PHENOLIC COMPOUNDS OF CRANBERRY GENOTYPES AND THEIR ANTIOXIDANT CAPACITIES AND BIOACTIVITIES

By

Gihan Abeywickrama

A thesis submitted to the School of Graduate Studies in

Partial fulfillment of the requirement of the degree of

Master of Science in Biology

Department of Biology

Memorial University of Newfoundland

August 2015

ABSTRACT

Cranberry species are a rich source of natural antioxidants with potential health benefits linked to the prevention of certain chronic and degenerative diseases. This study compared the antioxidant activity of phenolic constituents of five different cranberry genotypes (pilgrim, wild clone NL2, wild clone NL3, wild clone PEI, wild clone NL1) and market samples. Phenolic constituents were separated into free, esterified and bound fractions, the content of each fraction as well as that of their flavonoid counterparts and antioxidative efficacy were evaluated. The latter was assessed using trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (DRSC), hydroxyl radical scavenging capacity and reducing power (RP) as well as metal chelation assays. Among cranberry varieties, pilgrim genotype showed the highest phenolic content (30.02 mg of gallic acid equivalents (GAE)/g dried fruit weight). Similarly, pilgrim had the highest flavonoid content (19.94 mg of catechin equivalents (CE)/g of dried fruit weight). The phenolic compounds in cranberry were predominantly present in the esterified and free forms in the pilgrim and wild clone NL2. In addition, results showed that contribution of esterified phenolics to the antioxidant activity, metal chelating and reducing power was higher than the free and bound phenolics for all tested cranberry genotypes.

HPLC-MS analysis was performed for two of the rich sources of phenolics and antioxidant activity. For these, the results showed that caffeic acid, *p*-coumaric acid, epicatechin, proanthocyanin trimer A-type, proanthocyanin dimer B-type and quercertin 3-*O*-glucoside predominated in wild clone NL2, while chlorogenic, caffeic acid, epicatechin, proanthocyanidin dimer B-type, proanthocyanin trimer A-type and myricertin 3-*O*-arabinoside were predominant in pilgrim.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. F. Shahidi for his invaluable professional and financial (through NSERC) support and personal encouragement throughout this project, which are deeply appreciated. Thanks are extended to my supervisory committee members Dr. Samir Debnath and Dr. Andrei Igamberdiev. I would also like to extend my appreciation to the Department of Biology and the School of Graduate Studies at Memorial University of Newfoundland for the award of a fellowship. Special thanks go to Ms. Linda Winsor at the Centre for Chemical Analysis, Research and Training (C-CART), Memorial University of Newfoundland for her technical assistance. My sincere thanks are also extended to all the friends and colleagues in Dr. Shahidi's research team for creating an enjoyable working environment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

AAPH	2, 2'-Azobis (2-aminopropane) dihydrochloride
AH	Phenolic antioxidants
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CE	Catechin equivalents
CD	Conjugated diene
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DPPH	1, 1 -Dipheny1-2-picrylhydrazyl
DRSC	DPPH radical scavenging capacity
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
ESI	Electrospray ionization
ET	Electron transfer
FRAP	Ferric reducing antioxidant power

GAE	Gallic acid equivalents
GC-MS	Gas chromatography-mass spectrometry
HAT	Hydrogen atom transfer
HBA	Hydroxybenzoic acids
HCA	Hydroxycinnamic acids
HPLC	High performance liquid chromatography
LDL	Low density lipoprotein
MDA	Malondialdehyde
NL	Newfoundland and Labrador
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
OPD	O-Phenylenediamine
PAC	Proanthocyanidins
PAL	Phenylalanine ammonia lyase
p-AV	*para*-Anisidine value
PBS	Phosphate buffer solution
PEI	Prince Edward Island

PG	Propyl gallate
PV	Peroxide value
ROS	Reactive oxygen species
TAL	Tyrosine ammonia lyase
TE	Trolox equivalent
TBHQ	*tert*-Butylhyroquinone
TRAP	Total radical trapping antioxidant parameter
USDA	US Department of Agriculture
UTI	Urinary tract infections
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

The health beneficial properties of plants have been attributed to their bioactive compounds, known as phytochemicals. Most common phytochemicals include secondary metabolites such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds (Kris-Etherton *et al* 2002; Nigam, 2009). Naturally-occurring plant metabolites such as phytochemicals, including those in fruits and vegetables, have received widespread attention due to their health-promoting properties. These studies have consistently shown a direct relationship between consumption of fruits, vegetables and whole grains with a lower risk of developing chronic illnesses such as cardiovascular diseases, stroke, degenerative diseases, and certain types of cancer (Tulo *et al* 2014).

Among the phytochemicals, phenolic compounds have been extensively studied due to their diverse biological effects, including antioxidant activity and for preventing chronic inflammation, cardiovascular diseases, cancer and diabetes (Beatriz *et al* 2014). Crude extracts of fruits, vegetables, whole grains and other plant materials rich in phenolics are increasing interest in the food industry because they inhibit or retard oxidative degradation of lipids and improve the quality and nutritional value of products. Phenolic phytochemicals also have a wide range of biological actions, including the ability to act as antioxidants, anti-inflammatory agents, modulating enzyme activity, and regulating gene expression (McKay *et al* 2015).

Polyphenols can be considered as the most abundant antioxidants in the human diet (Manach *et al* 2004). The compounds that can delay or inhibit the effects of oxidation have been considered as antioxidants, including compounds that either inhibit specific oxidizing enzymes or react with oxidants before they damage critical biological molecules (Cote *et al* 2011). As antioxidants,

phenolic compounds prevent the formation of free radicals, which have deleterious health effects, or neutralize them once formed, and are therefore important in disease risk reduction (Shahidi, 2000). They have been demonstrated to have positive effects on certain types of cancer including cancer of the stomach, colon, prostate, and breast as well as various inflammatory disorders (Andriantsitohaina *et al* 1999).

Comparison of the antioxidant capacities of different foods provides a valid tool with which to rank their health benefits. Berries invariably rank highly due to their high content of phenolic compounds with powerful antioxidant effect.

Berries are highly appreciated as small fruits for their attractive colour, delicate texture and unique flavour. Berry fruits are consumed not only in the fresh and frozen forms, but also as processed and derived products, including dried and canned fruits, yogurts, beverages, jams, and jellies (Seeram, 2006), as well as in the powdered form in capsules and tablets. Small berries represent a very diverse group, including a variety of red, blue or purple small-sized and highly perishable fruits, also named as super fruits. Among the colourful fruits, berries such as strawberry, currant (black, red or white), gooseberry, blackberry, raspberry (black or red), blueberry, cranberry and others of minor economic importance (i.e. boysenberry, bilberry, jostaberry, cloudberry, loganberry, lingonberry) (Manganaris *et al* 2013) are popularly used in the human diet either fresh, sometimes frozen, or in the processed forms (Nile & Park, 2014).

