A FRUIT-BASED FUNCTIONAL BEVERAGE DESIGNED TO REDUCE THE RISK OF CARDIOVASCULAR DISEASE

by

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Signature of Author

DEDICATIONS

I would like to dedicate this thesis to my wife, Dharshani and my daughters, Dasunmi and Chiranmi.

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LIST OF ABBREVIATIONS USED

ABTS	2,2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid)
ACUC	Animal Care and Use Committee
CRD	Completely randomized design
CVD	Cardiovascular disease
DF	Dilution factor
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
ELAM	endothelial-leukocyte adhesion molecule
FW	Fresh weight
FRAP	Ferric reducing antioxidant power assay
GAE	Gallic acid equivalent
GSH	Glutathione
GSSG	Glutathione disulfide
HDL	High-density lipoprotein
IC_{50}	Half maximal inhibitory concentration
ICAM	Intercellular Adhesion Molecule
LC-MS	Liquid chromatography and mass spectrometry
LDL	Low-density lipoprotein
MF	Microfiltration
ND	Not detected
NO	Nitric oxide
ORAC	oxygen radical absorption capacity
ox-LDL	oxidized low-density lipoprotein
RDI	Recommended daily intake
RO	Reverse osmosis
ROS	Reactive oxygen species
SD	Standard deviation
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TPTZ	2, 4, 6-tripyridyl-s- triazine
UF	Ultrafiltration
UPLC/MS	Ultra performance liquid chromatography / Mass spectrometry
VCAM	Vascular cell adhesion protein
WHO	World Health Organization

ABSTRACT

A functional beverage, designed to be cardio-protective, was formulated, using a blend of juices of cranberry (*Vaccinium macrocarpon* L), blueberry (*Vaccinium angustifolium* Aiton.), apple (*Malus domestica* L.), ginger (*Zingiber officinale* Roscoe) and selected cardio-protective ingredients. Membrane filtration enhanced the antioxidant properties of the fruit juices. Ultrasound-assisted water extract of ginger showed potential antioxidant activities. The selected fruit juice combination, 50% blueberry; 12.5% cranberry; and 37.5% apple, showed higher consumer acceptability. Incorporation of functional ingredients at 10% RDI and 2% (v/v) ginger extract did not affect the sensory properties of the beverage. Phenolic concentration, FRAP value, and % LDL oxidation inhibition of the formulation were 1024 mg GAE/L, 3114 mg TE/L and 45%, respectively. Diet supplementation with the formulation resulted in lower serum and liver lipid levels in spontaneously hypotensive rats. Blood pressure was reduced by the formulation after two but not four weeks supplementation.

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CHAPTER 1.0 INTRODUCTION

Cardiovascular disease (CVD) is ranked as one of the leading causes of death in most of the developed nations. Various research findings have demonstrated that changes in oxygen utilization in the body and increased formation of reactive oxygen species (ROS) contribute to atherogenesis and CVD progression (Kaliora et al. 2006; Madamanchi et al. 2005). These ROS are generated intracellularly during the mitochondrial electron transport chain and are controlled by the antioxidant defense system (Ji, 1999). The endogenous antioxidant defence system is not totally adequate to counteract oxidative stress (Houston, 2010). Therefore, protection against oxidative stress depends partly on the adequacy of dietary antioxidants (Kaliora et al. 2006).

Fruits and vegetables contain significant amounts of polyphenolic antioxidants at varying concentrations (Kaur and Harish, 2001). For example, total polyphenol contents of some fruits are: apple (27-298 mg GAE/100 g FW), blueberry (135-280 mg GAE/100 g FW) and cranberry (77-247 mg GAE/100 g FW) (Bravo, 1998). Increases in the intake of fruits and vegetables rich in antioxidants are effective strategies to minimize oxidative stress (Liu, 2003; Liu, 2004; Vattem et al. 2005). However, significant variation in the amounts, bioavailability and functionality of phenolic antioxidants available in the diet suggest that development of innovative strategies is required for enriching the human diet with safe and efficacious levels of phenolic antioxidants. Consumption of such a beverage, which has a variety of bioactive compounds derived from fruits and medicinal herbs, may impart unique functional attributes (Vattem et al. 2005). Considering these facts, the introduction of a bioactive-rich ‘functional beverage’ could be one of the

dietary strategies to reduce the risk of oxidative stress linked chronic diseases such as CVD.

Functional foods can be described as "foods which are similar in appearance to, or may be a conventional food, is consumed as a part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic diseases beyond basic nutritional functions" (Health Canada, 1998). The North American market for functional beverages is rapidly growing. For example, in Canada, CAD$ 1.1 billion worth of functional beverages was sold in 2007, with the estimated sales to rise by 35% (1.5 billion) by 2012 (B. Berry-Ben, 2009). In general, there is an increasing demand for health promoting foods (Bagchi, 2002). Hence, development of functional beverages enriched with antioxidants is designed to promote nutritional benefits, health benefits and convenience (Granato et al. 2010).

Cranberry (*Vaccinium macrocarpon* L.), blueberry (*Vaccinium angustifolium* Aiton.) and apple (*Malus domestica* L.) are some of antioxidant-rich fruits available in Eastern Canada (Morton, et al. 2008). In recent years, the demand for ginger (*Zingiber officinale* Roscoe) has increased in North America because of its reported medicinal properties, including anti-hypertensive (Ghayur et al. 2008) and anti-atherogenic (Bhandari et al. 1998 and Nicoll et al. 2007) characteristics. There are possibilities of formulating a 'heart-healthy' functional beverage with a mixture of bioactive phytochemicals from selected fruits and ginger. Further enhancement of the health functionality of the beverage can be achieved through fortifying it with specific ingredients such as minerals, vitamins and amino acids.

Currently, there are a number of commercial ready-to-drink polyphenol-rich beverages available in the market (Mintel, 2007). However, it appears that beverages formulated with the aim of targeting the reduction of the risk of CVD, by using the combination of fruits rich in different classes of biologically active polyphenolics and isoprenoids and specific cardio-protective ingredients, are scarce or unavailable. Therefore, the aim of this study is to develop a functional beverage by combining Atlantic Canadian grown fruits, rich in different classes of phenolics, together with ginger extract and fortified with cardio-protective minerals, amino acids and vitamins, to enhance the physiological properties.

CHAPTER 2.0 OBJECTIVES

2.1 Research Hypothesis

Dietary intake of efficacious amounts of natural antioxidants, formulated as a beverage by combining selected classes of bioactives from juices of apple, cranberry, blueberry and ginger, has ability to reduce the risk of CVD in a spontaneously hypertensive rat model.

2.2 Overall Objective

The overall objective of this study was to develop and assess a functional beverage, formulated by combining multiple classes of polyphenols from selected fruits, ginger and minerals (Se, Zn, K), amino acids (taurine, arginine) and vitamin B6.

2.3 Specific Objectives

The specific objectives of this project were to:

1. Optimize extraction of ginger bioactive using response surface methodology;
2. Evaluate ginger extracts for their antioxidant protection against human LDL oxidation, *in vitro*;
3. Evaluate and optimize the processing parameters for concentration of phenolics of fruit juices, using a membrane processing technique;
4. Assess the *in vitro* antioxidant activity, physico-chemical and sensory properties of different fruit juice blends and design and formulate a functional beverage, targeted to reduce the risk of CVD and hypertension, by combining optimum blends of fruit juices, ginger extracts and cardio-protective ingredients;

5. Test the efficacy of the functional beverage using an experimental animal model of spontaneously hypertensive rats (SHR).

2.4 Research Approach and Methodology

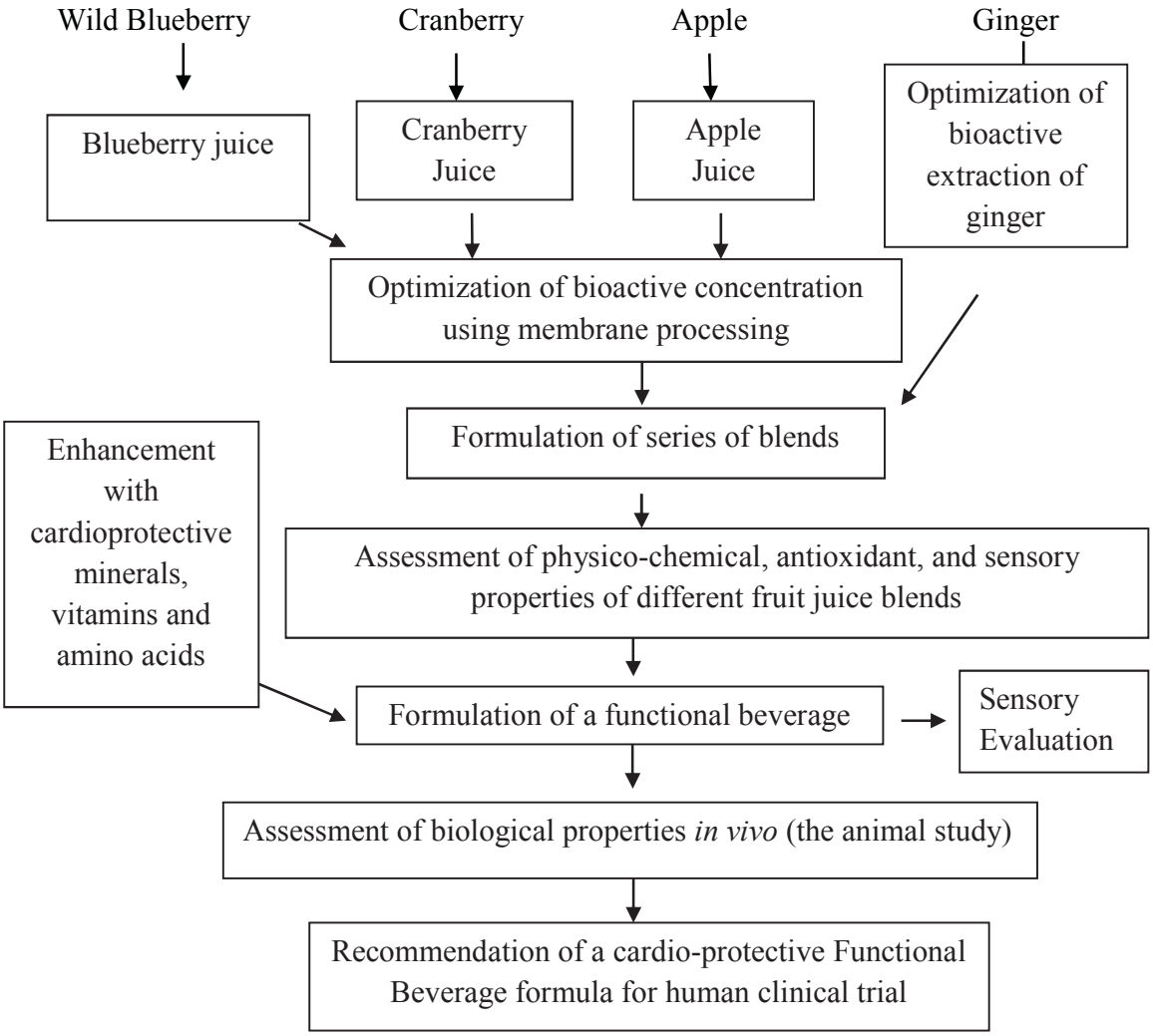


Figure 2.1. A schematic diagram to illustrate the research approach for the formulation of a cardio-protective functional beverage

CHAPTER 3.0 LITERATURE REVIEW

3.1 Cardiovascular Diseases and Hypertension

Cardiovascular diseases (CVD) are considered collectively as the one of the leading causes of death in most industrialized countries such as USA, UK and Canada (Gersh et al. 2010). CVD, mainly heart disease, stroke, atherosclerosis and hypertension (high blood pressure) are responsible for 35% of deaths in Canada annually (Mirolla, 2004). Hypertension, defined as blood pressure of 140/90 mm Hg or higher, is considered a major risk factor for myocardial infarction (Kearney et al. 2005). In 2007, 18.1% of the Canadian adult population reported having high blood pressure (Public Health Agency of Canada, 2009). Previous health reports have shown that these heart diseases exert a high burden of illness on Canadians, with a total annual direct cost, such as drugs, hospital care, physician care and other institutional care, of CAD$ 7.6 billion and an indirect cost (relating to mortality, and short and long term disability) of CAD$ 14.6 billion in 2000 (Public Health Agency of Canada, 2009). The World Health Organization (WHO) strongly advocates for the prevention, treatment, and management of hypertension and other CVD as a top priority (Kearney et al. 2005, WHO 2008). Atherosclerosis, accumulation of cholesterol in the arterial wall and formation of atherosclerotic plaque, has been known as a major pathophysiological process that leads to development of CVD (Stocker and Keany, 2004). There is now a consensus that atherosclerosis represents a higher oxidative stress characterized by lipid and protein oxidation in the vascular wall (Stocker and Keany, 2004).

3.2 Oxidative Stress and Atherosclerosis Development

Free radicals and reactive oxygen species (ROS) are highly reactive by-products of cellular metabolism and play a role in cell signalling and regulation (Thannickal and Fanburg**,** 2000). The most common free radicals and ROS of importance in living organisms include hydroxyl, superoxdes, singlet oxygen and hydrogen peroxides. Some of these reactive species can cause cell injury while attacking susceptible substrates such as nucleotides and lipids (Figure 3.1). However, cells have evolved with antioxidative defence mechanisms which consists of both enzymatic and non-enzymatic mechanisms to prevent injury caused by these ROS and free radicals. Enzymes, such as superoxide dismutase (SOD), glutathione peroxidase and catalase, are the main antioxidants involved in cellular antioxidant defense mechanism, whereas ascorbate, α-tocopherol and glutathione are some of the non-enzymatic antioxidants in the defence system (Madamanchi et al. 2005). However, in pathophysiologic circumstances, overproduction of these ROS can overwhelm the antioxidative capacity of the celluar antioxidant defense system and leads to oxidation stress and subsequent damage to cellular proteins, lipids, nucleic acids and membranes (Madamanchi et al. 2005).

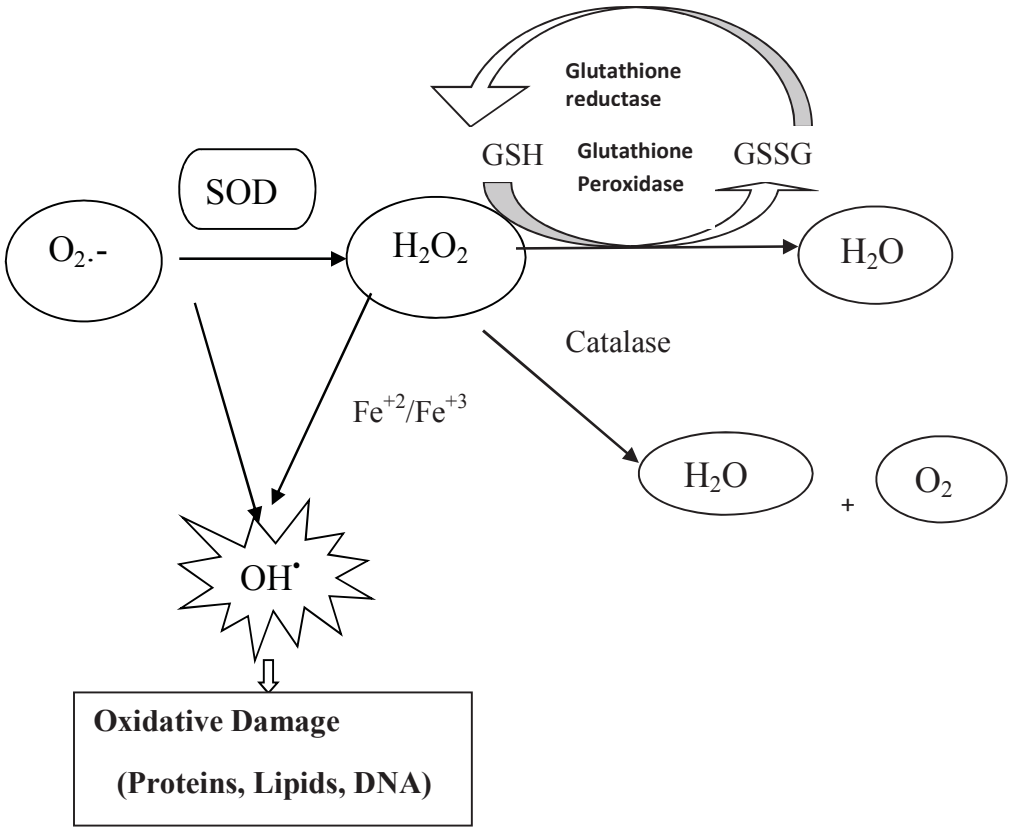


Figure 3.1. An imbalance between production of pro-oxidants and antioxidants could lead to excess generation of reactive oxygen species and severe cellular damage (adapted from Wakamatsu et al. 2008) (SOD; superoxide dismutase, GSH; glutathione, GSSG; glutathione disulfide, O_2.- ; superoxide radicals, OH•; hydroxyl radicals, H_2O_2; hydrogen peroxide)

Growing evidence indicates that chronic and acute overproduction of ROS under pathological conditions is integral in the development of CVD (Madamanchi et al. 2005). Various animal models of oxidative stress support that ROS plays a role in the development of atherosclerosis and other CVD (Lerouet, et al. 2002; Mertens, et al. 2003; Miyazaki et al. 2002). Conversion of LDL into oxidized LDL (ox-LDL) and transporation via the endothelium into the artery wall (Figure 3.2) are considered as the important initiation steps in the progression of atherosclerosis (Navab et al. 1996).

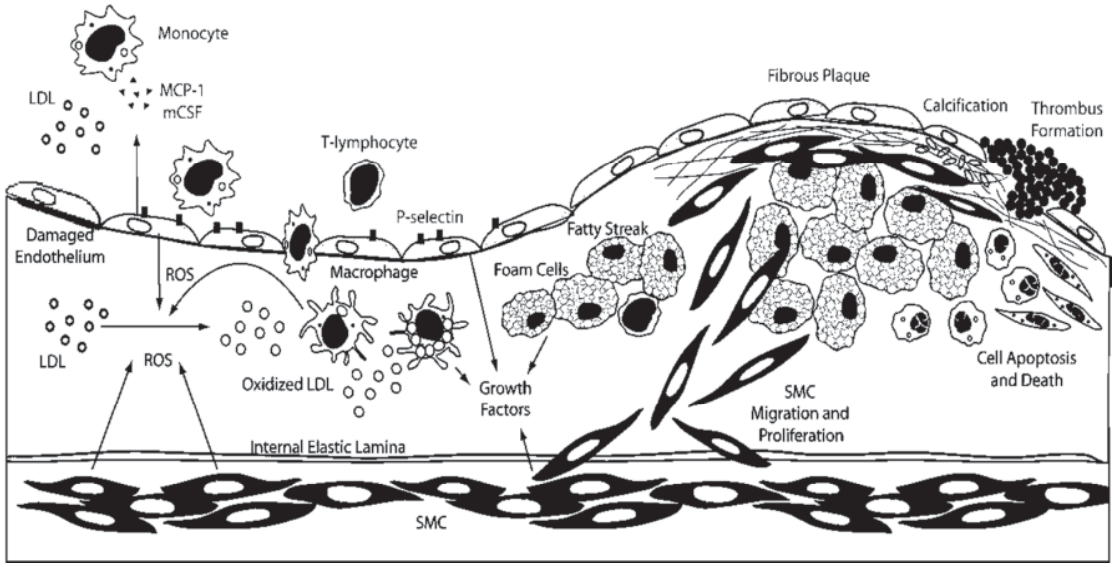


Figure 3.2. Atherosclerosis development (adapted from Madamanchi et al. 2005) (SMC; smooth muscle cells, MCP-1; Monocyte chemotactic protein-1, mCSF; Macrophage colony-stimulating factor

Ox-LDL damages the endothelial cells and induce the expression of adhesion molecules such as monocytes and promotes migration of these molecules into subendothelial spaces (Ross, 1993). According to Madamanchi et al. (2005), monocytes engulf the LDL and change into macrophages; in the macrophage these ox-LDL are further oxidized, accumulation and fusion of macrophages convert into foam cells. Foam cells combine with leucocytes to become fatty streaks and induce the smooth muscle cells to migrate into intima and proliferate. The continuous influx and propogation of monocytes and macrophages leads to the formation of more advanced endothelial lesions and ultimately, a fibrous plaque which protrudes into the arterial lumen. In acute coronary syndrome (e.g. myocardial infarction), rupturing of these fibrous plaques leads to occlusion of the vessels.

3.3 Diet, Antioxidants and Cardiovascular Disease

The main causes of CVD have been associated with lifestyle choices and one of the most important is diet (Boyer and Liu, 2004). Recent studies have demonstrated that increased formation of ROS contributes to CVD progression and atherogenesis (Kaliora et al. 2006). Animals including humans have a highly complicated antioxidant system; however, endogenous antioxidant defence is not adequate to counteract the oxidative stress in the body. Under the 'oxidation theory' for atherosclerosis, dietary antioxidants have gained considerable attention as preventive and therapeutic agents (Kaliora et al. 2006). It has been suggested that increasing antioxidant status of serum may reduce the risk of many chronic degenerative diseases (Kaplan and Aviram, 1999). Therefore, it is suggested that the dietary modification towards more access to antioxidants is an effective strategy to prevent the formation of atherosclerotic lesions (Kaliora et al. 2006).

Fruit and vegetable juices have been shown to be rich sources of bioaccessible antioxidants and are thought to be responsible for health benefits (Wootton-Beard et al. 2011). Consumption of a wide range of different fruits and vegetables provides the most comprehensive access to the vast number of polyphenolic antioxidant compounds which they contain (Wootton-Beard and Ryan, 2011). Antioxidant-rich foods are prepared most commonly from plant sources which contain a number of healthy components which make them a positive addition to the diet as supplement to the body's antioxidant defense system. Cranberry (Chu et al. 2005), blueberry (Smith et al. 2000) and apple (Vrhovsek et al. 2004) are some of antioxidant-rich fruits produced in Canada. Other than fruits and vegetables, antioxidant properties of herbs and spices such as ginger and garlic (*Allium sativum* L.) are of particular interest in view of the impact of oxidative modification of

LDL cholesterol in the development of atherosclerosis (Tapsell et al. 2006). Functional foods are one of the choices that people, researchers and industry are examining to enrich diets with bioactives with known functional properties, designed to attempt to combat oxidative stress related chronic diseases including CVD.

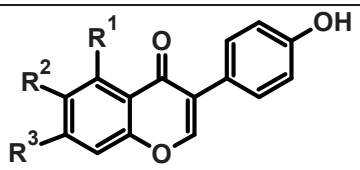
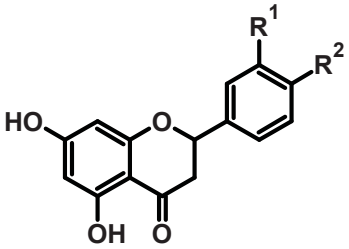
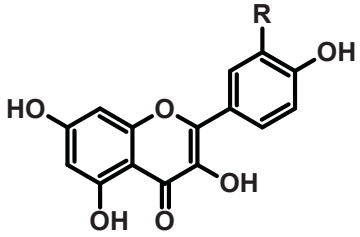
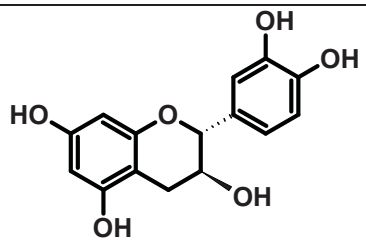
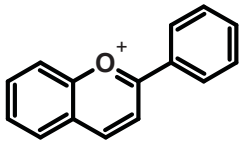
3.4 Functional Foods and Market Trends

Functional foods can be described as "a food which is similar in appearance to, or may be a conventional food, is consumed as a part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic diseases beyond basic nutritional functions" (Health Canada, 1998). The North American market for functional beverages is rapidly growing (14% per year since 2002) due to increasing health consciousness, obesity concerns and life style choices, along with an aging population; these have been the main market drivers for functional food (Mintel, 2007). When purchasing a functional beverage, about 60% of US consumers preferred antioxidant based products (Mintel, 2007). Fruit-based functional beverages are the largest segment of sales of functional beverages as they account for 58% of the functional beverages sold in America (Mintel, 2007). A recent market analysis study conducted in Nova Scotia revealed that consumers are willing to obtain nutritional and functional benefits through a food rather than dietary supplements in the form of a pill (McIntosh et al. 2006). Currently, there are number of commercial ready-to-drink beverages which claim to have high antioxidant potencies due to their perceived high content of polyphenolic antioxidants (Mintel, 2007).

3.5 Polyphenolic Antioxidants

Polyphenols are one of the most common and ubiquitously distributed plant secondary metabolites, of which flavonoids are a sub-class which has prompted growing interest because of these therapeutic properties (Bravo, 1998). According to the modification of the central-C ring, flavonoids can be divided into different sub classes (Table 3.1), including flavonols, flavones, flavan-3-ols, flavanones, isoflavones, and anthocyanidins (Fraga, 2009; Rupasinghe et al. 2008). Recent epidemiological studies suggest that adopting flavonoid-rich diets may protect against CVD (Huxley and Neil, 2003). However, mechanisms by which these phytochemicals exert their cardio-protective effects are still unknown. It is widely accepted that dietary flavonoids improve the cardiovascular health by inhibiting pathophysiological processes under oxidative stress (Nijveldt et al. 2001). Other mechanisms, which reduce the CVD risk, may be the ability of polyphenols to increase vasodilation by inducing relaxation of vascular smooth muscles (Stoclet et al. 2004) and regulation of hypertension by inhibiting the angiotensin converting enzyme by flavonoids (Balasuriya and Rupasinghe, 2011; Balasuriya and Rupasinghe, 2012). Various *in vitro* assays have demonstrated that flavonoids can potentially prevent ROS-related processes as they exhibit powerful antioxidant activities by scavenging a wide range of ROS (Santos and Mira, 2004), inhibiting ROS production (Selloum et al. 2001) and chelating pro-oxidant metal ions (Mira et al. 2002). The main non-flavonoid phenolic compounds of dietary significance, because of their abundance in the diet, are phenolic acids (e.g. ellagic acid), hydroxycinnamates (e.g. p-coumaric, caffeic, and ferulic acids) and their conjugated derivatives (e.g. chlorogenic acids, diaryl-hepanoids) and stilbenes (Crozier et al. 2007).

Table 3.1. Sub-classes of flavonoids and some examples

Sub-Classes	Structural formula	Examples
Isoflavones		genistein, daidzein, and glycitein
Flavanones		naringin, naringenin
Flavonols		quercetin, kaempferol, and myricetin
Flavanols		catechin, epicatechin, proanthocyanidins, gallocatechin
Anthocyanidin		cyanidin, petunidin, delphinidin

3.6 Phenolic Rich Fruits Produced in Atlantic Canada

3.6.1 Apple

Apples *(Malus domestica*) are widely consumed fruits and contain carbohydrates (8.25 g/100 g FW), ascorbic acid (14.25 mg/100g FW), dietary fiber (1.59 g/100 g FW), minerals (P 7.25 mg/100 g FW, K; 8.50 mg/100 g, Ca; 7.25 mg/100 g FW) and vitamins (niacin, thiamine, vitamin A) (Voicu et al. 2009). The phenolic compounds present in apples are of special interest as these act as a source of dietary antioxidants and contain the highest portion of free phenolics when compared to other fruits (Sun et al. 2002). The total polyphenol content ranges from 66.2 to 211.9 mg/100 g of fresh weight, depending on the variety (Vrhovsek et al. 2004). Flavonoids are the major class of apple polyphenols (71-90%) such as flavonols (quercetin and its glycosides) and flavanols catechin and epicatechin (Gliszczynska-Swiglo and Tyrakowska, 2003; Vrhovsek et al. 2004). The major non-flavonoid polyphenols in apple juice is chlorogenic acid (Gliszczynska-Swiglo and Tyrakowska, 2003). The main flavonols in apple are quercetin glycosides such as quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-arabinopyranoside, and quercetin-3-*O*-arabinofuranoside (Marks et al. 2007).

These apple bioactives have been shown to possess beneficial effects in increasing the antioxidant activity of the human plasma (Ko et al. 2005). Pearson and co-workers (1999) demonstrated that apple juice inhibits the Cu^{+2}-catalysed-LDL oxidation *in vitro*. Epidemiological studies have linked the consumption of apples with the reduced risk of some cancers, CVD, asthma, and diabetes (for review see Boyer and Liu, 2004). In an animal study, it was found that both apple juice and apple puree fed to hamsters for

12 weeks, increased the antioxidant capacity of the plasma and decreased the aortic fatty streaks area and plasma cholesterol level (Decorde et al. 2008). In a review of apple polyphenol and CVD (Weichselbaum et al. 2010), four studies of six demonstrated some favourable effects on blood lipid profiles in humans. Setorki and co-workers (2009) reported that supplementation of 10 mL apple juice per day along with high fat diet to rabbits for two months, decreased the total cholesterol, LDL, triacylgycerole, C-reactive protein in plasma and decreased the atherosclerotic lesion in both left and right coronary arteries.

3.6.2 Cranberry

Cranberry is one of the commercially important fruit native to North America and exhibits various health benefits, including the prevention of P. fimbriated E.coli-induced urinary tract infections (Foo et al. 2000), stomach ulcers (Burger et al. 2000), as well as improving oral hygiene (Weiss et al. 2002). Many of these biological effects have been linked to the presence of diverse phytochemicals (Seeram, et al. 2004). The phenolic content of cranberries is about 507–709 mg gallic acid equivalents (GAE)/100 g FW and most of the phenolics, about 91.3–96.2%, are present in a soluble free form (Vinson et al. 2001). Cranberries are a rich source of phenolic acids including benzoic, hydroxycinnamic, and ellagic acids and flavonoids such as anthocyanins, flavonols, and flavan-3-ols (McKay and Blumberg, 2007). Major anthocyanins present in cranberries are peonidin-3-galactoside, cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-arabinoside, peonidin-3-glucoside, and cyanidin-3-glucoside (McKay and Blumberg, 2007). The total proanthocyanidin content of whole cranberries has been reported as 418.8 mg/100 g FW (Gu et al. 2004). Quercetin, myricetin, and their glycosides are the

major flavonols present in cranberries (Bilyk and Sapers, 1986). Cranberries also contain stilbenes, such as resveratrol, which has several biological effects related to cardiovascular health, including quenching ROS, inhibiting platelet aggregation, and reducing inflammation (McKay and Blumberg, 2007).

Based on epidemiological and clinical evidence, dietary antioxidants are associated with a lower risk of CVD and hypertension (Young and Woodside, 2001). Therefore, recent research on cranberries and their components have focused on their use in the prevention and treatment of CVD. Cranberry extracts have been reported for potent antioxidant capacity *in vitro* in preventing LDL oxidation in a dose dependant manner (Wilson et al. 1998). It has been estimated that the antioxidant activity of 100 g cranberries against LDL oxidation is equivalent to 1000 mg vitamin C or 3700 mg vitamin E (Chu and Liu, 2004). Daily consumption of cranberry juice is associated with an increase in plasma HDL cholesterol concentrations in abdominally obese men (Ruel et al. 2006). In another study undertaken to investigate the effect of cranberry juice on LDL oxidation using a group of 21 men and it was found that consumption of cranberry juice (7 mL/kg of body weight) for two weeks decreased circulating plasma oxidized LDL and an increase in antioxidant capacity was observed (Ruel et al. 2005). However, Duthie et al., (2006) reported that short-term consumption of cranberry juice did not influence several biomarkers of blood lipid status in healthy young adults. Furthermore, the high ratio of potassium to sodium in cranberries may contribute to the promotion of lower blood pressure (Mckay and Blumberg, 2007). The vasodilatory effect of cranberry juice was demonstrated *in vivo* by Maher and co-workers (2003) in a rat model. In an atherogenic swine model on blood cholesterol level, it was found that feeding 150 g

cranberry juice powder per day decreased the total cholesterol and LDL by 20% and by 22%, respectively (Reed et al. 2001). The effect of cranberry juice consumption on lipid profile changes has also been investigated by Caron et al. (2005); the study found that consumption of 4.3 mL cranberry juice per kg body weight twice daily, for 28 days, significantly reduced the total cholesterol and LDL in humans. These findings suggest that the consumption of flavonoids-rich foods such as cranberry can be cardio-protective.

3.6.3 Blueberry

Atlantic Canada is the major low bush blueberry production region (60,000 tonnes in 2009) in the world (Agriculture and Agri-Food Canada). In 2010, 85% lowbush blueberry harvested in Canada was from the Atlantic Provinces. Nearly 38% of Canada's total wild blueberry harvest comes from Nova Scotia (Statistics Canada). Blueberry is a good source of dietary fiber (2.4 % FW), although it is not a very rich source of vitamin C (9.7 mg/100 g FW) (Agriculture Research Services). Polyphenolic content of blueberry is about 0.4% on a fresh weight basis (Kalt et al. 2001). Blueberry juice and fruits in general have received considerable attention recently (Prior et al. 2007) because they are well recognized for their potential health benefits due to their high anthocyanin and flavonoid contents (Smith et al. 2000). Total resveratrol content of wild blueberry is about 893 ng/g DW, which may contribute to the total antioxidant capacity of blueberry juice (Rimando and Cod, 2005). Furthermore, the blueberry skin is a rich source of pterostilbene (Rimando et al. 2005), a phenolic stilbene compound related to resveratrol and it is incorporated into some juices and found to lower the plasma LDL by 29% and increase the HDL by 7% in hypercholesterolemic hamsters, when fed a diet containing 25 ppm pterostilbenes (Rimando et al. 2005).

Research indicated that blueberry juice or fruit extract enhanced postprandial serum antioxidant status in humans (Kay and Holub, 2002; Pedersen et al. 2000) and inhibited the oxidation of human LDL, preventing various human diseases caused by oxidative stress (Ames et al. 1993). In an animal study, reduction in total cholesterol in pig plasma was observed as a result of feeding with blueberry added to the diet (Kalt et al. 2008). In a rat study, supplementing blueberries reduced the ischemic damage to the heart (Ahmet et al. 2009) and an anti-hypertension effect was exerted (Wiseman et al. 2011). In an animal study, a diet with 3% freeze dried blueberry decreased the hypertension in spontaneously hypertensive stroke-prone rats (SHRSP) (Shaughnessy et al. 2009). Blueberry improved the vaso-relaxation *ex vivo* (Kalea et al. 2009) and lipophilic antioxidative capacity *in vitro* (Wu et al. 2004). The effects of blueberry supplements on features of metabolic syndrome, lipid peroxidation and inflammation in obese men and women have been investigated and it was found that consuming 350 g blueberry for 8 weeks reduced the systolic and diastolic blood pressures and decreased the plasma oxidized LDL and serum malonaldehyde and hydroxynonenal concentrations (Basu et al. 2010).

3.7 Membrane Processing Technology as a Means of Concentrating Bioactives in Fruit Juices

Membrane processing provides a means of concentrating, fractionating and/or purifying fluids, generating two streams that are different in their compositional characteristics. Reverse osmosis (RO), ultrafiltration (UF) and microfiltration (MF) are the most common types of membrane filtration. UF and MF are involved with the separation of larger macromolecules, using a selective filtration process and RO is for

concentrating juices (Girard and Fukumoto, 1999). The use of an integrated membrane process, including RO, in preserving the natural antioxidant compounds in blood orange juice has been studied by Galaverna, et al. (2007) and found to be successful. Membrane processing techniques are relatively efficient in terms of energy because water is not removed by evaporation avoiding the need for intensive heat transfer equipment. These processes normally use lower temperatures in the range of 4 to 50 °C in order to minimize thermal damage of products rich in antioxidants (Girard and Fukumoto, 2000).

3.8 Blending of Fruit Juices as a Means of Improving Bioactive Profile

The profiles of phenolic phytochemicals determine the functionality of the whole food as a result of additive or synergistic interaction of constituent phenolic phytochemicals. A new healthy drink was developed by González-Molina and co-workers (2009) by combining lemon juice and pomegranate juice, resulting in a product which has better organoleptic properties, with an attractive red color, as well as an acceptable and enhanced bioactive content and antioxidant properties. The same group of scientists has developed another high antioxidative drink with lemon juice enriched with chokeberry (*Aronia melanocarpa* Michx.) (Gonzalez-Molina et al. 2008). It was found that an addition of 5% chokeberry juice could increase the antioxidant properties of lemon juice, as well as improve certain organoleptic characteristics, rendering an interesting beverage in the growing market of food for health. These findings demonstrated that novel antioxidant- rich beverages with improved organoleptic and bioactive profiles can be prepared by blending different classes of antioxidant-rich fruit juices.

