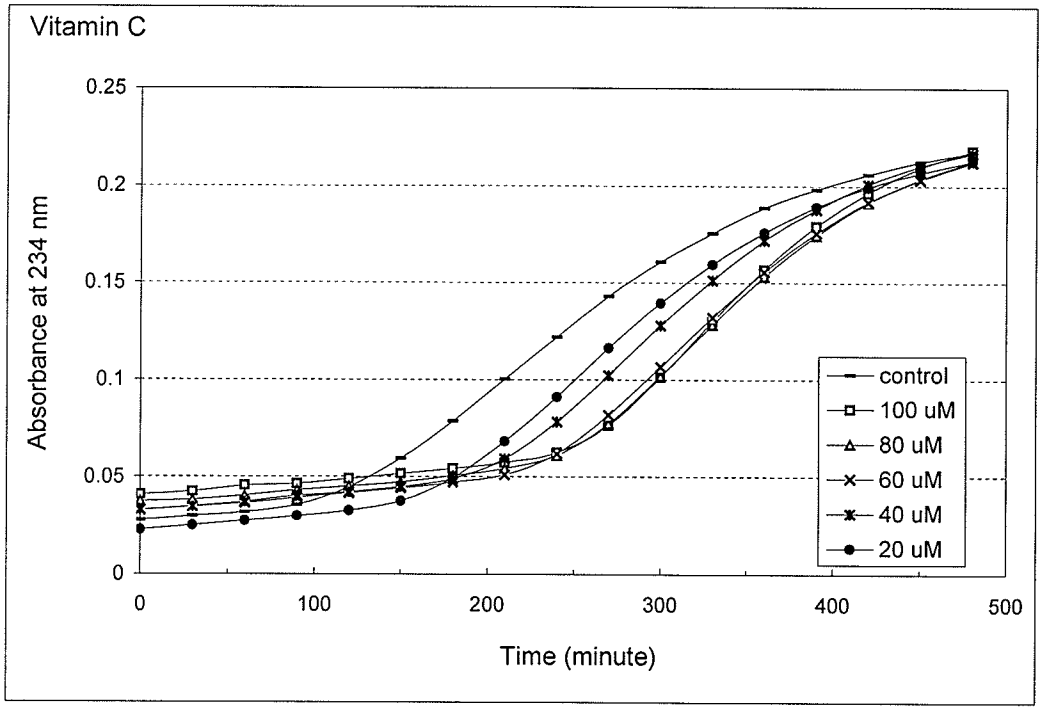


Figure 25. Oxidation – time curves of LDL in the hydrophilic free radical generating system. LDL (0.1 mg protein /ml) was co-incubated with 1.2 mM AAPH and Vitamin C at different concentrations for 6 hours at 37 ^{0}C. Each point on the curves represents the mean ± standard deviation of three independent experiments.

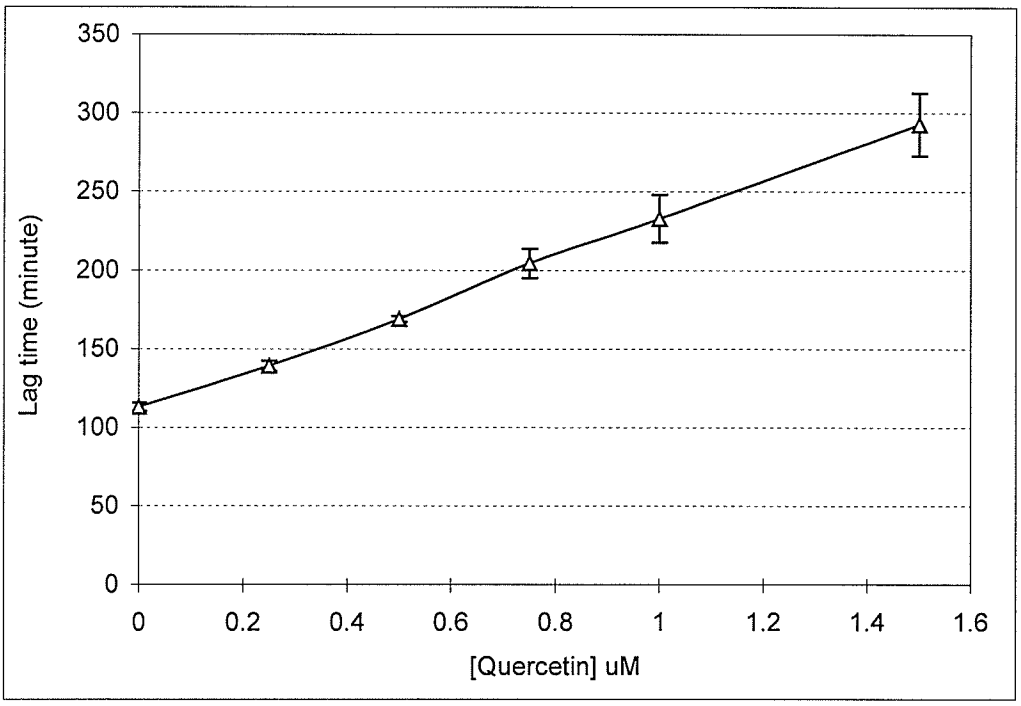


Figure 26. Concentration - response effect of quercetin on the lag time of AAPH induced – LDL peroxidation. $CLT_{50,}$ the antioxidant concentration increasing the lag time to 50% greater than that of the control, was determined graphically the dose-response curve. The CLT_{50} (μM) value of quercetin was 0.05 ± 0.02 (n = 3).

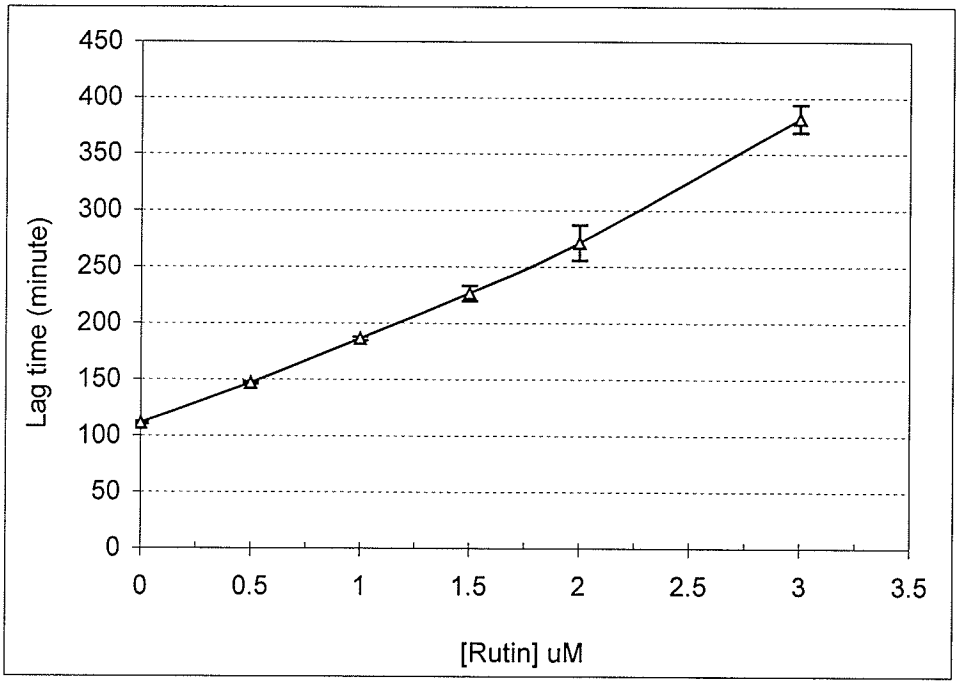


Figure 27. Concentration - response effect of rutin on the lag time of AAPH induced – LDL peroxidation. $CLT_{50,}$ the antioxidant concentration increasing the lag time to 50% greater than that of the control, was determined graphically the dose-response curve. The CLT_{50} (μM) value of rutin was 0.81 ± 0.03 (n = 3).

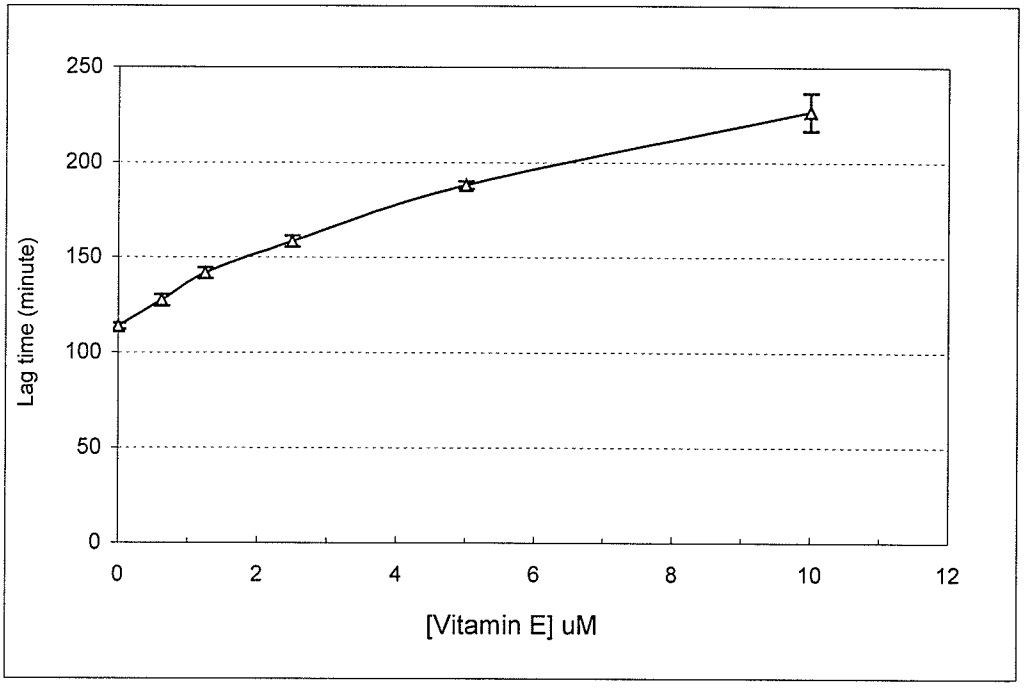


Figure 28. Concentration - response effect of α-tocopherol on the lag time of AAPH induced – LDL peroxidation. $CLT_{50,}$ the antioxidant concentration increasing the lag time to 50% greater than that of the control, was determined graphically the dose-response curve. The CLT_{50} (μM) value of vitamin E was 3.39 ± 0.08 (n = 3).

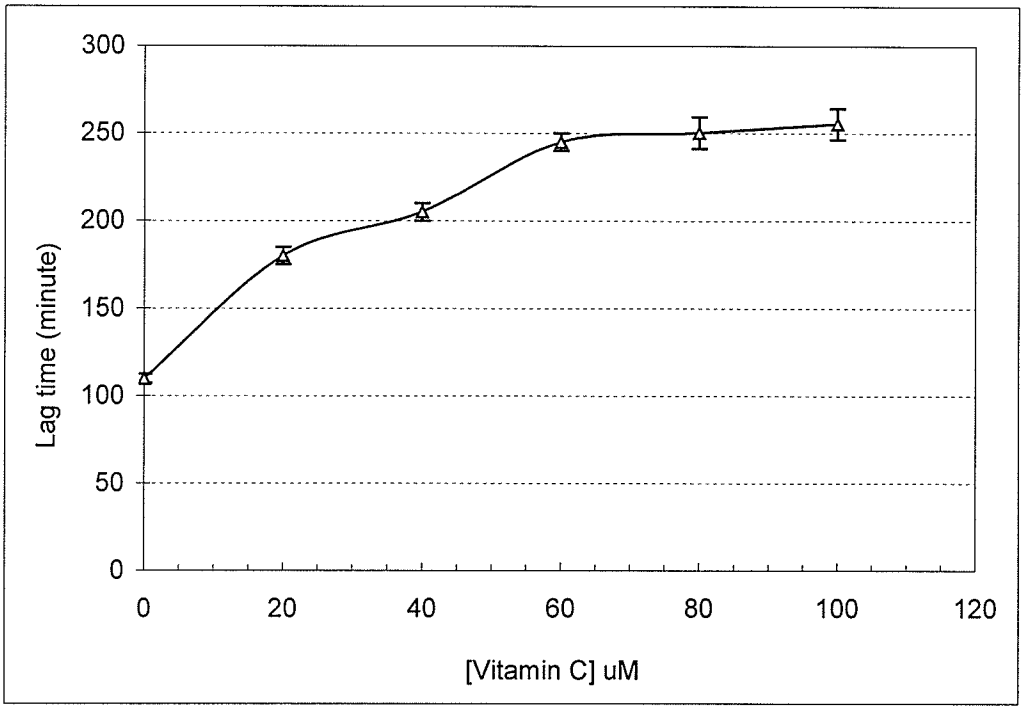


Figure 29. Concentration - response effect of ascorbic acid on the lag time of AAPH induced – LDL peroxidation. $CLT_{50,}$ the antioxidant concentration increasing the lag time to 50% greater than that of the control, was determined graphically the dose-response curve. The CLT_{50} (μM) value of vitamin C was 15.00 ± 0.72 (n = 3).

Table 20. IC_{50} values and area under the curve, lag time, slope for each compound at test concentrations relative to those of the control [1]

Antioxidants	Concentration (μM)	Relative AUC [2]	Relative slope [2]	Relative lag time	CLT_{50} of lag time [3] (μM)
Quercetin					0.50 ± 0.02 [c]
	0.25	0.91 *	1.04	1.23 *	
	0.5	0.81 *	0.93 *	1.50 *	
	0.75	0.75 *	0.93 *	1.81 *	
	1	0.69 *	0.90 *	2.06 *	
	1.5	0.55 *	0.93 *	2.59 *	
Rutin					0.81 ± 0.03 [c]
	0.5	0.87 *	0.91	1.21 *	
	1	0.78 *	0.92	1.55 *	
	1.5	0.68 *	0.95	1.90 *	
	2	0.57 *	0.85 *	2.26 *	
	3	0.32 *	0.84 *	3.19 *	
α-Tocopherol					3.39 ± 0.08 [b]
	0.63	0.96	0.98	1.13 *	
	1.25	0.93 *	0.95	1.27 *	
	2.5	0.88 *	0.94	1.37 *	
	5	0.79 *	0.79 *	1.72 *	
	10	0.70 *	0.73*	2.09 *	
Ascorbic acid					15.00 ± 0.72 [a]
	20	0.89 *	1.18 *	1.64 *	
	40	0.77 *	1.19 *	1.86 *	
	60	0.68 *	1.17 *	2.23 *	
	80	0.64 *	1.26 *	2.28 *	
	100	0.64 *	1.31 *	2.32 *	

[1] The control undergoes identical condition without added test compounds and has a relative value of 1.

[2] * Difference between control and test compound is significant based on the paired *t*-test ($P < 0.05$).

[3] Means sharing the same letter are not significantly different by Duncan ($P < 0.05$).