Berries provide significant health benefits because of their high contents of a diverse range of phytochemicals. These phytochemicals include a variety of beneficial compounds, such as essential minerals, vitamins, fatty acids, dietary fibres, provitamin A, vitamin C, and B-complex vitamins. The phenolic compounds in berries play many diverse biological functions including

roles in plant growth, development, and defense. They provide pigmentation, antimicrobial and antifungal functions, insect-feeding deterrence, ultraviolet radiation protection, chelation of toxic heavy metals, and antioxidant quenching of free radicals and reactive oxygen species (ROS) generated during photosynthesis (Beer *et al* 2004; Parry *et al* 2005).

Among the 20 most commonly consumed fruits in the North American diet, cranberry is ranked high among fruits in both antioxidant quality and quantity because of its substantial flavonoid content and a wealth of phenolic acids (Yan *et al* 2002). Cranberries are healthy fruits that contribute colour, flavour, nutritional value, and functionality in the daily diet. They constitute one of the native Northern American fruits. The North American cranberry (*Vaccinium macrocarpon*) has been recognized by the US Department of Agriculture (USDA) as the standard for fresh cranberries and cranberry juice cocktail. The European cranberry variety *(Vaccinium oxycoccus),* which is grown in parts of central Europe, Finland, and Germany, is a smaller fruit with anthocyanins and acid profiles slightly different compared to the North American variety (Girard & Sinha, 2006).

The composition of the cranberry fruit family, which includes the American and the European species, is rich in phenolic compounds and their flavonoid subclasses. The variety and concentration of antioxidants are highly dependent on the species and cultivar considered. Pre-harvest practices, environmental conditions, maturity stage at harvest, post-harvest storage and processing operations also play important roles in the phytochemical profiles of cranberries (Manganaris *et al* 2013).

Reactive oxygen species (ROS) are involved in the development of several oxidation-linked diseases such as cancer, cardiovascular diseases (CVD) and diabetes. Epidemiological studies, as

already noted have indicated that diets rich in fruits vegetables, and whole grains are associated with lower incidences of oxidation-linked diseases such as cancer, CVD and diabetes (Vattem *et al* 2005). Cranberry extracts, with their high content of phenolics, possess biological properties that provide human health benefits, as mentioned above, and may also be used to treat bladder and kidney ailments, relieve blood disorders, stomach ailments, liver problems, vomiting, appetite loss, scurvy, and cancer (Bodet *et al* 2008). Recently, cranberry extracts have received increasing attention in various areas of health research, including both infectious and non-infectious diseases.

Different studies have shown that cranberry juice consumption is beneficial in preventing urinary tract infections (Vasileiou *et al* 2013). Urinary tract infections (UTI) can occur due to the presence of a certain threshold number of bacteria in the urine. However, Urinary tract infections can be seen in both men and women, but are about 50 times more common in adult women compared to adult men due to the presence of a shorter urethra in women. The most UTI are the colonization of periurethral tissues with uropathogenic organisms and then followed by the passage of the bacteria through the urethra. *Escherichia coli* are responsible for most of the uncomplicated urinary tract infections. Low-dose antibiotic prophylaxis, used for preventing UTI, is related to the development of resistance to the causative microorganisms and the indigenous flora (Gupta, 2003). The beneficial effects of cranberries against urinary tract infections have been attributed to the prevention of bacterial adherence to uroepithelial cells that may reduce the development of urinary tract infections. The evidence from a different study suggests that consumption of cranberry products may control UTIs by preventing uropathogenic bacterial adhesion (Gauy, 2009). Cranberry may influence bacterial adhesion by altering cell surface properties of the bacteria (Habash *et al* 1982). Cranberries are great sources of dietary flavonoids, including anthocyanins and proanthocyanins (PACs) (Pedersen *et al* 2000). PACs are

the major compounds responsible for these beneficial effects. A-type cranberry PACs prevents the adhesion of uropathogenic *E. coli* to uroepithelial cells (Howell, 2007)

Yan *et al* (2002) reported that proliferation of MCF-7 and MDA-MB-435 breast cancer cells was inhibited by cranberry extracts possibly due to the fact that cranberries are a rich source of many bioactive compounds such as phenolic acids, anthocyanins, proanthocyanidin and flavonol glycosides (Cote *et al* 2010). Furthermore, researchers have found that a flavonoid fraction extracted from cranberry press cake was able to inhibit proliferation of 8 human tumour cell lines of multiple origins (Ferguson *et al* 2004). Bomser *et al* (1996) showed that extracts from vaccinium berry species were able to inhibit the induction of ornithine decarboxylase (ODC), an enzyme involved in tumour proliferation, and induction of quinone reductase, an enzyme that can inactivate certain carcinogens. Proanthocyanidin-rich cranberry extracts containing other flavonoids were reported to inhibit ODC induction in epithelial cells (Kandil *et al* 2002) and also render anti-angiogenic properties (Roy *et al* 2002).

The antioxidant properties of the phenolic compounds in cranberry play a major role in decreasing damage related to cardiovascular diseases including atherosclerosis and stroke, as well as some neurodegenerative diseases of aging (Neto, 2007). Numerous studies have demonstrated the effects of cranberry linked to the prevention of oxidative processes (Yan *et al* 2002) and decreased oxidative damage in stroke by reducing oxidation of lipoproteins, thus improving serum antioxidant status and lipid levels, and mitigating the effects of oxidative stress and inflammation on the vascular system (Porter *et al* 2001). Research findings have indicated that consumption of foods and beverages rich in flavonoids may decrease the risk of developing atherosclerosis due to the ability of these compounds to inhibit oxidation of low-density lipoprotein (LDL), platelet aggregation and adhesion, and inflammatory response of the vascular

tissues (Reed, 2002) due to the involvement of the phenolics in quenching free radicals from biological systems (Vattem *et al* 2005). The plasma antioxidant capacity of humans improved with consumption of cranberry products, which has implications for cardiovascular disease (Yan *et al* 2002).

In our study, phenolic constituents of cranberry were determined for the free, esterified, and bound forms using alkali hydrolysis prior to analysis. This would fill the existing gap in the literature with respect to different phenolic fractions of cranberries and would provide information about the antioxidant potential of cranberry in food systems. Specifically, this study compares five different cranberry genotypes along with two market samples, mature (red) and immature (reddish) fruits. These cranberry genotypes (*Vaccinium macrocarpon*) were wild clone NL2, pilgrim, wild clone NL3, wild clone PEI and wild clone NL1. These genotypes were collected from different locations and other details were shown here. Pilgrim was a hybrid between cultivars Prolific and McFarlin (Galletta & Ballington, 1996). The market samples were purchased from Costco in St. John's, NL; these were produced in Nova Scotia, Canada.