3.9 Ginger as another Source of Cardio-protective Bioactives

China and India are the world's most prolific producers of ginger. In recent years, the demand for ginger has been growing in North America, not only for use as a food ingredient, but also for its medicinal properties (Chrubasika et al. 2005). Ginger has been widely used in Chinese, Ayurvedic and Unani-Tibb medicines (Ali et al. 2008). Ginger has various pharmacological activities such as anti-emetic, anti-ulcer, anti-inflammatory, antioxidant, anti-platelet, glucose and lipid-lowering, cardio-protective and anti-cancer properties (Bhandari et al. 1998; Nicoll et al. 2009). Over 50 components in ginger oil have been characterized and these are mainly monoterpenoids and sesquiterpenoids. The pungency of fresh ginger is due primarily to the gingerols (Figure 3.3), which are a homologous series of phenols (Ali et al. 2008; Langner et al. 1998), whereas the pungency of dried ginger is mainly due to the presence of shogaols (Figure 3.3); for example, (6)-shogaol, which are dehydrated forms of gingerols.

Based on the findings in animal and *in vitro* research models, ginger has the potential to treat many aspects of CVD such as hyperlipidemia (Fuhrman et al. 2000), platelet aggregation (Young et al. 2006) and hypertension (Ghayur et al. 2008; Suekawa et al. 1984). The antiatherogenic effect of ginger is associated with reductions in plasma and hepatic LDL cholesterol levels, as well as their susceptibility to oxidation and aggregation (Fuhrman et al. 2000). In the cardiovascular system, both (6)-shogaol and (6)-gingerol are the two active components of ginger which produced a depressor response on blood pressure at lower doses (Suekawa et al. 1984).

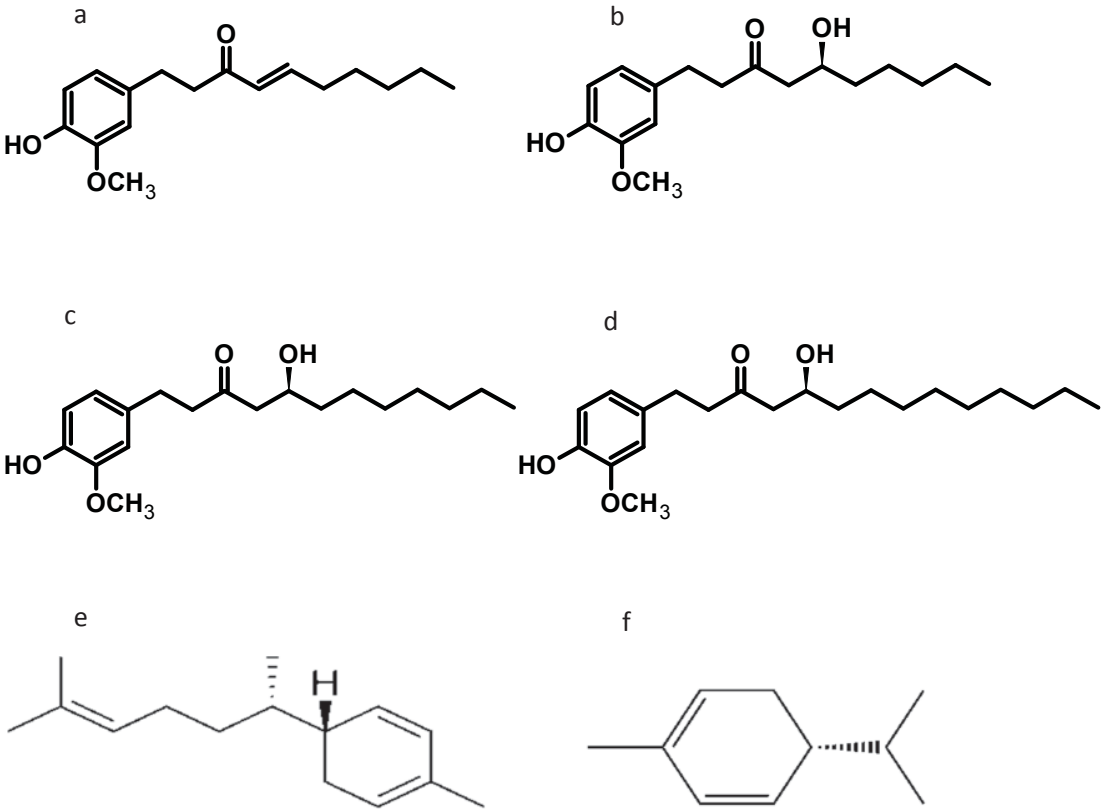


Figure 3.3. Structures of selected major bioactives of ginger: 6-shogaol (a), 6-gingerol (b), 8-gingerol (c), 10-gingerol (d) zingiberene (e) and phellandrene (f) present in ginger

3.10 Cardio-protective Minerals, Amino Acids and Vitamins

3.10.1 Selenium

Selenium (Se) has a clearly defined role in the antioxidant system as it is a functional component in glutathione peroxidase enzymes, which contribute to the antioxidant defence system (Barciela et al. 2008). An adequate supply of Se is necessary for normal health as an inadequate dietary supply has been associated with clinical conditions (Arthur and Beckett, 1994). An inverse relationship between low Se levels in blood and atherosclerosis has been investigated and Se appears to reduce the formation of free radicals (Reilly, 1998). A human clinical study has demonstrated the efficacy of daily intake of 110 μg Se in preventing postprandial oxidative stress, including

atherogenic formation of LDL (Natella et al. 2006). However, Se content of fruits and vegetables is less than 0.001-0.022 µg/g FW (Reilly, 1996). The US recommended dietary allowance (RDA) for Se is 70 µg/day for men and 55 µg/day for women. Sodium selenite, sodium selenate and selenomethionine are the forms of Se available for fortification (Reilly, 1998). However, selenium gives adverse health effect at higher levels of intake and has a narrow safety margin (Richardson, 1997).

3.10.2 Taurine

Taurine (2-aminoethanesulfonic acid) has numerous beneficial effects on the cardiovascular system (Houston, 2010) and is a key ingredient in energy drinks such as 'Red Bull®' and "Monster®" (Wojcik et al. 2010). Based on the evidence, the main biological actions of taurine include its ability to conjugate cholesterol into bile acids (Tsuji, et al. 1980), antioxidant properties (Winiarska et al. 2009), regulation of blood pressure (Harada et al. 2004), and anti-inflammatory effects (Wojcik, et al. 2010). In hypercholesterolemia, taurine supplementation *in vivo* has been found to improve the serum lipid profile (Ito and Azuma, 2004; Matsushima et al. 2003) and reduce the area of arterial lipid accumulation by 28% (Kondo et al. 2001). According to Zulli (2011), a major role of taurine is to act as an antioxidant and enhance the absorbtion of hypochlorous acid, but not the oxidative radicals. Toxicological evidence suggested that the upper level of taurine supplementation is 3 g per day (Shao and Hathcock, 2008).

3.10.3 Vitamin B6

Pyridoxine is the main form of vitamin B6 used in supplements and it is readily phosphorylated and oxidized to pyridoxal phosphate coenzyme which is active mainly in amino acid metabolism (Bender, 2011). Low serum pyridoxine levels are associated with

hypertension in human (Houston, 2010). Based on suggestive evidence with small trials, there are plausible mechanisms for an antihypertensive action of vitamin B6 supplementation (Bender, 2011). Epidemiological evidence has suggested that low dietary intake or reduced blood concentrations of vitamin B6 are associated with an increased risk of CVD (Friso et al. 2012). Elevated plasma homocysteine is a risk factor for atherosclerosis and conversion of homocyteine into cysteine involves two vitamin B6 dependent reactions. Therefore, a deficiency of vitamin B6 leads to impaired methionine metabolism (Bender, 2011). Some findings suggest that intake of vitamin B6 above the current RDA may be important in the alleviation of CVD among women (Rimm et al. 1998). The current RDA of vitamin B6 is 2 mg per day (Manore, 2000).

3.10.4 Potassium

The recommended intake of potassium (K^+) is 650 mEq/day with a K^+/Na^+ ratio of more than 5:1; however, the average American dietary intake of potassium is 45 mEq/d with a K/Na ratio of less than 1:2 (Houston and Harper, 2008). Epidemiological (Whelton and He, 1999) and clinical studies (Cappuccio and MacGregor, 1991) have suggested that an increase in potassium intake reduces the incidence of cardiovascular and blood pressure reduction. A human study suggested that 60 mmol daily supplement of potassium chloride reduces blood pressure by 5.0 mmHg in elderly hypertensive patients (Fotherby and Potter, 1992). Possible mechanisms of this hypotensive effect of potassium are decrease in aldosterone and vasopressin levels and reduction of their responsiveness to the pressor effects of angiotensin II, which is a potent vasoconstrictor (Tannen, 1983). This low response to angiotensin II could result from increased

prostaglandin production, receptor occupancy or a decrease in angiotensin II receptor affinity (Tannen, 1983).

3.10.5 Zinc

Zinc is involved in numerous biological processes such as catalysis, stabilization of cell membranes and regulation of gene expression (Samman, 2002). The RDA of Zn is 15 mg/day and low serum Zn levels correlate with hypertension, as well as hyperlipidemia, especially hypertriglyceridemia and low high-density lipoprotein (HDL) cholesterol and elevated lipoprotein (Houston, 2005). In a human clinical trial it was found that supplementation with 20 or 53 mg zinc per day decreased the plasma cholesterol concentrations (Boukaiba et al. 1993). An *in vitro* study has shown that zinc enhances thrombin activity of the common coagulation pathway (Marx and Eldor, 1985). An inverse correlation of blood pressure with serum zinc and zinc dependent enzyme–lysyl oxidase activity was observed. These effects, along with the membrane ion exchange and the rennin angiotensin system, may account for zinc is antihypertensive effects (Houston, 2007). Zinc is a cofactor for various forms of superoxide dismutases (SOD) and it contributes to the cellular antioxidant capacity and ensures sufficient nitric oxide (NO) is available for the maintenance of proper endothelial function (Ruz et al. 1992). Zinc deficiency is associated with lower SOD activity (Ruz et al. 1992).

3.10.6 Arginine

Although L-arginine is one of the non-essential amino acid, it is one of the main conditionally essential nutrients (CEN) in CVD therapy, based on human clinical studies (Berry, 2006). Conditionally essential nutrients are organic compounds that are usually produced by cells in amounts adequate to meet physiological requirements. However,

during certain stages of the life cycle or in some disorders, such as CVD, other physiological stressful conditions, their biosynthesis may be inadequate (Berry, 2006). Recently, the importance of L-arginine has become widely recognized by the cardiovascular research community as it serves as precursor for endothelial nitric oxide (NO) synthesis, via nitric oxide synthase. NO is a potent endogenous vasodilator and exhibits many other favorable cardiovascular effects, including inhibition of platelet aggregation, smooth muscle cells proliferation, oxidative enzyme activity and prevention of monocyte adherence and infiltration (Cooke & Dzau, 1997).

In a recent review of oral L-arginine supplementation, 12 of 16 human studies showed significant cardiovascular benefits, especially in those involving hypercholesterolemic subjects (Preli et al. 2002). Several human clinical studies showed positive outcomes with L-arginine supplementation, including the inhibition of platelet aggregation (Wolf et al. 1997), improvement in endothelial dependent vasodilation (Lerman et al. 1998), reduced monocyte-endothelial adhesion resulting in decreased atherogenesis (Chan et al. 2000), decreases in LDL cholesterol level and increases in HDL/LDL ratio (Hurson et al. 1995). Several animal studies also demonstrated the beneficial effects of L-arginine on cardiovascular system. For example, supplementation of dietary L-arginine improves the endothelial-dependent vasodilation in hypercholestemic rabbits (Cooke et al. 1992). In another study, it was found that alternation in NO activity played a critical role in atherogenesis in rabbits by regulating the endothelial adhesiveness for monocytes (Tsao et al. 1994). Aortic endothelium binding assay, measurement of NO release and histomorphometry (measurement of surface lesion area and intimal thickness) can be used for the evaluation of the effect of

L-arginine supplementation on the endothelium (Tsao et al. 1994). In summary; these studies strongly support favorable cardiovascular effects accruing from L-arginine supplementation, based on both animal and human subjects and patients with risk factors for CVD.

3.11 Evaluation of Cardio-protective Properties of Functional Foods

The majority of CVD results from atherosclerosis (Madamanchi et al. 2005). Oxidative modification of LDL leads to numerous pathological conditions such as atherosclerotic plaque formation. Many *in vitro* and *in vivo* models have been established for the evaluation of LDL oxidation for the screening of foods and nutraceuticals for cardio-protection. *In vitro* LDL oxidation models use isolated human plasma with a pro-oxidant (metal ions like copper or ions) or radical initiators such as AAPH or enzymes, including lipoxygenase (Benzie and Strain, 1999). A common method of measuring the inhibition of LDL oxidation is by determining the level of thiobarbituric acid reactive substances (TBARS) in response to the LDL oxidation (Xu et al. 2007). It has been suggested that in comparision to all other *in vitro* antioxidant assays, measuring LDL antioxidant activity is more physio-pathologically informative for screening antioxidant activity of foods for preventing atherosclerosis (Katsube et al. 2004).

Cell culture based *ex vivo* assays are also being carried out; these include various cell lines and induction of these cells through oxidation by different types of metal ions, radical initiators or sometimes, several cell types that are present in the sites of atherosclerotic plaque, such as macrophages, foam cells and smooth muscle cells (Dugas et al. 2000). Many animal studies and human clinical trials have been carried out to study hypercholesteromia and LDL oxidation (Thilakarathna et al. 2012; Wang et al. 2003).

Some of the most commonly used animals are rats, mice, hamster, pigs and rabbits. However, hamsters are considered particularly well suited for studying human lipid metabolism (Wang et al. 2003). Spontaneously hypertensive rat (SHR) is frequently used as model of cardiovascular disease (Gallaher, 1992).

3.12 CVD Biomarkers

A biomarker is defined as 'a characteristic' that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (Biomarkers definitions working group, 2001). The roles of plasma lipids in the etiology of atherosclerosis and coronary heart disease have been well studied. Important risk factors for the expression of coronary diseases are high plasma concentrations of total cholesterol, triacylglycerol, and LDL cholesterol and a low plasma concentration of HDL cholesterol. These plasma indices or biomarkers must be jointly considered in the assessment of CVD risk (Castro et al. 2005). Other important arterial vulnerability biomarkers are serum homocysteine and oxidized LDL levels. In a review of the health benefits of vegetarian diets, Benzie and Wachtel-Galor (2010) introduced plasma vitamin C as a novel biomarker of health status, presenting evidence from several epidemiological studies which demonstrate reductions in disease risk for CVD (40% reduction in risk for highest compared to lowest quintile) at the optimized plasma vitamin C levels.

Blood pressure, arterial stiffness and endothelial dysfunction are some of the functional markers for arterial vulnerability. Endothelial dysfunction biomarkers are vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM)-1 and endothelial leucocyte adhesion molecule (ELAM)-1 (Dessein et al., 2005). Elevated

circulating adhesion molecules are associated with cardiovascular risk factors (Ponthieux et al. 2004) and predict atherosclerosis and cardiovascular events (Hwang et al. 1997).

CHAPTER 4.0 OPTIMIZATION OF WATER BASED-EXTRACTION METHODS FOR THE PREPARATION OF BIOACTIVE RICH GINGER EXTRACTS USING RESPONSE SURFACE METHODOLOGY

4.1 Abstract

Bioactives present in ginger were of interest due to their various beneficial properties, including cardiovascular health. For the preparation of bioactive-rich ginger extract with water, conventional hot water extraction, ultrasonic-assisted extraction and high pressure homogenization-assisted extraction were evaluated. Response surface methodology was employed to optimize the extraction conditions of each method with respect to the highest phenolics, antioxidant capacity (ferric reducing antioxidant power; FRAP) and percent inhibition of low density lipoprotein (LDL) oxidation. Multiple response optimizations revealed that the optimum extraction conditions for each method were 60 min extraction time under 55 °C for hot water extraction, 15 min ultrasonication under 52 °C for ultrasound-assisted extraction and 62 °C under 140 MPa homogenization pressure for high pressure homogenization-assisted extraction. The extract prepared from the ultrasonic assisted-extraction method exhibited the highest phenolic recovery and antioxidant activity, compared to the extracts prepared from other two methods.

4.2 Introduction

Ginger has been demonstrated to have various pharmacological activities, such as anticancer, antioxidant, anti-platelet, antidiabetes and cardio-protective (Nicoll and Henein, 2009; Shukla and Singh, 2007). It has been demonstrated that gingerols are the most pharmacologically active component in ginger (Ali et al. 2008; Kim et al. 2005; Wang et al. 2011). In recent years, the demand for ginger has grown in North America, especially because of its anti-hypertensive (Ghayur et al. 2008) and anti-atherogenic medicinal properties (Bhandari et al. 1998; Nicoll and Henine, 2009). Therefore, there are possibilities of incorporating ginger as a functional ingredient for the formulation of functional foods containing multiple classes of bioactive molecules to obtain synergistic benefits.

Incorporation of bioactive-rich ginger extracts as value-added ingredients in functional foods can be achieved by identifying the appropriate extraction method. Consequently, novel extraction techniques, such as ultrasonic-assisted extraction (Chemat et al. 2004), microwave-assisted extraction (Brachet et al. 2002) high pressure homogenization-assisted extractions (Dong et al. 2011), have been introduced to increase the yield of water-extraction and to replace solvent-based bioactive extractions. Ultrasound-assisted extraction is a good alternative extraction method when compared to classical and conventional techniques because of its high efficiency and low energy requirement (Rodrigues et al. 2008). Ultrasonic is not only a rapid, efficient and reliable alternative to enhance the quality of food, but also has potential use in developing innovative new products with unique functionality (Soria and Villamiel, 2010). Homogenization-based extraction has been used to extract many different chemical

substances from different food materials and proven to be a more efficient practice (Torti et al. 1995). Use of hot water for the extraction of bioactives is a conventional technique and employed for the extraction of phenolics from ginger by Kishk and Sheshetawy (2010). However, according to Rupasinghe et al. (2011), most of the reported ultrasonic-assisted bioactive extraction protocols are based on "substandard processes" without optimization for specific bioactive constituents of interest. Therefore, optimization and standardization of bioactive extraction procedures from various plant sources are necessary for the development of natural health products. The aim of this study was to evaluate three different water-based bioactives extraction methods for fresh ginger. These methods include ultrasound-assisted extraction, high pressure homogenization-assisted extraction and hot water extraction. The optimum extraction conditions of each method, which yielded the greatest phenolic phytochemicals and antioxidant activity in terms of ferric reducing antioxidant power (FRAP) and percent LDL oxidation inhibition were estimated and compared using response surface methodology.

4.3 Materials and Method

4.3.1 Chemicals and Reagents

LDL isolated from human plasma (in 150 mM NaCl, 0.01% ethylene diamine tetra acetic acid (EDTA), pH 7.4) was purchased from EMD chemicals Inc. (Gibbstown, NJ, USA). Other chemicals used were of analytical grade and obtained from Sigma-Aldrich, Oakville, ON, Canada.

4.3.2 Sample Preparation

Fresh rhizomes of Chinese ginger were purchased from a local market in Truro, Nova Scotia, Canada. The rhizomes were cut into thin slices of 1-2 mm. Twenty five

grams of sliced ginger was used to make a homogeneous mass with 100 mL of deionized water at room temperature, using a food processor (Bead Beater; BioSpec Products, Inc. Bartlesville, OK, USA), at a maximum speed for 2 min; then the homogenates were transferred into 200 mL conical flasks. Homogenates were subjected to subsequent extraction based on the experimental design using three extraction methods: hot water extraction, ultrasound-assisted extraction and high pressure homogenization-assisted extraction. The extraction conditions used in each extraction method were based on previously reported findings (Kishk and Sheshetawy, 2010; Rodrigues et al. 2008; Yuan et al. 2008, Suárez-Jacobo et al. 2011).

4.3.3 Hot Water Extraction

Homogenized ginger in 200 mL conical flasks were placed in a shaking water bath (Shel Lab, Model: 23E GeneQinc., Montreal, Canada) at different time-temperature combinations (Table 4.2) for the extraction of phenolic bioactive constituents into the solution. The extracts were filtered with an 11 cm diameter glass fibre filter (G6), under reduced pressure, to remove the solids and subsequently stored at -20 °C for further analysis.

4.3.4 Ultrasound-assisted Extraction

The process for phenolics extraction from ginger was performed in an ultrasonic bath (Model 4HT-1524-12, Crest Ultrasonic Corp., Trenton, NJ, USA). The frequency and the power of the ultrasonic were 40 kHz and 150 W, respectively. Homogenized ginger in conical flasks was sonicated for different time periods at a specific temperature (Table 4.4). The extracts were filtered with an 11 cm diameter glass fibre filter (G6),

under reduced pressure, to remove the solids, and they were stored at -20 °C for further analysis.

4.3.5 High Pressure Homogenization-assisted Extraction

An EmulsiFlex-C3 high pressure homogenizer (Avestin Inc., Ottawa, ON, Canada) was used for the bioactive extraction from ginger. The filtered ginger extracts were pre-heated to the selected temperature (Table 4.6) in a water bath (Isotemp Model: 205, Fisher Scientific, Ubuque, IA, USA). Then, the preheated samples were loaded into a funnel and the flow rate was maintained at 3 L/hr. Each sample was run at each treated pressure (Table 4.6) for 3 cycles.

4.3.6 Determination of Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteu reagents assay (Singleton et al. 1999) with some modification, as described by Rupasinghe et al. (2008). Twenty µL of extract was added into the wells of the 96-well plate and then 100 µL of the Folin–Ciocalteu phenol reagent was also added. After 5 min, 80 µL of 7.5% sodium carbonate was added to the mixture. After 2 hours in darkness, the absorbance at 750 nm was measured using the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). The concentration of total phenols was expressed as mg gallic acid equivalent (GAE)/L of extract. The linear range used for the calibration was 10-250 mg GAE/L

4.3.7 FRAP (Ferric Reducing Antioxidant Power) Assay

Antioxidant capacity of ginger extracts were measured using FRAP assay, according to the Benzie and Strain (1999) method with some modifications as described by Rupasinghe, et al. (2008). Briefly, the reaction was carried out in a 96-well micro plate. The antioxidant capacities of the standards/extracts are estimated by the increase of

absorbance caused by the generated ferrous ions. The working FRAP reagent contained 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ), 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1. Freshly prepared FRAP working reagent was incubated to 37 °C. One hundred and eighty µL of this working solution was dispensed to each well of the micro plate. Then, addition of 20 µL diluted extracts initiated the reaction and absorbance was read after 10 min. FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA) was utilized to read the absorbance at 593 nm. Trolox was the standard and all measurements were performed in triplicate.

4.3.8 Percent LDL Oxidation Inhibition

4.3.8.1 LDL Preparation

LDL were dialyzed extensively against phosphate buffer saline (PBS) containing 138 mM NaCl and 27 mM KCl (pH 7.4) to remove inherent antioxidants using cellulose dialysis tubing (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) at 4 °C for 24 hours. The buffer was changed every six hours. The dialyzed LDL was immediately stored at -80 °C and used within two weeks. Protein content of the dialyzed-LDL was measured by the Lowry's method (Lowry et al. 1951), using bovine serum albumen as the standard.

4.3.8.2 Measurement of Percent LDL Oxidation Inhibition as TBARS

Briefly, 180 µL LDL (50 µg protein/mL) was incubated, with 20 µL ginger extracts or 20 µL blank, for 4 hours at 37 °C in 50 mM phosphate buffer saline (PBS) at pH 7.4, for a total volume of 200 µL. The reaction was initiated with the addition of 10 µM $CuSO_4$. The experimental units consisted of a blank, a positive control (induction with 10 µM $CuSO_4$, but without the antioxidant treatment), a negative control (without induction or treatment) and water extracts of ginger. Oxidation was terminated by adding

50 µL of 5 mM solution of EDTA to have 1 mM final concentration of EDTA in the 250 µL solution mixture. The LDL oxidation was determined by spectrophotometrically measuring the amount of TBARS using the method described by Xu et al. 2007, with minor modifications. Briefly, after terminating the LDL oxidation, TBA reagent (0.67% thiobarbituric acid and 20% trichloroacetic acid (TCA) in 0.2 M NaOH) was added to the reaction mixture. Then the mixture was incubated at 95 °C for 30 min to develop a pink chromogen. The samples were placed in the refrigerator for 10 min to cool down to room temperature. Then the tubes were centrifuged at 1500 g for 15 min and absorbance was measured at 532 nm using the FLUOstar OPTIMA plate reader. TBARS activity was determined as the percent inhibition of LDL oxidation with comparison of positive control.

$$\text{Percent inhibition (\%)} = \frac{(\text{Absorbance (positive control)} - \text{Absorbance (sample)})}{\text{Absorbance (positive control)}} * 100$$

4.3.9 Statistical Analysis

All data from the study were presented as mean ± SD of three replications, and means were compared using analysis of variance (ANOVA). Acquired data were manipulated to calculate statistical values such as mean and standard deviation (SD) using Microsoft Excel (Microsoft Inc., Redmond, WA, USA). The assumptions of normality and constant variance were tested using Anderson-Darling test and examining residual versus fits.

4.3.10 Experimental Design for the Optimization of Ginger Bioactive Extraction

A two factor and three level (-1, 0 and +1) central composite design was used for each of the three extraction techniques to achieve maximal information about the

process from a minimum number of possible experiments. For the hot water extraction and the ultrasound-assisted extraction, the independent variables were extraction temperature and extraction time, for the high pressure homogenization-assisted method, the extraction temperature and extraction pressure, were the independent variables. The dependent variables were total phenolics content (mg GAE/L), antioxidant capacity (mg TE/L) and percent inhibition of LDL oxidation and each variable was coded at three levels, -1, 0 and +1 (Table 4.1). This experiment was carried out separately for each extraction technique to identify the optimum extraction conditions. For data analysis, RSREG procedures of SAS software (SAS, 9.2, Cary, NC), as well as Minitab16 software, were used. Canonical analysis was performed to optimize the independent variables using SAS procedure. The assumptions of normality and constant variance were checked using Anderson-Darling test and confirmed. Adequacy of the model was determined by the ANOVA. Ridge analysis was performed to compute the ridge of the optimum response when the results showed a saddle point in the response surfaces. The contour plots and overlaid contour plots were generated using the Minitab software.

Table 4.1. The variables of three extraction processes and their levels in central composite design

Levels of operating parameters					
	Hot water extraction				
Coded value	-1.41	-1	0	+1	+1.41
Uncoded value					
Temperature (°C)	33.61	45	72.5	100	111.39
Time (min)	11.72	20	40	60	68.28
	Ultrasound-assisted extraction				
Coded value	-1.41	-1	0	+1	+1.41
Uncoded value					
Temperature (°C)	23.79	30	45	60	66.21
Time (min)	11.72	20	40	60	68.28
	High pressure homogenization-assisted extraction				
Coded value	-1.41	-1	0	+1	+1.41
Uncoded value					
Temperature (°C)	23.79	30	45	60	66.21
Pressure (MPa)	79.29	100	150	200	220.71

4.4 Results

4.4.1 Hot Water Extraction

The combined effects of temperature and time during hot water extraction of ginger root on total phenolics, FRAP value and percent inhibition of LDL oxidation, were investigated. The results of the central composite design are presented in Table 4.2. The statistical software SAS and Minitab were used to fit contour plots for the response variables. Regression coefficients of predicted models for the responses of phenolics and antioxidant activities are shown in Table 4.3. The assumptions of normality and constant variance were checked and found to be valid. Contour plots of phenolics (mg GAE/L) (a),

FRAP (mg TE/L) (b) and % LDL oxidation inhibition (c) of hot water extraction of ginger is shown in Figure 4.1.

Table 4.2. Response values for given levels of variables in hot water extraction of ginger root in RSM

Experiment No.	Coded		Uncoded		Phenolics	FRAP value	% LDL oxidation inhibition
	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)			
1	-1	-1	45	20	363.7	219.9	31.2
2	-1	+1	45	60	446.9	232.4	55.4
3	+1	-1	100	20	405.0	314.8	36.2
4	+1	+1	100	60	427.2	229.9	42.3
5	0	0	72.5	40	443.7	217.4	39.8
6	0	0	72.5	40	428.4	234.9	45.3
7	0	0	72.5	40	435.8	212.4	33.6
8	0	0	72.5	40	435.3	197.4	40.8
9	0	0	72.5	40	420.6	242.4	41.0
10	+1.41	0	100	40	418.5	259.9	41.5
11	-1.41	0	34	40	383.8	217.4	37.5
12	0	+1.41	72.5	68	454.5	292.4	34.7
13	0	-1.41	72.5	12	404.9	187.4	32.1

Phenolics (mg GAE/L), FRAP (mg TE/L)

Table 4.3. Estimated regression coefficients for predicted models and analysis of ANOVA for hot water extraction of ginger root

Regression coefficient	Phenolic content	FRAP value	% LDL inhibition
β_0	156.364	149.34	6.401
β_1	4.688	0.278	0.144
β_2	3.645	1.341	1.234
β_{11}	-0.022	0.0150	0.001
β_{12}	-0.007	-0.044	-0.005
β_{22}	0.028	0.029	-0.008
(p-values)			
Lack-of-fit	0.389	0.0722	0.1118

Phenolics (mg GAE/L), FRAP (mg TE/L), β_0; intercept, β_1; linear (temp.), β_2; linear (time), β_{11} & β_{22}; quadratic, β_{12}; interaction

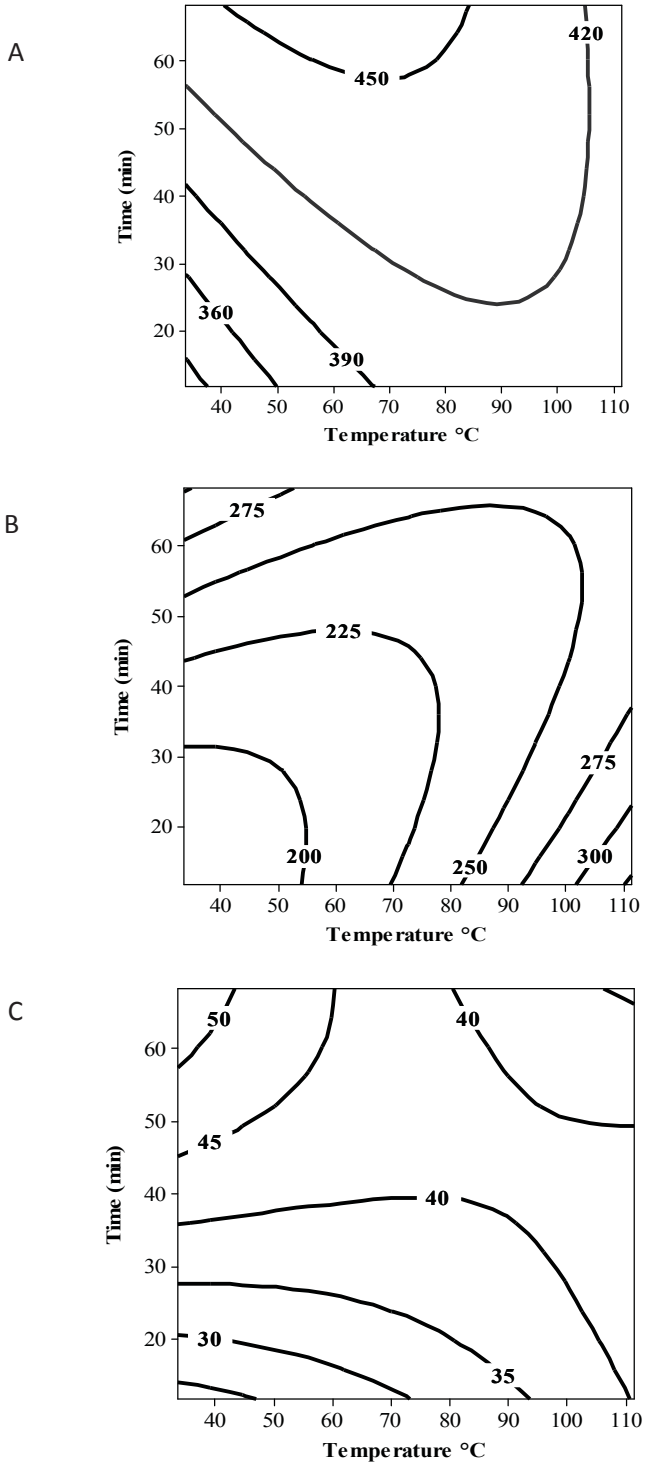


Figure 4.1. Contour plots of total phenolics (mg GAE/L) (a), FRAP value (mg TE/L) (b) and % LDL oxidation inhibition (c) of hot water extraction of ginger

4.4.2 Ultrasonic-assisted Extraction

Minitab software was used to fit response contour plots for total phenolics, FRAP and percent LDL oxidation inhibition in ultrasonic-assisted extraction of ginger. The response values obtained using response surface methodology and the central composite design using response surface methodology, is given in Table 4.4. Analysis of variance was performed on each response separately (Table 4.5). There was no significant lack of fit ($p>0.05$), indicating that the design of the experiment was enough to determine the effect of independent variables on the responses. Contour plots obtained for three response variables (phenolics, FRAP and % LDL oxidation inhibition), with respect to the ultrasonic-assisted extraction, are shown in Figure 4.2.

Table 4.4. Response values for given levels of variables in ultrasonic-assisted extraction of ginger root in RSM

Experiment No.	Coded		Uncoded		Phenolics	FRAP value	% LDL oxidation inhibition
	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)			
1	-1	-1	30	20	432.7	217.9	38.5
2	-1	+1	30	60	449.2	275.5	49.6
3	+1	-1	60	20	386.8	246.9	35.9
4	+1	+1	60	60	454.5	231.9	42.1
5	0	0	45	40	475.3	210.2	43.7
6	0	0	45	40	454.6	230.2	38.5
7	0	0	45	40	488.9	211.2	51.7
8	0	0	45	40	476.8	230.7	50.7
9	0	0	45	40	458.3	243.2	42.2
10	+1.41	0	66	40	454.2	173.8	25.2
11	-1.41	0	24	40	390.7	203.2	50.7
12	0	+1.41	45	68	485.5	231.9	21.6
13	0	-1.41	45	12	393.3	200.2	33.7

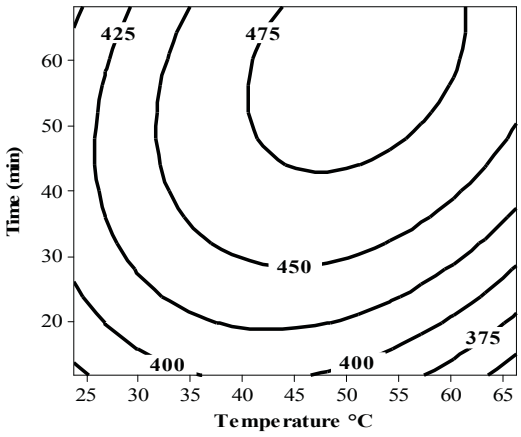
Phenolics (mg GAE/L), FRAP (mg TE/L)

Table 4.5. Regression coefficients and analysis of variance (ANOVA) for ultrasonic assisted-extraction of ginger root

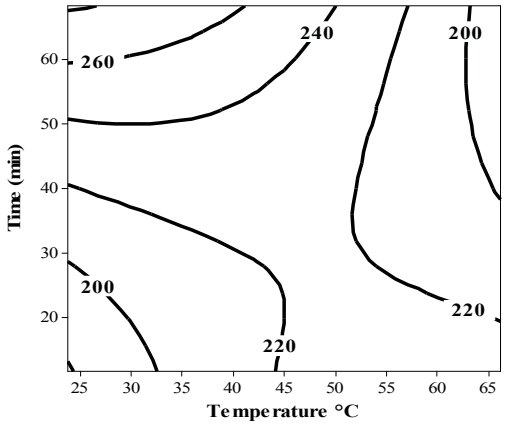
Regression coefficient	Phenolic content	FRAP value	% LDL inhibition
β_0	194.807	64.186	14.795
β_1	8.382	5.244	0.391
β_2	2.569	2.138	1.521
β_{11}	-0.108	-0.036	-0.007
β_{12}	0.043	-0.061	0.004
β_{22}	-0.039	0.014	-0.017
(p-values)			
Lack-of-fit	0.109	0.0629	0.107

Phenolics (mg GAE/L), FRAP (mg TE/L), β_0; intercept, β_1; linear (temp.), β_2; linear (time), β_{11} & β_{22}; quadratic, β_{12}; interaction

A



B



C

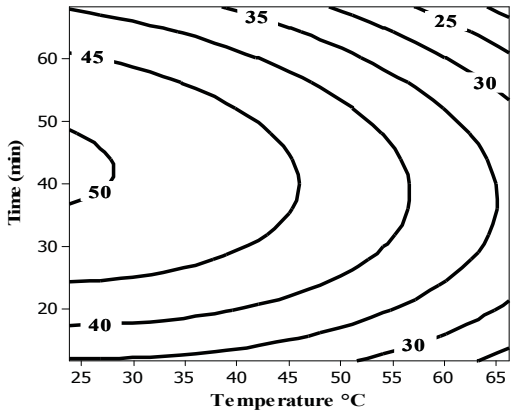


Figure 4.2. Contour plots of phenolics (mg GAE/L) (a), FRAP (mg TE/L) (b) and % LDL oxidation inhibition (c) of ultrasonic-assisted extraction of ginger

4.4.3 High Pressure Homogenization-assisted Extraction

Minitab statistical software was used to fit a response surface and to construct contour plots for the response variables (phenolic, FRAP and percent LDL oxidation inhibition) obtained at different extraction pressures and temperatures during the high pressure homogenization assisted-extraction method (Table 4.6). Testing of model adequacy, ANOVA and regression analysis for the predicted models are shown in Table 4.7. The assumptions of normality, constant variance were checked and found to be valid. Contour plots obtained for three response variables (phenolics, FRAP and % LDL oxidation inhibition) with respect to the high pressure homogenization-assisted extraction, are shown in Figure 4.3.