4.2.2 Protective effects of three buckwheat species against low-density lipoprotein peroxidation

This study was conducted to investigate the protective effects of extracts of *F. esculentum*, *F. tataricum,* and *F. homotropicum* against lipid peroxidation. Conjugated diene measurement was used to determine the cupric ion induced lipoprotein oxidation in isolated low-density lipoprotein (LDL) of rabbits. The samples of the three buckwheat species used here were as same as those described in 4**.1.1.** The buckwheat extracts were prepared as described in 3**.2.2.2. B**. One gram of buckwheat was extracted with 80% methanol to make the final volume of 10 ml. The concentration unit of buckwheat extract was expressed as mg buckwheat/ml, which gave the weight of buckwheat from which per ml of buckwheat solution was extracted. To investigated protective effects of the 11 buckwheat cultivars/lines against LDL peroxidation, the buckwheat extracts were made into the different concentration dependent on species: for *F. esculentum*, the buckwheat extract was condensed ten times to make the concentration at 1 g buckwheat /ml; for *F. homotropicum,* the buckwheat extract was condensed four times to make the concentration at 0.4 g buckwheat /ml; for *F. tataricum,* no condensation or dilution was required and the concentration was at 0.1 g buckwheat /ml. In the conjugated diene assay as described in **3.2.2.4**, 10 µl of the buckwheat extract was added to the LDL solution and made to a total volume of 5 ml (0.1 mg cholesterol /ml, 12 µM $CuSO_4$). The concentration of buckwheat extract in the LDL solution was 2 mg buckwheat/ml for *F. esculentum,* 0.8 mg buckwheat/ml for *F. homotropicum,* and 0.2 mg buckwheat/ml for *F. tataricum.*

Figure 30 shows the time plots of conjugated diene formation in LDL incubated with 12 µM $CuSO_4$ in the absence or presence of different buckwheat cultivars/lines. The area under

the curve (AUC) and the lag time are presented in **Table 21**. When compared to the control, *F. homotropicum* (0.8 mg buckwheat/ml) and *F. tataricum* (0.2 mg buckwheat/ml) demonstrated an inhibitory effect on copper-induced LDL oxidation with a reduction in AUC, averaging 75.6% and 16.7% of the control, respectively. No significant difference in AUC value was found between *F. esculentum* (2 mg buckwheat/ml) and the control. *F.tataricum* (0.2 mg buckwheat/ml) produced 4.9 times greater increase in lag time than the control, followed by *F. homotropicum* (0.8 mg buckwheat/ml) with 2.1 times increase, and *F. esculentum* (2 mg buckwheat/ml) with only a 1.5 times increase than the control. At the test concentration of buckwheat, linear regression analysis revealed a correlation between lag time and flavonoid content ($R^2 = 0.7731$, $\alpha = 0.01$) or lag time and rutin ($R^2 = 0.9755$, $\alpha = 0.01$) content, and an inverse correlation between AUC and flavonoid content ($R^2 = 0.7963$, $\alpha = 0.01$) or AUC and rutin content ($R^2 = 0.9712$, $\alpha = 0.01$) with all buckwheat cultivars/lines.

To investigate the dose-response effects of *F.esculentum*, *F.homotropicum,* and *F.tataricum* species on LDL peroxidation, the pooled samples were prepared by mixing the equal volume of each buckwheat cultivar/line extract of the same species. For example, equal volumes of K980956 extract, K980855 extract and K980854 extract were mixed well to make the pooled sample of *F.homotropicum.* Each pooled buckwheat extract was further condensed or diluted to prepare the buckwheat extract sample at three different concentrations. The time plots of conjugated diene formation in LDL co-incubated with 12 μM $CuSO_4$ and buckwheat extract at three different concentrations are shown in **Figure 31** (*F. esculentum)*, **Figure 32** (*F.homotropicum)*, and **Figure 33** (*F.tataricum)*, respectively. **Table 22** presents the AUC and lag time of the three buckwheat species at the three concentrations relative to those of the control, and their IC_{50} values of AUC and CLT_{50}

values of lag time. As compared to the control, the three buckwheat species at all test concentrations showed a decrease in AUC and an increase in lag time ($P < 0.05$). The three species exhibited a dose – response effect on the AUC as well as on the lag time of LDL peroxidation. The pooled buckwheat extract concentration that produced a 50% reduction in AUC relative to the control (IC_{50}) and the concentration increasing the lag time to 50% greater than that of control (CLT_{50}) were used as the criteria for comparing the antioxidant efficiency. Both IC_{50} of AUC and CLT_{50} of lag time were determined from the linear dose-response curves. *F. tataricum*, with the lowest IC_{50} value of 0.10 mg buckwheat/ml and the lowest CLT_{50} value of 0.03 mg buckwheat/ml, was the most effective in inhibiting LDL peroxidation, followed by *F. homotropicum* with an IC_{50} value of 1.55 mg buckwheat/ml and the CLT_{50} value of 0.41 mg buckwheat/ml. *F. esculentum* with the highest IC_{50} value of 7.2 mg buckwheat/ml and the highest CLT_{50} value of 2.0 mg buckwheat/ml showed the weakest ability to protect LDL against peroxidation.

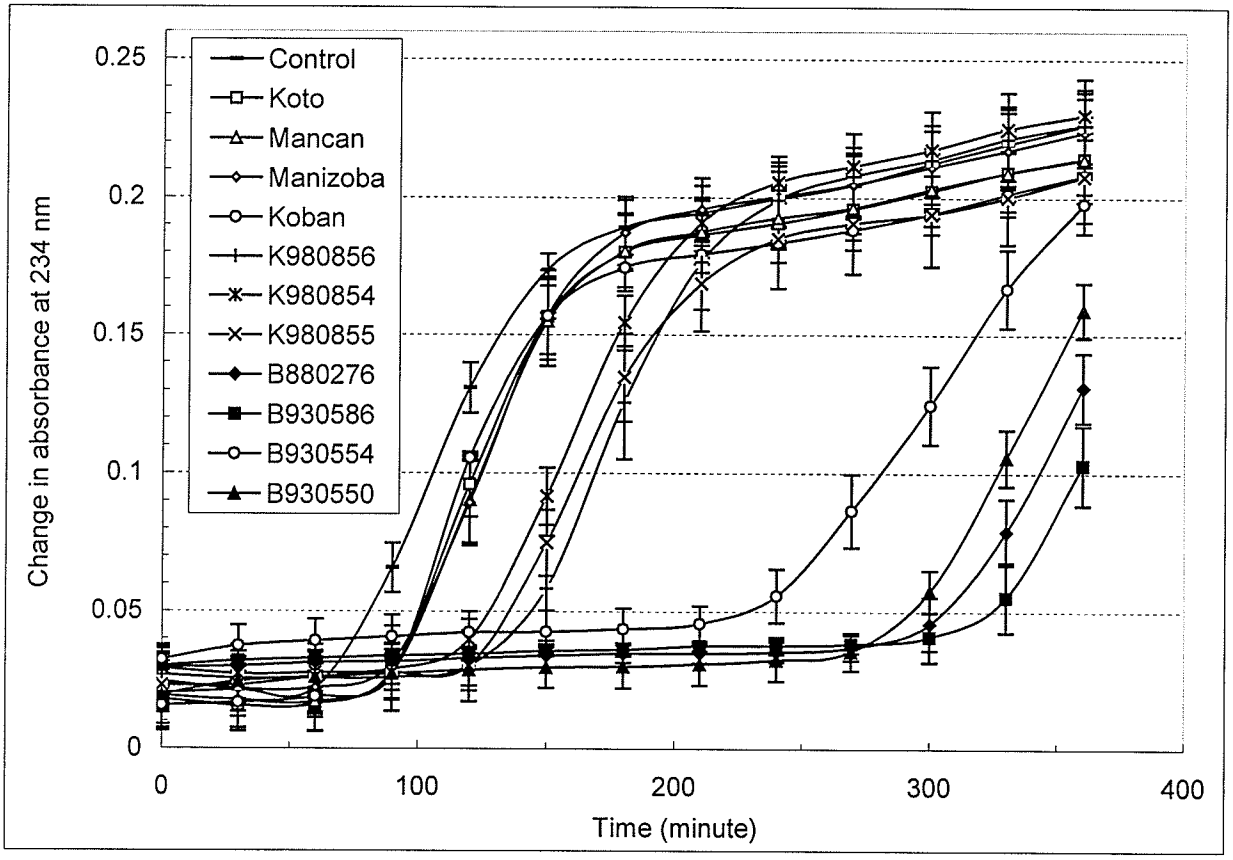


Figure 30. Copper- induced oxidative - time curves of LDL incubated with the three different buckwheat species. LDL (0.1 mg protein /ml) was incubated with 12 μM CuSO4 and buckwheat extract for 6 hours at 37 ^{0} C. The control underwent the identical conditions without buckwheat extract added. The concentration of buckwheat extract in the LDL solution were 2 mg buckwheat/ml for *F. esculentum,* 0.8 mg buckwheat/ml for *F. homotropicum,* and 0.2 mg buckwheat/ml for *F. tataricum.* Each point on the curves represents the mean ± standard deviation of three independent experiments.

Table 21. Area under the oxidation curve (AUC) and lag time of LDL incubated with different buckwheat cultivars/lines

Cultivar/lines	Individual AUC	Mean ± SD	Individual lag time (minute)	Mean ± SD
Control		45.4 ± 1.5 [a]		60 ± 3 [d]
F.esculentum		41.3 ± 0.6 [a]		88 ± 3 [c]
(2 mg buckwheat/ml)				
Koto	42.0		85	
Mancan	40.6		90	
Manizoba	41.5		90	
Koban	41.2		85	
F.homotropicum		34.3 ± 2.2 [b]		125 ±13 [b]
(0.8 mg buckwheat/ml)				
K980856	33.6		140	
K980855	36.7		120	
K980854	32.5		115	
F. tataricum		7.6 ± 4.6 [c]		291± 37 [a]
(0.2 mg buckwheat/ml)				
B930550	3.6		290	
B930586	4.5		325	
B930554	13.6		240	
B880276	8.5		310	

[a, b, c] Data are expressed as means ± standard deviation and the value within a column sharing the same letter are not significantly different by Duncan's multiple-range test ($P < 0.05$).

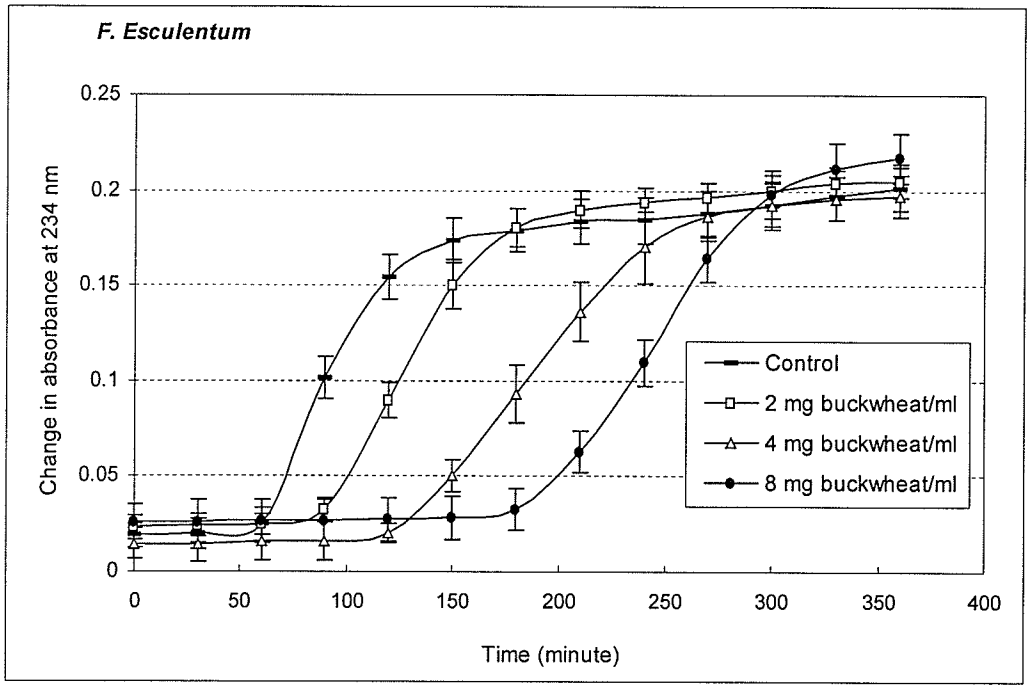


Figure 31. Dose - response effects of the pooled buckwheat extracts of *F. esculentum* on copper-induced LDL oxidation. LDL (0.1 mg protein /ml) was co-incubated with 12 μM $CuSO_4$ and the pooled *F. esculentum* extract at three different lconcentrations for 6 hours at 37 ^{0} C. The control underwent the identical conditions without a buckwheat extract added. Each point on the curves represents the mean ± standard deviation of three independent experiments.

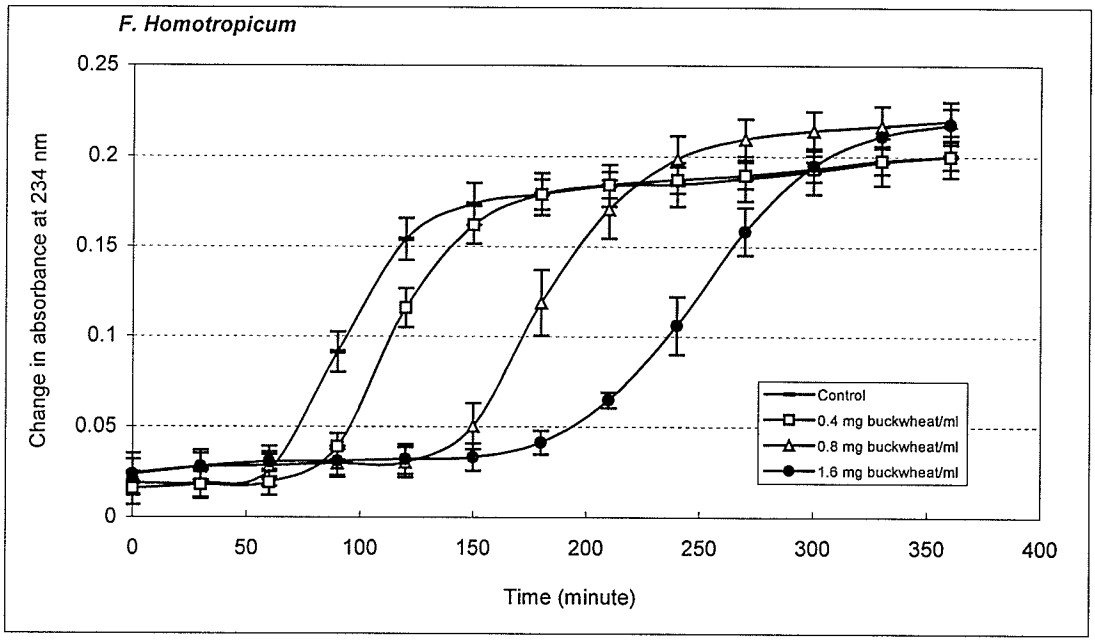


Figure 32. Dose - response effects of the pooled buckwheat extracts of *F. homotropicum* on copper-induced LDL oxidation. LDL (0.1 mg protein /ml) was co-incubated with 12 μM $CuSO_4$ and the pooled *F. homotropicum* extract at three different lconcentrations for 6 hours at 37 ^{0} C. The control underwent the identical conditions without a buckwheat extract added. Each point on the curves represents the mean ± standard deviation of three independent experiments.