Genotype	**Place of collection**	**Latitude (N)**	**Longitude(W)**
Wild Clone NL2	Point La Haye, NL	46°52'	53°36'
Wild Clone NL3	St. Brides, NL	46°55'	54°10'
Wild Clone PEI	Harrington, PEI	46°21'	63°10'
Wild Clone NL1	New Melbourne, NL	48°03'	53°09'

Their total phenolic contents and antioxidant activities were determined in order to shed light on their potential as a source of health promoting products and in reducing disease risk. Their antioxidant potential in food and biological model systems was also investigated to highlight their efficiency. The other objective of this study was to characterize the differences between the phenolic profiles among different genotypes using high-performance liquid chromatography (HPLC) and HPLC-mass spectrometry.

CHAPTER 2

LITERATURE REVIEW

The importance of antioxidants contained in foods is well appreciated for both preserving the foods themselves and for supplying essential antioxidants *in vivo* (Shi & Noriko, 2001). Antioxidants are known to act at different levels in the oxidative sequence (Shahidi, 1997). There is mounting interest in natural antioxidants due to safety concerns for synthetic antioxidants (Prior & Cao, 2000). The following sections describe the phenolic compounds and their origin, different categories, sub-categories, measurement of antioxidant activity, importance of fruits and vegetables as antioxidants, antioxidant mechanism, synthetic and natural antioxidants and extraction methods.

2.1. Phenolics and Polyphenolics

There are some 10,000 different plant phenols, which have unique biological, chemical and physical properties that make them powerful antioxidants (Chodak & Tarko, 2011; Fig. 2.1). Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Randhir *et al* 2004). They are produced from phenylalanine and to a lesser extent tyrosine via the removal of ammonia. They possess considerable physiological and morphological importance in plants and have a myriad of essential functions in the plants (Baidez *et al* 2007). Once consumed, they render health benefits that are related or unrelated to their antioxidant potential (Han *et al* 2007; Owen *et al* 2000; Surh, 2003). The antioxidant activities of phenolics are related to their chemical structures, generally depending on the number and positions of hydroxyl groups and

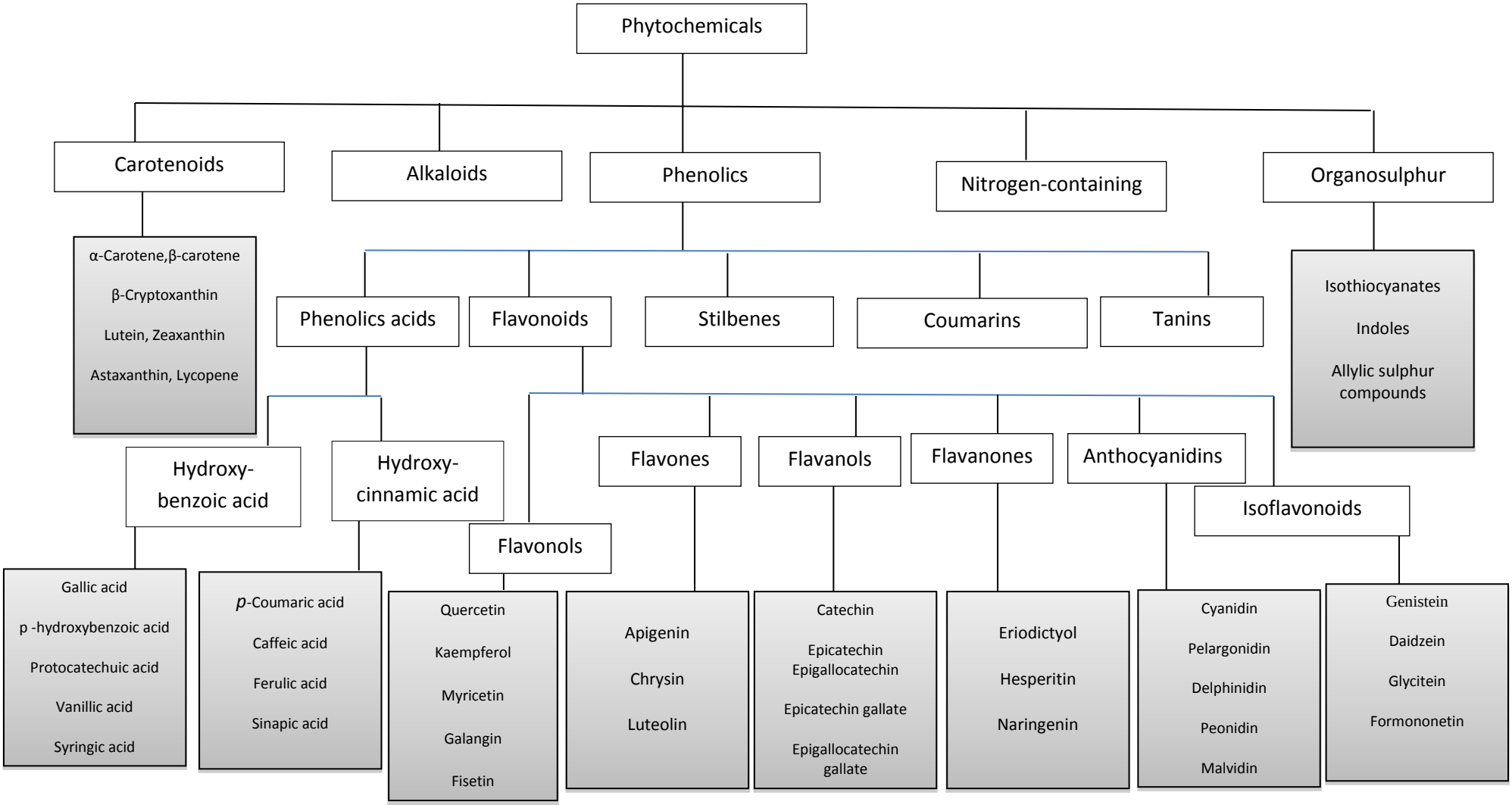


Figure 2.1: Classification of dietary phytochemicals (Adapted from Shahidi and Ho, 2007)

glycosylation or presence of other substituents in the respective molecules (Cai *et al* 2006; Heim *et al* 2002).

Phenolics are compounds possessing one or more aromatic rings bearing one or more hydroxyl group substituents, and range from simple phenolic molecules to highly polymerized compounds (Bravo, 1998). Phenolics are categorized as phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others, based on the number of phenolic rings and of the structural elements that link these rings (Fresco *et al* 2006). Phenolic compounds are present as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters and methyl esters (Harborne, 1989; Harborne *et al* 1999; Shahidi & Naczk, 1995).

2.1.1. Phenolic acids

Phenolic acids are a major class of phenolic compounds, are widely spread throughout the plant kingdom (Cai *et al* 2004). Phenolic acids, either occurring in the free or conjugated forms, usually appear as esters or amides (Huang *et al* 2009) in the bound form. Most of them are an integral part of the human diet, and are also consumed as medicinal preparations (Xu *et al* 2008). Substituted derivatives of hydroxybenzoic and hydroxycinnamic acids are the predominant phenolic acids in plants, with hydroxycinnamic acids being more common. These derivatives differ in the pattern of the hydroxylation and methoxylation in their aromatic rings (Shahidi & Naczk, 2004; Mattila & Hellstrom, 2007).