Table 4.6. Response values for given levels of variables in high pressure homogenization-assisted extraction of ginger root in RSM

Experiment No.	Coded		Uncoded		Phenolics	FRAP	% LDL oxidation inhibition
	Temp. (°C)	Press MPa	Temp. (°C)	Press MPa			
1	-1	-1	30	100	461.6	200.2	12.9
2	-1	+1	30	200	457.8	225.4	32.2
3	+1	-1	60	100	448.4	225.9	37.6
4	+1	+1	60	200	482.2	225.1	32.3
5	0	0	45	150	470.4	225.7	32.6
6	0	0	45	150	475.0	275.9	36.6
7	0	0	45	150	448.0	300.4	44.8
8	0	0	45	150	438.9	225.3	32.1
9	0	0	45	150	473.7	200.8	41.1
10	+1.41	0	66	150	482.3	275.1	39.4
11	-1.41	0	24	150	453.9	175.0	42.4
12	0	+1.41	45	220	478.7	325.1	17.1
13	0	-1.41	45	80	448.9	225.7	32.3

Table 4.7. Regression coefficients and analysis of variance (ANOVA) for high pressure homogenization-assisted extraction

Regression coefficient	Phenolic content	FRAP value	% LDL inhibition
β0			
β_1	123.279	416.321	-66.229
β_2	6.160	-0.628	-0.406
β_{11}	2.471	-2.067	1.521
β_{12}	0.061	-0.021	0.030
β_{22}	-0.067	0.019	-0.014
	0.002	0.003	-0.003
(p-values)			
Lack-of-fit	0.903	0.076	0.141

Phenolics (mg GAE/L), FRAP (mg TE/L), β_0, intercept, β_1; linear (temp.), β_2; linear (pressure), β_{11} & β_{22}; quadratic, β_{12}; interaction

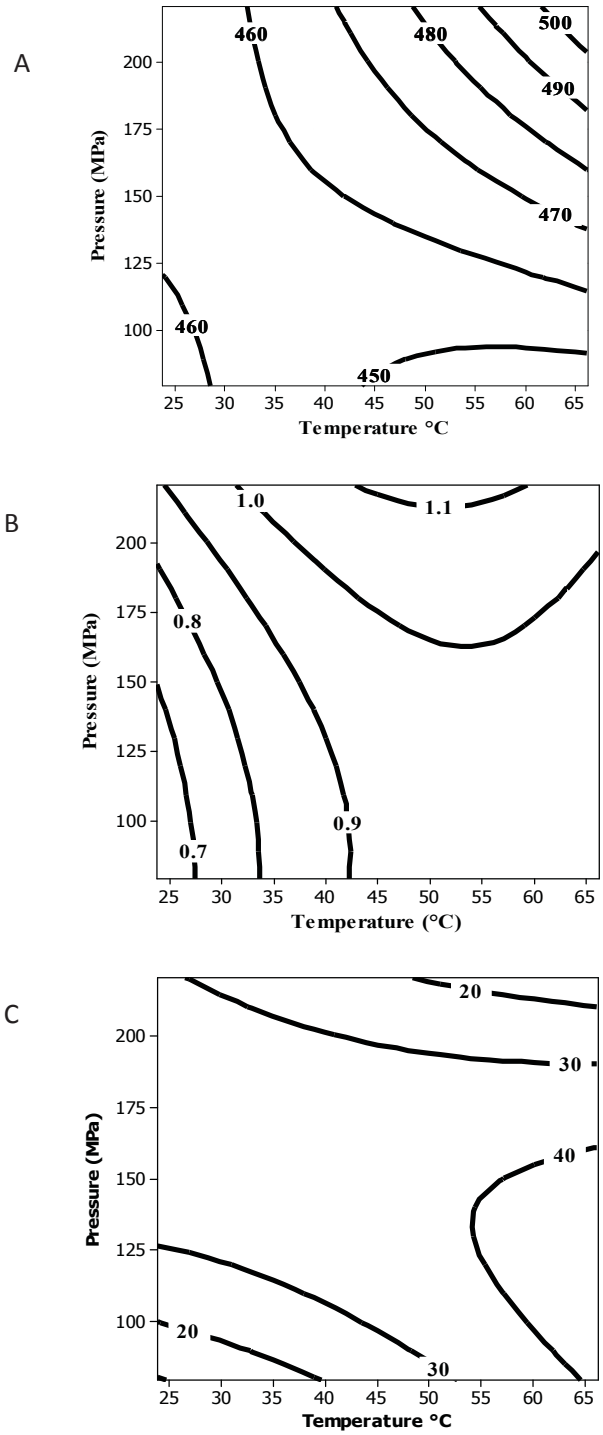


Figure 4.3. Contour plots of % phenolics (mg GAE/L), FRAP (mg TE/L, % LDL oxidation inhibition of high pressure homogenization assisted extraction of ginger

4.4.4. Optimization of Multiple Responses

To optimize the multiple responses of each extraction method, overlaid contour plots of phenolic, FRAP and percent LDL oxidation inhibition were generated using Minitab software (Figure 4.4). Table 4.8 shows the summary of the optimum extraction conditions for each extraction method, obtained from establishing overlaid contour plots.

Table 4.8. Optimum extraction conditions drawn from overlaid plots of different extraction methods

Extraction method	Optimum extraction conditions
Hot water extraction	Temperature: 55 °C Time: 60 min
Ultrasonic-assisted extraction	Temperature: 52 °C Time: 15 min
High pressure homogenization-assisted extraction	Temperature: 62 °C Pressure: 140 MPa

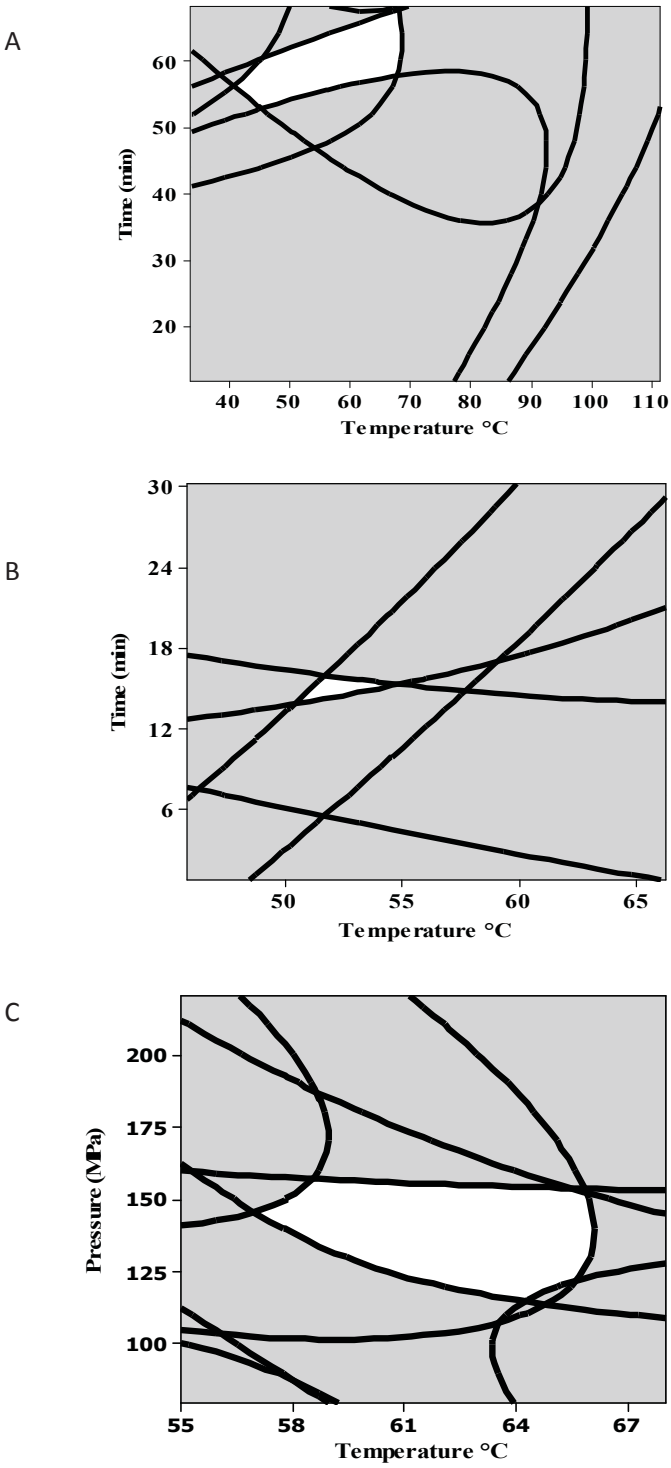


Figure 4.4. Overlaid contour plots of phenolics, FRAP and LDL oxidation inhibition of hot water extraction (a), ultrasound-assisted extraction (b) and high pressure homogenization-assisted extraction (c) of ginger

4.5 Discussion

Hot water extraction is a conventional technique used to isolate bioactives from plant sources. According to the extraction conditions used in the experiment, the phenolic yield varied between 364 to 455 mg GAE/L the FRAP ranged from 187 to 314 mg TE/L and % LDL oxidation inhibition ranged from 31% to 55%. Contour plots (Figure 4.1) demonstrated the saddle points for all predicted responses for hot water extraction and the estimated surfaces did not have unique optima. Therefore, ridge analysis was performed to determine the estimated ridge of the optimum response (Appendix A)**.** The ridge analysis indicated that maximum phenolic content can be extracted at above 60 °C for more than 60 min, whereas maximum FRAP value in the extract can be predicted to be obtained at 63 °C temperature and 26 minutes. Maximum LDL oxidation inhibition can be achieved at a relatively low temperature (about 44 °C) over a longer time (55 min). Analysis of contour plots (Figure 4.1) of phenolics, FRAP and percent LDL oxidation inhibition allows one to conclude that the high extraction temperature and shorter extraction time leads to an extract which is high in phenolic content together with high antioxidant activities, FRAP and LDL oxidation inhibition.

Ultrasonic-assisted extraction is highly effective because it produces higher yield phenolic constituents and antioxidant activity while taking less time (Morelli and Prado, 2012; Rupasinghe et al. 2011). The ultrasonication extraction exerts two types of physical phenomena: diffusion of the extractant through the cell walls and washing out the cell content through ruptured cell walls (Vinatoru, 2001). Ultrasound-assisted extraction conduction between 30-60 °C and 20-60 min time-temperature range yields 387-489 mg GAE/L phenolics, 174-276 mg TE/L antioxidant capacity and 22-52% LDL oxidation

inhibition. Contour plots (Figure 4.2a and 4.2c) illustrated that the predicted response for phenolics and percent LDL oxidation inhibition are maximum. Based on the canonical analysis, maximum phenolic content can be extracted from ginger by ultrasonication at 60 °C for 51 min. Higher phenolic extraction from ginger can be achieved under ultrasonic conditions of relatively higher temperature for an extended time duration (Figure 4.2a). However, predicted response for FRAP value at ultrasonic-assisted extraction of ginger is a saddle point. The contour plot (Figure 4.2b) of the predicted FRAP values confirmed this saddle point clearly. Therefore, ridge analysis was performed to determine the levels of the design variables that would produce maximum response for FRAP value in the extract. According to the ridge analysis (Appendix B), maximum FRAP value can be obtained at 35 °C for 65 min under ultrasonic conditions. The contour plot (Figure 4.2c) clearly shows that the maximum LDL oxidation inhibition can be achieved by selecting relatively low extraction temperatures for longer time, nearly 44 min in ultrasonic-assisted extraction.

Ginger extracts subjected to high pressure homogenization showed total phenolic content in the range of 412-510 mg GAE/L, antioxidant capacity from 186 to 308 mg TE/L and the LDL oxidation inhibition from 17 to 62%. Analysis of contour plots of phenolics (Figure 4.3a), FRAP (Figure 4.3b) and percent LDL oxidation inhibition (Figure 4.3c) demonstrated a saddle point for all predicted responses during high pressure homogenization-assisted extraction of ginger. Therefore, the estimated surfaces of phenolic, FRAP and LDL oxidation inhibition did not have unique optimum. Therefore, ridge analysis was performed to determine the estimated ridge of the optimum response. According to the ridge analysis (Appendix C), the maximum phenolic content can be

extracted at more than 108 °C and 62 MPa homogenization pressure, whereas it can be predicted that maximum FRAP value in the extract can be to obtained at 40 °C temperature for 82 MPa homogenization pressure. Maximum LDL oxidation inhibition can be achieved at 65 °C temperature and 126 MPa homogenization pressure.

Desirable results in terms of high phenolic content, together with high antioxidant activities, antioxidant capacity and LDL oxidation inhibition, are described for the ginger extract to be incorporated into a functional food. Overlaying these three responses provided the visual output required to select optimum extraction conditions. The optimum extraction conditions which yielded the greatest phenolics, FRAP and LDL oxidation inhibitions of hot water extraction, ultrasonic-assisted extraction and high pressure homogenization-assisted extraction were 55 °C for 60 min (Figure 4.4a), 52 °C for 15 min (Figure 4.4b) and 140 MPa homogenization pressure at 55 °C (Figure 4.4c) respectively. Based on the results, the highest phenolic content and antioxidant activity can be achieved with relatively at lower extraction conditions using ultrasonication compared with the other two methods. Homogenization pressure and temperature had significant effect on stability of nanoemulsion of bioactives in the extract (Yuan et al. 2008) and it improved the extractibility of bioactives as well (Dong et al. 2011). However, the predicted phenolic content and antioxidant activities associated with the high pressure homogenization-assisted extraction is lower than that of the ultrasonic assisted extraction, but higher than the hot water extraction.

4.6 Conclusion

The response surface methodology was used to determine the optimum extraction conditions for three extraction methods which give the highest phenolic content and antioxidant activities. Based on the results, ultrasonic-assisted extractions yielded highest predicted phenolic and antioxidant activities at lower extracting conditons, 52 °C for 15 min, compared with other two extraction methods tested.

CHAPTER 5.0 INHIBITION OF HUMAN LOW DENSITY LIPOPROTEIN OXIDATION *IN VITRO* BY GINGER EXTRACTS

5.1 Abstract

In this study, the effect of ethanol, methanol, ethyl acetate and hexane solvent extracts of ginger and pure major ginger constituents on Cu^{+2}-induced oxidation of human LDL *in vitro* were examined. Results showed that the phenolic content and the antioxidant capacity of ultrasonic-assisted water extracted ginger were lower than those of organic solvent extracts tested in all experimental conditions. The LDL oxidation inhibition by ethanol, methanol, ethyl acetate and hexane extracts of ginger were 71%, 76%, 67% and 67%, respectively at their optimum extraction conditions. The inhibition of oxidation by water extract of ginger, which was prepared by ultrasonic-assisted extraction conditions of 52 °C for 15 min, was about 43%. This study revealed that the ginger root extracted using differernt methods, inhibited the Cu^{2+}-induced LDL oxidation *in vitro* at varying levels. Ginger may be considered as a potential ingredient for functional foods designed for cardiovascular health.

5.2 Introduction

Oxidation of LDL has been suggested to play a significant role in the process of development of atherosclerosis (Madamanchi et al. 2005; Ryu, 2000; for review see Thilakarathna and Rupasinghe, 2012). Oxidized LDL in the intima is considered as more atherogenic than the un-oxidized LDL and plays a role as a chemo-factor in the recruitment of circulating monocytes and macrophages (Quinn et al. 1985). In the atherogenic process, oxidized LDL are engulfed by macrophage scavenger receptors and induce both the cellular and molecular level atherogenic consequences, such as cholesterol-ester accumulation, foam cell formation, inhibition of regular NO associated vasodilation (Kugiyama et al. 1990).

Epidemiological studies show that there is a positive correlation between the consumption of dietary antioxidants and a decrease of the incidence of coronary artery disease (Joshipura et al. 2001). The association of dietary antioxidant and CVD is explained by the "oxidative-modification hypothesis" of atherosclerosis which proposes that atherogenesis is initiated by the oxidation of the lipids in LDL (Ryu, 2000). Based on this hypothesis, antioxidants that inhibit peroxidation of lipids in LDL should also limit atherosclerosis and related clinical conditions, such as myocardial infarction and stroke (Yla-Hertuala et al. 1989). Recent studies have demonstrated the actions of oxidized LDL in atherosclerotic lesion formation (Mertens, et al. 2003, Miyazaki et al. 2002, Lerouet, et al. 2002) and also suggested that the antioxidant administration retards the progression of atherosclerosis (Libby et al. 2002).

Ginger is a medicinal herb, widely used in herbal medicines, and has been extensively studied for its biological activities (Langner et al. 1998). Currently, ginger

has received a renewed interest because of its pharmacological activities, such as anti-cancer (Shukla and Singh, 2007), anti-inflammatory (Grzanna et al. 2005), antioxidant (Kota et al. 2008) and cardio-protective properties (Bhandari et al. 1998; Nicoll and Henein, 2009). It has been suggested that ginger could be a promising antioxidant source with its ability to scavenge a number of free radicals (Srivastava and Mustafa, 1989). The active principles of ginger are mainly phenolics, a series of gingerols (Figure 3.3 in Chapter 3.0) (Jolad et al. 2004) and isoprenoids such as monoterpenoids and sesquiterpenoids (Melo et al. 2002). Recent research has shown that ginger is endowed with strong *in vitro* and *in vivo* antioxidant properties (Ali et al. 2008); for instance, methanolic extract of fresh ginger exhibits hypotensive (Ghayur and Gilani, 2005), endothelium-independent vasodilator (Ghayur and Gilani, 2005) and cardio-suppressant properties (Ghayur et al. 2005). There is, however, a paucity of information on the ability of ginger to inhibit LDL oxidation. Therefore, in this study, antioxidant protection of human LDL *in vitro* and FRAP activity by ginger extracts, were examined. Apart from the crude extracts, some pure pungent phenolic constituents of ginger, namely 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol and α-farnesene, were also tested on their ability to inhibit LDL oxidation.

5.3 Materials and Methods

5.3.1 Chemical and Reagents

LDL isolated from human plasma (in 150 mM NaCl, 0.01% EDTA, pH 7.4) was purchased from EMD chemicals Inc. (Gibbstown, NJ, USA). Pure ginger constituents were obtained from ChromaDex Inc. (Irvine, CA, USA). Other chemicals used were of analytical grade and purchased from Sigma-Aldrich, Oakville, ON, Canada.

5.3.2 Plant Materials and Sample Preparation

Fresh rhizomes of ginger were purchased from a local market in Truro, Nova Scotia, Canada. The rhizomes were cut into 1-2 mm thin slices. Phenolic compounds were extracted from 25 g of sliced ginger samples in 100 mL of deionized water or organic solvent, using a laboratory scale blender (Bead Beater; BioSpec Products, Inc. Bartlesville, OK, USA) at the maximum speed for 2 min. Water extract of ginger was prepared using ultrasonication at 52 °C for 15 min. These extraction conditions were selected based on the preliminary studies on optimization of ginger bioactive extraction, as described in Chapter 4. Four different organic solvents methanol, ethanol, ethyl acetate and hexane were used for the extraction. Optimum extraction conditions for each solvent such as solvent concentration (50, 70, 100%), extraction time (20, 40 min) and temperature (30, 45°C) of ultrasonication for each solvent were selected based on a preliminary factorial experiment (3 × 2 × 2), with total phenolic content and antioxidant activity of the extracts as responses. All extracts were homogenized and filtered with 11 cm-diameter glass fibre filters (G6) under reduced pressure, to remove the solids. The filtered extracts were stored at -20 °C until the analysis.

5.3.3 Determination of Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteu assay (Singleton et al. 1999), with some modifications as described by Rupasinghe et al. (2008). Twenty µL of extract was added into the wells of the 96-well microplate, followed by 100 µL of the Folin–Ciocalteu phenol reagents. After 5 min, 80 µL of 7.5% sodium carbonate was added to the mixture. After 2 hours in darkness at room temperature, the absorbance at 750 nm was measured using the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). The concentration of total phenols was expressed as mg gallic acid equivalent (GAE) per liter extract (mg GAE/L).

5.3.4. Analysis of Main Phenolic Bioactives of Ginger

Analysis of major phenolic bioactives of ginger were carried out using Ultra performance liquid chromatography (UPLC) (Model H-class system, Waters, Milford, MA, USA) equipped with an acuity UPLC BEH C18 coloumn (2.1 x 100 mm, 1.7 µm) according to the procedure reported by He et al. (1998).

5.3.5 Ferric Reducing Antioxidant Power (FRAP) Assay

Antioxidant capacity of ginger extracts were measured using FRAP assay, according to the Benzie and Strain method (1999), with some modifications, as described by Rupasinghe, et al. (2008).

5.3.6 Percent Inhibition of LDL Oxidation

LDL was dialyzed as described earlier and the percent LDL oxidation inhibition were measured, based on the method described in Xu et al. (2007), with slight modifications as mentioned in Chapter 4.0. Five main constituents of ginger, such as 6-gingerols, 8-gingerols, 10-gingerols and 6-shagoal and α-farnesene at 0.01, 0.1, 1, 10 and

50 mg/L concentrations, were also tested to investigate the IC_{50} level of LDL oxidation inhibition. Solvent extracts of ginger at different dilution levels (dilution factors: 1, 10, 100, 1000) were also evaluated for their ability to inhibit the LDL oxidation.

5.3.7 Statistical Analysis

All data from the study were presented as mean ± standard deviation of three replications. Samples were analyzed in triplicate and one way analysis of variance (ANOVA) was performed, using general linear model (SAS 9.2, Cary, NC). Differences were considered to be statistically significant if the probability values were less than 0.05 ($p< 0.05$). The assumptions of normality were tested using the Anderson-Darling test and constant variance using residual versus fits, and the independence assumptions were achieved through randomization. When there was a significant difference, multiple mean comparisons were carried out using the LSD method. To evaluate the relationship between the antioxidant activity and phenolic contents, the Pearson correlation coefficient analyses were performed using MINITAB 16 (State College, PA, USA).

5.4. Results

5.4.1 Total Phenolic Content

The highest phenolic concentration found in a ginger extract was with a solvent concentration and ultrasonic extraction conditions as follows, 70% ethanol at 45°C for 40 min; 100% hexane at 45 °C for 20 min; methanol 70% at 45°C for 40 min; and 100% ethyl acetate at 30 °C for 40 min (Table 5.1). Based on the results of Chapter 4, the optimum temperature and time for water-based extraction of ginger phenolics were 52 °C for 15 min.

The total phenolic content of different extracts of ginger, extracted at the optimum extraction conditions for each solvent, is shown in Figure 5.1a. The highest total phenolics level was observed in methanol extract, which was about 726 mg GAE/L and ethanol extract contained about 660 mg GAE/L. Similarly, ethyl acetate (649 mg GAE/L) as an extraction solvent provided a similar recovery of phenolics as hexane (632 mg GAE/L) in terms of total phenolics when compared with water at its optimum extraction conditions. Phenolic content of the water extract (482 mg GAE/L) was significantly ($p>0.05$) lower than those of organic extracts. The composition of main ginger bioactives of each solvent extract is shown in Table 5.2.

Table 5.1. Total phenolic content (mg GAE/L) of ginger extracts under different extraction conditions

Extraction conditions			Solvents			
Con. %	**Temp (°C)**	**Time (min)**	**Ethanol**	**Methanol**	**Hexane**	**Ethyl acetate**
50	**30**	**20**	512.2±7.3^{d}	581.1±15.4^{cd}	491.7±28.5^{d}	560.7±24.3^{bc}
50	**30**	**40**	529.8±15.8^{cd}	570.2±18.7^{d}	536.9±2.5^{c}	610.0±32.8^{ab}
50	**45**	**20**	563.3±15.5^{c}	618.9±24.4^{c}	601.5±7.9^{ab}	611.4±15.7^{ab}
50	**45**	**40**	559.1±22.6^{c}	643.4±10.4^{bc}	570.6±23.3^{b}	468.7±57.8^{d}
70	**30**	**20**	594.9±6.1^{b}	627.2±28.2^{bc}	578.8±4.9^{b}	552.1±16.1^{c}
70	**30**	**40**	613.3±24.6^{b}	693.4±72.9^{ab}	562.4±23.3^{bc}	579.6±13.1^{bc}
70	**45**	**20**	608.4±9.8^{b}	722.2±10.5^{a}	601.0±56.7^{ab}	600.2±20.4^{b}
70	**45**	**40**	**660.1±17.3^{a}**	**726.0±22.6^{a}**	527.6±9.7^{c}	608.4±32.3^{b}
100	**30**	**20**	577.1±41.0^{c}	617.5±30.2^{c}	505.9±10.2^{d}	593.3±12.6^{b}
100	**30**	**40**	554.1±14.8^{c}	675.9±30.7^{b}	544.3±9.9^{c}	**649.4±14.2^{a}**
100	**45**	**20**	547.6±36.3^{c}	561.5±27.1^{d}	**632.3±43.7^{a}**	624.8±59.4^{ab}
100	**45**	**40**	559.1±24.6^{c}	582.5±12.2^{d}	576.3±22.2^{b}	592.2±42.4^{b}

Data presented as mean±SD. Data with different superscripts in each column are significantly different. Comparisons were done for different solvent concentrations and extraction conditions (temperature and time). Con; concentration as a % (v/v), temp; temperature

Table 5.2. Composition of main bioactives of ginger extracts (mg/L)

Ginger extracts*	10-Gingerol	8-Gingerol	6-Gingerol	6-Shogaol
Ethanol extract	66.2±8.1^{b}	24.9±10.3^{c}	39.4±4.6^{c}	47.3±1.5^{d}
Methanol extract	121.7±13.6^{a}	58.2±1.5^{a}	65.3±3.0^{a}	55.3±1.2^{b}
Ethylacetate extract	13.5±1.9^{c}	12.4±1.8^{d}	28.5±1.9^{d}	48.6±1.2^{d}
Hexane extract	115.3±8.1^{a}	51.0±6.2^{b}	61.4±11.4^{a}	63.1±6.2^{a}
Water extract	23.2±19.3^{c}	17.9±5.7^{c}	47.7±1.1^{b}	51.6±2.1^{c}

Data presented as mean±SD. Data with different superscripts in each column are significantly different. *Solvent extract of ginger, extracted at optimum extraction conditions for phenolics of each solvent

5.4.2 Antioxidant Capacity

Antioxidant capacity of ginger extracts prepared using different concentrations of organic solvent levels are shown in Table 5.3. The optimum solvent concentrations (%) and extraction conditions (temperature and time) which showed the highest FRAP antioxidant capacity was as follows: 70% ethanol at 30 °C for 40 min; 50% hexane at 45 °C for 20 min; 70% methanol at 45 °C for 20 min; and 50% ethyl acetate at 45 °C for 40 min. Methanol extract of ginger showed the highest antioxidant capacity, 492 mg TE/L, compared with other organic acid extracts tested. Antioxidant capacity of ethanol, hexane and ethyl acetate extracts of ginger was 463, 392 and 367 mg TE/L, respectively. According to Figure 5.1b, water extract showed significantly ($p<0.05$) lower antioxidant capacity (264 mg TE/L) compared with that of organic solvent extracts. This antioxidant capacity assay was linearly correlated with the phenolic content ($R^2=0.72$).

Table 5.3. FRAP value (mg TE/L) of ginger extracts at different extraction conditions

Extraction conditions			Solvents			
Con. %	**Temp (°C)**	**Time (min)**	**Ethanol**	**Methanol**	**Hexane**	**Ethyl acetate**
50	**30**	**20**	400.5±10.4^{bc}	379.2±38.9^{bc}	355.9±21.3^{b}	313.6±4.8^{b}
50	**30**	**40**	374.2±10.8^{cd}	403.8±14.6^{b}	319.3±31.4^{bc}	318.2±8.2^{b}
50	**45**	**20**	357.7±12.1^{d}	400.9±18.5^{b}	**391.6±11.8^{a}**	364.0±43.6^{ab}
50	**45**	**40**	387.4±18.1^{c}	400.6±29.4^{b}	326.8±14.6^{c}	**366.9±33.1^{a}**
70	**30**	**20**	341.9±32.0^{d}	475.7±32.8^{a}	286.1±12.5^{d}	318.8±21.3^{b}
70	**30**	**40**	**462.5±38.0^{a}**	477.5±45.7^{a}	351.1±16.4^{b}	374.4±9.7^{a}
70	**45**	**20**	398.3±39.4^{bc}	**491.5±29.6^{a}**	331.1±11.6^{c}	345.9±31.7^{ab}
70	**45**	**40**	427.5±14.0^{b}	405.5±38.1^{b}	299.7±8.7^{d}	274.9±34.7^{c}
100	**30**	**20**	365.8±29.1^{bc}	348.6±23.9^{c}	275.9±21.4^{de}	293.0±24.4^{c}
100	**30**	**40**	371.8±29.5^{bc}	364.1±28.9^{c}	276.9±14.2^{de}	312.8±24.7^{bc}
100	**45**	**20**	297.4±39.5^{e}	298.2±19.8^{d}	279.3±13.2^{d}	232.7±21.3^{d}
100	**45**	**40**	260.0±40.7^{e}	235.4±16.8^{e}	264.1±10.7^{de}	243.7±57.1^{dc}

Data presented as mean±SD. Data with different superscripts in each column are significantly different. Comparisons were done for different solvent concentrations and extraction conditions (temperature and time). Conc.; concentration as a % (v/v), Temp.; temperature

5.4.3 Inhibition of LDL Oxidation

According to Table 5.4, the optimum solvent concentrations (%) and extraction conditions (temperature and time) which showed the highest LDL oxidation inhibition was as follows: 70% ethanol at 45 °C for 20 min; 100% hexane at 45 °C for 20 min; 70% methanol at 45 °C for 20 min; and 50% ethyl acetate at 30 °C for 40 min. Solvent extracts of ginger exhibited significantly ($p<0.05$) higher LDL oxidation inhibition ability compared with that of water extracts (Figure 5.1c). The percent LDL oxidation inhibited by ethanol, hexane, methanol and ethyl acetate extracts of ginger were 71±3.%, 67±2.9%,

76±7% and 67±1%, respectively at their optimum extraction conditions. Inhibition by water extract was about 43±2% at the optimum ultrasonic-assisted extraction conditions. The inhibition of LDL oxidation by extract correlates with the phenolic content of the extracts (R^2=0.86).

Table 5.4. Percent inhibition of Cu^{2+}-induced LDL oxidation of ginger extracts at different extraction conditions

Extraction conditions			Solvents			
Con. %	**Temp (°C)**	**Time (min)**	**Ethanol**	**Methanol**	**Hexane**	**Ethyl acetate**
50	**30**	**20**	51.6±5.9 ^{c}	63.1±1.4^{b}	54.1±2.2^{c}	61.5±1.4^{b}
50	**30**	**40**	64.4±0.9 ^{b}	62.9±0.8^{b}	62.3±2.8^{b}	**66.7±1.1^{a}**
50	**45**	**20**	63.7±1.1 ^{b}	62.2±2.8^{b}	61.3±2.3^{b}	62.4±0.3^{b}
50	**45**	**40**	64.3±0.6 ^{b}	64.5±4.8^{b}	57.9±1.4^{c}	60.6±1.2^{bc}
70	**30**	**20**	66.4±0.8 ^{a}	73.5±5.3^{a}	53.0±4.8^{c}	61.9±1.9^{bc}
70	**30**	**40**	69.1±1.0 ^{a}	69.9±1.5^{ab}	60.7±2.5^{b}	61.7±0.9^{b}
70	**45**	**20**	**71.1±3.0 ^{a}**	**75.7±6.8^{a}**	56.2±4.2^{bc}	62.6±0.5^{b}
70	**45**	**40**	66.9±1.6 ^{a}	68.1±4.3^{ab}	61.1±3.3^{b}	59.7±1.5^{bc}
100	**30**	**20**	58.8±2.1 ^{c}	64.2±3.9^{b}	55.9±2.1^{c}	58.4±2.3c
100	**30**	**40**	58.8±2.1 ^{c}	59.8±3.8^{bc}	62.6±1.0^{b}	59.8±1.3bc
100	**45**	**20**	60.6±6.1 ^{c}	61.5±6.4^{bc}	**67.2±2.9^{a}**	59.5±1.7b^{c}
100	**45**	**40**	60.6±3.7 ^{c}	54.5±5.9^{c}	65.1±2.8^{ab}	63.8±0.6^{b}

Data presented as mean±SD. Data with different superscripts in each column are significantly different. Comparisons were done for different solvent concentrations and extraction conditions (temperature and time). Con; concentration as a % (v/v), temp; temperature

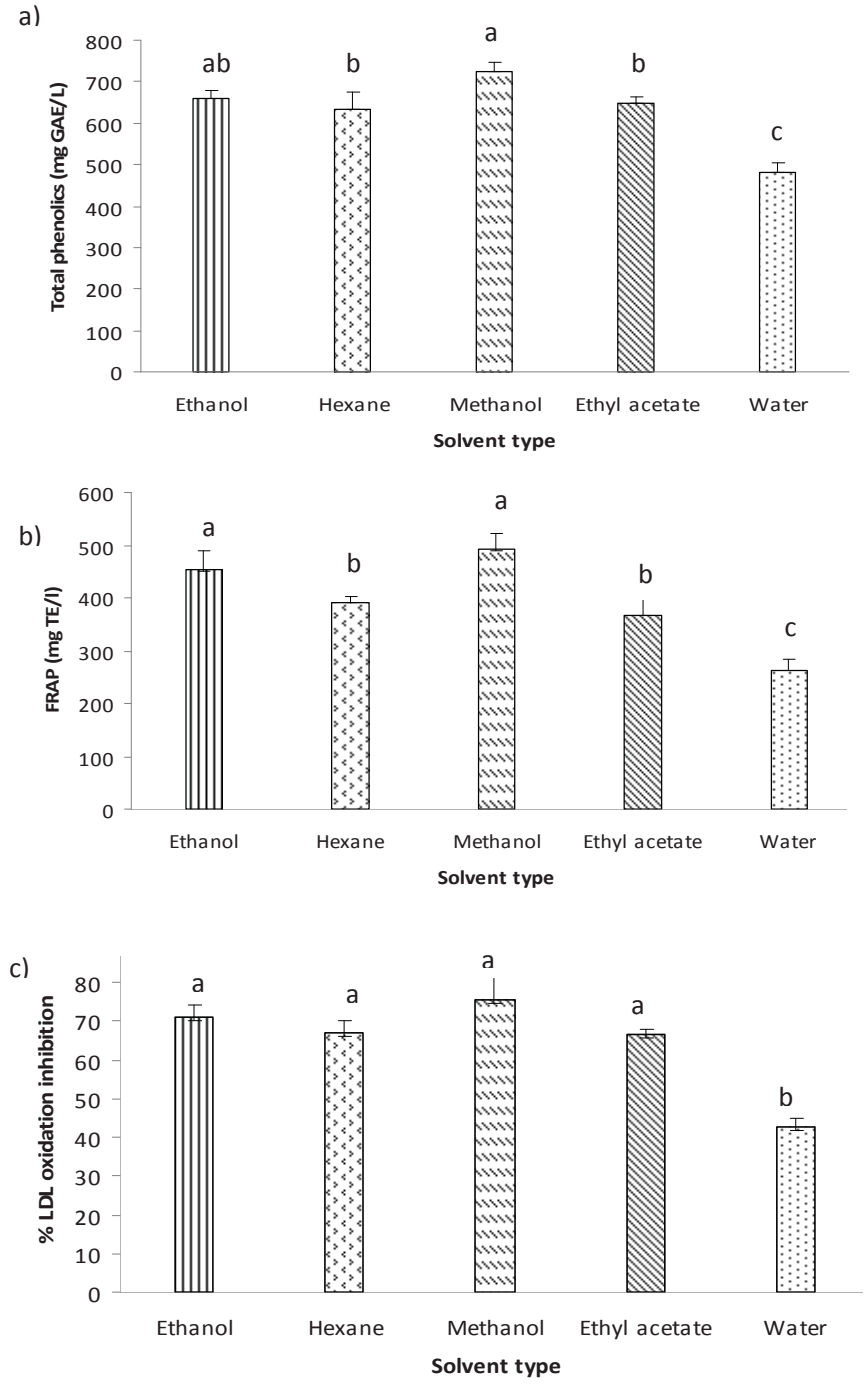


Figure 5.1. Total phenolic content (mg GAE/L), antioxidant capacity (mg TE/L) and Percent LDL oxidation inhibition by different solvent extracts of ginger extracted at optimum extraction conditions for each solvent. Bars with different letters in a graph are significantly different ($p<0.05$)

Percent LDL oxidation inhibition by different solvent extracts prepared at their optimum extraction conditions at different dilution levels are shown in Table 5.5. Results clearly indicate that as the dilution factor increased, antioxidant activity of the extract decreased. Ability of main ginger bioactive constituents to inhibit LDL oxidation in terms of IC_{50} are shown in Figure 5.2. Results clearly indicate that the phenolic bioactives; 6-gingerols, 8- gingerols, 10-gingerols and 6-shogaol, are active inhibitors of Cu^{2+}-induced LDL oxidation compared with α-farnesene.