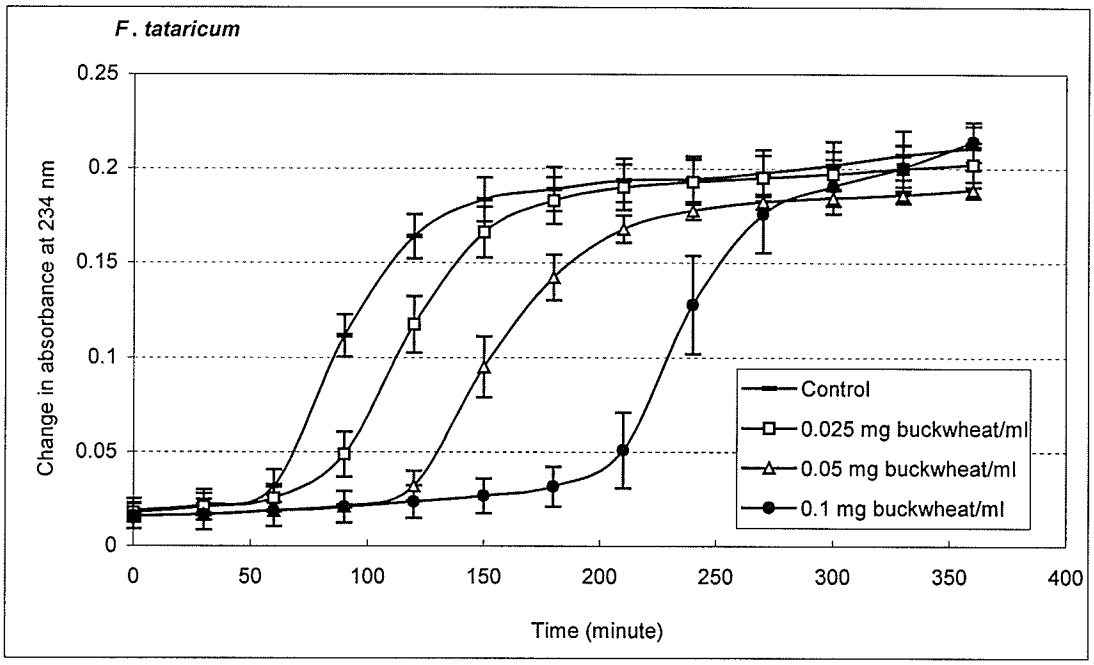


Figure 33. Dose - response effects of the pooled buckwheat extracts of *F.tataricum* on copper-induced LDL oxidation. LDL (0.1 mg protein /ml) was co-incubated with 12 μM $CuSO_4$ and the pooled *F.tataricum* extract at three different lconcentrations for 6 hours at 37 ^{0} C. The control underwent the identical conditions without a buckwheat extract added. Each point on the curves represents the mean ± standard deviation of three independent experiments.

Table 22. AUC and lag times of the three buckwheat species at different concentration relative to those of the control and their IC_{50} of AUC and CLT_{50}

Buckwheat species	Pooled extract concentration (mg buckwheat/ml)	Relative AUC (% Control) [1]	IC_{50} [2] (mg buckwheat/ml)	Relative lag time (minute) [1]	CLT_{50} [2] (mg buckwheat/ml)
F.esculentum			7.20 ± 0.26 [a]		2.00 ± 0.05 [a]
	8	46.4 ± 1.3		3.4 ± 0.2	
	4	69.1 ± 2.9		2.4 ± 0.1	
	2	89.4 ± 2.0		1.5 ± 0.1	
F.homotropicum			1.55 ± 0.05 [b]		0.41 ± 0.02 [b]
	1.6	48.4 ± 1.4		3.3 ± 0.2	
	0.8	76.6 ± 2.8		2.4 ± 0.1	
	0.4	95.2 ± 2.9		1.5 ± 0.1	
F.tataricum			0.10 ± 0.00 [c]		0.03 ± 0.00 [c]
	0.1	47.9 ± 1.6		3.5 ± 0.2	
	0.05	71.8 ± 2.9		2.1 ± 0.1	
	0.025	90.5 ± 2.0		1.4 ± 0.1	

[1] The control underwent identical conditions but without buckwheat extract added and has relative value of 1. Data are expressed as means ± standard deviation of three independent experiments. [2] IC_{50} and CLT_{50} are determined graphically from dose-response curves and their values within a column sharing the same letter are not significantly different by Duncan's multiple-range test ($P < 0.05$).

4.2.3 Effects of *F.tataricum* on low-density lipoprotein peroxidation in hydrophilic and lipophilic free radical generating systems

In this study, using both the thiobarbituric acid-reactive substances (TBARS) test and conjugated diene measurement with isolated LDL of rabbits, the antioxidant effectiveness of *F. tataricum* in aqueous and lipid environments were evaluated and compared to *F. esculentum*. The azo-compounds 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) were used as a hydrophilic and a lipophilic free radical generator, respectively. AMVN (4 mM) and AAPH (18 mM) generating a similar MDA level (see **4.2.1.1.)** were used for the TBARS test. AAPH (1.2 mM) was used for the conjugated diene assay (see **3.2.2.4.).** *F.esculentum,* a bulked sample of common buckwheat, and the *F. tataricum* lines B930555, K970848, B830222 and B770198 harvested in 1998 were selected for this study. The buckwheat extracts were prepared as described in **3.2.2.2, A**. Two gram of buckwheat was extracted to make the final volume of 50 ml. The rutin content in buckwheat seed and in buckwheat extracts of the test buckwheat cultivar/lines are presented in **Table 23**.

Table 23. Rutin content in different buckwheat cultivar/lines and their test extracts

Buckwheat cultivars/lines	Rutin content (g/ 100 g seed)	Rutin content in the test buckwheatextracts (mg/ml)
F. esculentum	0.0584	0.0234
F. tataricum:		
K970848	1.8344	0.7338
B930555	1.8504	0.7402
B770198	1.9507	0.7803
B830222	1.9721	0.7888

4.2.3.1 Antioxidant activities of *F. tataricum* in both aqueous and lipid environments of LDL using TBARS test

The AAPH – and AMVN - induced MDA productions in LDL incubated with or without the test buckwheat extracts are shown in **Figure 34** and **Figure 35**, respectively. AAPH – induced MDA production in all *F. tataricum* extract treatments were lower that that of control. However, *F.esculentum* extract did not show a significant inhibitory effect on AAPH-induced MDA production. No significant difference in AMVN – induced MDA production was found between the control and all treatments. The inhibitory effects of the test buckwheat extracts on LDL peroxidation in both hydrophilic and lipophilic free radical generating systems are summarized in **Table 24**. In an aqueous environment surrounding the LDL, all *F. tataricum* extracts were effective in inhibiting lipid peroxidation, with an average MDA production 43.1% of the control. There was no significant difference in the inhibitory effect between *F. tataricum* cultivars. In the lipid region within LDL, no test buckwheat extracts showed a protective effect against lipid peroxidation.

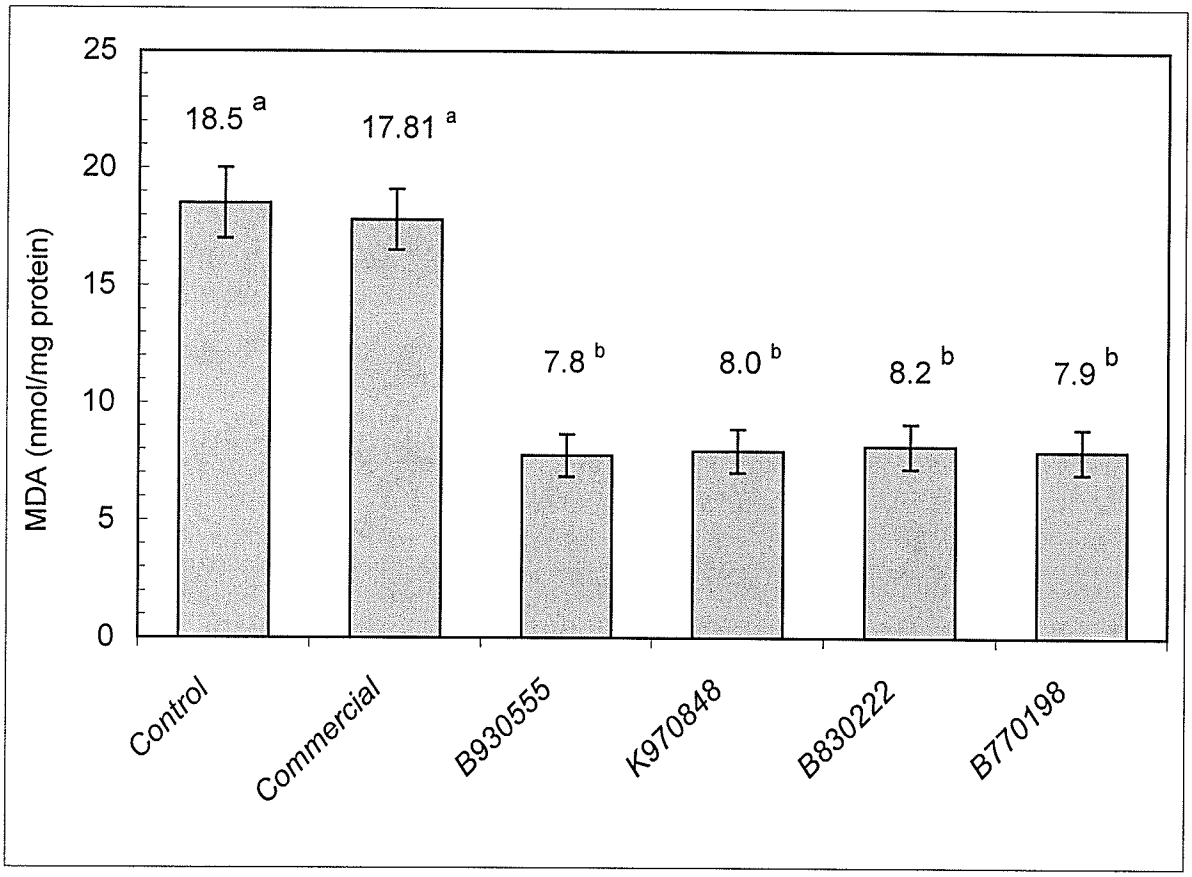


Figure 34. AAPH–induced MDA production in LDL incubated with or without buckwheat extract. LDL (0.5 mg protein / ml) was co-incubated with buckwheat extract (10 µl/ml) and 18 mM AAPH for 1 hour at 37 ^{0} C. The data are expressed as the means ± standard deviation of six independent experiments. ^{a,b,c} Values sharing the common superscript are not significantly different determined by Duncan multiple-range test ($P < 0.05$).

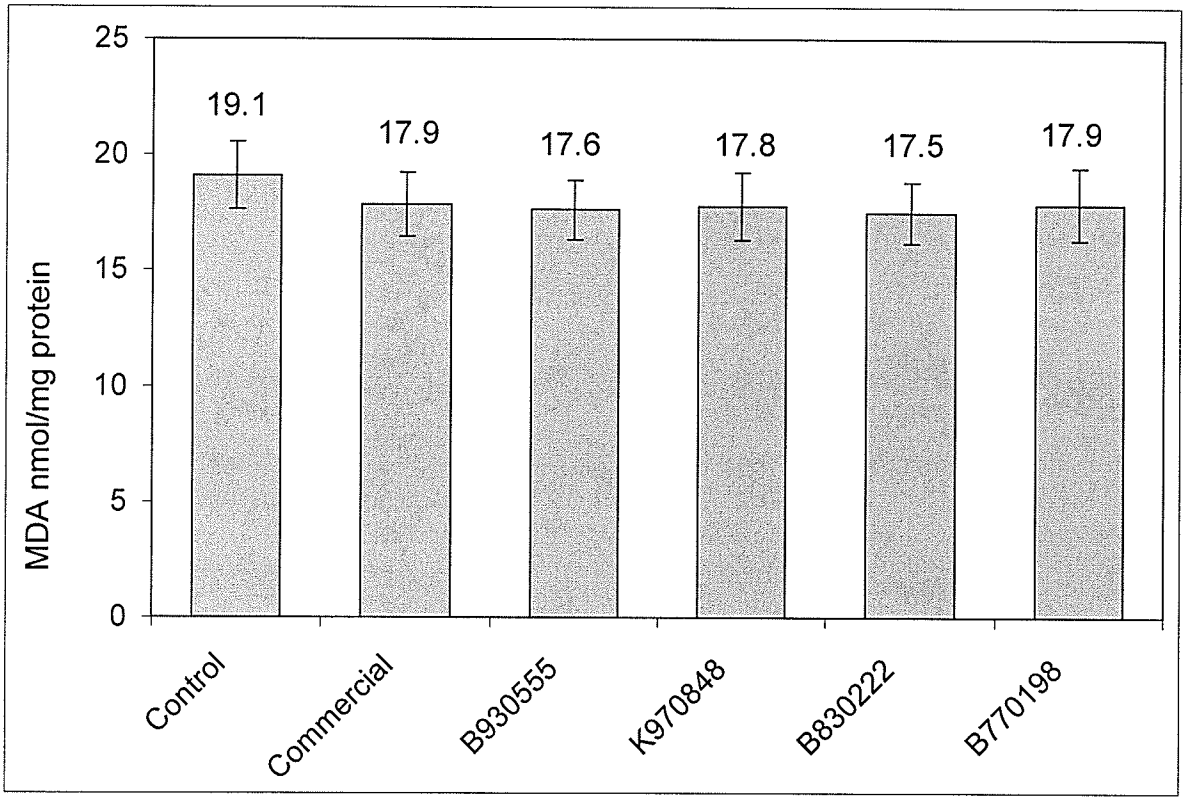


Figure 35. AMVN – induced MDA production in LDL incubated with or without buckwheat extract. LDL (0.5 mg protein / ml) was co-incubated with buckwheat extract (10 µl/ml) and 4 mM AMVN for 1 hour at 37 ^{0} C. The data are expressed as means ± standard deviation of six independent experiments. There is no significant difference in MDA production among six groups ($P < 0.05$).

Table 24. Effect of buckwheat extract on AAPH- or AMVN-induced MDA production in LDL [1, 2]

Buckwheat cultivar	AAPH-induced MDA production (% of control)	AMVN-induced MDA production (% of control)
F.esculentum	96.3 ± 2.7 [a]	97.9 ± 6.8
F.tataricum		
K970848	42.9 ± 3.5 [b]	88.6 ± 3.6
B930555	41.5 ± 1.9 [b]	94.7 ± 3.6
B770198	43.4 ± 2.4 [b]	88.4 ± 4.1
B830222	44.6 ± 2.4 [b]	90.8 ± 6.4

[1] The data are expressed as the means ± standard deviation from six different experiments. [2]

[a,b] The values sharing the same letter within column are not significantly different, determined by Duncan multiple range test ($P < 0.05$).

4.2.3.2 Protective effect of *F. tataricum* in an aqueous environment on the early stage of LDL peroxidation determined by the conjugated diene assay

In the conjugated diene assay as described in **3.2.2.4**, 10 µl of the buckwheat extract was added to the LDL solution and made the total volume of 5 ml (0.1 mg cholesterol /ml, 1.2 mM AAPH). The buckwheat extracts of *F. esculentum* and *F. tataricum* used in this assay were prepared as same as those uased in the TBARS test (**4.2.3.**). The AAPH – induced oxidative – time curves of LDL incubated with or without buckwheat extracts are shown in **Figure 36**. Results showing the inhibitory effects of the buckwheat extracts on LDL peroxidation are presented in **Table 25**. All *F. tataricum* extracts produced increases in lag time, averaging 2.9 times that of the control, while *F. esculentum* extract did not produce a significant increase in lag time as compared to the control. No significant differences in lag time increase were found between the *F. tataricum* extracts.

To compare the effect of *F. tataricum* extract on LDL peroxidation with that of rutin, the pooled *F. tataricum* extract and standard rutin were used in the conjugated diene assay. The pooled *F. tataricum* extract was prepared by mixing the equal volume of each buckwheat line extract: K970848, B930555, B770198 and B830222. The standard rutin was dissolved in methanol. 10 µl of the pooled *F. tataricum* extract or 10 µl of standard rutin solution was added to LDL solution and made the total volume of 5 ml (0.1 mg protein /ml, 1.2 mM AAPH). The rutin concentration in LDL solution incubated with the pooled *F. tataricum* extract or standard rutin was 2.5 µM rutin. The AAPH – induced oxidative – time curves of LDL incubated with the pooled *F. tataricum* extract and rutin solution were monitored for 7.5 hours at 37 ^{0} C (**Figure 37**). The pooled *F.tataricum* extract and the rutin solution produced 3 and 2.8 times respectively increase in lag time relative to the control. There was

no significant difference in inhibitory effect between *F.tataricum* extract and rutin solution at the same rutin concentration (**Table 26**).