Phenylpropanoids (C6-C3) are derivatives of cinnamic acid, produced from phenylalanine, in the presence of phenylalanine lyase (PAL), or from tyrosine via the action of tyrosine lyase (TAL) (Fig. 2.2). Benzoic acid derivatives are generally formed from C6–C3 compounds by loss of a two-carbon moiety. Chalcones are formed by condensation of C6–C3 compounds with 3 molecules of malonyl coenzyme A. The chalcone so formed could be further cyclized to produce flavone, flavonol, flavanone, flavanonol, and flavanol, among others (Fig. 2.2).

The most common hydroxybenzoic acids (HBA) are gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids (Fig. 2.3), which have common structure of C6–C1 (Bravo, 1998). Hydroxybenzoic acid derivatives are mainly present in foods in the form of glucosides (Shahidi & Naczk, 2004).

Gallic acid is widely distributed in plants, such as *Barringtonia racemosa, Cornus officinalis, Cassia auriculata, Polygonum aviculare, Punica granatum, Rheum officinale, Rhus chinensis, Sanguisorba officinalis,* and *Terminalia chebula* as well as spices such as thyme and clove (Cai *et al* 2004). Protocatechuic acid can be found in *Dolichos biflorus, Feronia elephantum*, *Paeonia lactiflora*, *Cinnamomum cassia, Lawsonia inermis,* dill, and grape. Vanillic acid is distributed in *Foeniculum vulgare, Ipomoea turpethum,* and *Picrorhiza scrophulariiflora*. *Ceratostigma willmottianum* and sugarcane straw are good sources of syringic acid (Shan *et al* 2005; Sampietro *et al* 2006; Stago *et al* 2006).

Hydroxycinnamic acids (HCA; C6–C3) include caffeic, ferulic, *p*-coumaric and sinapic acids (Fig. 2.4; Bravo, 1998), which frequently occur in foods as simple esters with quinic acid or glucose; ferulic, caffeic and *p*-coumaric acids are present in many medicinal herbs and dietary spices, fruits, vegetables, and grains (Cai *et al* 2004).

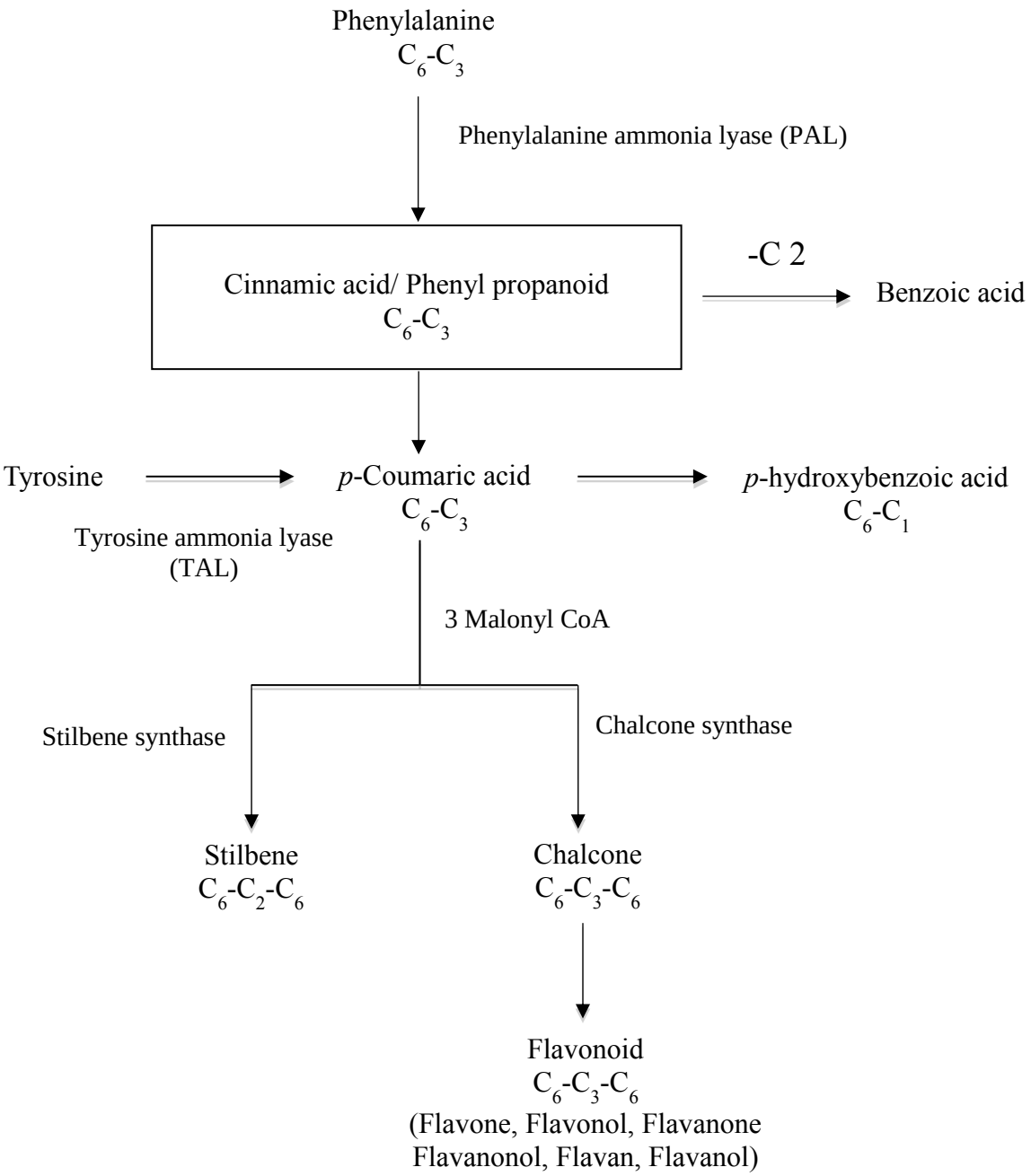
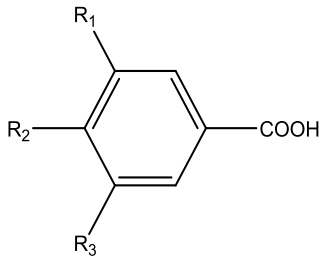
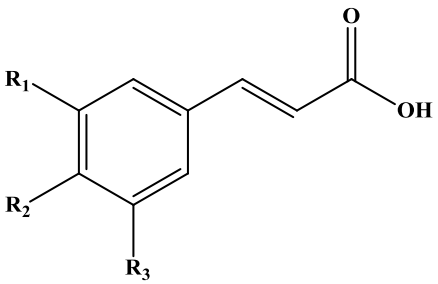


Figure 2.2: Basic pathways for the synthesis of phenolic compounds from aromatic amino acids



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

Figure 2.3. Chemical structures of common hydroxybenzoic acid derivatives



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

Figure 2.4. Chemical structures of common hydroxycinnamic acid derivatives

Hydroxycinnamic acids (HCA) such as caffeic, ferulic, and *p*-coumaric acids are widely distributed in berries such as cranberry, blueberry, blackberry, chokeberry, strawberry, red raspberry, sweet cherry, and elderberry (Bao *et al* 2005; Ren *et al* 2000). Probably the most well-known bound hydroxycinnamic acid is chlorogenic acid, which is combined caffeic and quinic acids. Chlorogenic acid is widely distributed in medicinal plants especially in the species of Apocynaceae and Asclepiadaceae (Huang *et al* 2007).