Table 5.5. Percent inhibition of LDL oxidation by water and solvent extracts (extracted at optimum extraction for each solvent) at different concentration levels

Dilution factor	Ethanol	Hexane	Methanol	Ethyl acetate	Water
1	72.9±1.7	63.3±1.3	74.5±1.6	63.4±1.9	45.8±1.1
10	69.2±2.4	60.4±3.0	64.0±2.6	44.3±2.1	39.3±2.6
100	46.5±10.2	47.4±2.3	44.5±4.9	46.2±5.3	31.5±1.1
1000	31.0±6.1	28.4±5.4	30.3±4.3	31.7±1.1	20.0±1.4

Data presented as mean±SD

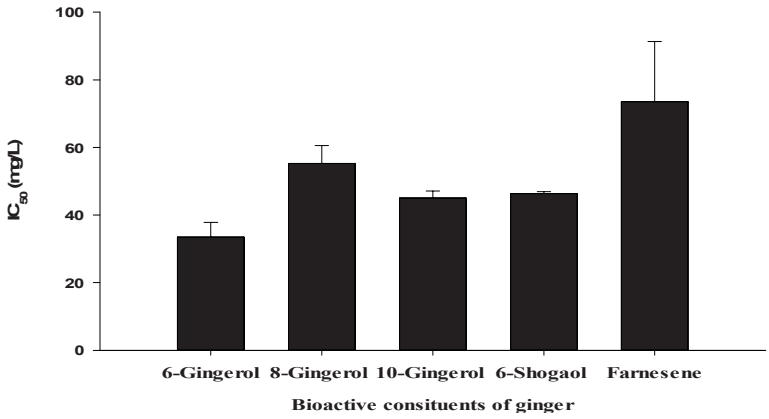


Figure 5.2. IC_{50} of ginger bioactive constituents for LDL oxidation inhibition

5.5 Discussion

Different solvent systems have been used for extraction of polyphenols from plant materials and extraction yield is dependent on the solvent and method of extraction (Goli, et al. 2004). Organic solvent extracts showed a significantly ($p<0.05$) higher amount of phenolic constituents, compared to water extract under the optimum ultrasonic-assisted extraction conditions, for each solvent (Figure 5.2). The total phenolic yield of organic solvent extracts kept increasing with water content, up to a certain level, and then decreased. A similar trend was reported in grapefruit (Spigno et al. 2006). Addition of water to the ethanol and other organic solvents improved the extraction rate, but too high water content brought an increased concomitant extraction of other compounds and lower phenols concentrations in the extracts (Spigno et al. 2006). Several studies have emphasized that the use of methanol was the best solvent, yet ethanol and water are the only accepted extraction solvents in food systems because of the need for method to be hygienic, non-toxic, environmentally friendly, low cost and abundant, as well as being compatible with health (Moure et al. 2001). In certain processes, water can replace organic solvents for food and pharmaceutical manufacturing, reducing the costs and eliminating the environmental problems associated with production and disposal of organic solvents (Moure et al. 2001).

The bioactive constituents in ginger are numerous and vary depending on the origin (Ali et al. 2008). The pungency of fresh ginger is due primarily to the gingerols which are a homologous series of phenolics. These ginger phenolics contain multiple hydroxyl groups and they are hydrogen-donating antioxidants and singlet oxygen quenchers (Middleton and Kandawasmi, 1993). These ginger phenolics have been shown

to protect against lipid peroxidation in various experimental models such as hemoglobin-catalyzed peroxidation of linoleic acid (Cao et al. 1993) and peroxynitrite-mediated damage in lipo-polysaccharide activated macrophages (Ippoushi et al. 2003). In the cardiovascular system, both 6-shogaol and 6-gingerol produced depressor response, at lower doses, on the blood pressure in a laboratory animal model (Suekawa et al. 1984). Results revealed that methanolic and hexane extracts of ginger have shown significantly higher levels of 6, 8, 10-gingerols and 6-shogaol compared to other extracts. However, water extracts of ginger have also shown comparable levels of the main phenolic constituents analysed.

FRAP is widely used in the evaluation of antioxidant capacity of dietary polyphenols, has been used with ginger extracts (Ghasemzadeh et al. 2010) and can be used to measure the total reducing capability of antioxidants based on the reaction principle described in the Materials and Methods section of Chapter 4.0. According to Thaipong et al. (2006), the FRAP assay is simple, rapid and highly reproducible method that shows the highest correlation with total phenolics compared with other antioxidant activity determination techniques such as 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorption capacity (ORAC). Maizura et al. (2011) reported a strong positive relationship between total phenolic content and antioxidant capacity in ginger. Several studies (Shan et al. 2005; Wong et al. 2006) indicated that phenolic compounds in spices and herbs, including ginger, contribute to their antioxidant properties. Stoilova et al. (2007) reported that the ethanolic extract of ginger exhibited an inhibiting effect with regards to hydroxyl radicals that was even better than that of that of quercetin.

It has been suggested that among all *in vitro* antioxidant capacity assays, measuring LDL antioxidant activity is more physiopathologically important and more informative for screening antioxidant activity of foods for preventing atherosclerosis (Katsube et al. 2004). A common method for measuring the inhibition of LDL oxidation *in vitro* is by determining the level of TBARS in response to lipid peroxidation in LDL mediated by copper ions. Freshly prepared commercial LDL has been widely used to analyze the antioxidant activity of pure compounds or crude extracts of natural products (Esterbauer et al. 1992). The phenolic content of water-based ginger extracts was significantly ($p<0.05$) lower than that of organic extracts; it showed significantly lower antioxidant activity towards the inhibition of LDL oxidation. However, in the present study, the LDL oxidation inhibition in water extract is about 44%. It is less than organic extracts, but it is more relevant because it is safe for ingestion.

As reported by Halliwell, (1995), antioxidant substances were not effective at lower or higher concentration levels, compared with their optimum concentration range. At low levels, antioxidant compounds were less active and provided unsatisfactory protection, whereas at high levels, sometimes they acted as pro-oxidants. This is because gingerols and shogaol are phenolic compounds having antioxidant properties and farnesene is an isoprenoid showing comparatively low antioxidant properties towards Cu^{+2}-induced LDL oxidation. The pure phenolic compounds tested as references and the different ginger extracts inhibited Cu^{+2}-induced LDL oxidation *in vitro*. Owing to the activities displayed by the reference molecules, it could be considered that phenolic content and the antioxidant capacity of ultrasonic-assisted water extracted ginger were lower than those of organic solvent extracts tested in all experimental conditions. Both

gingerols and shogaols show similar IC_{50} values and this could reflect their similar chemical structures.

This study showed that higher total phenolic content of ginger extracts resulted in higher antioxidant activities, as similarly described by Cai et al. (2004) and Wong et al. (2006). Ginger extract exhibited an inhibiting effect with regard to the hydroxyl radicals, better than that of quercetin (Stoilova et al. 2007). In addition, the present results demonstrate that antioxidant capacity of ginger extracts, measured by FRAP assay, had a correlation to LDL oxidation inhibition assay. This study revealed that ginger has potent antioxidant activity against LDL oxidation *in vitro*. In an *in vivo* study, Bhandari et al. (1998) found that ethanolic ginger extracts reduced the serum and tissue cholesterol and triglycerides levels in cholesterol-fed rabbits. High levels of serum and tissue cholesterol are other atherogenic factors and this anti-atherogenic effect of ginger is associated with the reduction of the basal oxidative state, as well as their susceptibility to oxidation and aggregation (Fuhrman et al. 2000 and Chrubasika et al. 2005).

5.6 Conclusion

These *in vitro* results showed that ginger extracts have antioxidant properties, including the ability to inhibit LDL oxidation, and suggesting that the water extract of ginger has the potential for use in value-added food products, especially those used in functional foods designed for cardiovascular health.

CHAPTER 6.0 PARTIAL CONCENTRATION OF BIOACTIVES OF FRUIT JUICES BY MEMBRANE FILTRATION AND EVALUATION OF THEIR PHYSICO-CHEMICAL AND ANTIOXIDANT PROPERTIES

6.1 Abstract

Reverse osmosis (RO), as a technique for partial concentration of juice solutes including phenolics present in cranberry, blueberry and apple juices was evaluated, for the formulation of fruit based-antioxidant rich functional beverage. The effects of temperature (20 °C and 40 °C) and trans-membrane pressure (25, 30 and 35 bars) on the flux of fruit juices were evaluated to optimize the operating parameters for each fruit juice. Samples of fresh and concentrated fruit juices were analysed for physico-chemical (colour index, pH, total soluble solids and titratable acidity), total phenolics and antioxidant properties (FRAP and % LDL oxidation inhibition). The results showed that there was no significant difference between single-strength juice in any of the quality parameters after juice concentration under specific operating parameters of temperature and pressure. However, the colour index, pH, total soluble solids and titratable acidity of the partially concentrated fruit juices increased in proportion to their volumetric concentrations. Results indicated that the antioxidant capacity of concentrated apple, blueberry and cranberry juice increased by 40%, 34%, and 30%, respectively, and the percentage LDL oxidation inhibition by concentrated blueberry and cranberry juice were around 41% and 45%, respectively, whereas the three fresh juices showed only about 35% inhibition. However, LDL oxidation inhibition by concentrated apple juice was not different ($p<0.05$) compared with that of single strength apple juice.

6.2 Introduction

Consumption of plant-based products, such as fruits and vegetables, are associated with a healthier life style with lower risk of chronic diseases. Apple (*Malus domestica* L.), cranberry (*Vaccinium macrocarpon* L.) and blueberry (*Vaccinium angustifolium* Aiton.) are some of the bioactive-rich fruits grown in Canada (Morton, et al. 2008). Many investigations have shown health benefits associated with the consumption of these fruits (Vanduyn and Pivonka, 2000; Duthie et al. 2006). In addition to characterizing and improving the nutritional and pharmacological interest in phenolic bioactives, research to improve process technologies for enhancing the bioactive profile in food has also been growing (Gorelik et al. 2008; Leontowicz et al. 2007).

RO is a membrane separation process in which a hydraulic pressure that is higher than the osmotic pressure of the solution is applied in such a way that permeation of water from high to low solute concentration occurs (Girard and Fukumoto, 2000). This process can be applied to concentrate bioactives of fruit juices, reducing the damage caused by thermal evaporation of water and resulting in the maintenance of their nutritional and sensory characteristics (Girard and Fukumoto, 2000). The use of RO in the concentration of many fruits is very promising and this technique partially promotes dehydration, resulting in an increase of total soluble solids (TSS), including bioactives (Gurak et al. 2010). This process has been used for various fruit juices such as orange (Jesus et al. 2007) and grape (Gurak, et al. 2010). The aim of this work was to evaluate RO as a process for the partial concentration of bioactives present in cranberry, blueberry and apple juices and to determine the effects of the processing parameters on physico-chemical and antioxidant properties of the concentrated fruit juices.

6.3 Materials and Methods

6.3.1 Raw Materials and Chemicals

Apple, blueberry, and cranberry juice were purchased from three commercial juice companies: apple juice were obtained from J. W. Mason & Sons Ltd, Windsor, NS, Canada; blueberry juice from Van Dyks Ltd, Caledonia, NS, Canada; cranberry juice from Cranberry Acres Ltd, Berwick, NS, Canada, respectively. All the chemicals used were of analytical grade and purchased from Sigma-Aldrich, Oakville, ON, Canada.

6.3.2 Membrane Processing of Fruit Juices

The membrane technology (GEA filtration model L pilot plant) was used to concentrate different bioactive constituents in order to preserve the natural antioxidants and to maintain a high total antioxidant activity of the juice. The system was equipped with a Dow filmtec BW30-2540 RO membrane of surface area of 2.6 m^2 (Dow Chemical Company, Minnesota, USA). The effects of temperature and trans-membrane pressure on flux of the fruit juices were evaluated to optimize the operating parameters for each fruit juice. Permeate flux and volumetric concentration factor (VCF) were calculated using following equations, where V is the volume permeated during determined time (t), A is the membrane surface area, X is the total suspended solids maintained on the feed side of the membrane and Y is the concentration of suspended solids in the influent water to the membrane system

$$\text{Permeate flux} = \frac{V}{A * t}$$

$$VCF = \frac{X}{Y}$$

6.3.3 Experimental Design

For the evaluation of the effects of temperature and trans-membrane pressure on the physico-chemical, nutritional and antioxidant properties of the partially concentrated fruit juices, complete factorial design was carried out with fruit juice temperatures (20 and 40 °C) and trans-membrane pressures (25, 30 and 35 bars) as independent variables, and permeate flux and parameters related to juice quality (physico-chemical and antioxidant), as dependent variables (Table 6.1). All assays were conducted in triplicate and the average values of these assays were used to analyze the data. The range of each processing parameter was chosen based on the scientific literature on concentration of fruit juice using membrane filtration (Gurak et al. 2010; Jesus et al. 2007).

Table 6.1. Process conditions for the reverse osmosis trials based on factorial design

Test	Temperature (°C)	Trans-membrane pressure (bar)
1	20	25
2	20	30
3	20	35
4	40	25
5	40	30
6	40	35

6.3.4 Analysis of Physico-Chemical and Antioxidant Properties of Fruits Juices

6.3.4.1 Color Index

Color of the fresh and membrane filtered fruit juices were determined by using a reflectance colorimeter (Model CR-300, Minolta Camera Co Ltd, Osaka, Japan), based on a*, b* and L* values. L* ranges, from 0 (completely opaque) to 100 (completely transparent). Positive and negative a* values indicate reddish and greenish respectively, whereas positive and negative b* values indicate yellowish and bluish, respectively (Lopez-Nicolas et al. 2007).

6.3.4.2 pH, Total Soluble Solids and Titratable Acidity

Samples of single-strength juice and concentrated juice were analyzed in triplicate for pH, using a standardized pH meter (Model Accumet® 10, Denver Instruments Co., Arvada, CO, USA) and total soluble solids (TSS) were tested using a hand-held digital refractometer (Model 300016, Super Scientific Ltd, Scottsdale, AZ, USA). The titratable acidity was measured using a semi-automatic titrator (DMP 785, Metrohm Ltd., Herisau, Switzerland) at pH 8.2, with 0.1N NaOH as titrant and was expressed as malic acid equivalents for apple juice, citric acid equivalent for blueberry juice, and quinic acid equivalent for cranberry juice. The predominant organic acids in apple (Gokmen et al. 2001), blueberry (Kalt and McDonald, 1996) and cranberry (Coppola et al. 1978) are malic acid, citric acid and quinic acid, respectively.

6.3.4.3 Determination of Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteu assay (Singleton et al. 1999), with some modifications, as described by Rupasinghe et al. (2008) as previously described in Chapter 4.0.

6.3.4.4 Ferric Reducing Antioxidant Power Assay (FRAP)

Antioxidant capacity of fruit juices was measured using FRAP assay, according to Benzie and Strain (1999) with some modifications, as described by Rupasinghe, et al. (2008) as previously described in Chapter 4.0.

6.3.4.5 LDL Oxidation Inhibition

Percentage LDL oxidation inhibition was determined spectrophotometrically using the thiobarbituric acid reactive substances (TBARS) assay (Xu et al. 2007) with slight modifications, as reported in Chapter 4.0.

6.3.5 Determination of Major Phenolic Compounds

6.3.5.1. Sample Preparation

Both fresh and membrane filtered fruit juice samples were rendered free of sugars and organic acids by using solid phase extraction and passing them through a C18 Bond Elute column® (Agilent Technologies, Mississauga, ON, Canada). The column was conditioned with 3 mL of 100% methanol and was washed with 3 mL deionized water. Ten mL of sample was loaded, followed by a wash of 6 mL deionized water. Phenolic compounds were eluted using 3 mL of 100% methanol. The elutes were filtered through 0.45 µm nylon filters before the HPLC analysis.

6.3.5.2 Analysis of Major Phenolics Profile

Polyphenolics of fruit juice samples after solid phase extraction were analysed using an ultra high performance liquid chromatography (UHPLC) (Model H-class system, Waters, Milford, MA, USA) equipped with an acuity UHPLC BEH C18 column (2.1 x 100 mm, 1.7 µm) (Waters, Milford, MA, USA). For the analysis of non-anthocyanin phenolics, gradient elution was carried out with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), with the flow rate of 0.2 mL/min. A linear gradient profile was used with the following proportions of solvent A applied at time t (min); (t, A%): (0, 94%), (2, 83.5%), (2.61, 83%), (2.17, 82.5%), (3.63, 82.5%), (4.08, 81.5%), (4.76, 80%), (6.75, 20%), (8.75, 94%), (12, 94%). The analysis of anthocyanins was performed as described below; the mobile phases were 5% (v/v) formic acid in water (solvent A) and 5% (v/v) formic acid in methanol (solvent B). The linear gradients used were as follows; (t, A %): (0, 10%), (8, 30%), (17, 40%), (19, 40%),

(20, 10%), (22, 10%). The flow rate was 0.2 mL/ min, with an injection volume of 2.0 µL.

6.3.5.3 MS/MS Analysis

MS-MS analysis was performed with a Micromass Quattro micro API MS/MS system, which is controlled by Masslynx V4.1 data analysis system (Micromass, Cary, NC, USA) as described by Rupasinghe et al. (2008). Electrospray ionization in negative ion mode (ESI-) was used for the ionization of the flavonol and flavan-3-ol compounds. The mass spectrometry conditions included capillary voltage of 3000 V with nebulizing gas (N_2) at a temperature of 375 °C. Electrospray ionization in positive mode (ESI+) was used for the analysis of anthocyanins. The mass spectroscopy conditions used were capillary voltage of +3500 V with nebulizer gas at 375 °C and a flow rate of 0.35 mL/min. The cone voltage (25-50 V) was optimized for each individual compound. Individual samples were identified using a multiple reactions monitoring mode and specific precursor-product ion with quantified calibration curves generated by external standards.

6.3.6 Statistical Analysis

The experiment was a complete randomized design with three replicates and expressed as mean±standard deviation. Membrane filtration experiments were conducted independently in triplicates. The assumptions of normal distribution and constant variance were verified by examining the residuals (Montgomery, 2005). Analysis of variance (ANOVA) was used to test the significance of each variable ($p<0.05$) and the multiple mean comparison was performed in General Linear Model (SAS V8, Cary, NC,

USA) using Tukey's test method where appropriate. The differences at the 5% level ($p < 0.05$) were considered statistically significant.

6.4. Results

6.4.1 Effect of Trans-membrane Pressure and Product Temperature on Physico-Chemical and Antioxidant Properties of Partially Concentrated Fruit Juices

Physico-chemical, total phenolics and antioxidant capacities of partially concentrated apple, blueberry and cranberry juices were given in Tables 6.2, 6.3 and 6.4, respectively. The results showed that there were no significant difference ($p<0.05$) in the physico-chemical, total phenolics and antioxidant capacities of any fruit juices that were partially concentrated under any of the operating parameters tested. The Brix of all concentrated fruit juices were 15°. The lightness (L*), red green value (a*) and blue yellow value (b*) of fruit juices were also reported as the measurement of color. Total acidity of the concentrated apple, blueberry and cranberry juices were in the range of 1.1-1.3% as malic acid equivalent, 1.3-1.5% as citric acid equivalent and 9.9-11.6% as quinic acid equivalent, respectively. The total phenolic content and antioxidant capacity of the concentrated apple juices were in the range of 703-719 mg GAE/L and 779-807 mg TE/L, respectively. The total phenolic and antioxidant capacity of concentrated blueberry juice were 2508-2656 mg GAE/L and 5946-6279 mg TE/L. Total phenolic content of concentrated cranberry juice was about 2647-2918 mg GAE/L and had an antioxidant capacity of 6279-6454 mg TE/L. The physico-chemical and antioxidant properties of fresh and concentrated juices are shown in Table 6.5. The results indicated that the TSS levels in all fruit juices increased by 50%. Total phenolics and antioxidant capacity of apple, blueberry and cranberry juices increased significantly in the partial concentration process.

Table 6.2. Physico-chemical, total phenolics and antioxidant capacity of partially concentrated apple juice at different operating conditions of reverse osmosis

Quality parameters		Temperature (°C)					
		20			40		
		Pressure (bars)					
		25	30	35	25	30	35
Color	a*	2.30±0.40	3.60±0.10	1.50±0.70	2.30±0.40	2.40±0.80	2.60±0.50
	b*	42.3±0.20	42.4±0.10	43.0±0.50	43.1±0.10	43.2±0.20	43.2±0.20
	L*	50.3±1.0	47.8±0.32	50.5±2.6	52.8±1.8	51.5±2.2	51.1±1.5
Soluble solids		15.1±0.10	15.3±0.30	15.2±0.10	14.9±0.20	15.2±0.40	14.9±0.50
pH		3.60±0.010	3.60±0.020	3.60±0.010	3.60±0.010	3.60±0.010	3.60±0.010
Total acidity[x]		1.10±0.040	1.30±0.10	1.20±0.040	1.20±0.10	1.30±0.10	1.10±0.10
Total phenolics[y]		704±17	713±21	714±37	728±27	703±49	719±17
FRAP[z]		779±20.	799.9±13	783.6±11	807.9±12	789.8±17	800.6±26

Values are presented as mean ± SD, n=3

[x] Total acidity- percentage of total acidity (mg malic acid equivalent /100mL)

[y] mg GAE/L mg gallic acid equivalent per litre

[z] mg TE/L mg Trolox equivalent per litre

Table 6.3. Physico-chemicals, total phenolics and antioxidant capacity of partially concentrated blueberry juice at different operating conditions of reverse osmosis

Quality parameters		Temperature (°C)					
		20			40		
		Pressure (bars)					
		25	30	35	25	30	35
Color	a*	5.800±1.6	8.500±0.80	7.900±0.80	7.500±1.8	11.50±3.8	7.100±0.30
	b*	2.700±0.10	2.900±0.10	2.900±0.10	2.800±0.20	3.400±0.50	2.800±0.10
	L*	14.10±0.10	14.30±0.10	14.30±0.10	14.20±0.20	14.70±0.40	14.20±0.20
Soluble solids		14.90 ±0.60	15.30±0.40	14.90±0.10	14.70±0.010	15.00±0.010	14.90±0.30
ph		3.460±0.010	3.470±0.010	3.460±0.010	3.470±0.010	3.460±0.010	3.460±0.010
Total acidity[x]		1.400±0.10	1.500±0.10	1.500±0.10	1.300±0.20	1.400±0.010	1.300±0.10
Total phenolics[y]		2627±25	2588±66	2591±79	2508±73	2630±2.9	2657±35
FRAP[z]		6036±23	6174±99	5946±94	6034±76	6253±215	6279.3±148

Values are presented as mean ± SD, n=3

[x]Total acidity- percentage of total acidity (mg citric acid equivalent /100mL)

[y]mg GAE/L mg Gallic acid equivalent per litre

[z]mg TE/L mg Trolox equivalent per litre

RT- room temperature

Table 6.4. Physico-chemical and total phenolics and antioxidant capacity of partially concentrated cranberry juice at different operating conditions of reverse osmosis

Quality parameters		Temperature (°C)					
		20	20	20	40	40	40
		Pressure (bars)					
		25	30	35	25	30	35
Color	a*	20.90±1.40	19.40±0.900	18.10±0.100	21.00±2.60	20.40±1.10	20.40±1.20
	b*	8.400±0.100	8.100±0.300	7.600±0.100	8.600±0.800	8.300±0.400	8.300±0.400
	L*	17.50±0.300	17.30±0.200	16.90±0.100	17.60±0.500	17.50±0.300	17.50±0.200
Soluble solids		14.80 ±0.200	15.00±0.100	15.40±0.100	15.10±0.200	15.50±0.100	15.20±0.200
pH		2.580±0.0100	2.570±0.0100	2.570±0.100	2.570±0.0100	2.570±0.0100	2.570±0.0100
Total acidity[x]		10.50±1.50	11.60±0.300	12.00±0.100	9.900±1.90	11.60±0.200	11.30±0.100
Total phenolics[y]		2919±57.5	2647±235	2889±149	2752±98.6	2734±117	2792±73.5
FRAP[z]		6359±137	6316±161	6313±287	6352±137	6454±79.2	6279±165

[x]Values are presented as mean ± SD, n=3

[y]Total acidity- percentage of total acidity (mg quinic acid equivalent /100mL)

[z]mg GAE/L mg gallic acid equivalent per litre

[d] mg TE/L mg Trolox equivalent per litre

Table 6.5. Physico-chemical, total phenolics and antioxidant capacity of fresh and concentrated fruit juice

Quality parameters		Apple		Blueberry		Cranberry	
		Fresh	Conc.	Fresh	Conc.	Fresh	Conc.
TSS		9.900±0.100	14.90±0.500	10.90±0.100	14.90±0.300	10.10±0.100	15.20±0.200
pH		3.600±0.010	3.600±0.01	3.500±0.010	3.46±0.010	2.6±0.020	2.57±0.0100
Total acidity[x]		1.100±0.10	1.100±0.1	0.9000±0.10	1.300±0.10	7.200±0.30	11.30±0.10
Color	a*	1.300±0.40	2.600±0.50	9.000±0.20	7.100±0.30	20.90±0.60	20.40±1.2
	b*	38.80±0.10	43.20±0.20	3.000±0.30	2.800±0.10	9.200±0.20	8.300±0.40
	L*	50.80±1.3	51.10±1.5	14.30±0.30	14.20±0.20	17.80±0.20	17.50±0.20
phenolics[y]		525.9±14	719.1±12	1927±11	2657±35	2029±119	2792±74
FRAP[z]		578.5±8.9	800.6±26	4704±51	6279±148	4830.±72	6279±165

Values are presented as mean ± SD, n=3

Operating conditions used for concentration of fruit juices were 40 °C and 35 bars trans-membrane pressure

[x]Total acidity (mg/100 mL)- (for apple, blueberry and cranberry as malic acid, citric acid and quinic acid equivalents)

[y]mg GAE/L mg gallic acid equivalent per litre

[z]mg TE/L mg Trolox equivalent per litre

*conc.; concentrated

6.4.2 Composition of Phenolic Constituents in Fruit Juices

Composition of selected major flavan-3-ols, flavonols, phenolic acids and anthocyanin content of fresh and concentrated apple, blueberry and cranberry juices are shown in Table 6.6. Operating parameters used for the reverse osmosis of fruit juices were 40 °C and 35 bars trans-membrane pressure. Results showed that selected anthocyanins (Table 6.6) were not detected in both fresh and concentrated apple juice. However, chlorogenic acid, catechin and epicatechin content of apple juice significantly ($p<0.05$) increased by 48%, 30% and 74%, respectively when compared with fresh juice. Among the phenolics analyzed, blueberry juice contained a higher amount of flavan-3-ols, anthocyanins and chlorogenic acids. Partially concentrated blueberry juice had significantly ($p<0.05$) higher amounts of catechin, epicatechin, chlorogenic acids, malvidin-3-glucoside, delphinidin-3-galactoside and malvidin-3-galactoside compared with fresh blueberry juice. Quercetin galactoside, quercetin glucoside, epigallocatechin, epicatechin and chlorogenic content of cranberry juice was significantly ($p<0.05$) after concentrating by RO treatment. Similarly, some of the anthocyanins analysed, such as peonidin-3-glucoside, malvidin-3-glucoside, cyanidin-3-galactoside and malvidin-3-galactoside, were also higher in concentrated cranberry juice than in fresh juice.

Table 6.6. Composition of phenolic compounds (mg/100 mL) in fruit juices before and after reverse osmosis

Phenolic Compounds	Apple		Blueberry		Cranberry	
	Fresh	Concentrated	Fresh	Concentrated	Fresh	Concentrated
Flavan-3-ols						
Quercetin-3-*O*-Galactoside	0.100±0.10	0.200±0.100	375±20.	381±50.	349±32.^{b}	421±16.^{a}
Quercetin3-*O*-Glucoside	0.100±0.10	0.100±0.10	42.0±2.6	45.0±2.2	2.90±0.50^{b}	4.10±0.40^{a}
Quercetin-3-*O*-Rhamnoside	0.400±0.10	1.10±0.40	41.0±10	43.0±20	66.0±80	78.0±70
Quercetin-3-*O*-Rutinoside	0.500±0.10	0.600±0.20	1470±124	1558±100.	1.900±1.30	2.500±2.00
Flavonol						
Epigallocatechin	0.100±0.010	0.100±0.010	2.10±0.30	1.50±0.30	0.800±0.20^{b}	1.10±0.00^{a}
Catechin	7.30±0.40^{b}	9.50±0.70^{a}	2.70±0.10^{a}	4.40±0.20^{b}	1.90±0.20	2.10±0.10
Epicatechin	6.30±1.4^{b}	11.0±0.10^{a}	1.80±0.30^{a}	3.00±0.10^{b}	12.1±1.6^{b}	15.0±0.50^{a}
Epigallocatechin gallate	9.70±1.2	13.3±0.50	0.400 ±0.00	0.600±0.20	0.200±0.00	0.400±0.50
Phenolic acids						
Chlorogenic acid	297±27^{b}	440±32^{a}	1113±83^{b}	1213±53^{a}	131±19^{b}	170±11^{a}
Caffeic acid	0.500±0.10	0.900±0.00	3.40±0.10	3.60±0.10	0.800±0.50	1.30±0.00
Anthocyanins						
Cyanidin-3-*O*-Glucoside	ND	ND	3.80±0.50	4.30±0.20	0.000	0.100±0.00
Petunidin-3-*O*-Glucoside	ND	ND	8.10±0.40	9.50±0.90	0.000	0.000
Delphinidin-3-*O*-Glucoside	ND	ND	4.40±0.90	5.30±0.30	0.000	0.000
Peonidin-3-*O*-Glucoside	ND	ND	2.00±0.10	2.20±0.20	0.900±0.10^{b}	1.20±0.10^{a}
Malvidin-3-*O*-Glucoside	ND	ND	9.10±0.90^{b}	11.4±0.90^{a}	0.000^{b}	0.500±0.40^{a}
Cyanidin-3-*O*-Galactoside	ND	ND	2.20±0.200	2.60±0.10	6.80±0.50^{b}	8.40±0.40^{a}
Petunidin-3-*O*-Galactoside	ND	ND	3.80±0.40	4.50±0.50	0.100±0.00	0.100±0.00
Delphinidin-3-*O*-Galactoside	ND	ND	3.00±0.10^{b}	3.60±0.10^{a}	0.000	0.000
Peonidin-3-*O*-Galactoside	ND	ND	1.20±0.10	1.30±0.00	8.20±0.40	10.4±0.20
Malvidin-3-*O*-Galactoside	ND	ND	6.60±0.60^{b}	8.10±0.70^{a}	0.600±0.00^{b}	0.800±0.00^{a}

Values are presented as mean ± SD of three replicates,

Data with different superscripts within row for each fruit type are significantly different ($p<0.05$), ND-not detected

6.4.3 Antioxidant Activity of Fresh and Concentrated Fruit Juices

The total phenolic content, antioxidant capacity and % inhibition of LDL oxidation of apple, blueberry and cranberry juices, before and after membrane processing, are shown in Figure 6.1. The total phenolic content of all fruit juices were increased significantly ($p<0.05$) due to the RO treatment (Figure 6.1a). Similarly, antioxidant capacity also increased in all fruit juices with the RO (Figure 6.1b). The percentage LDL oxidation inhibition was significantly higher ($p<0.05$) in concentrated blueberry and cranberry juice, compared with the respective fresh juices (Figure 6.1c). However, percentage LDL oxidation inhibition by apple juice did not increase significantly due to the RO process.

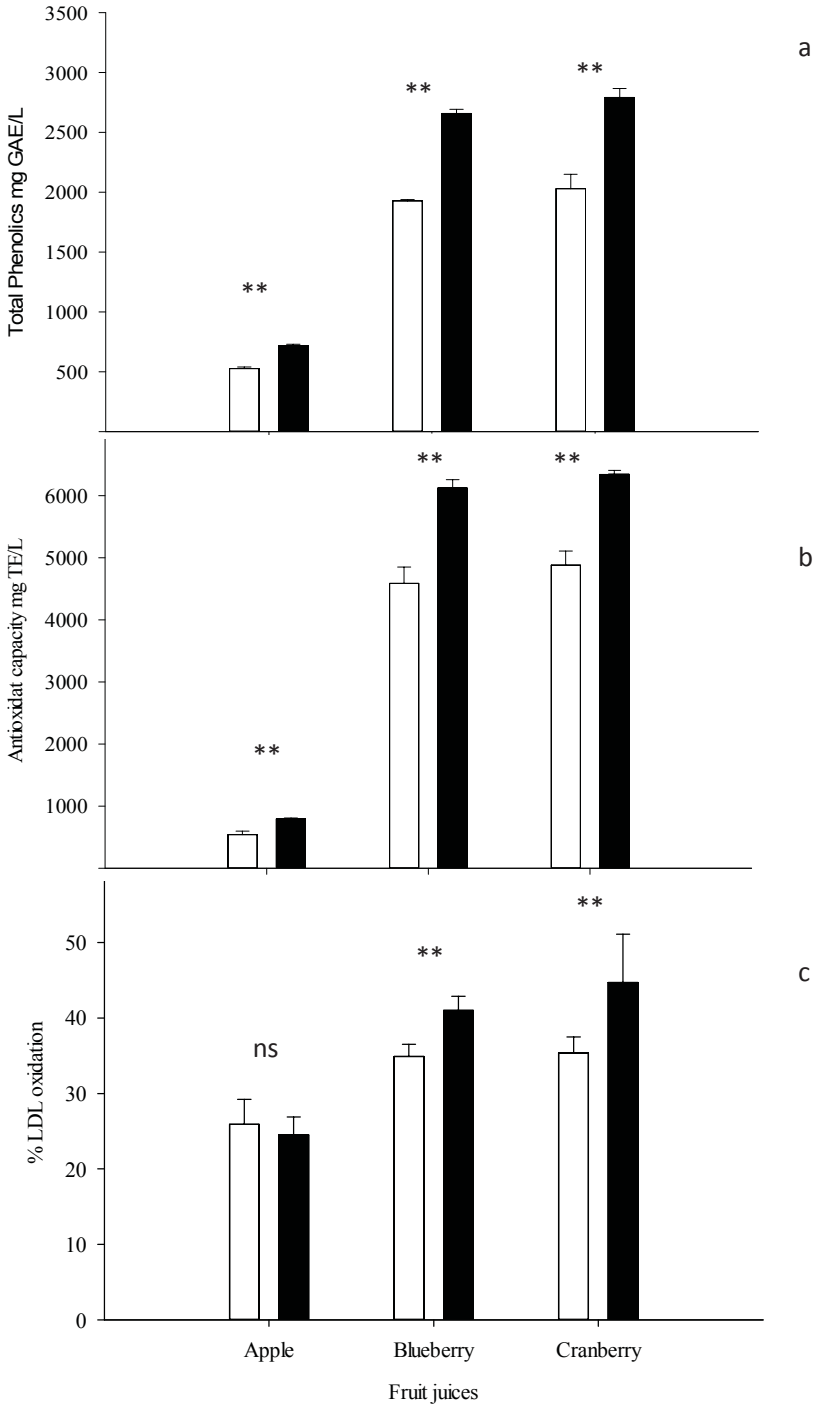


Figure 6.1. Total phenolic content (a), antioxidant capacity (b), %LDL oxidation inhibition (c), of fruit juices before (white) and after (black) membrane filtration. Fruit juice with ** are significantly different ($p<0.05$) between fresh and concentrated juice for each fruit type; ns-not significantly different

6.5 Discussion

The results indicated that the effects of trans-membrane pressure and juice temperature, under studied levels, have no effects on physico-chemical and antioxidant properties of all fruit juices studied (Table 6.2, 6.3 and 6.4). Similar results were observed in RO studies on grape (Gurak et al. 2010) and orange juice (Jesus et al. 2007). Processing parameters of 40 °C and 35 bars were selected based on the high permeate flux achieved (Appendix E). The concentrations of all fruit juices were carried out, up to Brix 15°, corresponding to a VCF of 1.5. This Brix value was selected as Saravanan and Aradhya (2011) have reported that this was the ideal Brix value for the formulation of nutraceutical food beverages. In terms of pH and total acidity, cranberry juice exhibited the lowest pH and the highest titratable acidity, compared with apple and blueberry juices. The major organic acids in the cranberry juice, quinic, malic and citric acids are mainly responsible for its high acidity (Coppola et al. 1978). The total acidity content of apple and blueberry juice is quite similar and according to Gokmen et al. (2001), malic acid is the main organic acid found in apple juice, whereas citric acid and malic acid are the predominant organic acid and chlorogenic is the major cinnamic acid ester in wild blueberry juice (Kalt and McDonald, 1996). Quality evaluation of concentrated fruit juices showed that there were increases in the titratable acidity, phenolic and antioxidant capacity of all three fruit juices that were proportional to the volumetric concentration factor.