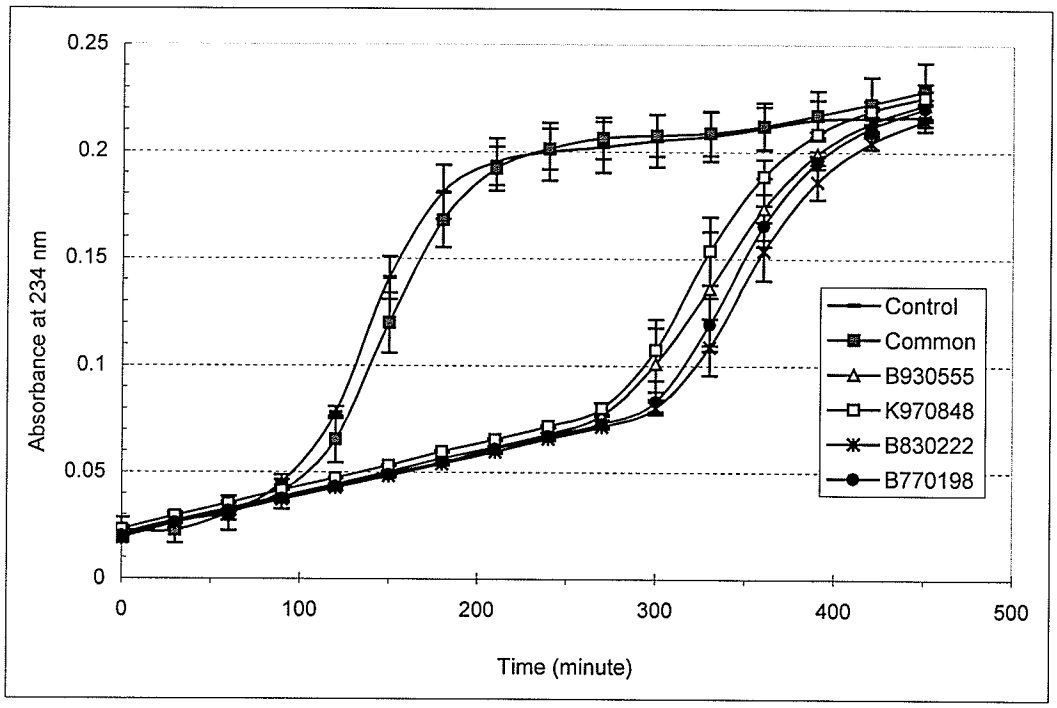


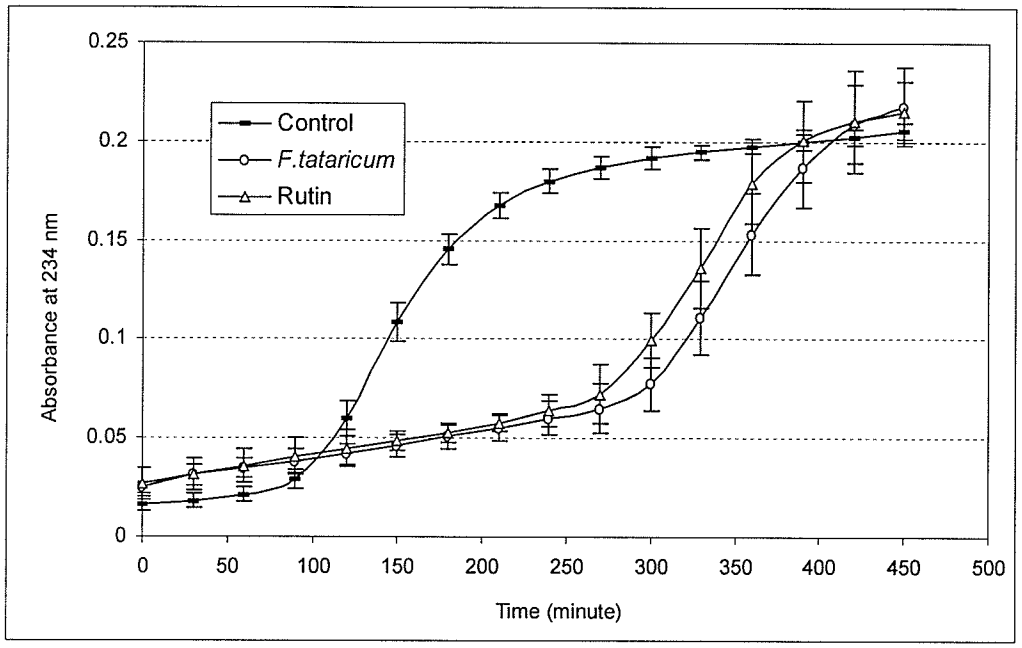
Figure 36. AAPH - induced oxidative - time curves of LDL incubated with Tartary buckwheat extracts. LDL (0.1 mg protein /ml) was incubated with 1.2 mM AAPH and buckwheat extracts at the test concentration (2 µl of buckwheat extract /ml) for 7.5 hours at 37 ^{0} C. Each point on the curves represents the mean ± standard deviation of three independent experiments.

Table 25. Effect of buckwheat extract on LDL peroxidation [1, 2]

Buckwheat cultivar	Lag time (minute)
Control	103 ± 5 [b]
F.esculentum	110 ± 8 [b]
F.tataricum:	
K970848	283 ± 15 [a]
B930555	290 ± 22 [a]
B770198	302 ± 10 [a]
B830222	307 ± 10 [a]

[1] The data are expressed as the mean ± standard deviation from three different experiments.

[2 a,b,c] The values sharing the same letter are not significantly different determined by Duncan multiple-range test ($P < 0.05$).



Figur 37. AAPH – induced oxidative - time curves of LDL incubated with or without buckwheat extract/rutin solution. LDL (0.1 mg protein /ml) was co-incubated with 1.2 mM AAPH and *F.tataricum* extract (containing 2.5μM rutin)/rutin solution (2.5 μM rutin) for 7.5 hours at **37** ^{0} C. Each point on the curves represents the mean ± standard deviation of three independent experiments.

Table 26. Comparison of the effects of *F.tataricum* extract and standard rutin on LDL peroxidation

Samples	Rutin concentration (μM)	Lag time (minute) [2]
Control	UD [1]	100 ± 5 [b]
F.tataricum	2.5	297 ± 17 [a]
Rutin	2.5	276 ± 15 [a]

[1] UD: undetectable. [2] The data are expressed as the means ± standard deviation from three different experiments. [a,b] Values sharing the same letter are not significantly different, determined by Duncan multiple range test ($P < 0.05$).

4.2.4 Effects of *F.homotropicum* on lipid peroxidation in an aqueous environment surrounding low-density lipoprotein

Applying the methods of both thiobarbituric acid-reactive substances (TBARS) test and conjugated diene measurement to isolated LDL of rabbits, two varieties of wheat Kyle CWAD and AC Barrie, commonly used for bread and noodle flour respectively in Canada, were selected as controls to evaluate the antioxidant effectiveness of the cross Q010001 and its parents K970851 and T990036 in an aqueous environments surrounding LDL. The azo-compound 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a hydrophilic free radical generator. AAPH (18 mM) and AAPH (1.2 mM) were used for the TBARS test and conjugated diene assay, respectively. Buckwheat extracts were prepared as described in **3.2.2.2.A**. Two gram of buckwheat or wheat was extracted to make the final volume of 50 ml. Then the extract was condensed 5 times and ready for an experiment. The rutin concentrations contained in buckwheat extracts in the two evaluation systems are presented in **Table 27**.

Table 27. Rutin concentrations in two evaluation systems

Sample	TBARS test Rutin (μM)	Conjugated diene assay Rutin ((μM)
Kyle CWAD wheat	UD	UD
AC Barrie CWKS wheat	UD	UD
K970851	8.8	1.7
T990036	2.5	0.5
Q010001	4.0	0.8

UD: undetectable.

4.2.4.1 Antioxidant activities of *F. homotropicum* in an aqueous environments of LDL evaluated by TBARS test

The effects of the test wheat and buckwheat in inhibiting MDA production are shown in **Figure 38**. The buckwheat extracts significantly decreased the MDA production in LDL, whereas the wheat extracts had no effect on MDA production. K970851 exhibited the most effective ability of inhibiting MDA production than T990036 and Q010001. No significant difference in inhibitory effect was found between T990036 and Q010001.

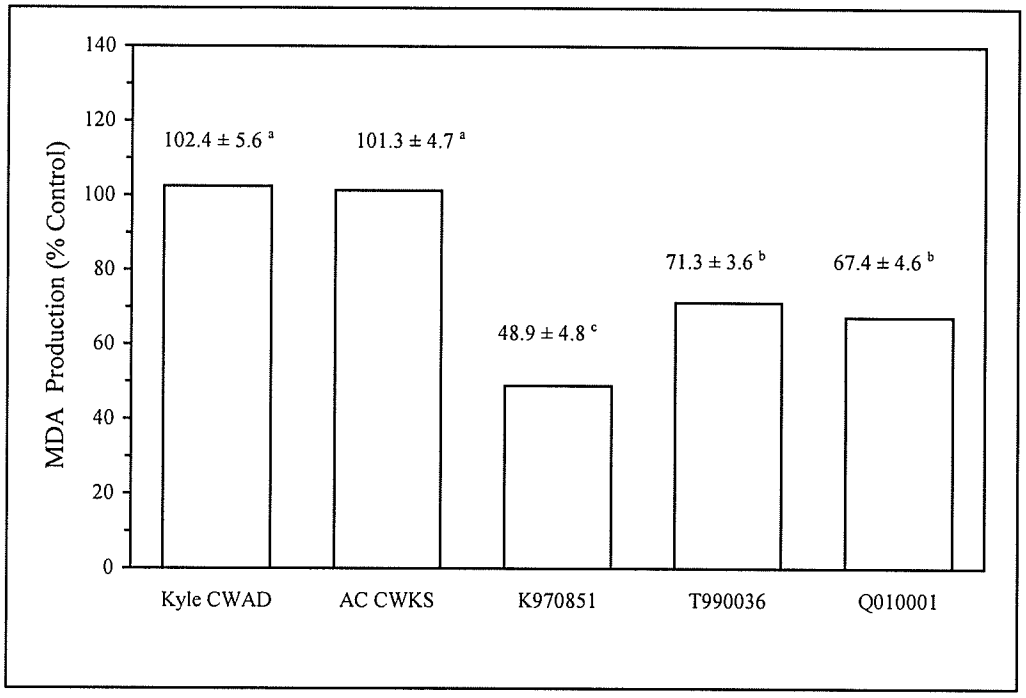


Figure 38. Effects of the test buckwheat extracts and wheat extracts on AAPH- induced MDA production in LDL. The data are expressed as means ± standard deviation from six different experiments. [a,b,c] Values sharing the same letter are not significantly different, determined by Duncan multiple range test ($P < 0.05$).

4.2.4.2 Protective effect of *F. homotropicum* in an aqueous area on the early stage of LDL peroxidation by the conjugated diene assay

The AAPH – induced oxidative – time curves of LDL incubated with or without wheat or buckwheat extracts are shown in **Figure 39**. The inhibitory effects of the buckwheat or wheat extracts on LDL peroxidation are presented in **Table 28**. As compared to control, all buckwheat extracts produced increases in lag time, whereas the wheat extracts gave similar lag time to that of the control. Among three buckwheat extracts, K970851 produced the greatest increase in lag time with 2.2 times lag time of control, followed by the cross Q010001 with 1.4 times lag time of the control, and T990036 which caused the least increase in lag time, 1.2 times control.

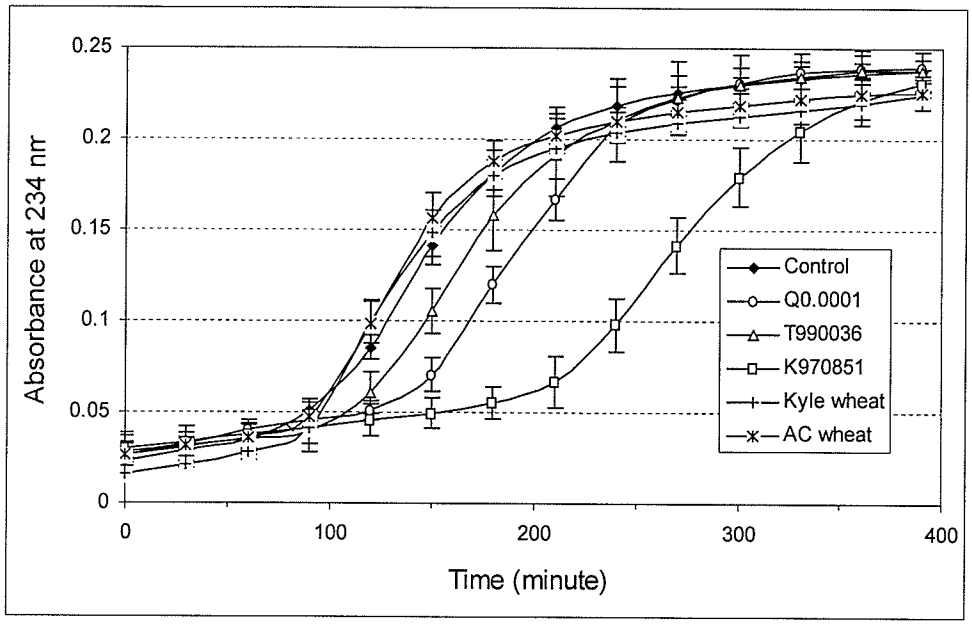


Figure 39 AAPH – induced lipid oxidative – time course of LDL (μg/ml) incubated with or without buckwheat extract at 37 ^{0} C. LDL (0.1 mg protein /ml) was co-incubated with 1.2 mM AAPH and buckwheat or wheat extracts for 7.5 hours at **37 ^{0} C.** Each point on the curves represents the mean ± standard deviation of three independent experiments.

Table 28. Effects of buckwheat and wheat on AAPH – induced lipid peroxidation [1, 2]

Test samples	Lag time (minute)
Control	95 ± 5 [d]
Wheat:	
Kyle CWAD wheat	92 ± 6 [d]
AC Barrie CWKS wheat	95 ± 5 [d]
Buckwheat:	
K970851	210 ± 10 [a]
T990036	115 ± 5 [c]
Q010001	135 ± 7 [b]

[1] The data are expressed as means ± standard deviation from six different experiments.

[2 a,b,c] Values sharing the same letter are not significantly different, determined by Duncan multiple range test ($P < 0.05$).

4.3 Effect of quercetin on the oxidative stress in cardiomyocytes of rat

A rapid laser exposure time of less than 2 seconds in the epifluorescent microscopy measurement minimized photo-oxidation of the internalized DCFH to DCF. The cardiomyocyte images of the control cells without any treatment, the H_2O_2 (1 mM) loaded cells, and the H_2O_2 (1 mM) loaded cells with the quercetin (5 μM) treatment of 24-hours pre-incubation are presented in **Figure 40**. H_2O_2 -induced DCF fluorescence of quercetin treated cells exhibited significantly lower mean fluorescence intensity (44.0) than that of cells without quercetin treatment (60.4) ($P < 0.05$), indicating that quercetin caused an inhibitory effect on hydrogen peroxide-induced 2', 7'-dichlorofluorescin oxidation in neonatal cardiomyocyte (**Figure 41**).