While both classes of phenolic acids act as antioxidants, hydroxycinnamic acids are more effective as free radical scavengers than their corresponding hydroxybenzoic acids (Shahidi & Wanasundara, 1992). Substituents on the aromatic ring affect the stabilization and therefore the radical-quenching ability of these phenolic acids which differ for different acids (Rice-Evans *et al* 1996).

2.1.2. Flavonoids

Flavonoids are the largest class of polyphenols that account for 60% of the total dietary phenolic compounds (Shahidi & Naczk, 2004). To date, more than 8000 flavonoids have been identified (Harborne & Williams, 2000). These are C15 compounds containing phenolic benzopyran structure (C6-C3-C6) where two aromatic rings A and B are attached to a heterocyclic ring C (Harborne & Simmonds, 1964). Flavonoids can be further subdivided into seven major subclasses, as already noted, based upon variations in the heterocyclic C-ring, including flavones, flavanones, flavonols, flavanonols, flavanols, isoflavones, and anthocyanidins (Tsao, 2010) (Fig. 2.2). The structural variations of flavonoid subclasses may arise from substitution patterns, such as hydroxylation, methoxylation, *O*-glycosylation, *C*-glycosylation and covalent

addition of prenyl or alkyl groups (Fig. 2.5; Aron & Kennedy, 2008). As for other phenolic compounds, flavonoids are essential for growth, development and defense system in plants. Some flavonoids are also responsible for imparting colour, flavour, and odour to the flowers, fruits and leaves (Gharras, 2009).

Flavonols are the most ubiquitous flavonoids in foods, and their main representatives are quercetin and kaempferol. These are widely distributed in onions, curly kale, leeks, broccoli and berries such as cranberry, blueberry, raspberry and blackberry (Manach *et al* 2004). Flavonols contain a 2, 3-double bond, 4-keto and 3-hydroxyl groups in the C ring. Flavonols are able to render high radical scavenging activity due to the presence of more hydroxyl groups in the molecule (Fig. 2.5; Cai *et al* 2006). Flavanones are characterized by a saturated C ring and a 4-keto group, and are precursors to all flavonoid structures and are among the most prevalent naturally occurring flavonoids (Fowler & Koffas, 2009). Flavanones are found in tomatoes and certain aromatic plants such as mint; citrus fruits are rich sources of flavanones (Simonetti *et al* 1997). Flavonoids lacking the double bonds and keto groups are called flavanols (Fig. 2.5) and are most widespread in plants. Isoflavones, differ from flavones in having their B ring attached at C-3 of the C ring, (Fig. 2.5) and are rich in soybean and its fermented products. Anthocyanidins (Fig. 2.5) with a central C ring are aglycones of anthocyanin pigments in flowers, leaves, fruits and roots of many plants. The six most common anthocyanidins are cyanidin, delphinidin, malvidin, perlagonidin, petunidin, and peonidin and their chemical structures are shown in Figure 2.7 (Kuhnau, 1976). Anthocyanins consist of an anthocyanidin molecule bound to one or more sugar moieties (Fig. 2.7) (Robards & Antolovich, 1997).

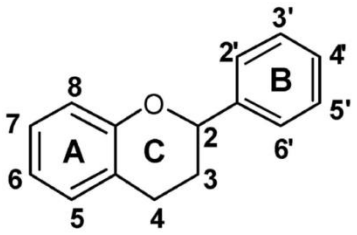


Figure 2.5. General structure of flavonoids

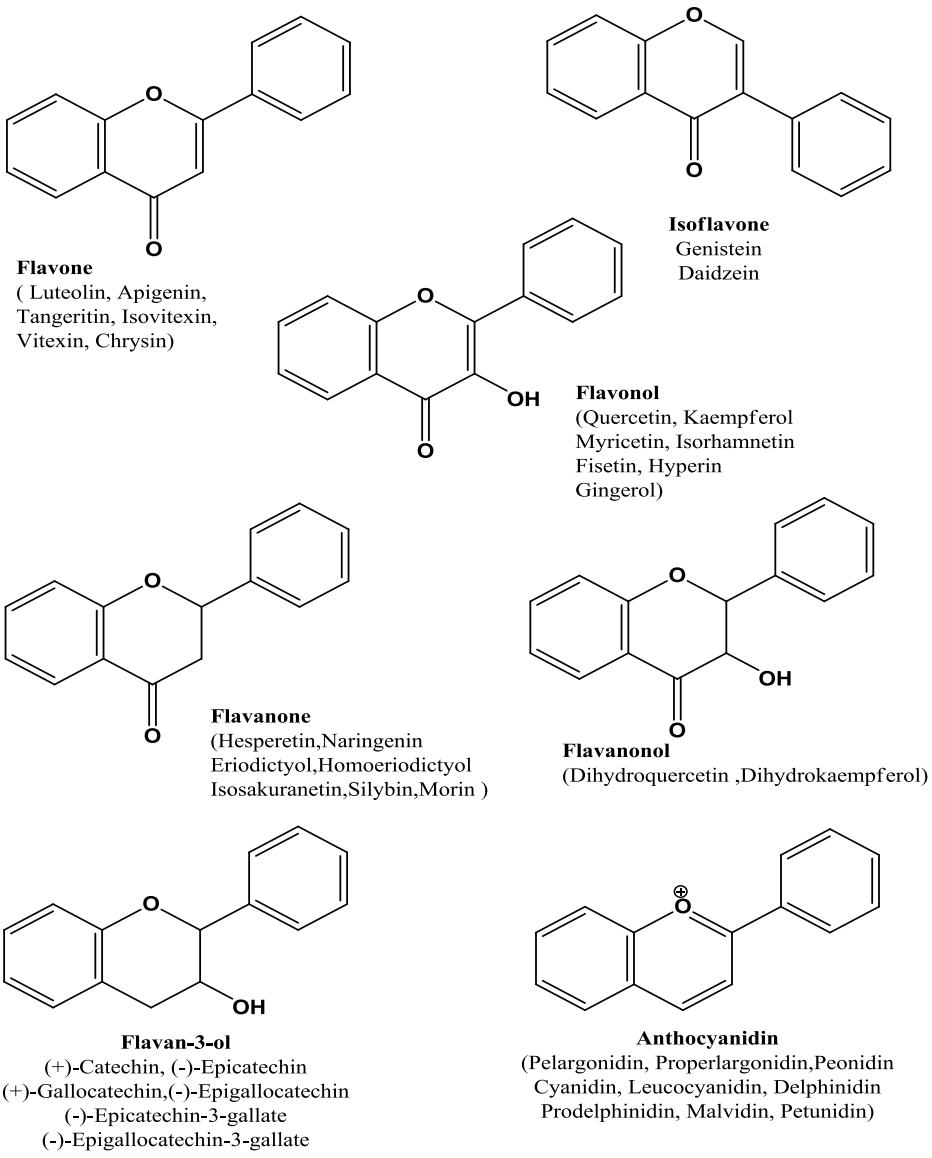
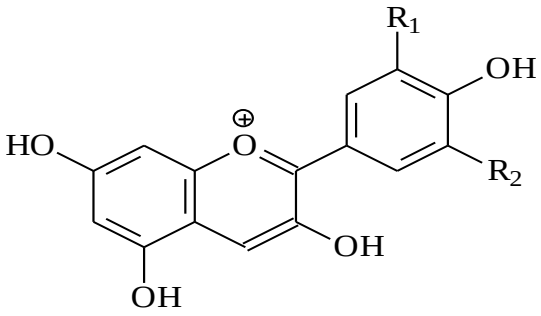
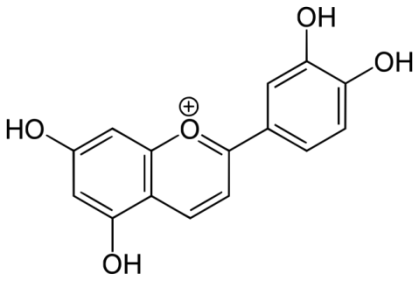