The total polyphenol content of concentrated apple juice ranged from 70.3-72.3 mg GAE /100 mL. Flavonoids are the major class of apple polyphenols (71-90%) (Vrhovsek et al. 2004). The major polyphenol of apple juice is chlorogenic acid, together

with flavonols (quercetin and its glycosides) and flavanols (catechin and epicatechin) (Gliszczynska-Swiglo and Tyrakowska, 2003). As flavonols, quercetin glycosides, such as quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, are common in apple juice (Marks et al. 2007). The results of present study indicated that due to the partial concentration of apple juice by reverse osmosis, the total chlorogenic acid, catechin and epicatechin content increased significantly. Total phenolic content of concentrated blueberry juice is about 251-266 mg GAE/100 mL on a FW basis. In general, blueberry has received considerable attention recently because of its well-recognized potential health benefits due to its high anthocyanin and flavonoid content (Kalt et al. 2008). Similarly, cranberries are also a rich source of phenolic acids including benzoic, hydroxycinnamic, and ellagic acids and flavonoids such as anthocyanins, flavonols, and flavan-3-ols (McKay and Blumberg, 2007). Based on the present findings, the amount of some flavanols (catechin and epicatechin), chlorogenic acid and some anthocyanins, has increased due to the partial concentration of blueberry juice and cranberry juice as a result of RO. In general, RO increased the total phenolic content in fruit juices, apple by 39%; blueberry by 38% and cranberry by 37% (Figure 6.1a).

Apple, blueberry and cranberry juices are reported to increase antioxidant activity in plasma (apple: Ko et al. 2005; blueberry: Kay and Holub, 2002; cranberry: Ruel et al. 2005), inhibit the LDL oxidation *in vitro* (apple: Pearson et al. 1999; blueberry: Ames et al. 1993; cranberry: Wilson et al. 1998) and improve the plasma lipid profile (apple in human: Weichselbaum et al. 2010; blueberry in pigs: Kalt et al. 2008; cranberry in rats: Reed, 2002; Ruel et al. 2006; Caron et al. 2005). The phenolic phytochemicals may contribute to these beneficial health effects because many of these compounds have

shown antioxidant activity that may help cells against oxidative damage caused by free radicals (Thilakarathna and Rupasinghe, 2012; Bazinet et al. 2012). Based on the current findings, it was observed that the partial concentration of these fruit juices increased the FRAP antioxidant capacity of apple by 39.8%; blueberry by 33.5% and cranberry by 30.0 % (Figure 6.1b). Percentage LDL oxidation inhibition of concentrated blueberry and cranberry juice were around 41 and 45%, respectively, whereas both fresh juices showed about 35% inhibition. However, there was no significant difference between fresh and concentrated apple juice in LDL oxidation inhibition due to high variability. The study revealed that partial concentration of apple, blueberry and cranberry improved the antioxidant status of the juice and this could be due to concentration effect of bioactives present in juices.

6.6 Conclusion

Reverse osmosis can be applied to enhance the bioactive concentration of fresh apple, blueberry and cranberry juices prior to using them for formulation of a functional beverage to increase their antioxidant properties. The results suggest that reverse osmosis of fruit juices, to raise them to a desirable Brix level, could provide more effective juice products and used in the formulation of noval fruit beverages.

CHAPTER 7.0 FORMULATION AND QUALITY EVALUATION OF FRUITS BEVERAGE FORTIFIED WITH CARDIO-PROTECTIVE INGREDIENTS

7.1 Abstract

Cranberry (*Vaccinium macrocarpon* L), blueberry (*Vaccinium angustifolium* Aiton) and apple (*Malus domestica* L.) together with water-extracts of ginger, and selected cardio-protective amino acids, vitamins, and minerals, were used for the formulation of a functional beverage targeted for potential reduction of the risk of cardiovascular diseases (CVD). A sensory analysis was conducted to identify the consumer acceptable combination of fruit juices and the amount of ginger extract to be incorporated in the formulation. Physico-chemical properties, antioxidant capacity and the ability to inhibit the oxidation of low density lipoprotein (LDL) *in vitro,* by the functional beverage, were assessed. It was found that up to 2% (v/v) ginger extract can be incorporated without compromising consumer acceptability. The fruit juice blend which scored the highest overall acceptability by the trained sensory panel was selected for the formulation. Sensory evaluation revealed that the fortification of L-arginine, taurine, Se, Zn, K and vitamin B6 at 10% RDI did not affect the sensory properties of the beverage. Phenolic content and *in vitro* antioxidant activities (FRAP and % inhibition of LDL oxidation) of the final functional beverage formulation were 1024±45 mg GAE/L, 3114±248 mg TE/L, 45.2±10%, respectively.

7.2 Introduction

CVD is the leading cause of death in most developed nations and recent research suggests that the changes in oxygen utilization and increased formation of reactive oxygen species (ROS) in the body contribute to atherosclerosis and CVD progression (Kaliora et al. 2006). Therefore, protection against oxidative stress depends partly on the adequacy of dietary antioxidants because the endogenous antioxidant defense system is not adequate to counteract the oxidative stress (Kaliora et al. 2006). Fruits and vegetables contain a significant amount of polyphenolic antioxidants at varying concentrations (Kaur and Harish, 2001). Epidemiological evidence suggests that the consumption of plant foods, such as fruits and vegetables, has routinely been associated with a lower risk of CVD (Stanner, 2005). Therefore, increased intake of fruits and vegetables rich in antioxidants, and their products, are a suggested strategy to prevent oxidative stress (Vattem et al. 2005).

Apple, blueberry and cranberry are some of the fruits produced in Atlantic Canada which are rich in antioxidant bioactives. These fruits are recognized for their health properties, especially for chronic diseases such as CVD, prompting the development of functional ingredients or products. Numerous *in vitro* and *in vivo* studies have been done to determine the cardio-protective properties of these fruits (apple: Boyer and Liu, 2004; Cranberry: Chu and Liu, 2004; Blueberry: Kalt et al. 2008; Shaughnessy et al. 2009). In addition, in recent years, the demand for ginger has grown in North America because of its medicinal properties as an anti-hypertensive (Ghayur et al. 2008) and anti-atherogenic agent (Bhandari et al. 1998 and Nicoll et al. 2007). Considering these facts, introduction of bioactive-rich 'functional beverages', based on these

bioactive-rich fruits, could be an alternative dietary strategy to reduce the risk of oxidative stress linked to chronic diseases such as CVD (Sirtori et al. 2009).

There are possibilities of formulating a 'heart-healthy' functional beverage with a balanced bioactive profile, using antioxidant rich fruits and ginger. Membrane processing can be applied to concentrate bioactive antioxidants in these fruit juices (Galaverna, et al. 2007). Blending different fruit juices with other functional ingredients like ginger can enhance the bioactive composition. The profile of phenolic phytochemicals determines the functionality of the whole food as a result of additive or synergistic interaction of phenolic phytochemicals (Liu, 2003). In addition, incorporation of cardio-protective ingredients such as minerals (Se, Zn, and K), pyridoxine (vitamin 6) and amino acids (arginine and taurine) into the formulation would enhance the ability of the functional properties of the formulated beverage (Houston, 2010). Therefore, the objectives of this present study were to develop an antioxidant-rich fruit-based functional beverage incorporating ginger and other cardio-protective nutrients, and to evaluate its antioxidant and sensory quality.

7.3 Materials and Method

7.3.1 Raw Materials and Chemicals

Apple juice, blueberry juice and cranberry juices were purchased from three commercial juice manufacturers (J. W. Mason & Sons Ltd, Windsor, NS, Canada; Van Dyks Ltd, Caledonia, NS, Canada; Cranberry Acres Ltd, Berwick, NS, Canada, respectively). All the chemicals used were of analytical grade and purchased from Sigma-Aldrich, Oakville, ON, Canada.

7.3.2 Preparation of Fruit Juice Blends

All the fruit juice were pre-concentrated using the food grade membrane filtration system (L model, GEA filtration system, Hudson, WI, USA) as described in Chapter 6.0. Fifteen liters of each juice were concentrated on a batch basis and the process was terminated at 15° Brix. The membrane was operated at 35 bars and 40 °C, based on optimum processing parameters obtained from results of Chapter 6.0. The equipment was cleaned in place after each membrane filtration process. Fruit juices were blended according to the Table 7.1 and pasteurized at 92±2 °C for 180 s and immediately bottled and cooled before storage at 4 °C.

Table 7.1. Blends of concentrated fruit juices (apple, cranberry and blueberry) mixed in different proportions (volume %)

Fruit juice	Blend 1	Blend 2	Blend 3	Blend 4	Blend 5	Blend 6
Apple (AJ)	33.33%	50%	25%	25%	50%	37.5%
Cranberry (CJ)	33.33%	25%	50%	25%	12.5%	12.5%
Blueberry (BJ)	33.33%	25%	25%	50%	37.5%	50%

7.3.3 Preparation of Ginger Extracts

Fresh rhizomes of ginger were purchased from a local market in Truro, Nova Scotia, Canada and the rhizomes were cut into thin slices of 2 mm. Water extracts of ginger were prepared from 250 g of sliced ginger in 1 L of deionized water using a commercial blender (Bead Beater, BioSpec Products Inc, Bartlesville, OK, USA) at a maximum speed for 2 min. Water extract of ginger was prepared using ultrasonication at 52 °C for 15 min and filtered through cheese cloth. These extraction conditions were selected based on the previous studies on optimization of ginger bioactive extraction in Chapter 4.0. This water extract of ginger was pasteurized and stored in sterilized glass bottles at 4 °C.

7.3.4 Physico-chemical Properties of Fruit Juice Blends

7.3.4.1 pH, Total Soluble Solids and Total Acidity

Samples of single-strength juice and concentrated juice were analyzed in triplicate for pH using a standardized pH meter (Model Accumet® 10, Denver Instruments Co., Arvada, CO, USA) and total soluble solids using a hand-held refractometer (Model 300016, Super Scientific Ltd, Scottsdale, AZ, USA). The titratable acidity was measured using semi-automatic titrator (DMP 785, Metrohm Ltd., Herisau, Switzerland) at pH 8.2 using 0.1N NaOH as titrant and was expressed as malic acid equivalent for apple juice, citric acid equivalent for blueberry juice and quinic acid equivalent for cranberry juice. The predominant organic acid in apple (Campo et al. 2006), blueberry and cranberry (Coppola et al. 1978) are malic acid, citric acid and quinic acid, respectively.

7.3.4.2 Color Index

Color of the fresh and membrane filtered fruit juices were determined by using a reflectance colorimeter (Model CR-300, Minolta Camera Co Ltd, Osaka, Japan) based on L*, a* and b* values (Gao and Rupasinghe, 2012). Lightness (L*) ranges from 0 (completely opaque) to 100 (completely transparent). Positive and negative a* values indicate reddish and greenish respectively, whereas positive and negative b* values indicate yellowish and bluish, respectively (Lopez-Nicolas et al. 2007).

7.3.4.3 Determination of Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteu assay (Singleton et al. 1999) with some modifications as described by Rupasinghe et al. (2008) as reported in Chapter 4.0.

7.3.4.4 Ferric Reducing Antioxidant Power Assay (FRAP)

Antioxidant capacity of fruit juices blends and formulated functional beverage were measured using FRAP assay according to the Benzie and Strain (1999) with some modifications described by Rupasinghe, et al. (2008) as mentioned in Chapter 4.0.

7.3.4.5 LDL Oxidation Inhibition

The percentage of LDL oxidation inhibition was determined spectrophotometrically by using the thiobarbituric acid reactive substances (TBARS) assay (Xu et al. 2007) with slight modifications as reported in Chapter 4.0.

7.3.5 Determination of Major Phenolic Compounds in Fruit Juice Blends

Sample preparation, analysis of major phenolic profile of phenolic compounds in the fruit juice blends, and LC-MS/MS were done according to the procedure described in Chapter 6.0.

7.3.6 Sensory Evaluation

7.3.6.1 Screening and Training of the Sensory Panel

Approval of the Research Ethics Board of Nova Scotia Agricultural College (Appendix F and G) was obtained prior to conducting this sensory study. Twenty five subjects were selected from the university community for the initial screening phase. A duo-trio discriminative test was employed and potential panelists for astringency, sourness and sweetness acuity (Meilgaard et al. 1991) were selected. Based on the results of the screening, panelists were identified for training sessions to develop and standardize organoleptic responses focused on astringency, sourness, sweetness and overall acceptability. The panelists were trained to do an unstructured descriptive test, using a

horizontal scale of 15 cm long with anchor points of 1.5 cm from each end. Food grade chemicals and commercial food samples were used for the training.

7.3.6.2 Evaluation of Sensory Properties of Fruit Juice Blends

Five juice blends were served in food grade (HDPE #2), transparent polyethylene cups on a tray. The samples on the trays were arranged in balanced and randomized order. Each sample had three digit codes to minimize the expectation error of the panelist (Meilgaard et al. 1991). One hundred twenty mili litre were provided to each panelist. Panelists evaluated the fruit juice blends for sweetness, sourness, astringency and overall acceptability and were asked to scale what they perceive in a horizontal 15 cm long line with two anchor points, 1.5 cm from each end, on the score sheet provided (Appendix G). Water and salt free crackers were provided as palate cleansing agents between samples. Panelists evaluated the fruit juice samples in standard sensory panel booths. The relative placements of the scores on the 15 cm line were recorded. These parametric data were subjected to statistical analysis using MINITAB and SAS, statistical software.

7.3.6.3 Determination of Levels of Ginger to be Incorporated into Functional Beverage by Using a Sensory Panel

The acceptable amount of ginger to be incorporated into the product was evaluated by incorporating four levels of ginger water extracts (0%, 1%, 2% and 3%, v/v basis) into selected fruit juice blends and evaluating their sensory properties using the same group of trained panelists described previously. Trained panelists evaluated the fruit juices with different levels of ginger for taste and odor and using a 15 cm unstructured scale (Appendix H).

7.3.6.4 Evaluation of Incorporation of Cardio-protective Ingredients into the Formulation on Sensory Properties

The fruit juice blends were fortified with selected cardio-protective ingredients separately at 10% RDI per 350 mL serving and were evaluated for taste and odor compared with unfortified fruit juice blend in order to determine whether any perceived taste was associated with this fortification The score sheet used for the evaluation of cardio-protective ingredients are shown in Appendix I.

7.3.6.5 Evaluation and Comparison of the Final Formulation with Commercial Beverages

Descriptive sensory testing was conducted on formulated fruit-based functional beverage and two commercial fruit based functional beverages for the attributes of odor, astringency, sourness, sweetness and overall acceptability using the same trained panelists and procedure described in 7.3.6.2. The score sheet used for the evaluation is shown in Appendix J.

7.3.7 Statistical Analysis

The experimental design was a completely randomized design with three replicates for the response variables of the fruit juice blends and beverage. The design for the sensory responses was the randomized block design, with panelists as the blocking factor and the juice samples as factor of interest. The assumptions of normal distribution and constant variance were verified by examining residuals (Montgomery, 2005). For the analysis of variance (ANOVA), the general linear model (GLM) procedure of SAS 9.1 was used. Tukey's Studentized Range test was used to detect significant difference among means.

7.4 Results

7.4.1 Physico-chemical and Antioxidant Properties of Fruit Juice Blends

Physico-chemical properties of fruit juice blends were analyzed (Table 7.2). All of the six juices blends had TSS levels close to 15° Brix and were not different ($p>0.05$) from each other. Blend 5 (AJ; 50%, BJ; 37.5% CJ; 12.5%) and blend 6 (AJ; 37.5%, BJ; 50%, CJ; 12.5%) showed significantly higher ($p<0.05$) pH values and significantly lower total acidity (mg citric acid eq/100 mL) levels compared with other blends. Color of the fruit juices were measured by a reflectance colorimeter based on a*, b* and L* values. In contrast, blend 5 and 6 had a significantly lower ($p<0.05$) lightness (L*), red green value (a*) and blue yellow value (b*) compare with the other blends. In terms of phenolic content of fruit juice blends, blend 2 and 5 had significantly lower ($p<0.05$) values and similarly those blends possed significantly lower ($p<0.05$) FRAP values compared with other blends. Blend 1 and 3 show significantly higher ($p<0.05$) phenolic content and FRAP/antioxidant capacity. Significantly lower percent LDL oxidation inhibition were observed in blend 2 while blend 3, 4 and 6 showed the highest percent inhibition among the fruit juice blends. Composition of selected major flavan-3-ols, flavonol, phenolic acids and anthocyanin content of fruit juice blends were shown in Table 7.3.

Table 7.2. Physico-chemical and antioxidant properties of fruit juice blends

Properties	Blend 1	Blend 2	Blend 3	Blend 4	Blend 5	Blend 6
Color L*	17.40±0.20 [a]	19.20±0.30 [ab]	18.20±0.30 [ab]	16.90±2.3 [b]	17.10±0.2 [0b]	16.70±0.20 [b]
a*	22.20±0.10 [bc]	29.20±1.2 [ab]	27.20±1.2 [ab]	32.20±5.7 [a]	22.30±0.6 [0bc]	21.00±1.2 [c]
b*	8.500±0.20 [b]	11.80±0.60 [ab]	10.80±0.60 [ab]	13.10±3.3 [a]	8.600±0.30 [b]	7.900±0.40 [b]
Soluble solids	14.70±0.20[a]	14.90±0.30[a]	14.70±0.30[a]	14.70±0.20[a]	14.80±0.20[a]	14.90±0.30[a]
pH	3.000±0.10[c]	3.000±0.10 [c]	3.000±0.10 [c]	3.000±0.00 [bc]	3.200±0.00 [a]	3.200±0.00 [ab]
Total acidity[x]	1.500±0.10 [a]	1.200±0.00 [b]	1.700±0.00 [b]	1.100±0.00 [b]	0.9000±0.00 [c]	0.8000±0.00 [c]
Total phenolics (mg GAE /L)[y]	1260±76 [ab]	1055±91[bc]	1338±51[a]	1125±71 [b]	1020±34[c]	1174±13 [bc]
FRAP (mg TE/L)[z]	3896±243 [a]	3335±86[bc]	3935±126 [a]	3635±226 [b]	3455±15 [b]	3766±228[b]
% LDL oxidation inhibition	41.30±1.9 [ab]	31.20±2.4 [b]	44.20±4.4 [a]	43.20±2.8 [a]	38.60±3.8 [ab]	44.80±1.8 [a]

Values are presented as mean ± SD, n=3
Blend 1- AJ 1: BJ1: CJ1
Blend 2- AJ2: BJ1: CJ1
Blend 3- AJ1: BJ1: CJ2
Blend 4- AJ1: BJ2: CJ1
Blend 5- AJ1: BJ0.75: CJ0.25
Blend 6- AJ0.75: BJ1: CJ0.25
[x]Total acidity- percentage of titratable acidity (mg citric acid equivalents /100 mL)
[y]mg GAE/L – mg gallic acid equivalent per liter
[z]mg TE/L- mg Trolox equivalent per litre
Data with different superscripts in each rows are significantly different ($p<0.05$)

Table 7.3. Polyphenolic composition (mg/100 mL) of fruit juice blends

Phenolic Compounds	Blend 1	Blend 2	Blend 3	Blend 4	Blend 5	Blend 6
Flavonol						
Epigallocatechin	1.20±0.20 ^{a}	0.800±0.10 ^{ab}	0.800±0.20 ^{ab}	0.900±0.40 ^{ab}	0.600±0.20 ^{b}	0.700±0.00 ^{ab}
Catechin	4.50±0.20 ^{ab}	5.10±1.00 ^{a}	3.60±0.50 ^{b}	3.70±0.10 ^{ab}	0.400±0.50 ^{ab}	3.60±0.20 ^{b}
Epicatechin	9.50±0.60 ^{a}	9.50±2.0 ^{a}	8.80±2.0 ^{ab}	6.00±1.3 ^{ab}	5.40±1.5 ^{b}	4.90±0.40 ^{b}
Epigallocatechin gallate	3.70±0.20 ^{bc}	5.40±1.5 ^{ab}	2.70±0.70 ^{c}	4.00±0.90 ^{abc}	6.30±1.0 ^{a}	4.70±0.30 ^{abc}
Epicatechin gallate	0.700±0.10 ^{a}	0.500±0.30 ^{ab}	0.400±0.20 ^{ab}	0.300±0.20 ^{ab}	0.200±0.00 ^{b}	0.200±0.00 ^{b}
Phenolic acids/ester						
Chlorogenic Acid	961±13 ^{a}	769±35 ^{ab}	593±151 ^{b}	904±89 ^{a}	913±138 ^{a}	988±45 ^{a}
Caffeic acid	2.80±0.20 ^{a}	2.40±0.20 ^{ab}	1.80±0.30 ^{b}	2.50±0.30 ^{ab}	2.30±0.40 ^{ab}	2.60±0.20 ^{a}
Dihydrochalcones						
Phloridzin	1.00±0.20 ^{ab}	1.40±0.40 ^{a}	0.800±0.10 ^{b}	0.900±0.10 ^{ab}	1.40±0.30 ^{a}	1.00±0.10 ^{ab}
Flavan-3-ols						
Quercetin-3-*O*-Galactoside	340±20 ^{a}	289±24 ^{ab}	306±64 ^{ab}	306±64 ^{ab}	208±45 ^{b}	254±3.4 ^{ab}
Quercetin-3-*O*-Glucoside	15.9±1.00 ^{a}	15.0±4.6 ^{ab}	10.7±3.4 ^{ab}	21.8±7.4 ^{ab}	15.1±5.0 ^{b}	21.2±1.4 ^{ab}
Quercetin-3-*O*-Rhamnoside	28.5±1.3 ^{a}	31.4±0.50 ^{ab}	46.2±0.40 ^{ab}	43.5±9.6 ^{ab}	31.1±6.8 ^{b}	33.9±3.2 ^{ab}
Quercetin-3-*O*-Rutinoside	43.3±2.5 ^{a}	40.2±18 ^{ab}	23.2±11 ^{ab}	67.0±24 ^{ab}	44.3±14 ^{b}	63.7±3.4 ^{ab}
Anthocyanins						
Cyanidin-3-*O*-Glucoside	2.20±0.10 ^{a}	1.60±0.50 ^{ab}	0.900±0.40 ^{b}	1.60±0.30 ^{ab}	1.20±0.30 ^{b}	1.60±0.10 ^{ab}
Petunidin-3-*O*-Glucoside	4.80±0.40 ^{a}	3.30±0.80 ^{abc}	2.00±0.80 ^{c}	3.80±0.60 ^{ab}	3.00±0.70 ^{bc}	3.50±0.40 ^{abc}
Delphinidin-3-*O*-Glucoside	2.70±0.20 ^{a}	1.90±0.70 ^{ab}	1.00±0.50 ^{b}	2.20±0.10 ^{a}	1.70±0.40 ^{ab}	2.00±0.30 ^{ab}
Malvidin-3-*O*-Glucoside	5.30±0.6 ^{a}	3.80±0.90 ^{ab}	2.20±0.80 ^{c}	4.00±0.40 ^{ab}	3.20±0.60 ^{bc}	4.00±0.20 ^{ab}
Cyanidin-3-*O*-Galactoside	6.20±0.30 ^{a}	3.90±0.30 ^{b}	3.50±0.60 ^{b}	2.90±0.50 ^{bc}	2.00±0.40 ^{c}	2.00±0.10 ^{c}
Petunidin-3-*O*-Galactoside	2.20±0.10 ^{a}	1.60±0.30 ^{abc}	0.900±0.40 ^{c}	1.80±0.20 ^{ab}	1.40±0.40 ^{bc}	1.70±0.20 ^{abc}
Peonidin-3-*O*-Galactoside	5.40±0.30 ^{a}	4.00±0.30 ^{b}	4.40±1.0 ^{ab}	3.20±0.50 ^{bc}	2.10±0.40 ^{c}	2.10±0.10 ^{c}
Malvidin-3-*O*-Galactoside	4.60±0.50 ^{a}	3.20±0.50 ^{b}	2.00±0.60 ^{b}	3.10±0.20 ^{b}	2.50±0.50 ^{b}	3.00±0.20 ^{b}

Values are presented as mean ± SD of 3 replicates; Blend 1- apple: blueberry: cranberry; 1:1:1 Blend 2; 2:1:1, Blend 3; 1:1:2, Blend 4; 1:2:1, Blend 5; 1:0.75:0.25, 7.Blend 6; 0.75:1:0.25. Data with different superscripts in each raw are significantly different to other blends ($p<0.05$)

7.4.2 Evaluation of Sensory Properties of Fruit Juice Blends

The results of the descriptive sensory evaluation of the fruit juice blends by trained panelists are shown in Table 7.4. Sensory scores for the astringency and sourness ranges from the 4.1 to 8.8 and from 5.0 to 10.5, respectively. Blend 5 obtained the lowest scores for sourness and astringency ($p<0.05$). Sensory properties of the blend 3 were not determined based on the preliminary in-house evaluation of the blends because of the intense sourness. The highest astringency and sourness were recorded in blend 1. In terms of sweetness and overall acceptability, Blend 5 and 6 were similar in sweetness intensity and received significantly higher scores for overall acceptability compared with other blends evaluated.

Table 7.4. Descriptive analysis of different fruits blends by trained panelists

Blends	**Sensory scores***			
	Astringency	**Sourness**	**Sweetness**	**Overall acceptability**
Blend 1	8.8 ± 3.6^{a}	10.5 ± 3.0^{a}	4.2 ± 1.9^{c}	4.8 ± 2.9^{c}
Blend 2	7.3 ± 3.2^{b}	9.3 ± 3.2^{ab}	5.1 ± 2.2^{bc}	6.6 ± 3.2^{b}
Blend 3	ND	ND	ND	ND
Blend 4	6.7 ± 3.0^{bc}	8.1 ± 3.3^{b}	6.2 ± 3.0^{b}	7.0 ± 2.9^{b}
Blend 5	4.1 ± 1.8^{d}	5.0 ± 2.9^{c}	9.9 ± 2.3^{a}	10.8 ± 2.1^{a}
Blend 6	5.6 ± 3.7^{c}	5.0 ± 3.0^{c}	9.0 ± 3.0^{a}	10.9 ± 1.9^{a}

Values are presented as mean ± SD, n=20
*Descriptive panel evaluation of attributes on 15 cm scale
Blend 1- AJ 1: BJ1: CJ1
Blend 2- AJ2: BJ1: CJ1
Blend 3- AJ1: BJ1: CJ2
Blend 4- AJ1: BJ2: CJ1
Blend 5- AJ1: BJ0.75: CJ0.25
Blend 6- AJ0.75: BJ1: CJ0.25
ND; not determined (rejection based on preliminary trial)
Higher value is more astringent/ sour/sweet/acceptable
Descriptors for blends with different superscripts in each column are significantly different from each other blends ($p<0.05$)

7.4.3 Determination of Levels of Ginger to be incorporated into Selected Fruit Juice Blend by Using a Sensory Panel

The descriptive analysis of taste and odor of ginger extracts incorporated fruit juice blend was done in order to determine the consumer acceptable amount of ginger to be incorporated into the functional beverage formulation (Table 7.5). Ginger extract up to 3% in the formulation was evaluated by the trained group of panelists. Based on the results, it was found that there was no significant difference among the different levels of ginger in terms of taste, however, results revealed that the incorporation of 3% ginger extracts into the fruit juice formulations scored significantly ($p<0.05$) lower scores for the attribute of odor.

Table 7.5. Descriptive analysis of taste and odor of selected fruit juice blend with different levels of ginger

Levels of ginger-water extract	Sensory scores*	
	Taste	Odor
0%	8.70±3.8 [a]	10.50±3.4[a]
1%	10.80±2.4 [a]	10.90±2.9 [a]
2%	9.90±2.7 [a]	10.70±2.2 [a]
3%	9.20±2.9 [a]	8.80±2.4 [b]

Values are presented as mean ± SD, n=20
Fruit juice blend - apple: blueberry: cranberry (0.75:1:0.25)
*Descriptive panel evaluation of attributes on 15 cm scale
Data with different superscripts in each columns are significantly different ($p<0.05$)

7.4.4 Effect of Cardio-protective Ingredients on Sensory Properties

Changes in sensory perception, taste and odor with the incorporation of 10% RDI level of cardio-protective ingredients into the formulation per 350 mL were evaluated using the same trained panelists and the results are shown in the Table 7.6. According to the descriptive sensory analysis of taste and odor, the results clearly showed that the

incorporation of 10% RDI level into the formulation do not effect taste and odor significantly ($p<0.05$) when compared with the same formulation without functional ingredients added. The sensory scores for the taste and the odor of the formulation with cardio-protective ingredients added were in the range of 8.7-9.2 and 10.3-10.5 in the descriptive testing respectively.

Table 7.6. Descriptive analysis of taste and odor of selected fruit juice blend with different cardio-protective ingredients (at 10% RDI per 350 mL serving)

Cardio-protective ingredients (10% RDA)	RDI	Sensory scores[x]	
		Taste	Odor
Vitamin B6	2 mg	8.90±3.1	10.5±2.7
Taurine	3000 mg	9.20±2.5	10.5±2.7
L-arginine	1500 g	8.70±2.8	10.5±3.3
Minerals[y]	(70 µg; Se, 3500 mg; K, 15 mg; Zn)	8.90±2.9	10.3±3.1
All ingredients[z]	-	9.20±3.0	10.4±2.9
Without ingredients	-	8.90±3.6	10.3±3.0

Values are presented as mean ± SD, n=20
Fruit juice blend - apple: blueberry: cranberry (0.75:1:0.25)
[x]Descriptive panel evaluation of attributes on 15 cm scale
[y]Se, K and Zn at 10% RDI
[z]Vitamin B6, taurine, L-arginine, Se, K, Zn at 10% RDI
Data with different superscripts in each columns are significantly different ($p<0.05$)

7.4.5 Formulation of Functional Beverage and Evaluation and Comparison of Final Formulation with Commercial Samples

Based on the physico-chemical, antioxidant and sensory evaluation of fruit juice blends, the best fruit juice blend and the levels of ginger extracts and cardioprotective ingredients for the formulation of functional beverage was determined. The composition and the physico-chemical and antioxidant properties of the formulated functional beverage are shown in Table 7.7 and 7.8. This fruit based functional beverage contained ginger extracts at the level of 2% and other cardio-protective ingredients such as vitamin

B6, taurine, arginine, minerals (Se, Zn and K) at 10% RDI per 350 mL levels. Total phenolic content of the beverage was about 1023 mg GAE/L and possesses 3113.5 mg TE/L antioxidant capacity and about 45.2 % inhibition of Cu^{2+}-induced LDL oxidation.

Table 7.7. Composition of the functional beverage (1L)

Ingredients	Amount
Cranberry juice*	125 mL
Blueberry juice*	500 mL
Apple juice*	375 mL
Ginger water extract	20 mL
Arginine	440 mg/L
Taurine	284 mg/L
Pyridoxine	0.6 mg/L
Se-methyl-L-selenocysteine	45.6 µg/L
K citrate	10.5 mg/L
Zn gluconate	13.1 mg/L

*concentrated fruit juice

Table 7.8. Physico-chemical and antioxidant properties of formulated functional beverage

Properties		Values
Color	**a***	43.10±1.1
	b*	20.00±0.1
	L*	28.00±1.4
Soluble solids		14.90±0.2
pH		3.200±0.1
Total acidity (%)[x]		0.8000±0.00
Total phenolics (mg GAE/L)		1024±45
FRAP (mg TE/L)		3114±248
% LDL oxidation inhibition		45.20±9.9

Values are presented as mean±SD, n=3; [x]as citric acid equivalent

The panelists rated and compared the formulated beverage with two commercial functional beverages for taste, appearance and overall acceptability. The evaluation of sensory properties of the newly formulated functional beverage in comparison with two commercial fruit-based functional beverages claiming 'antioxidant-rich' is shown in

Table 7.9. The formulated functional beverage received the highest scores for taste, appearance and overall acceptability compared with bioactive enriched commercial beverage 1. With reference to commercial beverage 2, formulated beverage received the high scores for taste, appearance and overall quality attributes although they are not significantly different ($p<0.05$). Commercial beverage 1 received a significantly high score for odor acceptability compared with formulated functional beverage.

Table 7.9. Evaluation of sensory properties of a functional beverage compared with commercial fruit-based functional beverage by using descriptive analysis

Functional Beverage	**Sensory scores***			
	Taste	Odor	Appearance	Overall acceptability
Formulated functional beverage	10.5±1.8[a]	9.80±2.7[ab]	11.4±1.7[a]	10.8±1.9[a]
Commercial beverage1	5.20±2.8[b]	8.90±3.8[b]	5.60±2.7[b]	5.10±2.6[b]
Commercial beverage2	10.4±2.2[a]	11.3±1.6[a]	10.3±2.6[a]	10.6±2.2[a]

Values are presented as mean ± SD, n=20
*Descriptive panel evaluation of attributes on 15 cm scale
Data with different superscripts in each coloumn are significantly different ($p<0.05$)

7.5 Discussion

TSS content of the fruit juices was maintained at 15° TSS in all the fruit juices used in this study. This TSS level was used based on the previous studies reviewed on fruit juices and functional beverages (Vazquez-Araujo et al. 2010; Saravanan and Aradhya, 2011). Results revealed that fruit juices blends containing 25% or more cranberry juice (blend 1, 2, 3 and 4) showed lower pH and higher total acidity compared with blend 5 and 6 which containing only 12.5% cranberry juice. In general, total acidity of cranberry juice is higher than the blueberry and apple juice (Main et al. 2011). Vazquez-Araujo et al. (2010) have also observed a decrease in acidity when blueberry

juice was added to pomegranate juice. Color of the fruit juices measured with a reflectance colorimeter showed a significantly lower ($p<0.05$) colormetric values (a*, b* and L*) in blends 5 and 6 as these blends containing 50 and 37.5% apple juice respectively and apple is lighter color fruit juice compared with blueberry and cranberry juice. Similar results were observed by Main et al. (2011).

Total phenolic content, FRAP values and % LDL oxidation inhibition were measured as an indicator of the antioxidant activity of the juice blends. FRAP is widely used in the evaluation of antioxidant capacity in dietary polyphenols (Benzie and Strain, 1999). The juice blends chosen for this study had phenolic content and FRAP values in the range of 1020-1337 mg GAE/L and 3335-3935 mg TE/L, respectively. It was observed that the juice blends 2 and 5 contain comparatively lower phenolic content and antioxidant capacity than other fruit juice blends. Apple juice contains lowest phenolic content among the fruit juices used in this study. Therefore, fruit juice blends contains low level of cranberry juice and higher level of apple juice shows relatively lower phenolic content and hence lower antioxidant capacity. Similarly, lower antioxidant activities were observed in blends 2 and 5 towards the Cu^{+2}-induced inhibition of LDL oxidation *in vitro*. Blend 3, 4 and 6 showed the highest inhibition of LDL oxidation among the fruit juice blends studied.

The sensory evaluation was performed to select the best apple/blueberry/cranberry juice blend for the formulation of a functional beverage. It was found that the blends 5 and 6 received scores indicating least astringency and sourness and scores for greatest sweetness and overall acceptability. Most favorable sensory ratings were based on low astringency and sourness and higher sweetness and

overall acceptability. It was observed that higher content of cranberry juice in the juice blend appeared to be correlated with a decrease in the overall acceptability of the blend as cranberry juice. According to the Vazquez-Araujo et al. (2010), the sourness perception of consumers was strongly related to the total acidity. In terms of antioxidant properties, Blend 6 showed higher phenolic content, antioxidant capacity and ability to inhibit LDL oxidation. Therefore, Blend 6 containing 37.5% apple, 50% blueberry and 12.5% cranberry juice was selected for the formulation of the functional beverage.