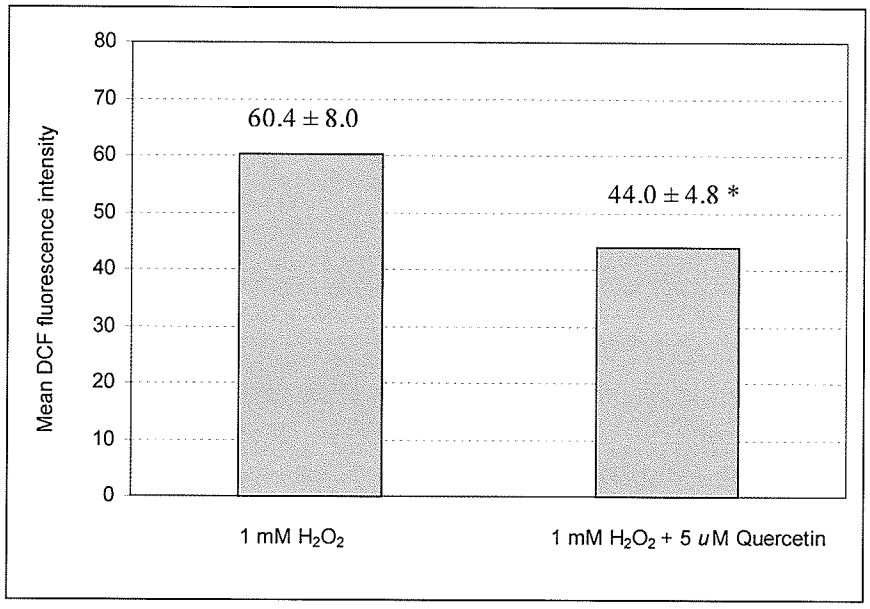
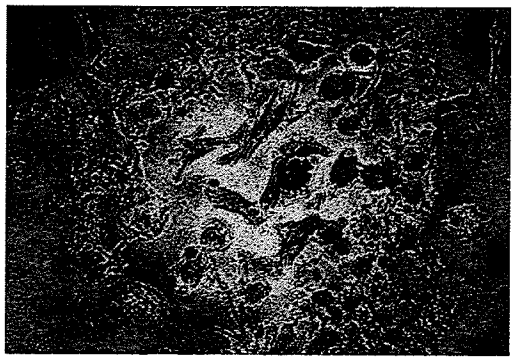
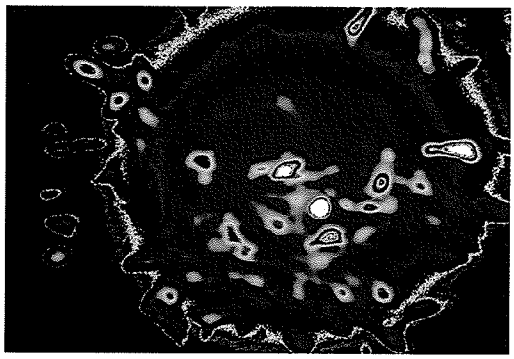


Figure 40. Effect of quercetin on hydrogen peroxide-induced 2', 7'-dichlorofluorescin oxidation in neonatal cardiomyocyte. Values are means ± standard deviation of six repeated experiments. In an experiment, each treatment group contained 8 cell wells. Mean fluorescence intensity in each well was calculated by averaing area intensities from 15-20 randomly selected cells. As compared to the H_2O_2 (1 mM) loaded cells, the quercetin (5 μM) treatment with 24-hours pre-incubation significantly reduced the oxidative stress in the cardiomyocytes (* $P < 0.05$).

A



B



C

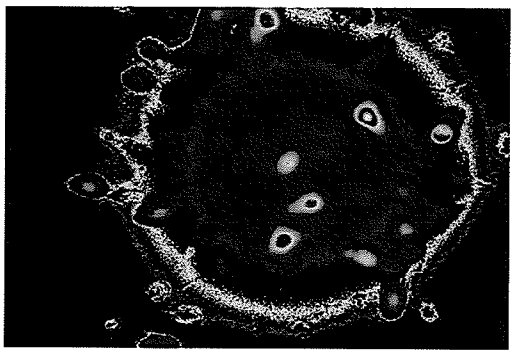


Figure 41. Representative cardiomyocytes images of DCF fluorescence of the three groups by epifluorescent microscopy. A: The control cells without any treatment. B: The 1 mM H_2O_2 loaded cells. C: The 1 mM H_2O_2 loaded cells with the quercetin (5 μM) treatment of 24-hours pre-incubation. A green fluorescent intensity reflects the oxidative stress of a cell. The degree of fluorescent brightness was directly proportional to the extent of oxidative stress in a cell.

Chapter V Discussion

5.1 Quantitative analysis of flavonoids in different buckwheat species

5.1.1 Total flavonoid and rutin contents in three buckwheat species

The solubility of flavonoids varies greatly due to their different structures. In general, flavonoid aglycones are soluble in methanol, ethanol, chloroform, ether and some organic solvents, but not soluble in water. Glycosylation of flavonoids increases the hydrophilicity and decreases the lipophilicity, making flavonoid glycosides readily soluble in hot water, methanol and ethanol but less soluble or insoluble in chloroform and ether. Methanol and ethanol are the most common solvents used for the extraction of flavonoids from plants. Their mixture with water at varying ratios can be applied to extracting different flavonoids.

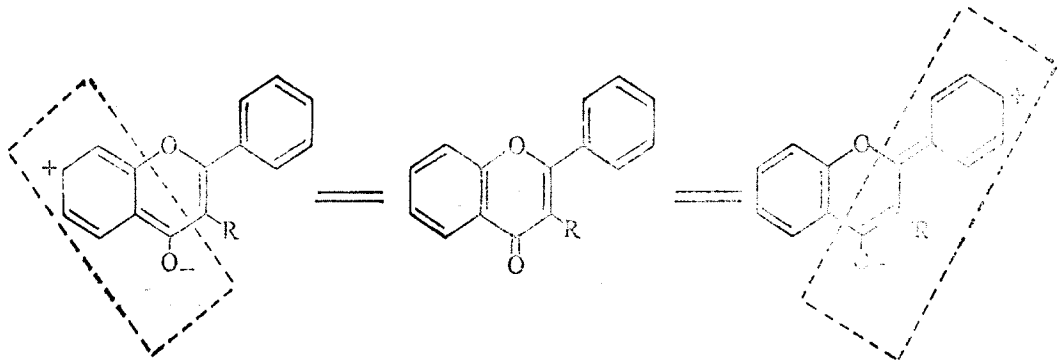
Most methods of rutin analysis in buckwheat use methanol and ethanol as extraction solvents. In these methods, there are differences in extraction parameters such as solvent concentration, extraction temperature, and extraction time. Methanol extraction by heating under reflux with the temperature range from 65 ^{0}C to 90 ^{0}C is widely used, but various methods employ different extraction times including 1 hour, 2 hours, or 3 hours and different methanol concentration including 100%, 80 %, or 50% (Holasova *et al.*, 2002; Steadman *et al.*, 2001; Oomah and Mazza, 1996; Kalinova and Dadakova, 2004; Park *et al.*, 2004). Ohsawa and Tsutsumi (1995) reported that the values of rutin content obtained from methanol extraction for 1 day at 35 ^{0}C correlated closely with the values obtained from extraction at 80 ^{0}C for 1 hour, indicating that this method of extraction was very convenient because of lower extraction temperature. A similar extraction method was used in a study by Im *et al* (2003) who reported the application of methanol extraction for two days at 35 ^{0}C for rutin content determination in buckwheat grit cakes. Kreft *et al.* (2002) also reported using a

solution of methanol : acetic acid : water (100 : 2 : 100 by volume) to extract rutin from buckwheat herb at room temperature for 1 hour. Some investigators have used ethanol as an extraction solvent for rutin analysis in buckwheat seed or grain, such as a solution of water : ethanol : ammonium hydroxide (35 : 60 : 5) mixture with shaking for 3 hours at 30 ^{0}C (Kreft *et al.*, 1999) or ethanol boiling under reflux (Dietrych-Szostak and Oleszek, 1999). Recently, Hinneburg and Neubert (2005) have reported a systematic investigation regarding the effects of extraction parameters on the determination of rutin content in buckwheat. In their study, ethanol concentrations of 30 % and 70 %, extraction temperatures of 25 ^{0}C and 60 ^{0}C, and extraction times of 2 hours and 24 hours were examined, showing that a high rutin extraction content was obtained with 30 % ethanol at 60 ^{0}C for 2 hours. Our extraction study, with 80 % methanol shaking at 70 ^{0}C, has shown that a half hour was the optimum time for extracting rutin from buckwheat seeds. Compared to other methods with 2 hours or 3 hours extraction time at the temperatures ranging from 65 ^{0}C to 90 ^{0}C, this extraction method was shown to be efficient.

A high-performance liquid chromatography assay has been used widely in the analysis of flavonoids in buckwheat. With the complicated gradient elution and the time-consuming characteristics, many of these HPLC methods quantitatively analyse flavonoids such as rutin, quercetin, quercetrin, orintin, vitexin, etc. in buckwheat seeds and herbs. Retention times of rutin in these methods were over 20 minutes and each injection ran at least 30 minutes (Dietrych-Szostak and Oleszek, 1999; Steadman *et al.*, 2001; Fabjan *et al.*, 2003). There are also some HPLC methods designed to determine only the rutin content in buckwheat. For example, to investigate rutin contents in buckwheat seeds and hulls, Oomah and Mazza (1996) used a typical assay whereby the methanol extract was dried under vacuum at 30 ^{0}C

and the residual was dissolved in methanol-water-oxalic solution before the HPLC analysis. Gradient elution was used and each injection required 40 minutes. The method developed and validated in our laboratory, with easy sample preparation, simple isocratic elution, and short run-time of each injection (retention time of rutin was 6.9 minutes) was very effective for routine analysis of rutin in buckwheat.

Flavonoid compounds have two key chemical structural features: cinnamoyl and benzoyl. The conjugated systems formed by cinnamoyl and benzoyl make flavonoid methanol solutions exhibit two absorption bands in the UV region (200 –400 nm): band I (300 – 400 nm) and band II (220 – 280 nm) (**Figure 42**). In our study, a rutin methanol solution showed a strong absorption at 360 nm that was in agreement with most rutin analytical methods where detection was set between 350 – 360 nm.



Band II (220 – 280 nm) Band I (300 – 400 nm)

Figure 42. The conjugated systems of flavonoids.

This figure is adapted from Yao X. S (1998).

Our method for quantitative analysis of rutin was validated. An excellent linearity of the calibration curve (R^2 = 0.9997) was obtained over the range 5 – 200 μg/ml for the rutin compound. The concentration range of our calibration curve was lower relative to those of other methods. For example, the calibration curve used to determine the rutin content in common buckwheat seeds was reported to range from 1 – 250 mg/ml (Oomah and Mazza, 1996). In buckwheat breeding work, some plants have very limited amounts of seed for samples. Therefore, the smallest possible sample amounts may be required for quantitative analysis for rutin in a buckwheat breeding program. One gram of common buckwheat seed sample was required for the HPLC assay of rutin in both the report by Oomah and Mazza (1996) and the report by Ohsawa and Tsutsumi (1995), although they used different extraction methods. About 0.1 g of tartary buckwheat seed sample was extracted for rutin analysis by HPLC in the study by Fabjan *et al.* (2003). In our method, the amounts of seed sample required were only 0.2 g for common buckwheat, 0.1 g for *F.homotropicu*m, and 0.02 g for Tartary buckwheat. As compared to other methods, the smaller amount of seed sample used in our method not only better meet the requirement for buckwheat breeding, but also improved the efficiency by reducing the work of dehulling and grinding the sample. No method validation for the quantitative determination of rutin in buckwheat has been reported. Wang *et al.* (2003) described the development and validation of a liquid chromatography/mass spectrometry (LC/MS) method for rutin in asparagus. The recovery was validated by spiking sample with a known concentration of rutin and the result was calculated to be 95.25 %. A capillary zone electrophoresis method to determine flavone contents in *Crataegus pinnatifida* was reported where the detection limit of rutin was 0.4 μg/ml and the recovery was 97.1 % (Liu *et al.*, 2003). In our method of validation, the

accuracies of both the analytical method and the HPLC system for the three buckwheat species were checked by spiking the sample extracts with three different levels of rutin. The recoveries of rutin for the analytical method were 92.0 – 96.0 % for *F. esculentum*, 91.8 – 95.4 % for *F. homotropicum* and 92.0 – 95.4 % for *F.tataricum*. The recoveries of rutin for the HPLC system were 98.7 – 103.8 % for *F. esculentum*, 99.0 – 102.2 % for *F. homotropicum* and 93.0 – 96.3 % for *F.tataricum*. The relative standard deviations (RSD, reproducibility) were 2.53% for *F. esculentum*, 4.85% for *F. homotropicum* and 1.88% for *F. tataricum.* These validation results show that our recommended method has good reliability and sensitivity and is acceptable for quantification of rutin in buckwheat.

The results from the determination of rutin in 11 buckwheat seed samples showed that the rutin content was significantly different in the three buckwheat species, decreasing in the order: *F. tataricum*, > *F. homotropicum* > *F. esculentum*. Many studies have investigated the difference in rutin content between common buckwheat and Tartary buckwheat. Examining the rutin contents in the two common buckwheat varieties, Mancan and Manor, and a Tartary buckwheat, Steadman *et al.* (2001) reported that the Tartary buckwheat hull contained a similar amounts of rutin (4.37 g/kg) as the hull of common buckwheat (4.41 g/kg in Manor and 0.84 g/kg in Mancan), but the rutin level was 300-fold higher in groats of Tartary buckwheat (81 g/kg) than those in groats of common buckwheat (0.19 g/kg in Manor and 0.18 g/kg in Mancan). Fabjan *et al.* (2003) also reported that three tartary buckwheat cultivars contained more rutin in the seed (0.8 – 1.7 % DM) than did common buckwheat seed (0.01 % DM). These data supported the previous study in which Tartary buckwheat seed had much higher rutin content (1.52 % DM) than common buckwheat seed (0.015 % DM) (Minami *et al.*, 1992). In a recent study involving the analysis of seven common buckwheat

cultivars and seven Tartary buckwheat cultivars in China, the average content of rutin in Tartary buckwheat (1.09%) was reported to be higher than that in common buckwheat (0.42%) (Chai *et al.*, 2004). Our results, with a rutin content 1.67 % DM in Tartary buckwheat seed and 0.02 % DM in common buckwheat seed, were consistent with the reports by Fabjan *et al.* (2003) and Minami *et al.* (1992). There have been no previous literature reports on rutin analysis in *F. homotropicum*. Our study first revealed that this buckwheat species contained 0.10 % rutin in seed, more than common buckwheat but less than tartary buckwheat.

Genetic variation of rutin content in common buckwheat seed within varieties or strains has been investigated extensively, revealing a wide inter-varietal variation of rutin content in common buckwheat (Kitabayashi *et al.*, 1995; Ohsawa and Tsutsumi, 1995; Kalinova and Dadakova, 2004, Chai *et al.*, 2004). Significant difference in rutin content with range of 16.9 – 25.2 mg/100 g seed was also found among common buckwheat cultivars in our study. This result was consistent with the previous studies by Kitabayashi *et al.* (1995), Ohsawa and Tsutsumi (1995), Kalinova and Dadakova (2004), where the rutin content ranged from 12.6 to 35.9 mg, 10.4 to 22 mg, and 14.0 to 24.0 mg per 100 g of seed, respectively. The inter-varietal variation of rutin content also exists in Tartary buckwheat seed. An obvious variation of rutin content in the three Tartary buckwheat cultivars: China 1, China 2, and Luxembourg was observed ranging from 1180 to 1660 mg per 100 g of seed (Fanjan *et al.*, 2003). Ten Tartary buckwheat varieties grown in China exhibited a wide range of rutin contents from 980 – 1160 mg per 100 g of seed (Chai *et al.*, 2004). The rutin contents of the four Tartary buckwheat lines in our study showed a significant difference with a range of 1570 – 1795 mg per 100 g of seed. A wide inter-varietal variation of rutin content in *F. homotropicum* was

first reported in our study, ranging from 74 – 143 mg per 100 g of seed. More research on the rutin content variation is needed on this wild species. Total flavonoids in 11 buckwheats in our study showed significant differences among the three species. Tartary buckwheat contained the highest amount of flavonoids in the seed, on average 2.04 % of DM, followed by *F. homotropicum* buckwheat (0.35 % DM). The lowest flavonoid content was found in common buckwheat seed, on average 0.04 % of DM. Significantly linear correlation between rutin content and total flavonoid content was found in these 11 buckwheat cultivars/lines (R^2 = 0.9952, α =0.01).