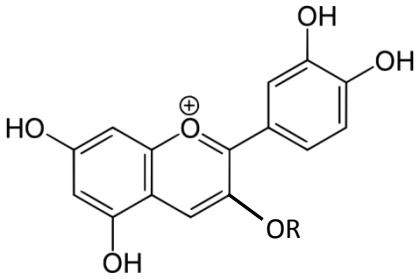
Figure 2.6. Classification of dietary flavonoids and their backbone structures (Adapted from Shahidi and Ho, 2007).



Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Pelargonidin	H	H
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OH	OCH_3



Anthocyanidin



Anthocyanin

Figure 2.7. Chemical structures of anthocyanidins and anthocyanins

In addition to the major flavonoid groups, other flavonoids such as neoflavonoids, which have a C-4 linkage of the B and C rings, and some minor flavonoids such as chalcones, aurones and auronols, with slightly varied structures have also been identified (Marais *et al* 2006). Among seven major groups of flavonoids, anthocyanins and catechins, known collectively as flavans, because of lack of the carbonyl group in the 3-position and flavan-3-ol and flavan-3,4-diols belong to this category (Shahidi & Naczk, 2004). Flavan-3-ols are found abundantly in green tea, grapes and blackberries whereas flavanones are exclusively found in citrus fruits in the glycosidic forms (Jaganath & Crozier, 2010).

2.3. Phenolics as antioxidants

Different studies have shown that exogenic antioxidants, especially those supplied in foods, are very important in inhibiting oxidative stress (Gray *et al* 1996; Halliwell *et al* 1995). Phenolic compounds such as flavonoids, phenolic acids, lignans, stilbenes, tocopherols, and tocotrienols are the largest groups of exogenic antioxidants (Gaikwad *et al* 2010).

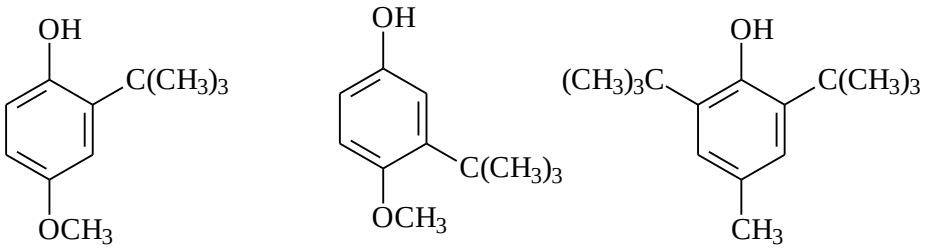
Antioxidants are able to inhibit or retard oxidation processes in different ways, in the first case, protecting target molecules from oxidation initiators by preventive reactive oxygen species (ROS) formation or scavenge species responsible for oxidation initiation. In the second case, they may act as chain breaking antioxidants, stalling the propagation phase by intercepting radical oxidation propagators or indirectly participate in stopping radical chain propagation (Dawidowicz *et al* 2012; Zhang *et al* 2006).

There have been numerous studies on the biological activities of phenolics, which are potent antioxidants and free radical scavengers (Naczk & Shahidi, 2004; 2006; Tung *et al* 2007; Zhong & Shahidi, 2011). Phenolic compounds are reported to neutralize free radicals by donating a hydrogen atom and/or an electron to them (Gaikwad *et al* 2010). Both concerted or stepwise chemical mechanisms may be involved in the transfer of an electron or a hydrogen atom. Antioxidant activities of phenolic compounds are based on the position and degree of hydroxylation, polarity, solubility and reducing potential (Millic *et al* 1998; Karadag *et al* 2009).

Antioxidants may be added intentionally to foods to prevent lipid oxidation and are either synthetic or natural in their origin (Halliwell & Gutteridge, 1999). Synthetic antioxidants that are approved for use in food include phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhyroquinone (TBHQ), and propyl gallate (PG) (Fig. 2.8), as well as non-phenolics such as ascorbic acid, erythrobic acid, and ascorbyl palmitate (Shahidi *et al* 1987; Frankel, 1996). Natural antioxidants include tocopherols and their derivatives, (Shahidi & Wanasundara, 1992; Hall, 2001), carotenoids, antioxidant enzymes and a large number of phenolic compounds of mainly plant origin. The importance of antioxidants contained in food is well appreciated for both preserving foods themselves and for supplying essential antioxidants *in vivo*.

2.5 Mechanism of action of phenolic antioxidants

The antioxidant potential of phenolic compounds depends on the number and arrangement of the hydroxyl groups in the molecules of interest (Cao *et al* 1997; Sang *et al* 2002).



2-BHA

3-BHA

Butylated hydroxyanisole

Butylated hydroxytoluene

Tertiary butylhydroquinone

Propyl gallate

Figure 2.8. Chemical structures of major synthetic antioxidants

Phenolic antioxidants (AH) can donate hydrogen atoms to lipid radicals and produce lipid derivatives and antioxidant radicals (Reaction I), which are more stable and less readily available to promote autoxidation (Kiokias *et al* 2008). The antioxidant free radicals may further interfere with the chain-propagation reactions (Reactions II and III).