Ginger is a medicinal herb widely used in herbal medicines (Langner et al. 1998) and currently has received a renewed interest because of its pharmacological activities towards chronic diseases including CVD (Shukla and Singh, 2007). Water extract of ginger has comparable antioxidant activities towards LDL oxidation inhibition and so can be recommended in cardioprotective beverage formulations. However, it is important to be cautious that the level of ginger extracts to be incorporated into the formulation does not adversely affect the sensory perceptions and functional properties. Therefore, four levels of ginger extracts in the formulation were evaluated for taste and odor parameters using trained sensory panelists (Table 7.5). It was found that up to 2% (v/v) ginger extract can be incorporated without compromising the consumer appeal.

Enhancement of the physiological functionality of the beverage can be achieved through fortifying with specific cardio-protective ingredients such as minerals (Se, Zn, and K) (Houstan, 2005; Barciela, et al. 2008), vitamins (pyridoxine) (Houston, 2005) and amino acids (taurine, arginine) (Tsuji, et al. 1980; Houston, 2005). Selenium is a functional component in glutathione peroxidase enzymes, which contributes to the antioxidant defense system (Barciela, et al. 2008). Taurine has beneficial effects on the

cardiovascular system such as conjugation of cholesterol into bile acids (Tsuji, et al. 1980), antioxidant properties (Winiarska et al. 2009) and regulation of blood pressure (Harada et al. 2004). Vitamin 6 has some antihypertensive properties (Bender, 2011). Based on the epidemiologic and clinical studies, potassium intake reduces the incidence of CVD and blood pressure (Whelton and He, 1999). Zinc is involved in a numerous biological processes like catalysis and low serum Zn level is associated with hypertension and hyperlipidemia (Houstan, 2005). L-arginine serves as precursor for endothelial nitric oxide (NO) synthesis via nitric oxide synthase and NO is a potent endogenous vasodilator and exhibits many other favorable cardiovascular effects (Cooke & Dzau, 1997). For the fortification of these functional ingredients, 10% RDI level per 350 mL beverage was used and sensory evaluation of the fortified formulation revealed that the level used do not affect the sensory properties of the beverage.

Total phenolic content of the beverage was 1024 mg GAE/L. Consumption of approximately one portion size of beverage for adult human (350 mL) will ingest about 350 mg GAE of polyphenols. The formulated beverage has *in vitro* antioxidant capacity about 3313 mg TE/L. According to the Ellington and Kullo (2008) chronically elevated oxidized LDL considered as the risk factor for the development of atherosclerosis and the formulated beverage has potential to inhibit *in vitro* LDL oxidation by 45% *in vitro* at concentrations of approximately 1024 GAE/mL.

The formulated functional beverage was compared with two commercial functional beverages for taste, odor, appearance and overall acceptability using trained panelists. It was observed that the formulated beverage was comparably acceptable in terms of the sensory properties to a commercial functional beverage; with various non-

fruits ingredient added product and another commercial beverage did containing a mixture of fruit juices. The formulated functional beverage requires further assessment of its consumer acceptance. Then efficacy and safety research including animal and human trials can be conducted as proof-of-concept evidence for potential health claims.

7.6 Conclusion

Based on the physico-chemical, antioxidant and sensory evaluation of fruit juice blends, the best fruit juice blend ideal for the formulation of a functional beverage was 37.5% apple, 50% blueberry, and 12.5% cranberry. It was found that 2% (v/v) ginger extract and selected cardio-protective nutrients (taurine, L-arginine, vitamin B6, Se, Zn and K) at 10% RDI per 350 mL serving, can be incorporated into the formulation without compromising the consumer acceptability. Phenolic content and *in vitro* antioxidant activities (FRAP and % inhibition of LDL oxidation) of the functional beverage formulation were 1024±45 GAE/L, 3114±248 TE/L, and 45±10%, respectively. The newly designed functional beverage has acceptable organoleptic properties and antioxidant properties. However, the cardio-protective properties of the formulated functional beverage need to be assessed in animal models or human clinical trials.

CHAPTER 8.0 EVALUATION OF THE CARDIO-PROTECTIVE PROPERTIES OF A FUNCTIONAL BEVERAGE USING AN ANIMAL MODEL OF SPONTANEOUSLY HYPERTENSIVE RATS

8.1 Abstract

This study was designed to test the effects of a newly formulated functional beverage on the blood pressure and serum and liver lipid profiles *in vivo*. Sixty spontaneously hypertensive (SH) rats were divided into five groups after 2 weeks adaptation. Rats were fed with a AIN-93G-diet as the normal control (NC), high cholesterol diet as atherogenic control (AC), and the AC diet supplemented with three different dosages (0.5X, 1X, 2X) of the functional beverage, where X is the equivalence of two portion sizes of the beverage for an adult (X=10 mL/kg BW/day). Blood pressure was measured at week two and four, respectively, using a non-invasive tail-cuff blood pressure system. Systolic and diastolic blood pressures of rats were lowered by the beverage at the dose of 1X or 2X after 2 week but the effect disappeared after 4 weeks treatments. Administration of two or four portion size of beverage significantly lowers total and free cholesterol levels in the liver and cholesterol, LDL and triacylglycerol levels in the serum. These results obtained in SH rats suggested that the formulated functional beverage does possess cardio-protective effects which may be related to the ability of the beverage to lower the serum and liver lipid levels.

8.2 Introduction

Polyphenols-rich foods have received a growing attention as the dietary antioxidants are may reduce the risk of many chronic diseases such as cardiovascular diseases (CVD) (Hertog et al. 1995). Any diseases that affect heart and blood vessels are referred to as CVD. CVD is a major cause of the death in developed countries (American Heart Association, 2003). The main pathophysiological process for the development of CVD is atherosclerosis, which is the deposition of cholesterol and lipids in arterial wall and development of atherosclerotic plaque (Stocker and Kenny, 2004). The role of plasma lipids in the etiology of atherosclerosis and CVD has been well defined (Castro et al. 2005). High concentrations of plasma cholesterol, triacylglycerol, and LDL cholesterol and a low plasma concentration of HDL cholesterol are considered important risk factors for the expression of CVD, and these biomarkers are used in the assessment of population risk (Kannel et al. 1979).

Growing evidence indicates that there is a negative correlation between intake of food and beverages that containing polyphenolic bioactives and CVD (Fki et al. 2005; Jalili et al. 2006; Morton et al. 2008). Much of the recent human nutritional research has focused on understanding the influence of various diets on chronic diseases such as CVD. In keeping with this, cardiovascular health benefits of some polyphenolic antioxidant rich fruits such as cranberry, blueberry and apple are increasingly being attributed, in part, to their polyphenolic bioactives content (Morton et al. 2008). Therefore, increasing intake of these fruit polyphenolics or products with antioxidants may be an effective strategy to prevent CVD (Thilakarathna and Rupasinghe, 2012; Vattem et al. 2005). Ginger, a widely used medicinal food ingredient also shows some cardio-protective properties

(Bhandari et al. 1998). According to Vattem and co-workers (2005), consuming a beverage with efficacious and safe levels of variety of bioactives derived from fruits and medicinal herbs may impart health benefits. Therefore, a bioactive enriched beverage was developed as an adjunct to reduce the risk of CVD as described in Chapter 7.

It is essential to assess the health functional properties of the newly formulated functional beverage *in vivo* in order to establish evidence for the efficacy of cardio-protection by the product. Many different animal species have been used as experimental models to investigate prevention and treatment measures for various chronic diseases. Among them, rats are frequently used to study the cholesterol and lipo-protein metabolism (Gollaher, 1992). The spontaneously hypertensive rats (SHR), is an animal model of human essential and primary hypertension, and has been extensively used to study hypertension and CVD (Kundu and Rao, 2008). As hypertension is also a major risk factor for CVD, SHR was used as the animal model for the evaluation of cardio-protective properties of the beverage. The present study was designed to investigate the potential cardio-protective effects of the newly formulated functional beverage using SHR fed an atherogenic diet with the aim of understanding the effects of different doses of functional beverage on blood pressure and serum and liver cholesterol levels in SHR.

8.3 Materials and Method

8.3.1 Chemical and Reagents

All feed ingredients were purchased from Dyets (Bethlehem, PA, USA). The chemicals and reagents required for serum lipid analysis were purchased from BioPacific Diagnostic, Inc. (British Colombia, Canada). For the liver triacylglycerol and cholesterol analysis, chemicals and reagents were purchased from BioVision, Inc., CA, USA. All

other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

8.3.2 Animal Model and Diet

The experiment was performed at National Research Council of Canada in Charlottetown, PEI. Ethical approval was obtained from the Animal Care Committee (ACC) of the University of Prince Edward Island. The experiment was conducted according to the guidelines of the Canadian Council for Animal Care.

Sixty male rats weighing 200-250 g were purchased from Charles River Laboratories Inc. (QC, Canada) and housed in individual cages and subjected to 12 hour light and dark cycle. They were given a regular rodent chow with free access to the diet and water for two weeks to acclimatize to the new environment. Subsequently, the rats were randomly divided into five groups, with twelve per group. The control group was fed a casein-corn starch-sucrose-based AIN-93G diet to serve as the normal control (NC, Table 8.1). The second group was fed the normal diet with 2.0 % cholesterol and 0.5% cholic acid to serve as an atherogenic control (AC, Table 8.1). The other three groups were fed the AC diet supplemented with three different doses of the functional beverage (0.5X, X and 2X). The dosage level X (mL) was equivalent to two portion size of beverage (700 mL) per day for an adult 70 kg human (X=10 mL/ kg BW/day). Feed intake was recorded daily and the fasted (12 hours) body weight of animals was measured weekly. The behavior of animals was observed and recorded throughout the experimental period.

Table 8.1. Composition of normal control and atherogenic diets

Ingredients	% Amount (w/w)	
	Control diet	Atherogenic diet
Casein	20.0	19.7
Corn starch	28.0	27.8
Sucrose	36.3	36.0
Oil[a]	5.00	5.00
Cellulose	4.90	4.90
DL-Methionine	0.500	0.500
Mineral mixture[b]	4.00	4.00
Vitamin mixture[c]	1.00	1.00
Choline bitartrate	0.200	0.200
Cholesterol	0.100	2.00
Butylated hydroxytoluene	0.0200	0.0200
Cholic acid	-	0.500

[a] 96% of oil was lard, 4% sunflower oil.
[b]5000 mg Ca, 1561 mg P, 3600 mg K, 1019 mg Na, 1571 mg Cl, 300 mg S, 507 mg Mg, 35 mg Fe, 6 mg Cu, 10 mg Mn, 30 mg Zn, 1 mg Cr, 0.2 mg I, 0.15 Se, 1 mg F, 0.5 mg B, 0.15 mg Mo, 5 mg Si, 0.5 mg Ni, 0.1 Li, 0.1 mg V per kg of the mix
[c]20 mg thiamin HCl, 15 mg riboflavin, 7 mg pyridoxine HCl, 90 mg niacin, 40 mg calcium pentothenate, 2 mg folic acid, 0.6 mg biotin, 10 mg cyanocobalamin, 4 mg menadione sodium bisulphate, 5000 IU vitamin A palmitate, 50 IU vitamin E acetate, 2400 IU vitamin D3, 100 mg inositol per kg of the mix

8.3.3 Preparation of Functional Beverage

Functional beverage was prepared by mixing concentrated (up to 15° Brix by membrane filtration) cranberry, apple and blueberry juices together with ginger extracts and cardio-protective ingredients including Se, Zn, K, vitamin B6, arginine, and taurine according to the procedure given in Chapter 7. The composition of the beverage is given in Table 8.2. The experimental treatment diets for rats were formulated with addition of functional beverage at a dose of 5 mL/kg body weight (0.5X), 10 mL/kg body weight/day (X) and 20 mL/kg body weight/day (2X) to the AC diet.

8.3.4 Blood Pressure and Heart Rate

The blood pressure was measured using CODA-6 a non-invasive tail-cuff blood pressure system (Kent Scientific, Torrington, CT, as described by Daugherty et al. (2009). The systolic and diastolic blood pressures of rats were measured during week two and four of the 40 day study, respectively, following the dietary treatment.

8.3.5 Collection and Storage of Tissue and Serum Samples

After 40 days of intervention, fasted (12 hours) rats were anesthetized by isoflurane inhalation. Blood samples were collected into serum tubes through cardiac puncture and allowed to clot at room temperature for 30 min and then placed on ice until centrifugation. The blood samples were centrifuged at 2500 g for 15 min and serum was collected and stored at -80 °C until later analysis. Liver tissues were dissected and rinsed in PBS to remove residual blood, weighed, and flash frozen in liquid nitrogen and stored at -80 °C until analysis.

8.3.6 Serum and Liver Lipids Analysis

Serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triacylglycerol levels and were determined using a Ponte-180 Chemistry Analyzer (Lincolin Park, MI, USA) following the manufacturer's instructions. For HDL cholesterol, non-HDL cholesterol was precipitated with dextran sulfate (Wamick et al. 1982). Duplicate sub samples of each serum sample were analysed. Data were expressed as mg cholesterol per dL serum.

For liver cholesterol analysis, about 10 mg of liver was extracted with 200 μL of chloroform: isoproponal: NP-40 (7:11:0.1) using a micro homogenizer (model FTH-115 PowerGen 125, Fisher Scientific, Pittsburgh, PA, USA). Extracts were spun for 10 min at

15,000 rpm in a centrifuge (model; Microlite, Thermoelectron cooperation, Whaltham, MA, USA) and dried at 50° C to remove chloroform. The organic phase was pooled transferred to a new tube and dried under nitrogen to remove chloroform. Dried lipid samples were dissolved in 200 µL cholesterol assay buffer by vortexing. Total and free cholesterol levels were determined using commercial kits (BioVision catalog number K603-100 (cholesterol/cholesteryl ester quantification kit) by measuring the florescence on a plate reader (Model; Varioskan Flash, Thermo Scientific, Whaltham, MA, USA).

For the analysis of liver triaylgycerol, about 100 mg of liver was extracted with 1 mL water containing 5% NP-40 and heated at 80° C in water bath for 2 min. This process was repeated and the resulting extract was centrifuged. Triacylglycerol levels were determined using commercial kits (BioVision catalog number K622-100) by measuring florescence on the plate reader. Each liver sample was extracted and analysed for cholesterol and tryglycerol in duplicate and data were expressed as µg lipids per mg liver tissue.

8.3.7 Statistical Analysis

Data are expressed as means (n=12) and their standard deviations. The normality and constant variance assumptions were tested using the Anderson-Darline test and by examining the residual versus fits. Data were analyzed by one-way ANOVA and Fisher's least significant difference test using the SAS program (SAS V8, Cary, NC, USA). Multiple mean comparison with least significant means test were carried out when there was a significant difference ($p<0.05$).

8.4 Results

8.4.1 Composition of the Beverage

The composition of the functional beverage is given in Table 8.2. The formulated beverage contains 50% blueberry juice, 37.5% apple juice, 12.5% cranberry juice, 2% ginger extract and selected cardio-protective ingredients.

Table 8.2. Composition of the functional beverage (1 L)

Ingredients	Amount
Cranberry juice	125 mL
Blueberry juice	500 mL
Apple Juice	375 mL
Ginger water extract	20 mL
Arginine	440 mg
Taurine	284 mg
Pyridoxine	0.6 mg
Se-methyl-L-selenocysteine	46 µg
K citrate	10.5 mg
Zn gluconate	13.1 mg

8.4.2 Body Weight, Feed Intake and Organ to Body Weight Ratio

The body weight of rats increased in all groups throughout the feeding trial without any significant differences ($p<0.05$) among the treatment groups including the normal control and atherogenic control (Table 8.3). Average daily feed intakes were similar among the experimental groups (Table 8.4).

Table 8.5 shows the ratios of liver and kidney weights to body weight. There were no significant differences among the three treatment groups. However, the liver/body weight ratio significantly ($p<0.05$) increased in rats fed the high cholesterol diet compared with the normal control, irrespective of the level of beverage consumed.

Table 8.3. The weekly average body weights of rats in each treatment groups during the experimental period

Treatment group	Feeding period (weeks)					
	0	**1**	**2**	**3**	**4**	**5**
Normal control	248.9±6.5	266.3±7.9	287.2±6.4	299.3±5.6	305.6±5.9	317.4±7.1
Atherogenic control	252.8±9.2	271.6±5.8	296.1±6.0	305.5±8.6	313.9±10	328.2±11
0.5X*	248.9±6.6	270.1±6.6	289.6±10.	302.6±12	311.9±12	325.2±11
X*	251.4±4.7	274.3±7.2	293.9±7.3	307.4±8.9	314.2±10	329.6±12
2X*	250.9±12	271.6±12	289.1±13	302.2±15	314.3±15	330.3±16

Values are presented as mean ± SD (g), n=12

*Different dosage of the functional beverage (0.5X, X and 2X). The dosage level X was estimated based on the equivalent of two portion size of beverage (700 mL) per day for an adult human

Table 8.4. The weekly average feed intakes of rats in each treatment groups during the experimental period

Treatment group	Feeding period (weeks)					
	1	**2**	**3**	**4**	**5**	**5+5 days**
Normal control	23.7±1.7	20.7±1.8	19.9±3.2	18.6±1.7	17.1±1.8	16.7±1.7
Atherogenic control	22.5±2.3	20.3±1.9	16.6±1.9	18.4±2.7	17.3±1.7	16.3±1.5
0.5X*	22.3±1.5	18.8±1.2	15.9±6.2	18.5±1.2	16.9±3.1	15.7±3.1
X*	22.9±1.8	18.6±2.2	16.2±2.6	19.1±2.6	16.8±3.0	16.4±2.8
2X*	20.2±1.8	19.2±1.8	18.4±3.4	19.3±1.9	17.8±2.4	17.3±2.6

Values are presented as mean ± SD (g), n=12, *Different dosage of the functional beverage (0.5X, X and 2X) mixed with feed. The dosage level X was estimated based on the equivalent of two portion size of beverage (700 mL) per day for an adult human.

Table 8.5. Organ weight to body weight ratio among treatment group at the end of 40 days period

Ratio	Normal control	Atherogenic control	0.5X *	1X *	2X *
Liver/BW	0.034±0.003[b]	0.054±0.003[a]	0.053±0.003[a]	0.054±0.003[a]	0.053±0.003[a]
Kidneys/BW	0.0070±0.001	0.0070±0.001	0.0070±0.001	0.0070±0.001	0.0070±0.001

Values are presented as mean ± SD (g), n=12

*Different dosage of the functional beverage (0.5X, X and 2X). The dosage level X was estimated based on the equivalent of two portion size of beverage (700 mL) per day for an adult human (X= 10 mL/kg BW/day).

Data with different superscripts in each columns are significantly different ($p<0.05$)

8.4.3 Blood Pressure

Table 8.6 shows the mean systolic and diastolic blood pressure at the second and fourth week of the experiment. In the second week, systolic blood pressures of rat groups fed with the functional beverage at 1X or 2X were significantly lower than that of normal and atherogenic control groups. There was no significant difference observed between rats fed the 0.5X functional beverage and the normal or atherogenic control rats. The diastolic blood pressures of rats fed the functional beverage at 2X were significantly lower than that of normal control groups and similar with other groups. However, there were no any significant difference ($p<0.05$) observed in either systolic or diastolic blood pressure at the fourth week of the treatment.

Table 8.6. Systolic and diastolic blood pressure of animals at the second and fourth weeks of the experiment

Blood pressure (mmHg)		Normal control	Atherogenic control	0.5X	1X	2X
Week 2	Systolic	158.4±5.1^{a}	159.3±5.1^{a}	149.4±7.0^{b}	152.9±8.8^{ab}	147.2±7.6^{b}
	Diastolic	103.7±16^{ab}	109.5±10.^{a}	102.3±18^{ab}	95.50±15^{b}	97.30±12.5^{b}
Week 4	Systolic	151.2±6.4^{a}	158.9±6.8^{a}	157.7±12^{a}	158.6±17^{a}	155.5±8.2^{a}
	Diastolic	92.00±18^{a}	105.5±12^{a}	98.40±9.5^{a}	95.60±8.1^{a}	91.20±8.7^{a}

Values are presented as mean ± SD (g), n=12
Data with different superscripts in each rows are significantly different ($p<0.05$)

8.4.4 Liver Lipid Profile

Figure 8.1 shows the total cholesterol (a), free cholesterol (b) and triacylglycerol (c) levels of liver at the end of experiment. Rats treated with 1X or 2X of the beverage had significantly ($p<0.05$) lower total (Figure 8.1a) and free cholesterol levels (Figure 8.1b) in the liver compared with the atherogenic control and group fed with 0.5X of the functional beverage. There was no significant difference in total and free cholesterol levels between atherogenic control and the rat group fed with half dosage levels (0.5X).

Normal control groups had the ($p<0.05$) lowest total cholesterol and free cholesterol levels in the liver than the other treatment groups.

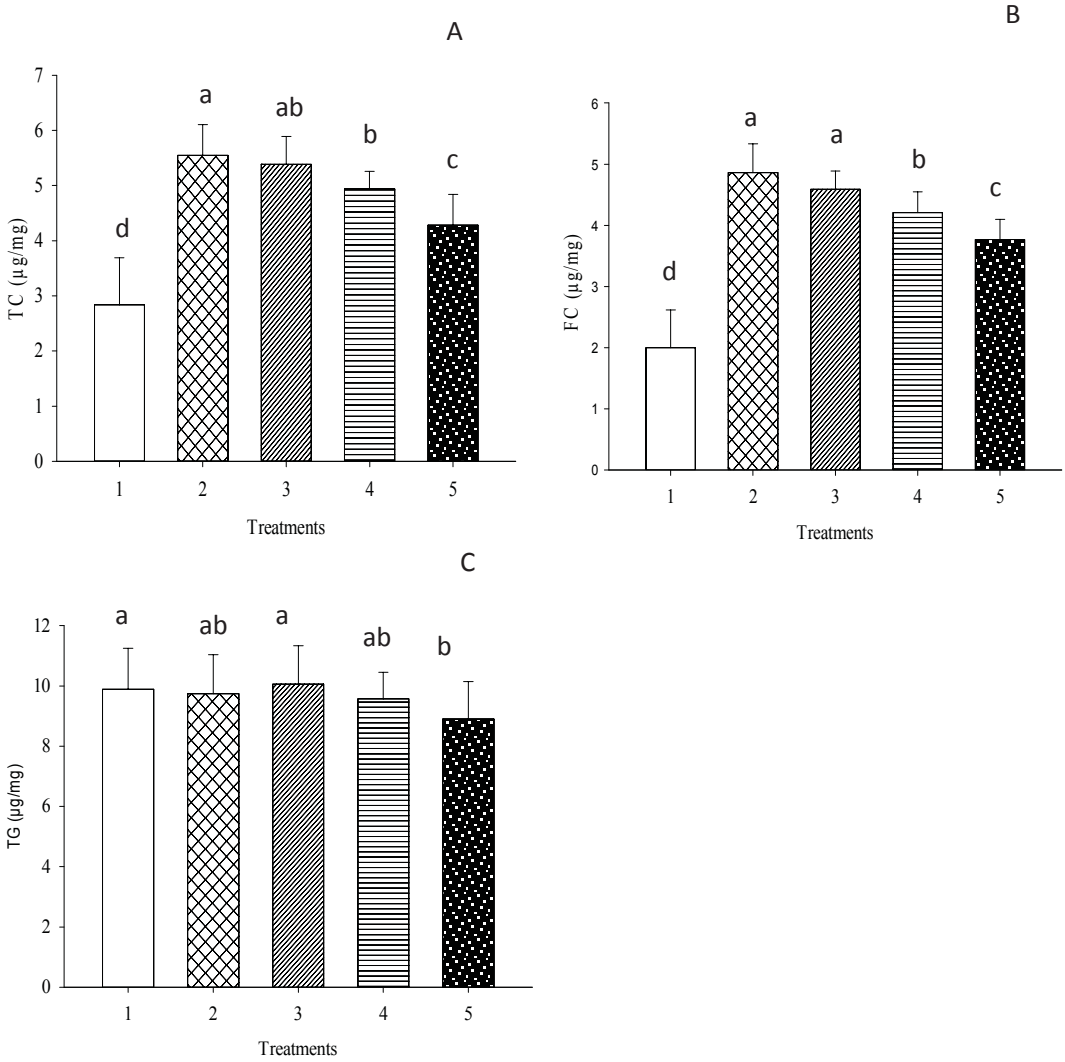


Figure 8.1. Effect of 40 days consumption of functional beverage dosage levels on the total cholesterol (A), free cholesterol (B) and triacylglyceride (C) levels in the liver. 1: normal control, 2: atherogenic control 3: 0.5X dosage of the functional beverage, 4: 1X dosage of the functional beverage, 5: 2X dosage of the functional beverage (The dosage level X was estimated based on the equivalent of two portion size of beverage (700 mL) per day for an adult human). Each bar represents mean ± SE from 12 rats. Bars with different letters differ, $p<0.05$.

8.4.5 Serum Lipid Profile

After 40 days of treatment, total cholesterol (Figure 8.2 a), LDL (Figure 8.2 c) and the triacylglycerol content (Figure 8.2 d) in the serum of SHR fed a high cholesterol diet was significantly ($p<0.05$) higher compared with SHR fed a normal control diet. However, results clearly indicated that the serum cholesterol, LDL and triglycerides levels of the rat group fed with high cholesterol diet together with functional beverage at the dosage level equivalent to two (1X) or four (2X) portion sizes of human daily consumption were significantly ($p<0.05$) lower than that of atherogenic control and the group fed with 0.5X dosage functional beverage. In comparison to the normal control diet, a significant ($p<0.05$) decrease in HDL cholesterol level was observed in all high cholesterol fed groups irrespective of the dosage of the functional beverage given (8.2 b).

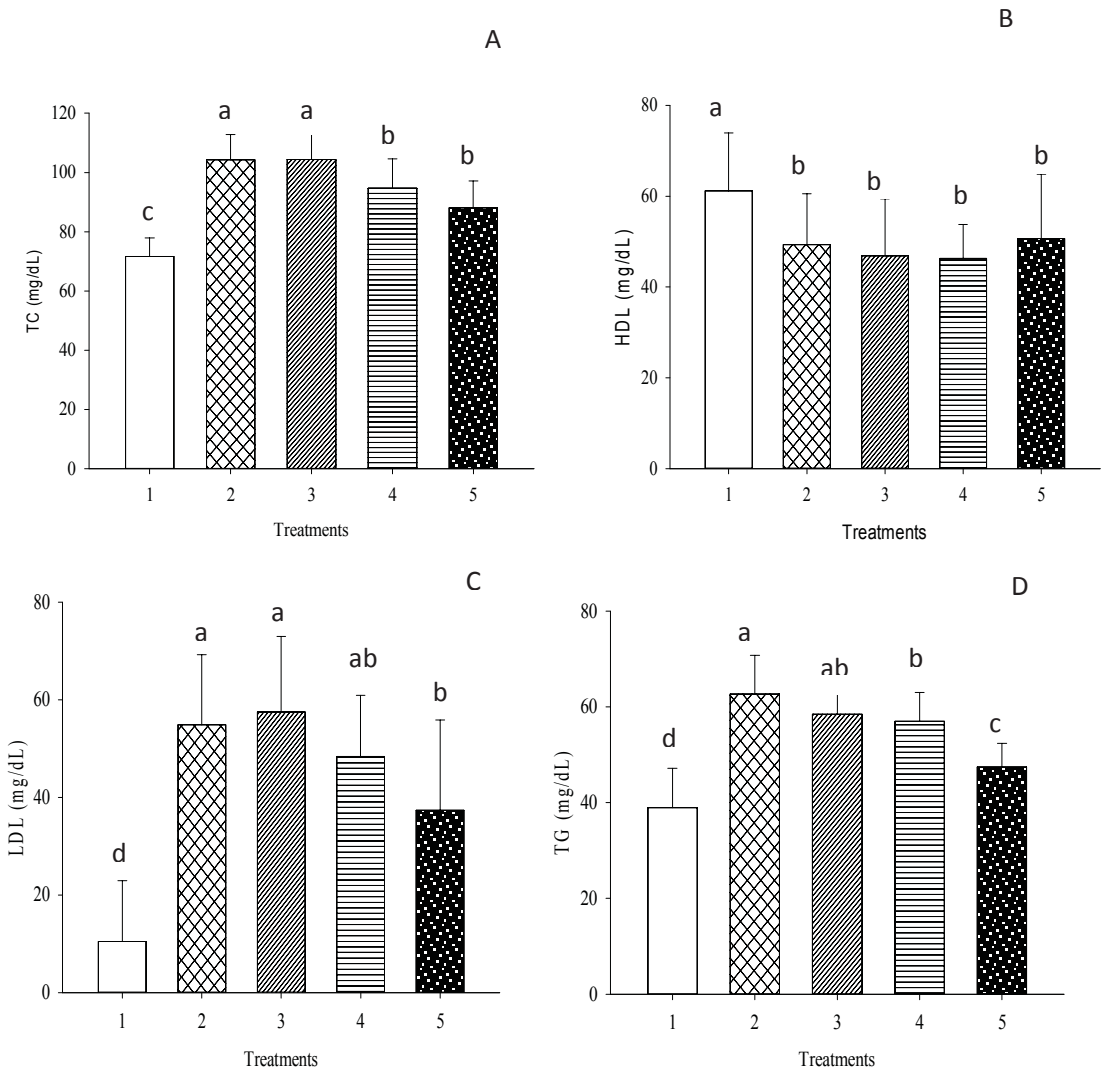


Figure 8.2. Effect of 40 days consumption of functional beverage dosage levels on the total cholesterol (A), HDL cholesterol (B), LDL cholesterol (C) and triacylglycerol (D) levels of the serum. 1: normal control, 2: atherogenic control 3: 0.5X dosage of the functional beverage, 4: 1X dosage of the functional beverage, 5: 2X dosage of the functional beverage (The dosage level X was estimated based on the equivalent of two portion size of beverage (700 mL) per day for an adult human). Each bar represents mean ± SE from 12 rats. Bars with different letters differ, $p<0.05$

8.5 Discussion

Atherosclerosis is a complex disease process that is often initiated by hypercholesterolemia and has been known as a major pathophysiological process that leads to the development of CVD (Stocker and Keany, 2004). A number of epidemiological studies have implied a role of polyphenols in reducing the risk of CVD (Thilakarathna and Rupasinghe, 2012). Cranberry, blueberry and apple are some of the polyphenol rich fruits available in eastern Canada and are good ingredients for functional beverage formulation (Morton, et al. 2008). Ginger also showed some cardio-protective properties (Bhandari et al. 1998). Arginine, taurine, potassium, zinc, selenium and vitamin B6 are functional ingredients widely used in cardio-protective formulations (Houston, 2010). The present study investigated the effect of the formulated fruit-based functional beverage on the blood pressure and cholesterol metabolism in rats fed with high cholesterol diet.

The SHR strain, in which development of hypertension is very similar to that in human, has been used extensively to test acute and chronic antihypertensive effects of functional foods and nutraceuticals (Gallaher, 1992). It is interesting that the SHR group fed with equivalent of two or four portion size of beverage per day equivalent for an adult human (70 kg BW) demonstrated a significant decrease in systolic blood pressure compared to atherogenic and normal control two week after the dietary supplementation. However, there was no significant difference among any groups at four week time point, although the average blood pressure measurements in functional beverage groups lower compared with atherogenic control groups but not significant ($p<0.05$). This may be due

to genetic factors responsible to serve hypertension in this particular rat group likely overwhelmed the beneficial effects of the beverage.

It is generally accepted that hypertension is accompanied by increased oxidative stress (Pu et al. 2003). Although the precise mechanism responsible for maintenance of healthy blood pressure values among chronic flavonoids consumption studies is not known, it is speculated that the two classes of phenolic compounds such as catechins and anthocyanins present in the beverage, may have effect in lowering blood pressure (Aviram and Dornfeld, 2001). Grassi et al. 2009 reported that the dietary flavonols reduce the blood pressure.

The total and free cholesterol levels in the liver were significantly reduced with the administration of all doses of the functional beverage for 40 days. The feeding of formulated functional beverage in the amount equivalent to two portion size of beverage (10 mL/kg bw/day; 1X) or four portion sizes (20 mL/kg bw/day; 2X) per day for an adult human, may affect the hepatic cholesterol metabolism of SHR. A review (Zern and Fernandez, 2005) of the effects of the polyphenols on CVD suggest that the polyphenols and their metabolites may modify hepatic cholesterol metabolism by reducing the cholesterol absorption, up-regulating the hepatic mRNA abundance for the LDL receptor and modifying the VLDL particle (Zern and Fernandez, 2005). Another study (Tebib and colleagues, 2003) found that addition of 2% polymeric tannins to rat's diet reduces the liver cholesterol by increasing the fecal excretion of cholesterol. Furthermore, the results the present study reveal that the intake of formulated beverage in a volume equivalent to the one serving size (5 mL/kg bw/day; 0.5X) daily for human adult was not enough to reduce the hepatic cholesterol level in SHR. However, there was no significant difference

($p<0.05$) in liver triacylglycerol levels among treated groups compared with atherogenic control groups. In this study, the cholesterol-rich atherogenic diet increased the liver weight significantly ($p<0.05$). This could be related to the accumulation of lipids such as triacylglycerol and cholesterol. A similar result was reported by Jemai et al. (2008) feeding high cholesterol diet to Wistar rats.

The results demonstrated serum lipid lowering effects of the formulated functional beverage in SH rats whose diet was supplemented with cholesterol. This formulated functional beverage was made with antioxidant flavonoids rich fruit juices such as cranberry, blueberry and apple. Previously, Chapter 6 described that the partial concentration of these fruit juices by membrane filtration increases the polyphenolic concentration. Several studies have shown that the flavonoids possess hypocholesterolemic and antioxidant activity specially when they administered with high doses, for example, grape seed extract rich in flavonoids in hamsters (Bagchi et al. 2003) and rutin in rats (Park et al. 2002).

In their review, Weichselbaum et al. (2010) reported that four out of six studies demonstrate some favorable effect of apple juice and its constituents on human blood lipid profile. In another trial, it was found that apple juice supplementation of 10 mL (per kg BW) for two months, reduced the total cholesterol and triacylglycerol levels (Setoki et al. 2009). Similarly, the effect of cranberry and blueberry juice consumption has been investigated using *in vivo* models and found that these fruits possess hypocholesterolemic properties (Caron et al. 2005; Kalt et al. 2008).

Ginger is a one of the ingredients in the formulated functional beverage. Various *in vivo* and *in vitro* research models demonstrate that ginger has anti-atherogenic effect

which is associated with a significant reduction in plasma and hepatic LDL cholesterol levels (Chrubasika et al. 2005). In concordance with the findings of ElRokh and co-workers (2010) in a rat model of hypercholesterolaemic, they have found that 200-400 mg fresh ginger per kg body weight daily for four weeks has an anti-hypercholesterolaemic effect which was comparable to the standard hypocholesterolaemic drug. Taurine, zinc and arginine are some of the ingredients known to have hypolipidemic effects and were used in the formulation. In hypercholesterolemia, taurine supplementation *in vivo* has been found to improve the serum lipid profile (Yokogoshi et al. 1999; Ito and Azuma, 2004; Matsushima et al. 2003). In a human clinical study, it showed positive outcomes with L-arginine supplementation as it decreased the LDL cholesterol level and increase in HDL/LDL ratio (Hurson et al. 1995a). Low serum Zn levels correlate with hyperlipidemia, especially hypertriglyceridaemia and low high-density lipoprotein (HDL) cholesterol (Houstan, 2005) and in a human clinical study it was found that Zn supplementation decreases the serum cholesterol level (Boukaiba et al. 1993).

8.6 Conclusion

The present study demonstrate that supplementation with the functional beverage at a level equivalent to 700 mL portion or 1400 mL portion sizes for adult humans daily had a hypolipidemic effect in cholesterol-fed SH rats as compared to an atherogenic control group. This treatment could significantly reduce the triacylglycerol and total cholesterol levels in the serum, and total and free cholesterol levels in liver. There were no significant difference in blood pressure among the functional beverage treated groups and atherogenic control groups after four weeks of intervention, although there was a

significant difference in these groups at 2 week time point. More studies are required to explain the potential hypolipidemic and hypocholestremic effects of formulated functional beverage.

CHAPTER 9.0 CONCLUSIONS

9.1 Background

CVD is considered as one of the leading causes of death, and recent research suggests that ROS-mediated processes play a role in the development of atherosclerosis. As the endogenous antioxidant defense system is not totally adequate to counteract the oxidative stress, protection against oxidative stress depends partly on the adequacy of dietary antioxidants. Therefore, it is suggested that intake of plant dietary antioxidants is an effective strategy to reduce the risk of atherosclerosis (Kaliora et al. 2006). We have explored whether bioactive-rich 'functional beverage' could be an alternative dietary strategy to reduce the risk of oxidative stress linked chronic diseases like CVD. Therefore, the overall objective of this study was to develop and assess the efficacy of biologically active compounds in a functional beverage, formulated by combining selected classes of polyphenols from apple, blueberry, cranberry and ginger together with cardio-protective minerals, amino acids and vitamins. The overall objective was subdivided into five specific objectives which were: optimization of aqueous extraction conditions of ginger bioactive and evaluation of aqueous and organic ginger extract in their antioxidant protection against human LDL oxidation *in vitro*; evaluation of membrane processing technique for the concentration of bioactives of fruit juices; formulation of a functional beverage targeted to reduce the risk of CVD and hypertension by combining optimum blends of fruit juices, ginger together with cardio-protective ingredients; and to test the efficacy of the functional beverage using a research rat model of spontaneous hypertension.