Flavonoids comprise the most common group of polyphenolic plant secondary metabolites. Their formation and accumulation can be affected by many factors. These factors include genotype such as species and variety, and environmental conditions such as location and cropping season. These may play an interactive role in the variation of rutin content. The effects of cultivar, location, year and their interactions on rutin content in common buckwheat seeds were comprehensively studied by Oomah and Mazza (1996). Their report showed that environmental effect, i. e., location, and year x location interaction had a much greater contribution to the variation in flavonoid and rutin contents than cultivar or year effects. The result was in agreement with a recent study by Chai *et al.* (2004), who reported that significant differences in rutin content in both common buckwheat and Tartary buckwheat were observed among different varieties as well as among different ecological environments at the experimental sites. In plants, flavonoids play an important role in UV-scavenging and their biosynthesis is also stimulated by UV-B radiation. Through the investigation of the effect of the cropping season on the rutin content of thirty common buckwheat cultivars, Ohsawa and Tsutsumi (1995) reported that the average rutin content in

late summer cropping was about half that of summer cropping. They suggested that the differences in the cumulative solar radiation received in the two seasons could be an explanation for the increase in rutin content in summer cropping. However, following the rutin analysis of three Tartary buckwheat cultivars grown in Slovenia and at the place of origin (China), Fabjan *et al.* (2003) argued that higher altitude associated with higher UV radiation did not necessarily cause an increase in rutin content in buckwheat seeds. Many different environmental conditions such as light, temperature, soil moisture, etc., are involved in the complexity of plant growth. Any or all these factors could affect flavonoid content.

In summary, inter-species or inter-varietal variation of rutin content in buckwheat seeds provides for selection and utilization of genotypes with higher rutin content through buckwheat breeding. The variation of rutin content in different ecological environments helps select the proper buckwheat production sites.

5.1.2 Rutin content in *F. tataricum*

Flavonoids contain one or more structural features which can be involved in complex formation with metals. In the case of flavonols, aluminum is rather unspecific in its chelating power. For example, quercetin (**Figure 43**) is capable to chelating with the 3', 4'-dihydroxyl groups, the 4-carbonyl and -5-hydroxy groups, and 4-carbonyl and -3-hydroxy groups (Cornard and Merlin J. C., 2002). The complex formation of flavonoids with aluminum results in a pronounced bathochromic shift of the spectral bands of flavonoids. Therefore, the spectrophotometric assay based on aluminum chloride complex formation is one of the most common analytical methods to determine flavonoid content. In the application of the aluminum chloride spectrophotometric method, rutin is usually selected to be the working

standard for determination of flavonoids in botanical drugs or medicinal plants (Runha *et al.*, 1999; Matyushchenko and Stepanova, 2003). This spectrophotometric assay could be also used to determine the rutin content in plants (Kreft *et al.*, 2002).

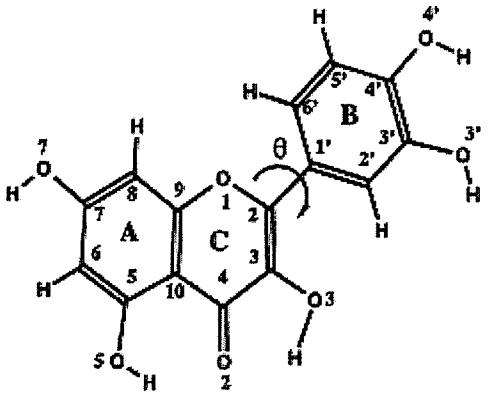


Figure 43. Atom numbering and preferential complex sites of quercetin

This figure is adapted from Cornard and Merlin J. C. (2002)

The spectrophotometric assay for rutin analysis in buckwheat seed and leaf is not only simple and fast but can be performed in most analytical facilities. In our study, the individual plants were examined for rutin content in the seeds and leaves (**Table 11** and **Table 12**). Within each line, the rutin content in the seeds was between 1.83 – 2.13 % for B830222, 1.68 – 2.06 % for K970848, 1.64 – 1.84 % for B930555, and 1.71 – 2.01 % for B770198. The difference in rutin content between the highest and the lowest in buckwheat seeds was 0.3 % in B830222, 0.3 % in B770198, 0.2 % in B930555, and 0.38 % in K970848. Within each line, the rutin content in the leaves ranged from 4.19 – 6.69 % for B830222, 4.74 – 6.69 % for K970848, 5.60 – 8.51 % for B930555, and 4.44 – 7.36 % for B770198. The difference in rutin content between the highest and the lowest in the buckwheat leaves was 2.5 % in

B830222, 2.92 % in B770198, 2.91 % in B930555, and only 1.95 % found in K970848. It is interesting to note that on average, the highest rutin content (6.59 % DM) was found in the line B930555 leaves, but by contrast, it showed the lowest rutin content (1.76 % DM) in its seeds. Reporting data for seven varieties of common buckwheat, Kalinova and Dadakova (2004) found that the rutin content in the seeds did not correlate to that found in the stems or in the flowers, but a positive correlation of rutin content was found in the seeds and in the leaves ($P < 0.01$). However, the rutin content among the Tartary buckwheat lines in our study did not show any correlation between seed and leaf (correlation coefficient $r = 0.2401$). Significant inter-varietal difference in rutin content of Tartary buckwheat in our study was supported by the results of other authors (Fanjan *et al.,* 2003, Chai *et al.*, 2004).

5.1.3 Rutin content in *F. homotropicum*

5.1.3.1 Selection of a *F. homotropicum* for the breeding with *F.esculentum*

Since *F. homotropicum,* a wild buckwheat species, was discovered in the 1990's, it has attracted great attention from breeding scientists due to its self-compatibility and ready ability to cross with common buckwheat. There have been, however, no reports on the rutin content in *F. homotropicum*. Our study investigated the rutin content in the seeds and leaves of individual plants of three *F. homotropicum* lines. The results showed the rutin content of the leaves, with an average of 1.86 % DM for the 990049 line or 1.90 % DM for the 990050 line, was much higher that that in the seeds, with an average of 0.044 % DM for the 990051 line (**Table 14**). No significant difference was observed in the rutin content of the buckwheat leaves between 990049 and 990050. As compared to Tartary buckwheat leaves as presented in **Table 11**, *F. homotropicum* leaves contained lower quantities of rutin ($P < 0.05$). *F.*

homotropicum (990051 line) seeds also had a much lower level of rutin than those of Tartary buckwheat seeds (**Table 12,** $P < 0.05$). Within each of homotropic buckwheat line, the rutin content of the leaves ranged from 0.80 – 3.49 % for 990049 and 0.66 – 3.22 % for 990050. The difference between the highest rutin level and the lowest in 990049 leaves was 2.69 % DM, which was similar to that found in 990050 leaves (2.56 % DM). The 990051 contained between 29 – 78 mg rutin per 100 g of seeds. In collaboration with the buckwheat breeding project at Kade Research, six *F. homotropicum* lines were examined to allow selection of the buckwheat line with a higher rutin content. Our result showed a significant variation of rutin content among the different homotropic buckwheat lines, and a wide range was observed between 55 – 266 mg per 100 g of seeds (**Table 15**). The line K970851, with the highest rutin level in the seed as well as other desirable characters such as non-shattering seed habit (Wang *et al.*, 2004) was chosen for use in the breeding program.

5.1.3.2 A new cross Q010001 between *F. homotropicum* and *F. esculentum*

Through breeding of *F. homotropicum* line, K970851 and self-pollinating buckwheat T990036, a new cross Q010001 was developed by Kade Research. The rutin content of the F_1 in this cross (121 mg per 100 g of seeds) was 1.57 times higher than that in T990036 and 2.2 times lower than that in K970851. In the second generation population of the cross Q010001, a wide variations of rutin contents in both leaves and seeds were observed ranging from 0.89 – 5.39 % DM and 4 mg to 258 mg / 100 g DM, respectively. The rutin content in the seed did not correlate to that in the leaves (correlation coefficient $r = 1.87$). However, the rutin content in the second generation did not fit a normal distribution as was expected from a quantitative trait. The trend of the progeny of the second generation skewed toward the low-

rutin parent. More than 50 % of the plants had a lower rutin content than those found in the low-rutin parent, suggesting that low rutin content may have a dominant effect in crossing. Studies with larger population sizes and further genetic studies are required to obtained further information in this area and fully elucidate the breeding efficiency (Wang *et al.*, 2004).

5.2 Protective effects of flavonoids and buckwheat against lipid peroxidation in low-density lipoprotein of rabbits

5.2.1 Effects of rutin and quercetin on low-density lipoprotein peroxidation in hydrophilic and lipophilic free radical generating systems

Antioxidant activities have been the most studied among the multiple biological effects of flavonoids. Quercetin is one of the most active antioxidative flavonoids and is the most effective in the flavonol class (Rice Evans, 1996). It is ubiquitous in plants in the form of glycoside such as rutin. Studies indicate that as naturally occurring antioxidants, these flavonols exhibit antioxidative activities in lipid oxidation during the initiation and the propagation phases. However, the relative antioxidant activity of quercetin and its glycosides has been controversial. Quercetin has been reported to be more effective than rutin in suppressing lipid peroxidation in human low-density lipoprotein (Hou *et al.*, 2004), rat liver microsomes (Yang *et al.*, 2001), hepatic lysosomal fractions of mice (Kazuo Nakagawa *et al.*, 2000), and nonpolar methyl linoleate (Hopia and Heinonen, 1999; Pekkarinen *et al.*, 1999). However, the opposite result was obtained with linoleic acid in an aqueous medium (Torel *et al.*, 1986) and in mouse liver homogenates (Yuting *et al.*, 1990). In Cu^{+}-induced human LDL oxidation, rutin demonstrated the same antioxidant activity as quercetin

(Teissedre *et al.*, 1996). The different methodologies used in these studies contributed to these controversial results (Hopia and Heinonen, 1999). Many factors should be taken into account when evaluating the antioxidant potential of a compound. These factors include the mechanism of the antioxidant action (free radical scavenging, metal ion chelating, or others), location (in aqueous phase, within lipid domain, or both), lipid system (polar lipid model or nonpolar lipid model), and the method used to detect the extent of lipid oxidation. In view of the pathological relevance to atherosclerosis, our study used LDL from rabbit plasma. Lipid peroxidation is accompanied by the formation of conjugated diene structures and results in the production of thiobarbituric acid reactive substances (TBARS). These two methods, measurement of conjugated diene formation and determination of MDA production, are used widely to assess antioxidant ability in oxidizing lipid systems (Halliwell and Chirico, 1993).

Figure 44 shows the chemical structures of azo-compounds 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN), which were used in our study to generate hydrophilic and lipophilic free radicals, respectively.

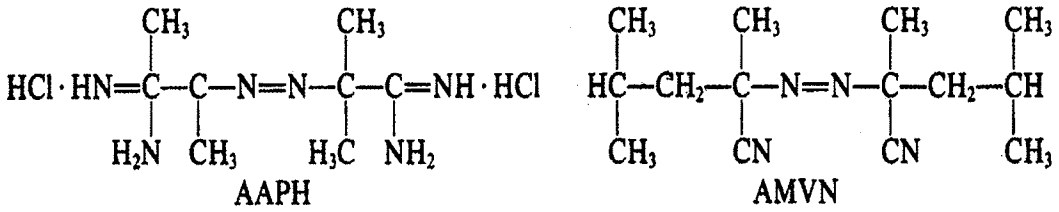


Figure 44. Chemical structures of azo-compounds

This figure is adapted from Etsuo Niki (1990).

Azo-compounds decompose thermally without biotransformation to produce a nitrogen molecule and two carbon radicals (Equation 1). The carbon radicals react readily with oxygen molecules to generate peroxyl radicals (Equation 2) (Niki, 1990), which in turn initiate lipid peroxidation by abstracting a hydrogen atom from the polyunsaturated fatty-acid side-chain in a lipid molecule (Equation 3). The propagation chain reaction of lipid peroxidation can be expressed in equations (2) and (3). Flavonoids are capable of preventing propagation by donating a hydrogen atom to a peroxyl radical, which terminates the

$$R—N=N—R \rightarrow R^{\cdot} + N_2 + R^{\cdot} \qquad (1)$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\bullet} \qquad (2)$$

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot} \qquad (3)$$

radical chain reactions (Equation 4) (Torel, 1986). A hydrophilic azo-compound AAPH and a

$$ROO^{\bullet} + AOH \rightarrow ROOH + AO^{\bullet} \qquad (4)$$

lipophilic azo-compound AMVN have been used successfully to provide a constant rate of peroxyl free radical production in the aqueous phase and within the lipid region, respectively (Niki, 1990).

Results of the TBARS test in our study demonstrated that the antioxidant efficiency against AAPH induced LDL peroxidation decreased in the order: quercetin > α-tocopherol > rutin > ascorbic acid. However, even at the highest concentration in this study, quercetin (30 μM), rutin (30 μM) and ascorbic acid (400 μM) did not show any protective effect against AMVN induced-LDL peroxidation. Lipophilicity of both antioxidants and free radicals has been reported to be an important factor in determining the extent of lipid peroxidation in LDL (Massaeli *et al.*, 1999). With its high lipophilicity, α-tocopherol is located within the

lipid region of LDL where lipophilic radicals are generated. Quercetin and rutin are more hydrophilic than α-tocopherol and are localized near the surface of LDL, scavenging the radicals formed in the aqueous phase or on the polar surface of LDL. Our data were supported by the results obtained from a lipophilic initiator α, α'- azoisobutyronitrile (AIBN) induced methyl linoleate oxidation, which indicates that the reactivity of quercetin as H-atom donors toward the peroxyl radicals was not comparable to that of α-tocopherol in the *tert*-butyl alcohol, a "water-like solvent" (Pedrielli *et al.*, 2001). Consistent with the result reported by Massaeli *et al.* (1999), our study showed that α-tocopherol was more effective in scavenging the peroxyl radicals in an aqueous phase than those in a lipid region. α-Tocopherol comprises a chroman head group and a phytyl side chain. Within a lipid bilayer, the chroman head group is orientated toward the surface with the function of scavenging free radicals, and the phytyl side chain is buried within the hydrophobic region fixing α-tocopherol in the lipid bilayer (Van Acker *et al.*, 1993). This characteristic of α-tocopherol within LDL may partly account for the difference in antioxidant capacity between the aqueous and the lipid phases.

The CLT_{50} values of lag time obtained from the measurement of conjugated diene formation indicated that in a hydrophilic free radical induced-LDL peroxidation, the inhibitory capacity of the test compounds decreased in the order: quercetin = rutin > α - tocopherol > ascorbic acid. In comparison to the other three compounds, ascorbic acid is hydrophilic and the weakest antioxidant in these systems. The CLT_{50} of ascorbic acid was 30 times higher than that of quercetin. With the exclusion of ascorbic acid, the statistical analysis focused on quercetin, rutin and α - tocopherol. The CLT_{50} values revealed that activity of antioxidants decreased in the order of quercetin > rutin > α - tocopherol.