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

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

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \quad (III)$$

As bond energy of hydrogen in a free radical scavenger decreases, the transfer of hydrogen to the free radical is more energetically favourable and thus more rapid (McClements & Decker, 2007). Any compound that has a reduction potential lower than the reduction potential of a free radical (or oxidized species) is capable of donating its hydrogen atom to that of the free radical unless the reaction is kinetically unfeasible. The phenoxyl radical is stabilized by delocalization of its unpaired electron around the aromatic ring (Fig. 2.9), which participates in the termination reaction.

Gorden (1990) reported that substitution at the *para* position with an ethyl or n-butyl group rather than a methyl group improves the activity of the antioxidant; however, the presence of chain or branched alkyl groups in this position decreases the antioxidant activity. The stability of the phenoxyl radical is further increased by bulky groups in the 2 and 6 positions as in 2,6-di-*tert*-butyl-4-methylphenol (BHT), since these substituents increase the steric hinderance in the region of the radical and thereby further reduce the rate of propagation reactions involving the antioxidant radical (Reactions IV, V, VI).

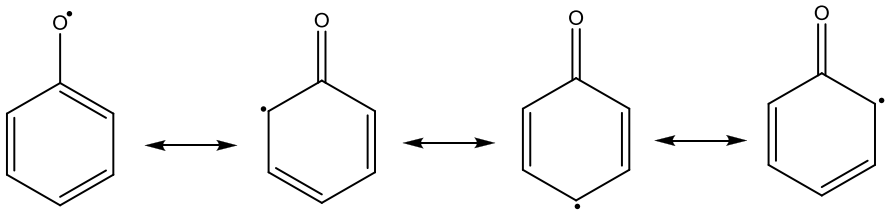


Figure 2.9. Resonance stabilization of phenoxyl radical

$$\dot{A} + O_2 \longrightarrow AOO^{\cdot} \quad \text{(IV)}$$

$$AOO^{\cdot} + RH \longrightarrow \dot{R} + AOOH \quad \text{(V)}$$

$$\dot{A} + RH \longrightarrow AH + \dot{R} \quad \text{(VI)}$$

The effect of antioxidant concentration on autoxidation rates depends on many factors, including the structure of the antioxidant, oxidation conditions, and the nature of the sample being oxidized (Shahidi & Naczk, 2004). Often phenolic antioxidants lose their activity at high concentrations and behave as prooxidants (Gorden, 1990) by involvement in initiation reactions (Reactions VII, VIII).

$$AH + O_2 \longrightarrow \dot{A} + HOO^{\cdot} \quad \text{(VII)}$$

$$AH + ROOH \longrightarrow RO^{\cdot} + H_2O + \dot{A} \quad \text{(VIII)}$$

Phenolic antioxidants are more effective in extending the induction period when added to any oil that has not deteriorated to any great extent. However, they are ineffective in retarding decomposition of already deteriorated lipids (Mabarouk & Dugan, 1961). Thus, antioxidants should be added to foodstuffs as early as possible during processing and storage in order to achieve maximum protection against oxidation (Shahidi & Wanasundara, 1992).

2.6. Measurement of antioxidant activity

The need to measure antioxidant activity is well documented; these are carried out for meaningful comparison of foods or commercial products and for provision of quality standards for regulatory issues and health claim (Shahidi & Ho, 2007). Lipid oxidation is conventionally

studied by determination of peroxide value (PV), thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), or *para*-anisidine value (*p*-AV) or by assessing volatile compounds (Kristinova *et a.*, 2009).

There are numerous methods for measuring antioxidant activity; these may be classified into two categories. The first category measures the ability of antioxidants in inhibiting oxidation in a model system by monitoring the associated changes using physical, chemical or instrumental means. Radical scavenging assays include methods based on hydrogen atom transfer (HAT) or electron transfer (ET) mechanisms.

Oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays are the major methods that measure HAT while trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays represent ET-based methods (Shahidi & Ho, 2007). HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation while ET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls and radicals (Shahidi & Zhong, 2005, 2007). Table 2.1 summarizes the methods commonly used to measure antioxidant activity and the units they carry.

2.7. Fruits and vegetables as sources of antioxidants

Numerous naturally occurring, biologically active, compounds have been identified in plant sources and vegetable extracts that impart health benefits beyond basic nutrition (Oomah &

Table 2.1. Antioxidant activity measurement methods and units

Methods	Measurement units
Peroxide value (PV)	Milliequivalents of oxygen per kilogram of sample (meq/kg)
Conjugated dienes and trienes	Conjugable oxidation products (COPs)
Thiobarbituric acid reactive substances (TBARS)	Milligrams of malondialdehyde (MDA) equivalents per kilogram sample or micromoles of MDA equivalents per gram of sample (meq/g)
p-Anisidine value (*p*-AnV)	Absorbance of a solution resulting from the reaction of 1 g of fat in isooctane solution (100 ml) with *p*-anisidine
Electrical conductivity	Oil stability index (OSI) value, which is defined as the point of maximal change of the rate of oxidation
Oxygen radical absorbance capacity (ORAC)	µmol of trolox equivalents
Total radical-trapping antioxidant parameter (TRAP)	µmol per litre peroxyl radical deactivated
Trolox equivalent antioxidant capacity (TEAC)	mM trolox equivalent to 1 mM test substance
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	EC_{50} (Concentration to decrease concentration of test free radical by 50%); T_{EC50} (Time to decrease concentration of the test free radical by 50%); AE (Antiradical efficiency $(1/EC_{50})$ T_{EC50})
Ferric reducing antioxidant power (FRAP)	Absorbance of Fe^{2+} complex at 593 nm produced by antioxidant reduction of corresponding tripyridyltriazine Fe^{2+}complex

Mazza, 2000). Common fruits such as cranberries, apples, blueberries, grapes, raspberries, and strawberries and their beverages like red wine, apple and orange juices are rich sources of phenolic phytochemicals. In addition to fruits, vegetables such as cabbage and onion; food grains such as wheat, sorghum, millet, barley, as well as legumes such as peas, lentils and others are also described as important sources of phenolics (Vattem *et al* 2005). The World Health Organization (WHO) has noted that a minimum consumption of 400 g of fruit and vegetables per day is needed, to help prevent chronic disease and nutritional deficiencies (Rekhy & McConchie, 2014). The above-mentioned foods are major sources of antioxidants that increase the plasma antioxidant capacity resulting in inhibition of atheroscelerosis related diseases in humans (Cao *et al* 1998). Researchers have noted highly significant negative correlation between total intake of fruits and vegetables, cardio- and cerebrovascular diseases and mortality (Acheson & Williams, 1983; Verlangieri *et al* 1985). According to Ziebland and Roe (2002), the increase in plasma antioxidant level has a clinical significance as it can reduce the risk of coronary heart disease, atherosclerosis, epithelial cancer and stroke.