9.2 Conclusions

For the preparation of bioactive enriched water extract of ginger, ultrasonic-assisted extractions yielded the highest predicted phenolic and antioxidant activities at lower extraction conditons compared with hot water extraction and high pressure homogenization-assisted extraction methods. *In vitro* studies on ability to inhibit LDL oxidation by water extract of ginger showed that ginger has potential in the prevention of CVD and suggesting that ginger extracts have potential for use in value-added food products, especially to be used in functional foods.

The results showed that there was no significant difference in any of the quality parameters of juices concentrated under any of the operating parameters tested (20-40 °C temperature and 25-35 bars transmembrane pressure). The physico-chemical properties of the partially concentrated fruit juices increased in proportion to the volumetric concentration. The results indicated that the antioxidant capacity of concentrated apple, blueberry and cranberry juice increased by 40%, 34% and 30%, respectively and percentage LDL oxidation inhibition by concentrated blueberry and cranberry juice were around 41% and 45%, respectively, whereas both fresh juices showed only about 35% inhibition. RO can be applied to partial concentration of fresh apple, blueberry and cranberry juice prior to the formulation of beverage to enhance their antioxidant activities.

The partially concentrated apple, blueberry and cranberry juice together with water-extracts of ginger and some cardio-protective ingredients were used for the formulation of a functional beverage. Based on the physico-chemical, antioxidant and sensory evaluation of fruit juice blends, the best fruit juice blend for the formulation of a

functional beverage was apple 37.5%, 50% blueberry, and 12.5% cranberry. It was found that 2% (v/v) ginger extract and selected cardio-protective nutrients at 10% RDI can be incorporated into the formulation without compromising consumer appeal. Phenolic concentration and *in vitro* antioxidant activities (FRAP and % inhibition of LDL oxidation) of the functional beverage formulation were 1024±45 GAE/L, 3114±248 TE/L, 45±10%, respectively. The newly designed functional beverage had good organoleptic properties and enhanced concentration bioactive and high antioxidant activity.

An animal study was designed to test the effects of newly formulated functional beverage on the blood pressure and serum and liver lipid profile. Results of the animal study demonstrated that the functional beverage at a dosage level equivalent to 700 mL or 1400 mL for adult human (70 kg) daily has a very pronounced lipid lowering effect as compared to atherogenic control group. It could significantly reduce the triacylglycerols and total cholesterol levels in the serum and total and free cholesterol levels in the liver. Furthermore, blood pressure was lower at week two of the feeding trial in the SH rat groups fed with functional beverage at 1X and 2X. However, due to high variability, the effect was not significant at the week four of the feeding trial although average diastolic and systolic blood pressure values were lower than that of atherogenic control group.

9.3 Recommendation for Future research

The present study indicates that the ginger extracts, concentrated fruit juices and formulated functional beverage have antioxidant properties in the inhibition of Cu^{2+}-induced LDL oxidation *in vitro*. Therefore, it is worthwhile to further investigate the mechanisms of action by the each component with potential cell culture models. Furthermore, in this study we have focused mainly the phenolic bioactive constituents of ginger. There are other ginger bioactive constituents, for example, isoprenoids, mainly zingiberene that could be examined.

Current trends and changing consumer interests show a great opportunity for innovation of new functional foods targeting chronic diseases like CVD. The formulated new product shows potential health benefits *in vitro* and *in vivo* towards the reducing the risk of CVD. However, the formulated product requires further assessment for their consumer acceptance, and their efficacy and safety through human clinical trials and to develop evidence for potential future health claims. Further, it is worth to note the pilot scale processing trials are necessary with novel processing approaches such as non-thermal pasteurization to maintain the nutritional and bioactive properties before it comes to industrial scale.

REFERENCES

Agbor, A. G., Oben, J. E., Ngogang, J. Y., Xinxing, C. and Vinson, J. A. Antioxidant capacity of some herbs/spices from Cameroon: A comparative study of two methods. *J. Agric. Food Chem*. **2005**, *53(17)*, 6819-6824

Agriculture and Agri-Food Canada. Statistical Overview of Canadian Horticulture 2009-2010. http://www4.agr.gc.ca/AAFC-AAC

Agriculture Research Service, US Department of Agriculture. *Nutrient Data Laboratoiy* (searchable database). Available at: http://www-nal.usda.gov/fnic/foodcomp/search. Accessed March, **2011**

Ahmet, I., Spangler, E., Shukitt-Hale, B., Juhaszova , M., Sollott, S. J., Joseph, J. A., Ingram, D. K. and Talan, M. Blueberryenriched diet protects rat heart from ischemic damage. PLoS One 4, **2009**, *4(6)*. 1-10

Ali, B. H., Blunden, G., Tanira, M. O. and Nemmar, A. Some physico-chemical, pharmacological and toxicological properties of ginger (*Zingerber officinale*)-a review. *Food and Chem. Toxico.* **2008**, *46,* 409-410

American heart association. Heart Disease and Stroke Statistics-2003 Update. American Heart Association, Dallas, TX. **2003**

Ames, B. M., Shigena, M. K., Hagen, T. M. Oxidants, antioxidants and the degenerative diseases of aging, *proc. Nat. Acad. Sci.*, U.S.A. **1993**, *90*, 7915-7922

Arthur, J. R. and Beckett, G. J. Newer aspects of micro-nutrients in at risk groups: new metabolic roles for selenium. *Proc. Nutr. Soc*. **1994**, *53*, 615-624

Aviram, M. and Dornfeld, L. Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. *Atheroscl*. **2001**,*158*, 195–198

Berry-Ben, B. Health and wealth trends for Canada and the world. *Agric. and agri-food Canada*, **2009**, April, http://www.ats.agr.ca/info/4367-eng.pdf. (Accessed 1 February, 2011)

Bagchi, D. Bagchi, M., Stohs, S. J., Ray, S. D., Sen, C. K. and Preuss, S. J. Cellular Protection with Proanthocyanidins Derived from Grape Seeds. Alcohol and wine in health and disease, *Ann. of the New York Academy of Sci*. **2002,** *957*, 260–270

Balachandran, S., Kentish S. E., Mawson, R., Ashokkumar, M. Ultrasonic enhancement of the supercritical extraction from ginger. *Ultrason. Sonochem.* 2006**,** *6*, 471-479

Balasuriya, B. W. N. and H. P. V. Rupasinghe. Plant flavonoids as angiotensin converting enzyme inhibitors in regulation of hypertension. *Func. Foods in Health and Dis.* **2011**, *5*, 172-188

Balasuriya, N. and Rupasinghe, H. P. V. Antihypertensive properties of flavonoid-rich apple peel extract. *Food Chem*. **2012**, *135(4)*, 2320–2325

Barciela, J., Herrero, C., García-Martín, S., Peña, R. M. A brief study of the role of selenium as antioxidant. *EJEAFChe.* **2008***, 7 (8),* 3151-3155

Basu, A., Du, M., Leyva,M. J., Sanchez, K., Betts, N. M. Wu, M., Aston, E. C. and Lyons, T. J. Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. *J. Nutri*., **2010,** 140(9), 1582-1587

Bazinet, L., Brianceau, S., Dubé, P., Desjardins, Y. Evolution of cranberry juice physico-chemical parameters during phenolic antioxidant enrichment by electrodialysis with filtration membrane. *Sepa. and Purifica. Tech.* **2012**, *87(5)*, 31–39

Bender, D. A. Vitamin B6: Beyond Adequacy. *J Evidence-Based Complemen. & Alternative Medi.* **2011**, *16 (29),* 29-39

Benzie I. F., **Wachtel-Galor, S**. Vegetarian diets and public health: biomarker and redox connections. *Antioxid Redox Signal*. **2010**, *13(10),* 1575-1591

Benzie, I. F. F and Strain, J. J. Ferric reducing (antioxidant) power as a measure of antioxidant capacity: the FRAP assay. In: Packer L (ed) Oxidants and Antioxidants.**1999**, *299* of Methods in Enzymology. Academic Press, Orlando, 15–27

Berry, S. K. Supplemental conditionally essential nutrients in cardiovascular disease therapy. *J. of Cardiov. Nurs*. **2006**, *21(1*), 9-16

Bhandari, U., Sharma, J. N. and Zafar, R. The protective action of ethanolic ginger (Zingiber officinale) extract in cholesterol-fed rabbits. *J. Ethnopharmacol*. **1998**, *61*, 167-171

Bilyk, A. and Sapers, G. M. Varietal differences in the quercetin, kaempferol, and myricetin contents of highbush blueberry, cranberry, and thornless blackberry fruits. *J Agri. Food Chem*. **1986**, *34*, 585–588

Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther*. **2001**, *69*, 89 –95

Boukaiba, N, Flament, C., Acher, S., Chappuis, P., Piau, A., Fusselier, M., Dardenne, M., Lemonnier, D. A physiological amount of zinc supplementation: effects on nutritional, lipid, and thymic status in an elderly population. *Am. J. Clin. Nutr*. **1993**, *57,* 566–572

Boyer, J. and Liu, R. H. Apple phytochemicals and their health benefits-a review. *Nutri. J.* **2004**, *3(5),* 1-15

Brachet, A., Christen, P. and Veuthey, J. L. Focused microwave-assisted extraction of cocaine and benzoylecgonine from coca leaves. *Phytochem. Analy*., **2002**, *13,* 162-169

Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance, a review. *Nutri. Review.* **1998**, *56(11)*, 317-333

Burger, O., Ofek, I., Tabak, M., Weiss, E. I., Sharon N. and Neeman, I. A high molecular constituent of cranberry juice inhibits *Helicobactor pylori* adhesion to human gastric mucus. *FEMS Immunol Med Microbiol.* **2000**, *29*, 295–301

Cai, Y., Luo, Q., Sun, M. and Corke, H. Antioxidant activity and phenolic compounds of 112 Chinese medicinal plants associated with anticancer. *Life Sci.* **2004**, *74*, 2157–2184

Campo, G., Berregi, I., Caracena, R., Santos, J. I. Quantitative analysis of malic acid, citric acid in fruit juices using proton nuclear magnetic resonance spectroscopy. *Analytic Chemica Acta*. **2006**, *556*, 462-468

Cao, Z. F., Chen, Z. G., Guo, P., Zhang, S. M., Lian, L. X., Luo, L., Hu, W. M. Scavenging effects of ginger on superoxide anion and hydroxyl radical. *Zhongguo Zhong Yao Za Zhi*. **1996**. *18*, 750-75

Cappuccio, F. P., Caron A. D., Kautza B. C., Wilson T. Cholesterol lowering effect of low calorie cranberry juice in humans. *FASEB J*. **2005**, *19*, 1009–1010

Cappuccio, F. P, MacGregor, G. A. Does potassium supplementation lower blood pressure? A meta-analysis of published trials. *J. Hyperten.* **1991**, *9*, 465–473

Caron, A. D., Kautza, B. C., Wilson, T. Cholesterol lowering effect of low calorie cranberry juice in humans. *FASEB J.* **2005**, *19*, 1009–1010

Castro, I. A., Barroso, L. P. and Sinnecker, P. Functional foods for coronary heart disease risk reduction: a meta-analysis using a multivariate approach. *Am. J. Clin. Nutr.* **2005**, *82,* 32– 40

Chan, J. R., Bode-Boger, S. M., Tangphao, O. Asymmetric dimethylglycine increases mononuclear cell adhesiveness in hypercholesterolimic humans. *Arterioscl. Thromb. Vasc. Biol.* **2000**, *20*, 1040-1046

Chan, S., Chang, C., Hsu, J., Lee, Y. and Shen, C. Homocysteine, Vitamin B6, and Lipid in Cardiovascular Disease. *Nutri.* **2002**, *18*, 595–598

Chemat, S., Lagha, A., AitAmar, H., Bartels, P. V. and Chemat, F. Comparison of conventional and ultrasound-assisted extraction of carvone and limonene from caraway seeds. *Flavor Frag. J.* **2004**, *19*, 188-195

Chrubasika, S., Pittlerc, M. H. and Roufogalis, B. D. Zingiberis rhizoma: A comprehensive review on the ginger effect and efficacy profiles, *J. Phytomedicine*. **2005***, 12*, 684–701

Chu, Y. and Liu, R. H. Cranberries inhibit LDL oxidation and induce LDL receptor expression in hepatocytes. *Life Sci*. **2005**, *77(15)*, 1892–1901

Cooke, J. P. and Dzau, V. J. Nitric oxide synthase: role in the genesis of vascular disease. *Annual Rev. of medi***. 1997**, *48*, 489-509

Cooke, J. P., Singer, A. H., Tsao, P., Zera, P., Reed, A. Rowan, R. A. and Billinghamt, M. E. Antiatherogenic Effects of L-Arginine in the Hypercholesterolemic Rabbit. *J. Clinic. Invest*. **1992** *90*, 1168-1172

Coppola, E. D., Conrad, E., C., Cotter, R. High pressure liquid chromatographic determination of major organic acids in cranberry juice. *J. Assoc. Off. Anal. Chem.* **1978**, *61(6)*, 1490-1492

Crozier, A., Clifford, M. C. and Ashihara, H. Phenols, Polyphenols and Tannins: An Overview. In: *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet.* Alan Crozier, Indu B. Jaganath, Michael N. Clifford. **2007**, Blackwell Publishing, 1-24

Daugherty, A. Rateri, D., Hong, L. and Balakrishnan, A. Measuring blood pressure in mice using volume pressure recording, a tail-cuff method. *JoVE*, **2009,** http://www.jove com

Decorde, K., Teissedre, P., Auger, C., Cristol, J. and Rouanet, J. Phenolics from purple grape, apple, purple grape juice and apple juice prevent early atherosclerosis induced by an atherogenic diet in hamsters. *Mol. Nutr. Food Res*. **2008**, *52*, 400 – 407

Dessein, P. H., Joffe, B. I. and Singh, S. Biomarkers of endothelial dysfunction, cardiovascular risk factors and atherosclerosis in rheumatoid arthritis. *Arthritis Res. & Therapy***. 2005**, *7*, 634-643

Dong, X., Zhao, M., Shi, J., Yang, B., Li , J., Luo, D., Jiang, G. and Jiang, Y. Effects of combined high-pressure homogenization and enzymatic treatment on extraction yield, hydrolysis and function properties of peanut proteins. *Innov. Food Sci. and Emerging Tech.,* **2011**, *12*, 478–483

Dugas, T. R., Morel, D. W. and Harrison, E. H. Novel cell culture medium used in oxidation esperiments provides insight into mechanism of endothelial cell-mediated oxidation of LDL. *In vitro cell div. Bio.-Animal*. **2000**, *36*, 571-577

Duthie, S. J., Jenkinson, A. M., Crozier, A., Mullen, W., Pirie, L., Kyle, J., Yap, L. S., Christen, P. and Duthie, G. G. The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. *Eur. J. Nutr*. **2006**, *45*, 113–122

Ellington A. A. and Kullo, I. J. Atherogenic lipoprotein sub profiling. *Adv. Clinic. Chem*. 2008, *46*, 295-317

ElRokh, E. M., Yassin, N. A. Z., El-Shenawy, S. M. A., Ibrahim, B. M. M. Antihypercholesterolaemic effect of ginger rhizome (Zingiber officinale) in rats. *Inflammopharmacol.* **2010**, *18*, 309–315

Esterbauer, H., Gebiki, J., Puhl, H. and Jurgens, G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic. Biol. Med*. **1992***, 13*, 341-390

Fki I., Bouaziz, M., Sahnoun, Z., Sayadi, S. Hypocholesterolemic effects of phenolic-rich extracts of Chemlali olive cultivar in rats fed a cholesterol-rich diet. *Bioorg Med. Chem*. **2005**, *13*, 5362–70

Foo, L. Y., Lu, Y., Howell, A. B. and Vorsa, N. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated Escherichia coli in vitro. *Phytochem.* **2000**, *54*, 173–181

Fotherby, M. D. and Potter, J. F. Potassium supplementation reduces clinic and ambulatory blood pressure in elderly hypertensive patients. *J. of Hyperten*. **1992**, *10(11),* 1403-1408

Fraga, C. S. Plant Phenolics and Human Health, John Wiley and Sons, Hoboken, USA, **2009**

Friso, S., Lotto, V., Corrocher, R. and Choi, S. W. Vitamin B6 and cardiovascular disease. *Sub-cellular biochem.*, **2012**, *56*, 265-290

Fuhrman, B., Rosenblat, M, Hayek, T., Coleman, R. and Michael Aviram, M. Ginger Extract Consumption Reduces Plasma Cholesterol, Inhibits LDL Oxidation and Attenuates Development of Atherosclerosis in Atherosclerotic, Apolipoprotein E-Deficient Mice. *J. of Nutri.* **2000,** *130,* 1124-1131

Galaverna, G., Silvestro, G. D., Cassano, A., Sforza, S., Dossena, A., Drioli, E., Marchelli, R. A new integrated membrane process for the production of concentrated blood orange juice; effect on bioactive compound and antioxidant activity. *Food Chem.* **2008***,106*, 1021-1038

Gallaher, D. D. Animal Models in Human Nutrition Research. *Nutr. Clin. Pract.* **1992**, *7(1),* 37-39

Gao, J. And Rupasinghe, H. P. V. Nutritional, Physicochemical and Microbial Quality of Ultrasound-Treated Apple-Carrot Juice Blends. *Food and Nutri. Sci*., **2012**, *3*, 212-218

Gersh, B. J., Sliwa, K., Mayosi, B. M. & Yusuf, S. Novel therapeutic concepts: the epidemic of cardiovascular disease in the developing world: global implications. *Eur. Heart J.* **2010**, *31*, 642–648

Ghasemzadeh, A., Jaafar, H. Z. E.and Rahmat A. Antioxidant Activities, Total Phenolics and Flavonoids Content in Two Varieties of Malaysia Young Ginger (*Zingiber officinale* Roscoe). *Molecul.* **2010**, *15*, 4324-4333

Ghayur, M. N. and Gilani, A. H. Ginger lowers blood pressure through blockade of voltage-dependent calcium channels. *J. Cardiovasc.Pharmacol.***2005**, *45*, 74– 80

Ghayur, M. N., Gilani, A. H. and Janssen, J. L. Ginger attenuates acetylcholine-induced contraction and Ca^{+2} signalling in murine airway smooth muscle cells. *Can. J. Physiol. Pharmocol.* **2008,** *86*, 264-271

Ghayur, M. N., Gilani, A. H., Afridi, M. B., Houghton, P. J. Cardiovascular effects of ginger aqueous extract and its phenolic constituents are mediated through multiple pathways. *Vascul. Pharmacol*. **2005**, *43*, 234–241

Girard, B. and Fukumoto, G. R. Membrane processing of fruit juice and beverages. *Crit. review in Food sci. Nutri.* **2000,** *40(2)*, 91-157

Girard, B. and Fukumoto, L. R. Apple juice clarification using microfiltration and ultrafiltration polymeric membrane, *Lebensm,-wiss u-technol.* **1999***, 32,* 290-298

Gliszczynska-Swiglo, A. and Tyrakowska, B. Quality of Commercial Apple Juices Evaluated on the Basis of the Polyphenol Content and the TEAC Antioxidant Activity. *J. of Food Sci*. **2003**, *68(5),* 1844–1849

Gokmen, V., Artik, N., Acar, J., Kahraman, N., Poyrazoglu. Effects of various clarification treatments on patulin, phenolic compound and organic acid compositions of apple juice. *Eur. Food Res. Technol.* **2001,** *213*, 194–199

Goli, A. H., Barzegar, M., & Sahari, M. A. Antioxidant activity and total phenolic compounds of pistachio (*Pistachia vera*) hull extracts. *Food Chem*. **2004**, *92*, 521–525

Gollaher, D. D. Animal models in human nutrition research. *Nutri. in Clinical Prac*. **1992**, *7 (1),* 37-39

González-Molina, E., Moreno, D. A. and García-Viguera, C. Genotype and Harvest Time Influence the Phytochemical Quality of Fino Lemon Juice (*Citrus limon* (L.) Burm. F.) for Industrial Use. *J. Agric. Food Chem.* **2008**, *56* (5), 1669–1675

González-Molina, E., Moreno, D.A. and García-Viguera, C. A new drink rich in healthy bioactives combining lemon and pomegranate juices. *Food Chem.* **2009**, *115*, 1364–1372

Gonzalez-Molina, E., Moreno, D.A. and Garcia-Viguera, C. Aronia-enriched lemon juice: A new highly antioxidant beverage. *J. Agric. Food Chem.* **2008**, *56,* 11327–11333

Gorelik, S., Ligumsky, M., Kohen, R. and Kanner, J. A novel function of red wine polyphenols in humans: prevention of absorption of cytotoxic lipid peroxidation products. The *FASEB J. Research Communication*, **2008**, 22, 41–46

Granato, D., Branco, G. F., Nazzaro, F. Cruz, A. G. and Faria, A. F. Functional foods and non dairy probiotic food development trends, concepts and products. *Compreh. review in Food Sci. Food Safety.* **2010,** *9*

Grassi, D., Desideri, G., Croce, G., Tiberti, S., Aggio, A. and Ferri, C. Flavonoids, vascular Function and Cardiovascular Protection. *Cur. Pharm. Design*, **2009**, *15(10)*, 1072-1084

Grzanna, R., Lindmark, L. and Frondoza, C. Ginger-an herbal medicinal product with broad anti-inflammatory action. *J. Med. Food*. **2005**, *8*, 367-371

Gu, L., Kelm, M. A. and Hammerstone, J. F. Concentration of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr*. **2004**, *134*,613–617

Gurak, P. D., Cabral, L. M. C., Rocha-Leão, M. H. M., Matta, V. M. and Freitas, S. P. Quality evaluation of grape juice concentrated by reverse osmosis. *J. Food Engin*. **2010**, *96 (3)*, 421–426

Halliwell, B. The definition and measurement of antioxidants in biological systems. *Free Rad. Biol. Med*. **1995**, *18*, 125-126

Harada, H., Tsujino, T., Watari, Y., Nonaka, H., Emoto, N., Yokoyama, M. Oral taurine supplementation prevents fructose-induced hypertension in rats. *Heart Vess.*, **2004**, *19*,132-136

He, X., Bernart, M. W., Lian, L., Lin, L. High-performance liquid chromatography–electrospray mass spectrometric analysis of pungent constituents of ginger. *J.of Chromat. A*.**1998**, *796(2)*, 327–334

Heath Canada. Nutraceutical/functional foods and health claims on foods: policy paper. (Available online: http://www.hc-sc.gc.ca/fn-an/label-etiquet/claims-reclam/nutra-func_foods-nutra-funct_aliment-eng.php) (Accessed on Oct. 20, 2010)

Hertog, M. G. L, Kromhout D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F.,Giampaoli, S., Jansen, A., Menotti, A. and Nedeljkovic, S. Flavonoid intake and long term risk of cardiovascular disease in the Seven Countries Study. *Arch. Intern. Med*. **1995**, *155*, 381–386

Houston, M. C. The role cellular micronutrient analysis, nutraceuticals, vitamins and antioxidants and minerals in the prevention and treatment of hypertension and cardiovascular disease. *Therap. adv. in cardiovas. Dis.* **2010**, *4 (3)*, 165-183

Houston, M. C. and Harper, K. J. Potassium, magnesium, and calcium: their role in both the cause and treatment of hypertension. *J. Clin. Hypertens*, **2008**, *10*, 3-11

Houston, M. C. Nutraceuticals, vitamins, antioxidants and minerals in the prevention of hypertension. *Progr. in Cardiovas. Dis.* **2005***, 47(6)*, 336-449

Houston, M. C. The role of mercury and cadmium heavy metals in vascular disease, hypertension, coronary heart disease, and myocardial infarction**.** *Altern. Ther. Health Med*. **2007**, *13*, 128–133

Hurson, M. Regan, M. C. and Kirk, S. J. Metabolic effects of arginine in a healthy elderly population. *JPEN*. **1995**, *19*, 227-230a

Hurson, M., Regan, M. C., Kirk, S. J., Wasserkrug, H. L. and Barbul, A. Metabolic effects of arginine in a healthy elderly population. *JPEN J. Parenter. Enter. Nutr***. 1995**, *19*, 227–230b

Huxley, R. R. and Neil, H. A. W. The relation between dietary flavonol intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies. *Europ. J. Clin. Nutri.* **2003**, *57*, 904–908

Hwang, S. J, Ballantyne, C. M., Sharrett, A. R., Smith, L. C., Davis, C. E., Gotto, A. M. and Boerwinkle, E. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases. *Circul.* **1997**, *96,* 4219-4225

Ippoushi, K., Azuma, K., Ito, H. Horie, H. and Higashio, H. [6]-Gingerol inhibits nitric oxide synthesis in activated J774.1 mouse macrophages and prevents peroxynitrite-induced oxidation and nitration reactions. *Life Sci*. **2003**, *73(26)*, 3427-3437

Ito, T and Azuma, J. Taurine is a possible anti-atherosclerotic agent. *Nippon Yakurigaku Zassh.,* **2004**, *123*, 311-317

Jalili, T., Carlstrom, J., Kim, S., Freeman D., Jin, H., Wu, T. C., Litwin, S. E. and David, S. J. Quercetin-supplemented diets lower blood pressure and attenuate cardiac hypertrophy in rats with aortic constriction. *J. Cardiov. Pharmacol*. **2006**, *47*, 531-541

Jemai, H., Fki, I., Bouaziz, M., Bouallagui, Z., El Feki, A., Isoda, H., Sayadi, S. Lipid lowering and antioxidant effects of hydroxytyrosol and its triacylated derivative recovered from olive tree leaves in cholesterol-fed rats. *J. Agric. Food Chem*. **2008**, *56,* 2630-2636

Jesus, D. F., Leite, M. F., Silva, L. F. M., Modesta, R. D., Matta, V. M. and Cabral, L. M. C. Orange (*Citrus sinensis*) juice concentration by reverse osmosis. *J. Food Engin*. **2007,** *81 (2),* 287–291

Ji, L. L. Antioxidants and oxidative stress in exercise. *Proc. Soc.Exp. Biol. Med.* **1999**, *222*, 283–292

Jiaial, I. and Grundy, S. M. The effect of dietary supplementation with alpha-tocopherol in the oxidative modification of LDL. *J. Lipid Res.* **1992**, *33*, 889-906

Jolad, S. D., Lantz, R. C., Solyon, A. M., Chen, G. J., Bates, R. B. and Timmermann, B. N. Fresh organically grown ginger (Zingiber officinale): composition and effects on LPS-induced PGE2 production. *Phytochem.* **2004**, *65*, 1937–1954

Joshipura, K. J., Hu, F. B., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., Colditz, G., Ascherio, A., Rosner, B., Spiegelman, D. and Willett, W. C. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann. Intern. Med.* **2001**, *134*, 1106–1114

Kalea, A. Z., Clark, K., Schuschke, D. A. and Klimas.zakas. D. Vascular Reactivity Is Affected by Dietary Consumption of Wild Blueberries in the Sprague-Dawley Rat**.** *J. of Medi. Food*. **2009**, *12(1),* 21-28

Kaliora, A. C., Dedoussis, G. V. Z and Schmidt, H. Dietary antioxidants in preventing atherogenesis-A review. *J. Atherosc.* **2006** *187*, 1-17

Kalt, W. and Dufour, D. Health functionality of blueberries. *HortTech*. **1997**, *7*, 216–221

Kalt, W. and McDonald, J. E. Chemical Composition of Lowbush Blueberry Cultivars**.** *J. Am. Soc. Hort. Sci.* **1996**, *121(1)*, 142-146

Kalt, W., Fillmore, S. A. E., Mcrae, K. B., Foote, K., Lyon, M., and Van Lunen, T. A. Effect of blueberry feeding on plasma lipids in pigs. *British J of Nutri*. **2008**, *100(1),* 70-78

Kalt, W., Ryan, D. A. J., Duy, J. C. Prior, R. L. Ehlenfeldt, M. K. and Kloet, S. P. V. Interspecific Variation in Anthocyanins, Phenolics, and Antioxidant Capacity among Genotypes of Highbush and Lowbush Blueberries (*Vaccinium* Section *cyanococcus* spp.) *J. Agric. Food Chem.*, **2001**, *49* (10), 4761–4767

Kannel, W. B, Castelli, W. P. and Gordon, T. Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham study. *Ann. Intern. Med* .**1979**, *90*, 85–91

Kaplan, M. and Aviram, M. Oxidized low density lipoprotein: Atherogenic and proinflammatory characteristics during macrophage foam cell formation. An inhibitory role for nutritional antioxidants and serum paraoxonase. *Clinical Chem. & Lab. Medi*., **1999**, 37, 777–787

Katsube, T., Tabata H. and Ohta, Y. Screening for antioxidant activity in edible plant products; comparison of LDL oxidation assay, DPPH assay and FC assay. *J. Agric. Food Chem*. **2004,** *52,* 2391-2396

Kaur, C. and Harish, C. K. Antioxidants in fruits and vegetables-the millennium's health. *Intern. J. Food Sci.Tech.* **2001,** *36*, 703-725

Kay, C. D. and Holub, B. J. The effect of wild blueberry (Vaccinium angustifolium) consumption on postprandial serum antioxidant status in human subjects. *British J. Nutri.* **2002,** *88*, 389–397

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K., and He, J. Global burden of hypertension: analysis of worldwide data*. Lancet.* **2005**, *365(9455)*, 217–223

Kim, S. O., Kundu, J. K., Shin, Y. K., Park, J. H., Cho, M. H. and Kim, T. Y. [6]-Gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-kappaB in phorbol ester-stimulated mouse skin. *Oncogene*. **2005**, *24*, 2558–2567

Kishk, Y. F. M. and Sheshetawy, H. E. Optimization of ginger (*Zingiber officinale*) phenolics extraction conditions and its antioxidant and radical scavenging activities using response surface methodology. *World J. of Dairy & Food Sci.* **2010**, *5(2),* 188-196

Kita, T., Ishii, K. Yokode, M., Kume, N. Nagano, Y. and Arai, H. and Kawai, C. The role of oxidized low density lipoprotein in the pathogenesis of atherosclerosis**.** *Eur Heart J***. 1990,** *11*, 122-127

Kitts, D. D., Wijewickreme, A. N. and Hu, C. Antioxidant properties of a North American ginseng extract. *Mole. and Cellular Biochem*. **2000**, *203(12)*, 1-10

Ko, S. H., Choi, S. W., Ye, S. K., Cho, B. L., Kim, H. S. and Chung, M. H. Comparison of the antioxidant activities of nine different fruits in human plasma.*J. Med. Food.* **2005**, *8*, 41–46.

Kondo, Y., Toda, Y., Kitajima, H., Oda H., Nagate, T., Kameo, K. and Murakami, S. Taurine inhibits development of atherosclerotic lesions in apolipoprotein E-deficient mice. *Clin. Exp. Pharmacol. Physiol.* **2001,** *28(10),* 809-815

Kota, N., Krishna, P., and Polasa, K. Alterations in antioxidant status of rats following intake of ginger through diet. *Food Chem*. **2008**, *106*, 991−996

Kugiyama, K., Kerns S. A., Morrisett, J. D., Roberts, R., Henry, and P. D. Impairment of endothelium -dependent aerial relaxation by lysolecithin in modified low density lipoproteins. *Nature***. 1990**, *344*, 160-162

Kundu, S. and Rao, J. P. The story of spontaneously hypertensive rat (SHR): a review. *Al Ameen J. Med. Sci.* **2008**, *1(1),* 65-66

Langner, E., Greifenberg, S. and Gruenwald, J. Ginger: history and use. *Adv. Ther*. **1998,** *15*, 25– 44

Leontowicz, M., Leontowicz, H., Drzewiecki, J., Jastrzebski, Z., Haruenkit, R., Poovarodom, S, Park, Y. S., Jung, S. T. Kang, S. G., Trakhtenberg, S., Gorinstein, S. Two exotic fruits positively affect rat's plasma composition, *Food Chem*. 2007, *102*, 192–200

Lerman, A., Burnett, J. C. and Higano, S. T. Long-term L-arginine supplementation improves the small vessal coronary endothelial function in humans. *Circul***. 1998***, 98*, 2123-2128

Lerouet D., Beray-Berthat V., Palmier B., Plotkine M. and Margaill I. Changes in oxidative stress, iNOS activity and neutrophil infiltration in severe transient focal cerebral ischemia in rats. *Brain Res*. **2002**, *958*, 166 –175

Libby, P., Ridker, P. M. and Maseri, A. Inflammation and atherosclerosis. *Circul.* **2002**, *105*, 1135-1143

Liu, R. H. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemical.*Am. J. Clin. Nutr.* **2003**, *78(3)*, 517-520

Liu, R. H. Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. *J. Nutr*. **2004**, *134(12)*, 3479-3485

Lopez-Nicolas, J. M., Perez-Lopez, A. J., Carbonell-Barrachina, A. and Garcia-Carmona, F. Use of natural and modified cyclodextrins as inhibiting agents of peach juice enzymatic browning. *J. of Agri. and Food Chem*. **2007**, *55*, 5312–5319

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Boil. Chem*, **1951**, *193*, 165-275

MacGregor, G. A. Does potassium supplementation lower blood pressure? A meta-analysis of published trials. *J. of Hypert.* **1991**, *9(5),* 465-473

MacIntosh, R. I. W., Rupasinghe, H. P. V., Embree C. and Erith, D. Apple-based bio-products: Market and industry development analysis. Phase 1: Overview of existing market for traditional apple products. Nova Scotia Fruit Growers Association, Nova Scotia, Canada, **2006,** *79*

Madamanchi, N. R., Vendrov A. and Runge, M. S. Oxidative Stress and Vascular Disease. *Arterioscl. Thromb Vasc. Biol,* **2005**, *25*, 29-38

Maher, M. A, Mataczynski, H., Stefaniak, H. M. and Wilson, T. Cranberry juice induces nitric oxide-dependent vasodilation in vitro and its infusion transiently reduces blood pressure in anesthetized rats. *J. Med. Food*. **2000**, *3,* 141–147

Main, G., Faupel, M., Morris, J. and Mcnew, R. Quality and stability of blueberry juice blended with apple, grape and cranberry juice. *J. of Food Quali.* **2011**, *24*, 111-125

Maizura, M., Aminah, A. and Wan Aida, W. M. Total phenolic content and antioxidant activity of kesum (*Polygonum minus*), ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) extract. *Interna. Food Res. J.* **2011***, 18, 529-534*

Manore, M. M. Effect of physical activity on thiamine, riboflavin and vitamin B-6 requirements. *Am. J. Clini. Nutri*. **2000**, *72 (2),* 598-606

Manuel, D. G., Leung, M., Nguyen, K., Tanuseputro, P. and Johansen, H. Burden of cardiovascular disease in Canada. *Can. J. Cardiol*. **2003**, *19(9),* 997-1004

Marks S. C., Mullen W. and Crozier, A. Flavonoid and chlorogenic acid profiles of English cider apples. *J. Sci. Food Agric.* **2007***, 87*,719–728

Marx G. and Eldor A. The procoagulant effect of zinc on fibrin clot formation. *Am. J. Hematol*. **1985**, *19*, 151–159

Matsushima, Y., Sekine, T., Kondo, Y., Sakurai, T., Kameo, K. Tachibana, M. and Murakami, S. Effects of taurine on serum cholesterols and development of atherosclerosis in spontaneously hyperlipidaemic mice. *Clinical and Experi. Pharmaco. and Physic.* **2003**, *30,* 295–299

McKay, D. L. and Blumberg, J. B. Cranberries (*Vaccinium macrocarpon*) and cardiovascular disease risk factors. *Nutr. Rev*. **2007**, *65(11),* 490-502

Meilgaard, M., Civille, G.V. and Carr, B. Sensory Evaluation Techniques, 2nd Ed., **1991**, Boca Raton, Florida, USA

Melo, M. D., Grespan, G. A. R., Fonseca, J., Farinha, T., Silva, E. L. D. and Romero, A. Inhibitory effects of ginger (Zingiber officinale Roscoe) essential oil on leukocyte migration in vivo and in vitro. *J. Nat. Med*. **2011**, *65*, 241-246

Mertens A., Verhamme P., Bielicki, J., Phillips, M., Quarck, R., Verreth, W., Stengel, D., Ninio, E, Navab, M., Mackness, B., Mackness, M. and Holvoet, P. Increased low-density lipoprotein oxidation and impaired high-density lipoprotein antioxidant defense are associated with increased macrophage homing and atherosclerosis in dyslipidemic obese mice:LCAT gene transfer decreases atherosclerosis. *Circul.*, **2003**, *107*, 1640–1646