Quercetin is generally reported to be more active than rutin in nonpolar lipid models (Hopia and Heinonen, 1999; Pekkarinen *et al.*, 1999), but their relative activity is controversial in polar lipid systems due to variable experimental conditions. In our study, the IC_{50} value from TBARS assay and the CLT_{50} value, demonstrated that quercetin was more effective in protecting LDL from peroxidation than rutin. This result was supported by a recent study, in which quercetin possessed a stronger protective effect against AAPH- and Cu^{2+}- initiated LDL peroxidation than rutin, when using either the uptake of oxygen method or the TBARS assay (Hou *et al.*, 2004). A hydroxyl group at the position C-3 of the C ring is critical for maximizing free radical scavenging activity of flavonoids (Cook and Samman, 1996; Bors *et al.*, 1990). Hedrickson *et al.* (1994) reported that a hydroxyl group in the C_3 position was more oxidizable than hydroxyl groups in the A ring based on the measurement of redox potential, and the oxidation rate of quercetin was faster than that of rutin. This suggests that the lower antioxidant activity of rutin relative to quercetin may be attributed partly to its lower oxidizability due to the presence of disaccharide in the C_3 position. According to the rate constant of reaction of antioxidants with peroxyl radicals, quercetin was found to be less active than α - tocopherol in linoleic acid micelles using AAPH as an initiator (Pryor *et al.*, 1993). This is in agreement with the report that as a H-donor to methyl linoleate peroxyl radicals initiated by α, α'-azoisobutyronitrile, α - tocopherol had higher reactivity than quercetin in both a nonpolar and a polar solvent (Pedrielli *et al.*, 2001). However, by monitoring the methyl linoleate hydroperoxide formation, hydroperoxide isomer distribution and hydroperoxide decomposition, Hopia *et. al.* (1999) and Pekkarinen *et al.* (1999) showed that although α - tocopherol was a more effective hydrogen donor, quercetin was a more effective antioxidant in inhibiting methyl linoleate oxidation. Both the TBARS test and the

conjugated assay in this study demonstrated that quercetin was more protective against AAPH induced LDL peroxidation than α - tocopherol. With respect to the relative activity of rutin and α - tocopherol, the two methods showed opposite results. Rutin was less effective in the TBARS test but more effective in the conjugated diene assay. This may be partially attributed to the different methods employed. TBARS test is simple but non-specific. During the test, high temperature and low pH may cause artefactural formation of lipid peroxidation products. On the other hand, not all lipid peroxides will form TBA-reactive material (Halliwell and Chirico, 1993). Therefore, two methods were used in the evaluation of antioxidant activities in this study.

In summary, although α - tocopherol expressed the antioxidant activity in the lipid region within LDL (AMVN system), whereas the flavonol compounds quercetin and rutin did not, quercetin was more effective than its glycoside rutin and α - tocopherol in suppressing hydrophilic free radical induced LDL oxidation (AAPH system). By measuring conjugated diene formation, rutin demonstrated higher oxidation inhibitory capacity than α - tocopherol in the water-soluble free radical generating system. It has been suggested that LDL oxidation is most likely initiated in the aqueous environment surrounding the lipoprotein particle (Frei, B., 1995). The evidence presented in our study reveals the potential of quercetin and rutin to prevent the initiation of lipid peroxidation.

The dietary intake of flavonoids is much higher than those of vitamin E and β-carotene. Quercetin, a major flavonoid consumed in the diet, is widely distributed in vegetables such as onions, broccoli and French beans; fruits such as apple and grape; and in various beverages such as red wine, black tea and green tea (Hertog *et al.*, 1992; Hertog *et al.*, 1993). However, the beneficial effects of flavonoids are dependent upon their bioavailablities in the human

body. Therefore, much attention has been paid to the pharmacokinetics of flavonoids with a particular focus on their absorption and metabolism. An early study on the pharmacokinetics of quercetin reported that no measurable plasma concentrations could be detected after oral administration, questioning the value of oral administration of flavonoids (Gugler *et al.*, 1975). In a study on the bioavialability of quercetin and rutin in rats, Manach *et al.* (1995) reported that the concentration of circulating flavonols was approximately 115 mmol/L in rats fed diets containing 16.4 mmol quercetin or rutin/kg, suggesting dietary flavonols were recovered in rat plasma as conjugated metabolites in non-negligible concentrations. They further reported that the rate of elimination of quercetin metabolites in rats was low, and high plasma metabolite concentrations were easily maintained with a regular supplementation of quercetin or rutin in the diet (Manach *et al.*, 1997). Many studies have indicated that the sugar moiety in quercetin glycosides affects their absorption and bioavailabilities. According to the study on the absorption of quercetin glycosides in man, Hollman *et al.* (1999) reported that the peak concentration of quercetin in plasma was 20 times higher and reached more than ten times faster after intake of the quercetin glucoside than after intake of the quercetin rutinoside. This result supported their earlier study on the bioavailability of quercetin from different dietary sources, in which peak quercetin plasma concentrations were achieved less than 0.7 of an hour after intake of onions containing quercetin glucosides, 2.5 h after intake of apples containing both glucose and non-glucose quercetin glycosides, and 9 h after intake of pure rutinoside; the half-lives of elimination were 23 hours for apples and 28 hours for onions, suggesting that repeated consumption of quercetin-containing foods could result in accumulation of quercetin in the blood (Hollman *et al.*, 1997). The absorption of quercetin or quercetin glycosides was found to be higher from onions (up to 53%) than those from pure

quercetin aglycone, apples, buckwheat teas, black teas and red wine (Hollman *et al.*, 1995; De Vries *et al.*, 1998; De Vries *et al.*, 2001; Graefe *et al.*, 2001). Quercetin was found to be in the conjugated form with a glucuronide moiety, sulfate or methyl group (Danny *et al.*, 2003). Some metabolites including quercetin-7-glucuronide, quercetin-3-glucuronide, isorhamnetin-3-glucuronide, and quercetin-3'-sulfate, were effective in inhibiting LDL oxidation (Justino *et al.*, 2004). Therefore, quercetin and rutin, as nutraceuticals, have the potential of protecting against coronary heart disease. More *in vivo* studies in animals and clinical research in humans are still required in this area in the future.

5.2.2 Protective effects against low-density lipoprotein peroxidarion of the three buckwheat species

Having investigated 11 cultivars/lines of the three buckwheat species, our study showed that Tartary buckwheat, with the highest flavonoid and rutin contents as compared to *F. homotropicum* and common buckwheat (**Table 8** in 4.**1.1.1** and **Table 10** in 4.**1.1.2**), exhibited the most effective inhibition of LDL peroxidation that was superior to *F. homotropicum* and common buckwheat (**Table 21** in 4.**2.2**). Our study revealed an interspecies linear correlation between rutin content and flavonoid content and also indicated that both flavonoid and rutin content in buckwheat seeds correlated to the protective effect against Cu^{2+}-induced LDL peroxidation in all cultivars/lines of the three species. Among the six flavonoids: rutin, orientin, vitexin, quercetin, isovitexin, and isoorientin which have been isolated and identified in common buckwheat grain, rutin and isovitexin are the only flavonoid components of buckwheat seeds while the hulls contain all six of the identified compounds. The total flavonoid concentration in the seeds was 18.8 mg/100 g of DM and

rutin attributed most of this flavonoid content (Dietrych-Szostak and Oleszek, 1999). According to the study on the antioxidant activities of the isolated compounds from common buckwheat hulls, Watanabe *et al.*(1997) reported that vitexin and isovitexin did not show peroxyl scavenging activity in buckwheat hull extracts. In addition, an antioxidant activity of mixed varieties of common buckwheat was also reported to significantly correlate to total phenolics or to rutin content in the evaluation of antioxidant activities of buckwheat seed, dehulled seed, hull, straw and leaf (Holasova *et al.*, 2002). All these findings support our results and suggest an important role of rutin in the antioxidant capacity of buckwheat. However, according to Oomah and Mazza (1996), the antioxidant activity in common buckwheat seeds demonstrated a weak association with total flavonoids and no correlation to rutin content, suggesting that components other than flavonoids present in buckwheat seed may contribute substantially to the antioxidant activities. Four catechins were observed to be abundant as was rutin in common buckwheat groats, but their antioxidant activity was superior to rutin (Watanabe *et al.*, 1998). In comparison of total phenolics, flavanol, proanthocyanidin, and flavonoid in a French common buckwheat variety 'La Harpe' with the antioxidant capacity of scavenging hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hypochlorous acid (HOCl), Quettier-Deleua *et al.* (Quettier-Deleua *et al.*, 2000) reported that the higher efficiency of buckwheat flour should be mainly attributed to flavanol-type, with a complementary contribution by the flavonoids, rather than solely to rutin. These controversial results may be attributed to use of different species or varieties in the various studies or the difference in methodology of the antioxidant activity measurement. Nevertheless, further studies are required to resolve the controversial results.

The oxidative modification of low-density lipoprotein is pathologically relevant to atherosclerosis, and monitoring conjugated diene formation in isolated LDL is commonly used in evaluation of the antioxidant capacity due to its simplicity and reliability (Esterbauer *et al.*, 1992; Halliwell *et al.*, 1993). The transition metal ions such as iron and copper salts greatly accelerate the decomposition of preformed lipid peroxides if present in LDL to give both peroxy and alkoxy radicals capable of abstracting hydrogen, thus continuing the chain reaction of lipid peroxidation (Esterbauer *et al.*, 1992; Frei and Gaziano, 1993). Therefore, copper ions are widely used to peroxidize LDL in many *vitro* studies. With the model of copper-induced isolated LDL oxidation, our study revealed that total flavonoids, especially rutin content in buckwheat seeds correlated to the protective effect against Cu^{2+}-induced LDL peroxidation in all test cultivars/lines of the three species. *F. tatarium*, with the highest rutin content as compared to *F. homotropicum* and *F. esculentum*, exhibited the most effective inhibition of LDL peroxidation than *F. homotropicum* and *F. esculentum*. *F. esculentum*, with the lowest rutin content, showed the weakest antioxidant ability. The findings in our present study suggested buckwheat and its flavonoids, especially rutin content have the potential to benefit the cardiovascular system. However, more *in vivo* studies in animals and clinical research in humans are required prior to development of functional foods containing high rutin cultivars.

5.2.3 Effects of *F.tataricum* on low-density lipoprotein peroxidation in hydrophilic and lipophilic free radical generating systems

5.2.3.1 Antioxidant activities of *F. tataricum* in both aqueous and lipid environments of LDL using TBARS test

In our study, all Tartary buckwheat lines exhibited an inhibitory effect on AAPH-induced MDA production in LDL, whereas the common buckwheat did not show the inhibitory effect (**Figure 34**). Tartary buckwheat intake has been reported to reduce blood lipid, glucose and cholestrol levels in clinical trials (He *et al.*, 1995; Zhao and Qiu, 1998; Lin *et al.*, 2004). Various tartary buckwheat health foods have been developed such as Tartary buckwheat kernel tea, Tartary buckwheat red koli vinegar, Tartary buckwheat vinegar soft-capsules, Tartary buckwheat noodle, and Tartary buckwheat flavonoid extracts (Bian *et al.*, 2004). Wang *et al.* (1998) reported that ingestion of Tartary buckwheat leaf extract markedly increased activities of superoxide dismutase (SOD) and GSH-peroxidase (GSH-Px) in blood, liver, and heart of mice, and decreased the amount of MDA in these organs. However, little research on inhibitory effect of Tartary buckwheat seed on lipid oxidation has been reported. Our data revealed that all Tartary buckwheat extracts tested were effective in inhibiting AAPH-induced lipid peroxidation in LDL. From the data on the antioxidant activity in buckwheat seeds, dehulled seeds, hulls, straws and leaves, Holasova *et al.* (2002) reported a significant correlation between total phenolics content as well as rutin content and antioxidant activity of buckwheat material ($R^2 = 0.987$, $P< 0.002$ and $R^2 = 0.972$, $P< 0.002$, respectively). Upon examination of the antioxidant capacity of honey from various floral sources, including buckwheat honey, a linear correlation was observed between the phenolic content and antioxidant activity ($R^2 = 0.9497$, $P< 0.0001$) (Gheldof and Engeseth, 2002). The four tested Tartary buckwheat extracts in the present study had similar levels of rutin and did not show any difference in protecting against AAPH-induced lipid peroxidation. The high rutin content (average of 1.90 g/100 g DM) in Tartary buckwheat seed, which is 32 times more than in common buckwheat seed (0.06 g/100 g DM) (**Table 23 in 4.2.3**), contributed to

the potent antioxidant capacity of the Tartary buckwheat seed. Neither Tartary buckwheat nor common buckwheat caused a significant inhibitory effect on AMVN-induced lipid peroxidation (**Figure 35**). The degree of lipid peroxidation and the protectant ability of antioxidants are modified by the lipophilicity of the reagents generating free radicals and the solubility of antioxidants in lipid or water. AAPH and AMVN generate peroxyl free radicals in the aqueous phase and within the lipid region, respectively. When using the TBARS test in hepatic lysosomal fractions of mice, Nakagawa *et al.* (2000) reported that quercetin and rutin exhibit protection against AAPH-induced lipid peroxidation more effectively than that induced by AMVN, suggesting that the affinities of antioxidant compounds and the radical generators in the lipid phase influenced their antioxidant abilities. Rutin is relatively hydrophilic and localizes near the surface of LDL, scavenging the radicals in the aqueous phase generated by AAPH. In the study by Nakagawa *et al* (2000), the production of lipid peroxidation was 80% of the control when rutin was added at a concentration of 100 μM in a lipophilic free radical generating system. In our study, the finding of no significant inhibition in AMVN-induced lipid peroxidation by rutin may partially be due to the low rutin concentration obtained by the addition of Tartary buckwheat extract to the LDL system (12.5 μM). When the rutin concentration was increased to over 30 μM, a precipitate was found in the LDL reaction system, but which has not yet been explained.

5.2.3.2 Protective effect of *F. tataricum* in an aqueous environment on the early stage of LDL peroxidation determined by the conjugated diene assay

The results of conjugated diene assay showed that all Tartary buckwheat extracts produced increase in lag time as compared to the control, whereas the common buckwheat extract and

the control produced a similar lag time (**Figure 36 and Table 25** in **4.2.3.2**). Four Tartary buckwheat extracts had a similar rutin level with an average concentration of 2.5 μM in the AAPH-induced lipid peroxidation system, and also exhibited a similar protection against lipid peroxidation in an aqueous area surrounding the LDL. Zielinski and Kozlowska (2000) reported that 80% methanolic extract of common buckwheat exhibited an ability to inhibit AAPH-induced lipid peroxidation *in vitro* using the phosphatidylcholine (PC) liposome system. However, the results from both TBARS test and the conjugated diene assay showed that the common buckwheat extract did not have antioxidant activity. This may be due to the very low rutin concentrations in our study, 0.38 μM in TBARS test and 0.08 μM in the conjugated diene assay. The comparison of the antioxidant activities of the *F. tataricum* extract and rutin demonstrated that Tartary buckwheat extract and standard rutin solution, both containing 2.5 μM of rutin in LDL system, had a similar protective effect against LDL peroxidation (**Figure 37** and **Table 26** in **4.2.3.2**). This finding suggested that rutin plays a major part in antioxidant activity in Tartary buckwheat seed.

5.2.4 Effects of *F.homotropicum* on lipid peroxidation in an aqueous environment surrounding low-density lipoprotein

The three buckwheat lines evaluated showed a significant difference in the rutin content of their seeds in the decreasing order of K970851 (266 mg/100 g DM) > Q010001 (121 mg/100 g DM) > T990036 (77 mg/100 g DM) with the rutin content as obtained from HPLC analysis being presented in **Table 16** in 4.**1.3.2**. No rutin was detectable in the wheat samples, Kyle CWAD or AC Barrie that were evaluated.

The rutin concentration in the TBARS test by adding buckwheat extracts to the LDL and AAPH reaction system was 8.8 μM in K970851, 2.5 μM in T990036 and 4.0 μM in Q010001 (**Table 27 in 4.2.4**). The result obtained from TBARS test indicated that the three buckwheat extracts had a protective effect against AAPH-induced lipid peroxidation, resulting in MDA production of LDL 48.9 %, 71.3 %, and 67.4 % of the control in K970851, T990036 and Q010001, respectively. When compared to the control, Kyle CWAD or AC Barrie did not show any significant difference in MDA production (**Figure 38 in 4.2.4.1**).

The result of the conjugated assay showed that all three buckwheat extracts exhibited an inhibitory effect on LDL peroxidation with an increase in lag time, whereas the two wheat extracts produced a similsr lag time to that of the control. In the conjugated diene assay, the rutin concentration as determined by adding buckwheat extracts to the LDL and AAPH reaction system was 1.75 μM in K970851, 0.50 μM in T990036 and 0.80 μM in Q010001 (**Table 27 in 4.2.4**). Our results from the conjugated diene assay demonstrated the protective capacity against LDL peroxidation of the three buckwheat lines significantly decreased in the order: K970851 > Q010001 > T990036, and also suggested that rutin played an important role in the antioxidant activity of the buckwheat. It is considered that the specificity of the TBARS test is relatively low, as aldehydes other than MDA can form chromogens with absorbance at 532 nm and many different aldehydes may be formed in peroxidizing lipid materials (Esterbauer, *et al.*, 1991). This may partially explain why no difference in antioxidant acivity was found between the buckwheat Q010001 and T990036 when using the TBARS test. Our present finding, however, reveals the potential for developing *F. homotropicum* lines with increased rutin content and the subsequent transfer of this desired

trait to common buckwheat lines to protect lipid peroxidation in an aqueous environment surrounding low-density lipoprotein.

5.3 Effect of quercetin on the oxidative stress in cardiomyocytes of rats

Fluorescein is the simplest of the fluorine dyes. Numerous fluorescein derivatives, including 2' 7' –dichlorofluorescin (DCFH), are in common use as fluorochromes or fluorescent probes. The chemical structures of DCF and its derivatives, and the mechanism to measure the intracellular oxidation are presented in **Figure 45.** The diacetate ester of 2' 7' –dichlorofluorescin (DCFH-DA), an esterified form of 2' 7' –dichlorofluorescin, is nonpolar and nonionic. It is a stable lipid-soluble compound that can easily penetrate cell membranes and is then enzymatically hydrolyzed by intracellular esterases to nonfluorescent 2' 7' –dichlorofluorescin (DCFH) within the cells. DCFH is trapped in the cytosol and oxidized by reactive oxygen species (ROS) to emit the green fluorescent 2', 7' –dichlorofluorescein (DCF). Therefore, the DCF fluorescence can be used as a sensitive cytosolic marker for oxidative stress (LeBel, *et al.*, 1992). However, this method is limited by a low specificity, because it could be an indicator of cellular redox status rather than a real estimaton of ROS production. Some substances are involved in the oxidation of DCFH to DCF, including peroxidase, peroxynitrite, and other reactive intermediates (Batandier, *et al.*, 2002). In addition, among ROS hydroxyl radical, superoxide, and hydrogen peroxide, only hydrogen peroxide is responsible for DCFH oxiation and the emitted fluorescence is directly proportional to the concentration of hydrogen peroxide (LeBel, *et al.*, 1992). Despite these considerations and limitations, this method is still commonly performed with a fluorimeter or image analysis system.

Among the methods utilized to measure oxidative stress or to evaluate antioxidant capacities, most are based on the *in vitro* chemical assay systems such as the widely used thiobarbituric acid reactive substances (TBARS) test, conjugated diene assay, and oxygen radical absorbing capacity (ORAC) determination. The application of 2', 7'-dichlorofluorescin diacetate (DCFH-DA), a cell-based fluorescent method, makes it possible to measure the oxidative stress in live cells and to feedback *in vitro* results into *in vivo* evaluation. Moreover, when connected with a microscope imaging system, this technology enables visualization of the *in vivo* situation at the cellular level. Dichlorofluorescein (DCF), as the fluorescent marker, has been used to study the role of reactive oxygen species (ROS) in various pathophysiological and physiological phenomena, because both *in vitro* and *in vivo* studies have indicated that dichlorofluorescin (DCFH) can be oxidized to dehydrodichlorofluorescin (DCF) by various ROS, such as H_2O_2 and several lipid hydroperoxides. ROS are recognized as important causal factors for cardiovascular diseases, such as heart ischemia-reperfusion injury, left ventricular hypertrophy, induction of preconditioning, and cardiac arrhythmias. However, up to now, researchers who reported the application of DCFH method for the evaluation of natural antioxidants at cell level, almost always used the human promyelocytic HL-60 cell line for their studies (Lin *et al.*, 2000; Bestwick and Miline, 2001; Takamatsu *et al.*, 2003). When considering the pharmacological relevance, our present study employed the dichloroflurescin method to investigate the effect of quercetin on H_2O_2-induced oxidative stress in cardiomyocytes.

With respect to the effect of quercetin or other flavonoids on the oxidative stress within HL-60 cells using DCF as an indicator, results were controversial. Bestwick and Miline (2001) reported that incubation with 1-100 μM quercetin reduced the extent of the

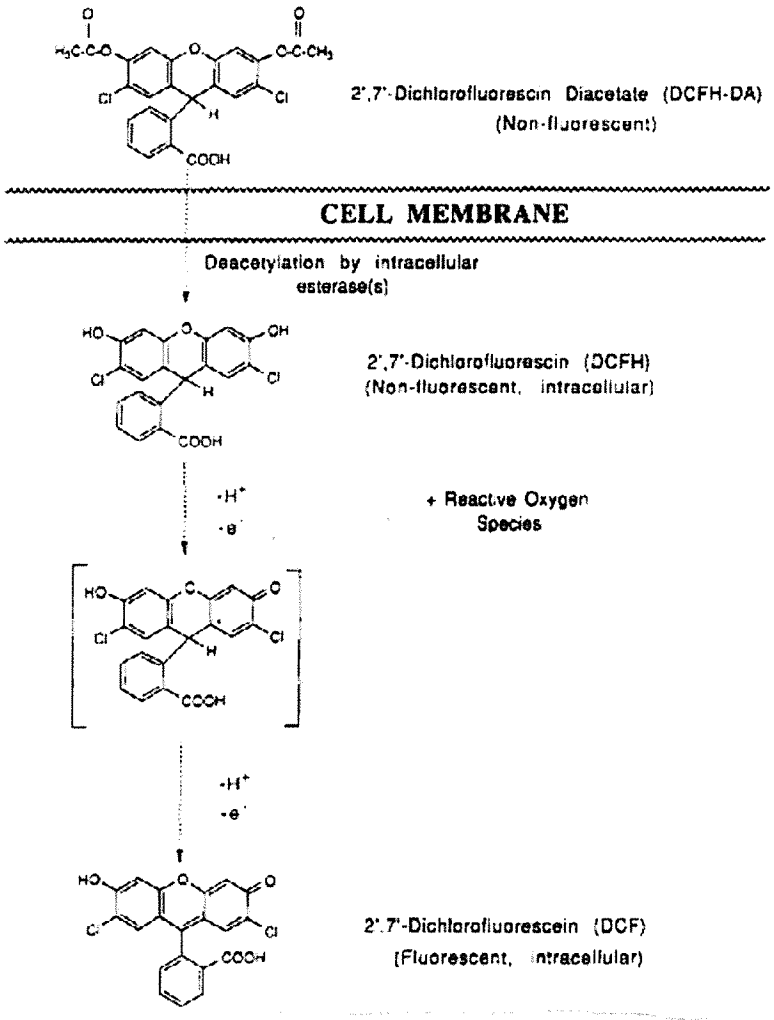


Figure 45. Proposed mechanism of entry of DCFH-DA into cells

This figure is adapted from LeBel C. P., *et al,* (1992).

menadione-induced increase in DCF in HL-60 cells. However, in the study of the inhibitory effects of flavonoids on exogenous cytoplasmic ROS-catalysed oxidation of DCFH in HL-60 cells by the treatment of phorbol 12-myristate 13-acetate (PMA), Takamatsu *et al.* (2003) reported that quercetin did not show apparent antioxidant activity measured by a microplate assay. He explained perhaps because it was a coloured sample in a dissolved condition. Our study demonstrated that 24-hour pre-incubation with 5 μM quercetin significantly reduced the H_2O_2-induced oxidative stress in cardiomyocytes. Similar observations were reported by Ramadass, *et al.* (2003) -in a study of the effects of dietary flavonoids on polychlorinated biphenyls-induced oxidative stress in vascular endothelial cells, quercetin was found, in a concentration-dependent manner, to strongly inhibit oxidative stress as measured by DCF fluorescence. Recently, protective effect of selected flavonoids against doxorubicin-induced toxicity in rat cardiomyocytes has been reported (Psotova, *et al.*, 2004). In this study, the cardiomyocyte protection was assessed by the extracellular lactate dehydrogenase (LDH) activity and the production of cellular ADP and ATP, and quercetin showed significantly better protective effect than dexrazoxan, an agent currently used for adjuvant therapy during doxorubicin. The latest report also demonstrated that quercetin after 24-hours of co-incubation with daunorubicin significantly increased the cardiomyocyte survival assessed by MTT test ant extracellular LDH detection (Mojzisova, *et al.*, 2006). Our study, using DCF as the fluorescent marker and a microscope imaging system, visualized the situation within cardiomyocytes. The result was consistent with the notion that the use of quercetin may be beneficial in preventing the cardiotoxicity associated with ROS-induced oxidative stress. However, the toxicological and pharmacokinetic property of quercetin or other flavonoids remain unclear and need to be further studies *in vivo*.

Chapter VI Conclusion

In this thesis, we developed an accurate, rapid, and simple high-performance liquid chromatographic assay for rutin in different buckwheat species that can be utilized in a buckwheat breeding program. With good reliability and sensitivity, this HPLC method is acceptable for routine rutin analysis of different buckwheat species. The results obtained from the determination of the rutin content by HPLC and total flavonoid content by spectrophotometric assay in 11 buckwheat cultivar/lines showed that both rutin content and total flavonoid content were significantly different in the buckwheat seed of the three species, decreasing in the order of: *F. tataricum*, > *F. homotropicum* > *F. esculentum*. On average, the rutin content in *F. tataricum* seed was 17 times that in *F. homotropicum* seed and 83 times that in *F. esculentum* seed. The total flavonoid content in *F. tataricum* seed was 6 times that in *F. homotropicum* seed and 55 times that in *F. esculentum* seed.

Forty-two individual plants of four *F. tataricum* lines were used to investigate the rutin content in the leaves and another 46 individual plants of the same cultivars were utilizes for evaluation of the rutin content in the seeds. The results obtained by the spectrophotometric assay indicated that on average, the rutin content in *F. tataricum* leaves was 4.8 times that in *F. tataricum* seeds, and a significant inter-cultivar difference in rutin content was found in both *F. tataricum* seeds and *F. tataricum* leaves ($P < 0.05$). Among *F. tataricum* lines evaluated, no correlation between the rutin content of the seed and leaf was observed in our study (correlation coefficient $r = 0.2401$). Thirty individual plants of two *F. homotropicum* lines were used to investigate the rutin content in the leaves and another 10 individual plants of one *F. homotropicum* lines were utilized to determine the rutin content of the seeds. The results obtained by a spectrophotometric assay showed that on average, rutin content in *F.*

homotropicum leaves was 42.7 times that in *F. homotropicum* seeds, but no significant inter-cultivar difference in rutin content was found in *F. homotropicum* leaves.

Our study on the rutin content of the new cross Q010001 developed by Kade Research and its parents, a *F. homotropicum* line and a self-pollinating buckwheat line exhibited the hybridization potential between *F. homotropicum* and *F. esculentum.* The results obtained from rutin analysis by HPLC in the F_2 generation of the cross Q010001 with a population of 117 individual plants demonstrated wide variation in rutin content in both the leaves and seeds. However, no correlation of rutin content was found between the leaves and seeds (correlation coefficient 0.1866). The distribution of rutin content in the F_2 generation was normal in the leaves but not normal in the seeds as expected for a quantitative trait, and the trend of the seed population was skewed toward the low-rutin parent. Investigation with a larger population sizes and more genetic research are required to obtain further information in this area.

In respect to the protective effects of quercetin and rutin against LDL oxidation induced by hydrophilic free radical generator AAPH or lipophilic free radical generator AMVN, the results obtained from TBARS test showed that in an AAPH system, the antioxidant activity of rutin was higher than that of the hydrophilic antioxidant ascorbic acid but lower than that of the lipophilic antioxidant α-tocopherol, and that quercetin demonstrated the strongest oxidation-inhibitory capacity among the four test antioxidants. However, over the range of the test concentration, only α-tocopherol exhibited an antioxidant activity in the AMVN system. The results of the conjugated diene assay indicated that in the AAPH system, no significant difference in antioxidant activity was found between quercetin and rutin, and that both of them were more effective in inhibiting LDL oxidation than ascorbic acid and α-tocopherol. It has been suggested that LDL oxidation is most likely initiated in the aqueous

environment surrounding the lipoprotein particle (Frei, B., 1995). Therefore, the evidence observed in our study reveals the potential of quercetin and rutin to prevent the initiation of lipid peroxidation.

Eleven buckwheat cultivars/lines were used to investigate the protective effects of buckwheat against copper-induced LDL oxidation, with these representing three buckwheat species. The results obtained from the conjugated diene assay showed an inhibitory effect on LDL oxidation of the three buckwheat species, decreasing in the order: *F. tataricum* > *F. homotropicum* > *F. esculentum*. The antioxidant activity of the buckwheat cultivars/lines positively correlated to their total flavonoid content as well as to rutin content. All three buckwheat species exhibited a dose-response effect of buckwheat methanol extract against LDL oxidation.

To examine the antioxidant activity of *F. tataricum* in a hydrophilic AAPH or a lipophilic AMVN system, four *F. tataricum* lines were used in this study and one *F. esculentum* cultivar in commercial use was utilized as a comparison. The results of the TBARS test indicated that all *F. tataricum* lines were effective in inhibiting lipid oxidation, whereas the commercial *F. esculentum* cultivar had no an antioxidant activity in the AAPH system. However, both *F. tataricum* and *F. esculentum* did not show a protective effect against lipid oxidation in the AMVN system. The results of a conjugated diene assay in the AAPH system also demonstrated that all *F. tataricum* lines exhibited an antioxidant activity, whereas the commercial *F. esculentum* cultivar did not show a protective effect against lipid oxidation. Furthermore, no significant difference in antioxidant activity was found between the *F. tataricum* extract and the standard rutin solution, when both of them contained the same concentration of rutin.

The antioxidant effectiveness of the cross Q010001 and its parents K970851 and T990036 in the AAPH system was studied, with two commercial wheat varieties: Kyle CWAD and AC Barrie being used for comparison. The TBARS test indicated that three buckwheat lines, with rutin in their extracts, had a protective effect against lipid peroxidation, while Kyle CWAD or AC Barrie, in which no rutin was detected, did not demonstrate any antioxidant effect. The result of a conjugated assay showed an inhibitory effect on LDL peroxidation by all three buckwheat lines with their antioxidant ability being in the order: K970851 > Q010001 > T990036. The two wheat varieties used in the conjugated diene assay did not show a inhibitory effect on LDL peroxidation.

When dichloroflurescin (DCFH) was utilized as a cellular fluorescent marker in neonatal cardiomyocytes, the effect of quercetin on H_2O_2-induced oxidative stress showed that quercetin treated cells exhibited significantly lower fluorescence intensity than that of the control cells. This indicated that quercetin had an inhibitory effect on oxidative stress in neonatal cardiomyocytes.

In summary, quercetin and its glycoside rutin possessed a protective property against lipid oxidation in the aqueous environment surrounding plasma LDL. Quercetin also exhibited an *in vitro* inhibitory effect on oxidative stress of neonatal cardiomyocytes. The rutin content in seed of the three buckwheat species decreased in the order *F. tataricum* > *F. homotropicum* > *F. esculentum,* and their antioxidant activity decreased in the same order. The rutin content in buckwheat seed was positively associated with their antioxidant activity, but no correlation for rutin content was found between buckwheat leaves and buckwheat seeds. Developing buckwheat lines from *F. homotropicum* was possible and meaningful from the perspectives of rutin and antioxidant ability, and further studies in this aspect are required and deserved. The

present study demonstrated that *F. homotropicum* can be used for buckwheat breeding development, and an improved buckwheat line with high rutin content will have potential as a functional food for preventing cardiovascular disease.

Chapter VII References

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