Middleton, E. and Kandaswamy, C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer, in *The Flavonoids: Advances in Research Since 1986* (Harbone J. B., ed), **1993**. 619–652. Chapman & Hall, London

Mintel, Functional beverages-US August. Market research report of Mintel International Group Ltd, US, **2007**

Mira, L., Fernandez, .M. T., Santos, M., Rocha, R., Florêncio, M. H. and Jennings, K. R. Interactions of Flavonoids with Iron and Copper Ions: A Mechanism for their Antioxidant Activity. *Free Radi, Res*. **2002**, *36 (11),* 1199-1208

Mirolla, M. The Cost of Chronic Disease in Canada. *The Chronic Disease Prevention Alliance of Canada (CDPAC).* **2004**

Miyazaki, H., Shoji, H. and Lee, M. C. Measurement of oxidative stress in stroke-prone spontaneously hypertensive rat brain using in vivo electron spin resonance spectroscopy. *Redox Rep*. **2002**, *7,* 260 –265

Miyazaki, T., Shimada, K., Sato, O., Kotani, K., Kume, A., Sumiyoshi, K., Sato, Y., Ohmura, H., Watanabe, Y., Mokuno, H. and Daida, H. Circulating malondialdehyde-modified LDL and atherogenic lipoprotein profiles measured by nuclear magnetic resonance spectroscopy in patients with coronary artery disease. *Atheroscler.* **2005**,*179*, 139 –145

Montgomery, D. C. Introduction to Factorial Designs, *Design and Analysis of Experiments*, John Wily and Sons, Inc., New York, **2005**

Morelli, L., L., L. and Prado, M. A. Extraction optimization for antioxidant phenolic compounds in red grape jam using ultrasound with a response surface methodology. *Ultrasonics Sonochem*.**2012,** in press

Morton, L. W. Caccetta, R. A. Puddey, I. B. and Croft, K. D. Chemistry and biologically effects of dietary phenolic compounds relevance to cardiovascular disease. *Clin. and Experim. Pharmaco. Physio.* **2008**, *27(3)*, 152-159

Moure, A., Cruz, J. M., Franco, D., Domı´nguez, J. M., Sineiro, J., Domı´nguez, H., Nu´n˜ez, M. J. and Parajo´, J. C. Natural antioxidants from residual sources. *Food Chem*. **2001**, *72*, 145-171

Moure, A., Franco, D., Sineiro, J., Dominguez, H., Nunes, M. J. and Lema, J. M. Antioxidant activity of extracts from Gevuina avellana and Rosa rubiginosa defatted seeds. *Food Res. Intern.* **2001**, *34*, 103–109

Natella, F., Fidale, M., Tubaro, F., Ursini, F. and Scaccini, C. Selenium supplementation prevents the increase in atherogenic electronegative LDL (LDL minus) in the postprandial phase. *Nutri. Metabol. Cardiovas. Dis.* **2007,** *17*, 649-656

Navab, M., Berliner J. A., Watson A. D., Hama, S. Y., Territo, M. C., Lusis, A. J., Shih, D. M., Van- Lenten, B. J., Frank, J. S., Demer, L. L., Edwards, P. A., and Fogelman, A. M. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. *Arterios. Thromb. Vasc Biol*. **1996**, *16*, 831– 842

Neto, C. C. Cranberry and blueberry: Evidence for protective effects against cancer and vascular diseases. *Mol. Nutri. & Food Res.* **2007,** *51(6)*, 652–664

Nicoll, R. and Henein, M. Ginger (*Zingeber Officinale* Rescore) a hot remedy for cardiovascular disease? *Intern. J. Cardiol*. **2009**, *131*, 408-409

Nijveldt, R. J., Nood, E. V., Hoorn, D. E. C. V, Boelens, P. G., Norren, K. V. and Leeuwen, P. A. M. V. Flavonoids: a review of probable mechanisms of action and potential applications**.** *Am. J. of Clin. Nutri.* **2001**, *74(4),* 418-425

Park, S., Bok, S., Jeon, S., Park, Y. B., Lee, S., Jeong, T. and Choi, M. Effect of rutin and tannic acid supplements on cholesterol metabolism in rats. *Nutri. Res.* **2002**, *22(3)*, 283–295

Pearson, D. A., Tan, C. H., German, J. B., Davis, P. A. and Gershwin, M. E. Apple juice inhibits human low density lipoprotein oxidation. *Life Sci.* **1999**, *64(21*), 1913–1920

Pedersen, C. B., Kyle, J., McE Jenkinson, A., Gardner, P. T., McPhail D. B. and Duthie, G. G. Effects of blueberry and cranberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. *Europ. J. of Clinical Nut*. **2000**, *54*, 405-408

Pérez-Conesa, D., García-Alonso, J., García-Valverde, V., Iniesta, M. D., Jacob, K., Sánchez-Siles, L. M., Ros, Gaspar and Periago, M. J. Changes in bioactive compounds and antioxidant activity during homogenization and thermal processing of tomato puree. *Innova. Food Sci. & Emerging Tech*. **2009**, *10*, 179–188

Pingret, D., Fabiano-Tixier, A., Bourvellec, C. L. Renard, C.M.G.C. and Chemat, F. Lab and pilot-scale ultrasound-assisted water extraction of polyphenols from apple pomace. *J. Food Engin.* **2012**, *111*, 73–81

Ponthieux, A., Herbeth, B., Droesch, S., Haddy, N., Lambert, D. and Visvikis, S. Biological determinants of serum ICAM-1, E-selectin, Pselectin and L-selectin levels in healthy subjects: the Stanislas study. *Atheroscle.* **2004**, *172*, 299-308

Preli, R. B., Klein, K. P. and Herrington, D. M. Vascular effects of dietary L-arginine supplementation. *Atheroscle.* **2002**, *162*, 1-15

Prior, R. L., Gu, L., Wu, X., Jacob, R. A., Sotoudeh, G., Kader, A. A., Cook, R. A. Plasma antioxidant capacity changes following a meal as a measure of the ability of a food to alter in vivo antioxidant status. *J. Am. Coll. Nutr*. **2007**, *26*, 170–181

Pu, Q., Neves, F., Virdis, A., Touyz, R. M. and Schiffrin, E. L. Effect of endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. *Hyperten*.**2003**, *42*, 49-55

Public Health Agency of Canada. Tracking heart disease and stroke in Canada 2009, available on Internet at the following address: www.phac-aspc.gc.ca

Quinn, M.T., Parthasarathy, S. and Steinberg, D. Endothelial cell-derived chemometric activity for mouse peritoneal macrophages and the effects of modified forms of low density lipoprotein. *Proc. of the National Academy of Sci. of the USA*. **1985***, 82*, 5949-5953

Ramachandran, S. and Vasan, R. S. Biomarkers of Cardiovascular Disease: Molecular Basis and Practical consideration. *Circul*. **2006**, *113*, 2335-2362

Reaven, P. D. and Witztum, J. L. Oxidized Low Density Lipoproteins in Atherogenesis: Role of Dietary Modification. *Annual Review of Nutri.* **1996***, (16)*, 51-71

Reed, J. Cranberry flavonoids, atherosclerosis, and cardiovascular health. *Crit. Rev. Food Sci. Nutr*. **2002**, *42*, 301–316

Reed, J. D., Krueger, C. G., and Porter, M. L. Cranberry juice powder decreases low density lipoprotein cholesterol in hypercholesterolemic swine, *FASEB J.* **2001**, *15(45),* 54

Reilly, C. Selenium in Food and Health. **1996**, Blackie Academic and Professional, London

Renaud, D. and Lorgiril, M. D. Wine, alcohol, platelets, and the French Paradox for coronary heart disease. *Lancet.* **1992***, 339*, 1523-1525

Richardson, D. P. The addition of nutrients to foods. *Proc. Nutr. Soc*. **1997**, *56*, 807-825

Rimando A.M. and Cod, R. Determination of Stilbenes in Blueberries. *LCGC North Ameri.*, **2005**, *23(11),* 1192-1200

Rimando, A. M., Nagmani, R., Feller, D. R. and Yokoyama, W. Pterostilbene, a new agonist for the peroxisome proliferatoractivated receptor a-isoform, lowers plasma lipoproteins and cholesterol in hypercholesterolemic hamsters, *J. Agric. Food Chem*. **2005**, *53*, 3403–3407

Rimm, E. B., Willett, W. C., Hu, F. B., Sampson, L., Colditz, G. A., Manson, J. E., Hennekens, C. and Stampfer, M. J. Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women. *JAMA*. **1998**, *279(5),* 359-364

Rodrigues, S., Pinto, G. A. S. and Fernandes, F. A. N. Optimization of ultrasound extraction of phenolic compounds from coconut (*Cocos nucifera*) shell powder by response surface methodology. *Ultrason. Sonochem*. **2008**, *15*, 95–100

Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. **1993**, *362*, 801– 809

Ruel, G., Pomerleau, S., Couture, P., Lamarche, B., and Couillard, C. Changes in plasma antioxidant capacity and oxidized low-density lipoprotein levels in men after short-term cranberry juice consumption. *Metabol.* **2005,** *54(7)*, 856-61

Ruel, G., Pomerleau, S., Couture, P., Lemieux, S. and Lamarche, B. Favourable impact of low calorie cranberry juice consumption on plasma HDL-cholesterol concentration in men. *British J. Nutri*. **2006**, *96*, 357-364

Rupasinghe , H. P. V, Kathirvel, P. and Huber, G. M. Ultra-Sonication-Assisted solvent extraction of quercetin glycosides from 'Idared' apple peels. *Molecul.* **2011**, *16*, 9783-9791

Rupasinghe, H. P. V. The role of polyphenols in quality, postharvest handling, and processing of fruits. In: *Postharvest Biology and Technology of Fruits, Vegetables and Flowers* (Ed.) Paliyath, G., Murr, D.P., Handa, A.K., and S. Lurie, S. **2008**, Wiley-Blackwell Publishing. 260-281

Rupasinghe, H. P. V., Wang, L., Huber G. M. and Pitts, N. L. Effect on baking on dietary and phenolics of muffins incorporated with apple skin powder. *Food Chem.* **2008**, *107*, 1217-1224

Ruz M., Cavan K. R., Bettger W. J., Fischer P. W. and Gibson R. S. Indices of iron and copper status during experimentally induced, marginal zinc deficiency in humans. *Biol Tr Elem Res.* **1992**, *34,* 197–212

Ryan, L. and Prescott, S. L. Stability of the antioxidant capacity of twenty-five commercially available fruit juices subjected to an in vitro digestion. *Intern. J of Food Sci. and Tech.* **2010**, *45(6),* 1191–1197

Ryu, B. H. Low Density Lipoprotein (LDL), Atherosclerosis and Antioxidants. *Biotechnol. Bioprocess Eng*. **2000**, *5*, 313-319

Samman, S. Zinc and copper. In Mann and Truswell A**S** (eds): "Essentials of Human Nutrition," 2nd ed. New York: Oxford University Press, **2002**, 159–166

Santos, M. R. and Mira, L. Protection by Flavonoids Against the Peroxynitrite-mediated Oxidation of Dihydrorhodamine. *Free Radi, Res*. **2004**, *38 (9*), 1011-1018

Saravanan, K. and Aradhya, S. **M.** Potential nutraceutical food beverage with antioxidant properties from banana plant bio-waste (pseudostem and rhizome). *Food Func.* **2011**, *2*, 603-610

Seeram, N. P, Aviram, M., Zhang, Y., Henning, S. M., Feng, L., Dreher, M. and Heber, D. Comparison of Antioxidant Potency of Commonly Consumed Polyphenol-Rich Beverages in the United States. *J. Agric. Food Chem.* **2008**, *56,* 1415–1422

Selloum, L., Reichl, S., Müller, M., Sebihi, L. and Arnhold, J. Effects of Flavonols on the Generation of Superoxide Anion Radicals by Xanthine Oxidase and Stimulated Neutrophils. *Arch. of Biochem. & Biophy*. **2001,** *395(1)*, 49–56

Setorki, **M., Asgary**, **S**., **Eidi, A.**, R**ohani, A. H**., **and Esmaeil, N.** Effects of apple juice on risk factors of lipid profile, inflammation and coagulation, endothelial markers and atherosclerotic lesions in high cholesterolemic rabbits. *Lipids in Health. and Dis.* **2009**, 8*(39)*, 1518-1525

Shan, B., Cai, Y. Z., Sun, M. and Corke, H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J. of the Agr.l and Food Chem.* .**2005**, *53*, 7749–7759

Shao, A. and Hathcock, J. N. Risk assessment of for the amino acids taurine, L-glutamine and L-arginine. *Regul. Toxico. Pharmocol*. **2008,** *50,* 376-399

Shaughnessy, K. S., Boswall, I. A., Scanlan, A. P., Gottschall-Pass, K. T. and Sweeney, M. I. Diets containing blueberry extract lower blood pressure in spontaneously hypertensive stroke-prone rats. *Nutri. Res.* **2009**, *29*, 130–138

Shukla, Y. and Singh, M. Cancer preventive properties of ginger: A brief review. *Food Chem. Toxico.* **2007**, *45*, 683–690

Singleton, V. L., Orthofer, R. and Lamuela-Raventos, R. Analysis of total phenols and other oxidation substrates and antioxidants by means of FC reagent. *Methods in Enzymo.* **1999,** *29,* 152-178

Siró, I., Kápolna, E., Kápolna, B., Lugasi, A. Functional food. Product development, marketing and consumer acceptance, a review. *Appetite*. **2008**, *51(3)*, 456–467

Sirtori, C. R., Galli, C., Anderson, J. W., Sirtori, E. and Arnoldi, A. Functional foods for dyslipidaemia and cardiovascular risk prevention. *Nutri. Res. Revi.* **2009**, *22(2)*, 244-261

Smith, M. A. L., Marley, K. A. Seigler, D. Singletary, K. W. and Meline, B. Bioactive properties of wild blueberry fruits. *J. Food Sci.* **2000***, 65(2)*, 352-356

Soria, A.C. and Villamiel, M. Effect of ultrasound on the technological properties and bioactivity of food: a review. *Trends in Food Sci. & Tech.* **2010**, *21*, 323-331

Spigno, G., Tramelli, L., Faveri, and D. M. D. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *J. of Food Engin*. **2007**, *81*, 200–208

Srivastava K. C. and Mustafa T. Ginger (*Zingiber officinale*) and rheumatic disorders. *Med Hypoth*. **1989**, *29(1),* 25–28

Stanner, S. ed. Cardiovascular Disease: Diet, Nutrition and Emerging Risk Factors. The Report of the British Nutrition Foundation Task Force. **2005**, Blackwell Science for the British Nutrition Foundation: Oxford, UK

Statistics Canada. 2011. Available from http://www.statcan.gc.ca/pub/22-003-x/2010001/t030-eng.htm **[accessed 18 December 2011]**

Stocker, R. and Keaney, J. F. Role of Oxidative Modifications in Atherosclerosis. *Physiol Rev*. **2004***, 84*, 1381-1478

Stoclet, J., Chataigneau, T., Ndiaye, M., Oak, M., Bedoui, J. E., Chataigneau, M. and Schini-Kerth, V. B. Vascular protection by dietary polyphenols. *Europ. J Pharm.* **2004**, *500*, 299– 313

Stoilova, I., Krastanov, A., Stoyanova, A., Denev, P. and Gargova, S. Antioxidant activity of a ginger extract (Zingiber officinale*). Food Chem.* **2007**, *102*, 764-770

Suárez-Jacobo, A., Rüfer, C. E., Gervilla, R., Guamis, B., Roig-Sagués, A. X. and Saldo, J. Influence of ultra-highpressure homogenisation on antioxidant capacity, polyphenol and vitamin content of clear apple juice. *Food Chem.* **2011**, *127(2),* 447–454

Suekawa, M., Ishige, A., Yuasa, K., Sudo, K., Aburada, M., Hosoya. Pharmacological studies on ginger. I. Pharmacological actions of pungent constituents, (6)-gingerol and (6)-shogaol. *J. Pharmacobiodyn*. **1984**, *7*, 836–848

Sun, J., Chu, Y., Wu, X. and Liu, R. H. Antioxidant and antiproliferative activities of common fruits**.** *J. Agric. Food Chem.* **2002,** *50***,** 7449-7454

Tannen, R. L. Effects of potassium on blood pressure control. *Ann. Intern. Med.* **1983**, *98*, 773-780

Tapsell, L. C., Hemphill, I., Cobiac, L., Patch, C. S., Sullivan, D. R., Fenech, M., Roodenrys, S., Keogh, J. B., Clifton, P. M., Williams, P. G., Fazio, V. A. and Inge, K. E. Health benefits of herbs and spices: the past, the present, the future. *Med. J. Aust.* **2006,** *185(4)*, 4-24

Tebib, K., Bitri, L., Besançon, P. and Rouanet, J. Polymeric grape seed tannins prevent plasma cholesterol changes in high-cholesterol-fed rats. *Food Chem.* **1994,** *49 (4)*, 403–406

Thaipong, K., Boonprakoba, U., Crosbyb, K., Cisneros-Zevallosc, L. and Byrnec, D. H. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J. of Food Comp. and Anal.* **2006**, *19*, 669–675

Thannickal, V. J. and Fanburg, B. L. Reactive oxygen species in cell signaling *AJP - Lung Physiol.* **2000***, 279(6), 1005-1028*

Thilakarathna, S. H., Rupasinghe, H. P. V. Anti-atherosclerotic effects of fruit bioactive compounds: A review of current scientific evidence, *Can. J of Plant Sci.* **2012**, *92(3),* 407-419

Thilakarathna, S. H., Wang, Y., Ghanam, K., Rupasinghe, H. P. V. Apple peel flavonoid and triterpene- enriched extracts differentially affect cholesterol homeostasis in hamsters, *J. of Func. Food.* **2012**, *4,* 963-971

Torti, S., Kursar, T. and Dearing, M. Extraction of phenolic compounds from fresh leaves: a comparison of methods. *J. of Chem. Ecol.* **1995**, *21(2),* 117-125

Tsao, P. S., McEvoy, L. M., Drexler, H., Butcher, E. C. and Cooke, J. P. Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. *Circul*. **1994**, *89*, 2176-2182

Tsuji, K., Iwao, H., Nakagawa, Y. and Seki, T. Influence of dietary taurineon distribution and fecal excretion of cholesterol in hypercholesterolemic rats. *Sulfer-Containing A.A.* **1980**, *3*, 165-171

Vanduyn, M. A. and Pivonka, E. Overview of the health benefits of fruit and vegetable consumption for the dietetics professional: selected literature. *J. of the Am. Dietetic Associa*. **2000**, *100 (12)*, 1511–1521

Vattem, D. A., Ghaedian, R. and Shetty, K. Enhancing health benefits of berries through phenolic antioxidant enrichment: focus on cranberry. *Asia Pacific J. Clinic. Nutri.* **2005***, 14(2)*, 120-130

Vazquez-Araujo. L., Chambers I. V. E., Adhikari K., Carbonell-Barrachina A. A. Sensory and physico-chemical characterization of juices made with pomegranate and blueberries, blackberries and raspberries. *J. Food Sci.* **2010**, *75(7),* 398-404

Vinatoru, M. An overview of ultrasonically assisted extraction of bioactive principles from herbs. *Ultrason. sonochem*. **2001,** *8*, 303-313

Vinson J. A., Su, X., Zubik, L. and Bose, P. Phenol antioxidant quantity and quality in foods: fruits. *J. Agric. Food Chem*. **2001**, *49*, 5315–5321

Voicu, A., Campeanu, G., Bibicu, M., Mohora, A., Negoita, M., Catana, L. and Catana, M. Achievement of a fortifying products based on apples, quinces and sea buckthorn. *Not. Bot. Hort. Agrobot. Cluj*. **2009***, 37(1)*, 224-228

Vrhovsek, U. Rigo, A. Tondon, D. and Mattivi, F. Quantification of polyphenols in different apple varieties. *J. Agric Food Chem.* **2004***, 52*, 6532-6538

Wakamatsu, T. H., Murat Dogru, M. and Tsubota, K. Tearful relations: oxidative stress, inflammation and eye diseases. *Arq. Bras. Oftalmol*. **2008**, *71(6),* 72-79

Wamlck, G. R., Benderaon, J. and Albers, J. J. Dextran sulfate-Mg++ precipitation procedure for quantitation of high density lipoprotein cholesterol. *Clin. Chem*. **1982**, *28*, 1379-1388

Wang, X., Zheng, Z., Guo, X., Yuan, J. and Zheng, C. Preparative separation of gingerols from Zingiber officinale by high-speed counter-current chromatography using stepwise elution. *Food Chem*. **2011**, *125*, 1476–1480

Wang, Y. W., Jones, P. J. H., Pischel, I. and Fairow, C. Effects of Policosanols and Phytosterols on Lipid Levels and Cholesterol Biosynthesis in Hamsters. *Lipids*, **2003**, *38(2),* 165-170

Wang, Y., Catana, F., Yang, Y., Roderick, R. V. and Breemen, R. B. An LC-MS method for analyzing total resveratrol in grape juice, cranberry juice, and in wine. *J. Agric. Food Chem*. **2002**, *50*, 431–435

Weichselbaum, E., Wyness, L. and Stanner, S. Apple polyphenols and cardiovascular disease- a review of the evidence. British Nutrition Foundation. *Nutri. Bulletin*. **2010**, *35*, 92–101

Weiss, E. L., Lev-Dor, R., Sharon, N. and Ofek, I. Inhibitory effect of a high molecular-weight constituent of cranberry on adhesion of oral bacteria. *Crit. Rev. Food Sci. Nut.* **2002**, *42*, 285–292

Whelton, P. K. and He, J. Potassium in preventing and treating high blood pressure. *Seminars in Nephro.* **1999**, *19(5)*, 494-499

WHO. **2008**. World Health Organization [online]. Available from http://www.who.int/topics/cardiovascular_diseases/en/ [accessed 1 December 2011]

Wilson, T., Porcari, J. P. and Harbin, D. Cranberry extract inhibits low density lipoprotein oxidation. *Life sci*. **1998**, *62(24)*, 381–386

Winiarska, K., Szymanski, K., Gorniak, P., Dudziak, M. and Bryla, J. Hypoglycemic, antioxidative and nephroprotective effects of taurine in alloxan diabetic rabbits. *Biochimie.* **2009**, *91*, 261-270

Wiseman, W., Egan, J. M., Slemmer, J. E., Shaughnessy, K. S., Ballem, K., Gottschall-Pass, K. T., Sweeney, M. I. Feeding blueberry diets inhibits angiotensin II-converting enzyme (ACE) activity in spontaneously hypertensive stroke-prone rats. *Can. J. of Physio. & Pharm.* **2011**, *89(1)*, 67-71

Wojcik, O. P., Koenig, K. L., Zeleniuch-Jacquotte, A., Costa, M. and Chen, Y. The potential protective effects of taurine on coronary heart disease. *Atheroscler.* **2010**, *208*, 19-25

Wolf, A., Zalpour, C. and Theilmeier, G. Dietary L-argignine supplementation normalizes platelet aggregation in hypercholerolemic humans. *J. Am. Coll. Cardiol.* **1997**, *29*, 479-485

Wong, C., Li, H., Cheng, K. and Chen, F. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chem*. **2006,** *97*, 705-711

Wootton-Beard, P. C. Moran, A., Ryan, L. Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after *in vitro* digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Res. Intern*. **2011**, *44(1)*, 217–224

Wootton-Beard, P. C., Ryan, L.Improving public health? The role of antioxidant-rich fruits and vegetable beverages. *Food Res. Intern*. **2011**, *44*, 3135-3148

Wu, X., Beecher, G. R. Holden, J. M., Haytowitz, D. B., Gebhardt, S. E. and. Prior, R. L. Lipophilic and Hydrophilic Antioxidant Capacities of Common Foods in the United States**.** *J. Agric. Food Chem.* **2004**, *52(12)*, 4026–4037

Xu, B. J., Yuan, S. H. and Chang, S. K. C. Comparative studies on the antioxidant activities of nine common food legumes against copper induced LDL oxidation *in vitro. J. of Food Sci.* **2007***, 72(7),* 522-527

Yla-Hertuala, S., Palinski, W., Rosenfeld, M. E., Parhasrathy, S., Carew, T. E., Butler, S., Wiztum, J. L. and Steinberg, D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest*. **1989**, *84*, 1086-1095

Yokogoshi, H., Mochizuki, H., Nanami, K., Hida, Y., Miyachiand, F. and Oda, H. Dietary Taurine Enhances Cholesterol Degradation and Reduces Serum and Liver Cholesterol Concentrations in Rats Fed a High-Cholesterol Diet. *J. Nut*. **1999**, *129(9),* 1705-1712

Young, I. S. and Woodside, J. V. Antioxidants in health and disease. *J. Clan. Pathos*. **2001**, *54*, 176–186

Young, H. Y., Liao, J. C., Chang, Y. S., Luo, Y. L., Lu, M. C. & Peng, W. H. Synergistic effect of ginger and nifedipine on human platelet aggregation; a study in hypertensive patients and normal volunteers. *Am. J. Chin. Med.* **2006**, *34(4)*, 545-551

Yuan, Y., Gao, Y., Maoa, L. and Jian Zhao, J. Optimization of conditions for the preparation of b-carotene nanoemulsions using response surface methodology. *Food Chem.* **2008**, *107*, 1300–1306

Zern, T. L. and Fernandez, M. L. Cardioprotective effects of dietary polyphenols. *J. Nutr*. **2005**, *135(10)*, 2291-2294

Zulli, A. Taurine in cardiovascular disease. *Current Opinion in Clini. Nutri. and Metabolic Care.* **2011**, *14*, 57–60

APPENDIX A: RIDGE ANALYSIS FOR MAXIMIZING THE INDIVIDUAL RESPONSE VALUE IN HOT WATER EXTRACTION OF GINGER

Estimated values at coded radius 1.0	Phenolics (mg GAE/L)	FRAP value (mg TE/L)	% LDL oxidation inhibition
Responses	467.47	1.12	45.30
Temp. (°C)	67.39	63.29	43.79
Time (min)	67.93	26.26	55.07

APPENDIX B: RIDGE ANALYSIS FOR MAXIMIZING THE FRAP VALUE IN ULTRASONIC-ASSISTED EXTRACTION OF GINGER

Estimated values at coded radius 1.0	FRAP value (mg TE/L)
Responses	1.05
Temp. (°C)	35.37
Time (min)	64.88

APPENDIX C: RIDGE ANALYSIS FOR MAXIMIZING THE INDIVIDUAL RESPONSE VALUE IN HIGH PRESSURE HOMOGENIZATION-ASSISTED EXTRACTION OF GINGER

Estimated values at coded radius 1.0	Phenolics (mg GAE/L)	FRAP value (mg TE/L)	% LDL oxidation inhibition
Responses	488.8	1.12	44.8
Temp. (°C)	60.3	48.6	64.9
Pressure (MPa)	197.9	218.9	127.9

APPENDIX D: PERMEATE FLUX OF FRUIT JUICES DURING RO

Processing conditions	Permeate flux (mL/min*m^2)		
	Apple	Blueberry	Cranberry
20 °C/25 bars	150.0±18	136.2±17	99.51±13
20 °C/30 bars	147.6±5.4	136.2±8.4	112.8±11
20 °C/35 bars	136.2±8.4	133.3±6.0	129.2±19
40 °C/25 bars	173.4±17	133.6±11	106.2±9.0
40 °C/30 bars	178.2±6.6	162.0±12	127.6±10
40 °C/35 bars	189.1±9.0	198.6±11	161.4±12

APPENDIX E: RESEARCH ETHICS BOARD APPROVAL FOR SENSORY EVALUATION OF FRUIT JUICE BLENDS

Nova Scotia Agricultural College
Research Ethics Board

October 21, 2011

Dear Prasanna Gunathilake:

Your project entitled, "Determination of the best fruit juice ratio and level of ginger for the formulation of a functional beverage" has been approved by the NSAC Research Ethics Board. The Board appreciated your response to its concerns.

There are a few administrative details that you should be aware of:

1) If the methodology or instruments of the study change, then please send the revisions to the Chair of the REB as soon as possible. The Chair will determine if the changes need to be reviewed and approved.

2) If an adverse event occurs such as a violation of privacy or a complaint by a respondent, please inform the REB within a week of the occurrence.

3) Please ensure that you keep a complete record of all material to this project in a secure location accessible for review by the REB or Tri-Council auditors including a copy of the submission, as well as correspondence with and from the REB such as those related to adverse events and amendments. Please also keep copies of the original signed consent forms and the data forms as outlined in the REB submission.

4) You are required to submit an annual and/or final report updating the REB about the progress of the research study. The form on which this report is completed can be found on the REB website.

Best of luck with your research.

Sincerely,

Steven Dukeshire
Chair, NSAC REB

cc Carolyn Terry

APPENDIX F: RESEARCH ETHICS BOARD APPROVAL FOR SENSORY EVALUATION OF FRUIT-BASED FUNCTIONAL BEVERAGE

Nova Scotia Agricultural College

Research Ethics Board

PO Box 550
Truro, Nova Scotia
Canada B2N 5E3
Ph: 902-893-6623
Fax: 902-893-1849
nsac.ca/reb

October 24, 2011

Dear Prasanna Gunathilake:

Your project entitled, "Evaluation of sensory properties of a fruit-based functional beverage" has been approved by the NSAC Research Ethics Board. The Board appreciated your response to its concerns.

There are a few administrative details that you should be aware of:

1) If the methodology or instruments of the study change, then please send the revisions to the Chair of the REB as soon as possible. The Chair will determine if the changes need to be reviewed and approved.

2) If an adverse event occurs such as a violation of privacy or a complaint by a respondent, please inform the REB within a week of the occurrence.

3) Please ensure that you keep a complete record of all material to this project in a secure location accessible for review by the REB or Tri-Council auditors including a copy of the submission, as well as correspondence with and from the REB such as those related to adverse events and amendments. Please also keep copies of the original signed consent forms and the data forms as outlined in the REB submission.

4) You are required to submit an annual and/or final report updating the REB about the progress of the research study. The form on which this report is completed can be found on the REB website.

Best of luck with your research.

Sincerely,

Steven Dukeshire
Chair, NSAC REB

cc Carolyn Terry

NSAC. Embrace Your World.

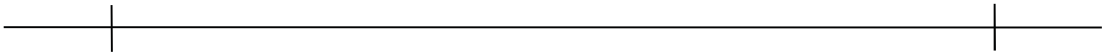
APPENDIX G: SCORE SHEET FOR SENSORY ANALYSIS OF FRUIT JUICES BLENDS

Please select your age range (please put X in the box):

☐	☐	☐	☐
18-25	26-35	36-45	above 45

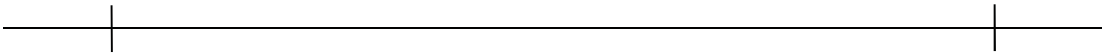
Please evaluate and score the products for astringency, sourness, sweetness and overall acceptability of the products. Code numbers are mentioned on the containers.

1. Please rate the following for **astringency**:



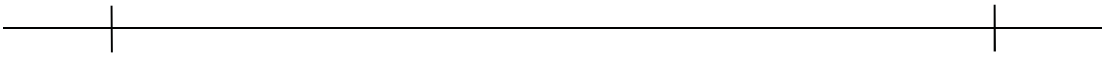
not astringent very astringent

2. Please rate the following for **sourness**:



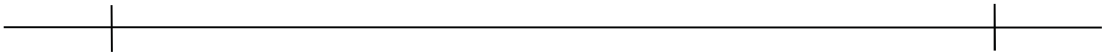
not sour very sour

3. Please rate the following for **sweetness**:



not sweet very sweet

4. Please rate the following for **overall acceptability**:



not acceptable very acceptable

Comments:……
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…….

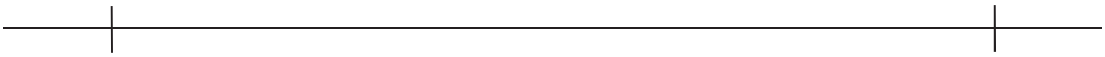
APPENDIX H: SCORE SHEET FOR SENSORY ANALYSIS OF FRUIT JUICES BLENDS WITH DIFFERENT LEVELS OF GINGER JUICE

Please select your age range (please put X in the box):

18-25 ☐ 26-35 ☐ 36-45 ☐ above 45 ☐

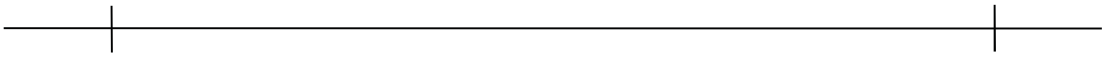
Please evaluate and score the products for odor of the products. Code numbers are identified on the containers.

1. Please rate the following for acceptability of the **odor**



not acceptable very acceptable

2. Please rate the following for the acceptability of the **taste**:



not acceptable very acceptable

Comments:……
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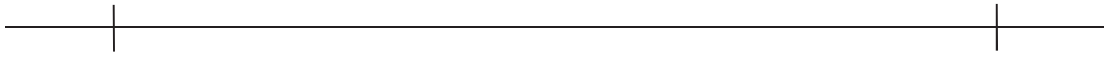
APPENDIX I: SCORE SHEET FOR SENSORY ANALYSIS OF FRUIT JUICES BLENDS WITH CARDIO-PROTECTIVE INGREDIENTS (10% RDI)

Please select your age range (please put X in the box):

☐ 18-25 ☐ 26-35 ☐ 36-45 ☐ above 45

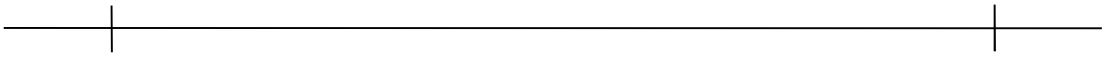
Please evaluate and score the products for odor of the products. Code numbers are identified on the containers.

1. Please rate the following for acceptability of the **odor**



not acceptable very acceptable

2. Please rate the following for the acceptability of the **taste**:



not acceptable very acceptable

Comments:……
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APPENDIX J: SCORE SHEET FOR SENSORY ANALYSIS OF EXPERIMENTAL FUNCTIONAL BEVERAGE AND TWO COMMERCIAL FUNCTIONAL BEVERAGES

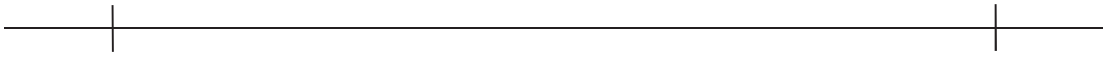
Please select your age range (please put X in the box):

18-25 ☐ 26-35 ☐ 36-45 ☐ above 45 ☐

Odor evaluation

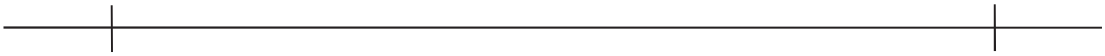
Please evaluate and score the products for odor. Code numbers are indicated on the containers.

Please rate the following for **odor:**



Not pleasant smell very pleasant smell

For **odor only**, please rate the acceptability of the products.



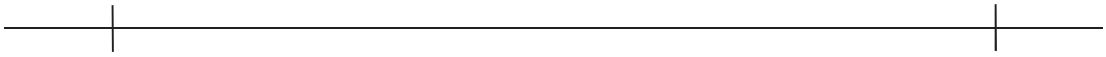
Not acceptable very acceptable

Comments:……

Flavour evaluation

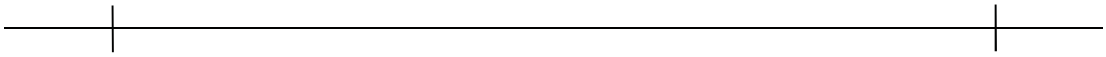
Please evaluate and score the products for astringency, sourness, sweetness and overall acceptability of the products. Code numbers are indicated on the containers.

1. Please rate the following for **astringency**:



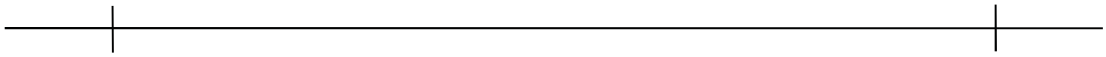
Not astringent very astringent

2. Please rate the following for **sourness**:



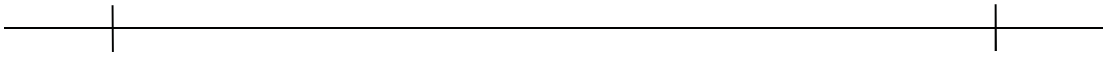
Not sour very sour

3. Please rate the following for **sweetness**:



Not sweet very sweet

4. Please rate the following for overall acceptability:



Not acceptable very acceptable

Comments:…….