Fruits and vegetables have antioxidative effects that boosts defense among patients with type 2 diabetes. It has been noted that patients with type 2 diabetes suffer from oxidative stress due to diminished antioxidative defense (Asgard *et al* 2007). These result in further complications among patients, including kidney problem, eye problem and problems associated with the circulatory system. This diminished defense is attributed to the formation of hyperglycaemia which leads to the development of ROS. Asgard *et al* (2007) also noted that patient suffering from type 2 diabetes had higher levels of peroxidation products. The study revealed that high vegetable and fruit intake can lead to a significant reduction in the level of inflammation.

Dietary components in fruits and vegetables acting as antioxidants are fibre, polyphenols, favonoids, linoleic acid, vitamins A, B, C, E, tocopherols, calcium, selenium, chlorophylls, alipharin, sulphides, and tetrahydrocurcumin (Karakaya & Kavas, 1999). These compounds may act independently or in combination as anticancer or cardioprotective agents by a variety of mechanisms. Fruits and vegetables contain hundreds of different antioxidant substances and phytochemicals (Thiel, 2010). These antioxidant substances are useful in managing oxidative stress which is caused by an imbalance between free radicals within the body and the antioxidant defense mechanism (Asgard *et al* 2007).

2.8. Cranberry as a rich source of phenolic compounds

Berries, including raspberries, blueberries, black currants, red currants, and cranberries, are a rich source of dietary antioxidants. Among them, cranberries (*Vaccinium macrocarpon*) are a rich source of phenolic compounds, particularly phenolic acids (including hydroxybenzoic, hydroxycinnamic and ellagic acids) and flavonoids (including flavonols, flavan-3-ols, anthocyanins, and proanthocyanidins [PACs]) (McKay *et al* 2015). Phenolic compounds in cranberry are present in the free, esterified and bound forms. They are mostly substituted derivatives of hydroxycinnamic acid as free form and hydroxybenzoic acid as bound form (Shahidi & Naczk, 2004).

Phenolic acids present in berries include cinnamic acid derivatives, namely caffeic, chlorogenic, ferulic, sinapic, and *p*-coumaric acids that appear to be more active antioxidants than

hydroxybenzoic acid derivatives, such as *p*-hydroxybenzoic, vanillic, and syringic acids. Flavonols can be found in abundance in Ericaceae family such as cranberry, blueberry, bilberry and raspberry (Nijveldt *et al* 2001; Heinonen, 2007). They are present mainly in the skin of fruits (Price *et al* 1999). Cranberry is one of the leading fruit sources of flavonols on a weight basis compared to other berries. The average flavonol content in cranberry is 20 - 30 mg/100 g fresh fruit weight, although contents as high as 48 mg/100 g have also been reported (Neto, 2007).

2.9. Extraction of phenolics

The chemistry of phenolics varies in different classes and this is one of the important factors influencing their extraction. Extraction of phenolics is also dependent on factors such as sample particle size, solvent system, extraction method, storage time, temperature and presence of other substances (Naczk & Shahidi, 2004). The different extraction solvents commonly used for phenolics are methanol, ethanol, propanol, acetone, water, ethyl acetate and their various combinations with each other or with water. The extraction of phenolics can be improved by adjusting sample-to-solvent ratio (Naczk & Shahidi, 1991). Sample particle size was found to significantly influence tannin recovery from dry beans (Deshpande, 1985). Optimization of polyphenolic extraction is essential due to large variation in their polarity and biochemical modifications such as glycosylation and esterification that affect the extraction output (Pellegrini *et al* 2007). Michiels *et al* (2012) evaluated various solvent to sample ratios and found that higher extraction of polyphenolics can be achieved at higher solvent-to-sample ratios. The proposed extraction conditions included extraction solvent, acetone-water-acetic acid mixture (70:29.5:0.5, v/v/v) with a solvent-to-solid ratio of 20:1 (v/w) and extraction for 1 h at 4^{o}C.

Komes *et al* (2011) demonstrated that hydrolysed extracts of medicinal plants (using 60% ethanol and 5 mL of 2M hydrochloric acid) had higher total phenolic content than non-hydrolysed extracts.

Krygier *et al* (1982), extracted free and esterified phenolic acids from oilseeds using a mixture of methanol-acetone-water (7:7:6, v/v/v) at room temperature. Firstly, the free phenolics were extracted with diethyl ether, and then the extract was treated with 4M NaOH under nitrogen. The resultant material was acidified and the liberated phenolic acids were extracted with diethyl ether. After exhaustive extraction with a mixture of methanol-acetone-water, the left-over sample was treated with 4M NaOH under nitrogen to liberate insoluble bound phenolics.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Cranberry samples, namely wild clone NL2, pilgrim, wild clone NL3, wild clone PEI and wild clone NL1 were obtained from the Research Centre of the Agriculture and Agri-Food Canada, St. John's, NL. Market samples were purchased from Costco in St. John's, NL; these were produced in Nova Scotia, Canada.

Folin-Ciocalteu's reagent, ferulic acid, *p*-coumaric acid, catechin, aluminum chloride, sodium nitrite, 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonate)(ABTS), sodium chloride, potassium ferricyanide, 2,2-azobis (2methylpropionamidine) dihydrochloride (AAPH), trichloroacetic acid (TCA), ferric chloride, ferrous chloride, ethylenediaminetetraacetic acid (EDTA), mono- and dibasic potassium phosphates, BHT, and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulphonic acid sodium salt (Ferrozine) were purchased from Sigma-Aldrich Canada Ltd. (Oakville,ON). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Acros Organics (Fair Lawn, NJ, USA). Organic solvents and reagents such as diethyl ether, and ethyl acetate, as well as acetic acid, formic acid, hydrochloric acid, sodium hydroxide, and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON). 2,2-diphenyl-1-picrylhydrazyl (DPPH), and all phenolic compound standards with a purity of ≥96% were obtained from Sigma-Aldrich Canada Ltd. Hydrogen peroxide, sodium hydroxide, BHA, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), ferrous sulphate as well as mono- and dibasic sodium

and potassium phosphates, deoxyribonucleic acid (DNA) of pBR 322 (*E.coli* strain RRI) and human LDL cholesterol were also purchased from Sigma-Aldrich Canada Ltd.

3.2 Methods

3.2.1. Preparation of crude extracts

Each cranberry samples were selected according to their morphological characters such as size, shape, colours and weight and then homogenized in a Waring blender (model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA). Homogenized samples were then kept frozen at -20ºC until used within one week for extraction of phenolics.

3.2.2. Extraction of phenolics

Free, esterified, and insoluble-bound phenolic compounds were extracted and fractionated as described by Krygier *et al* (1982) and modified by Naczk and Shahidi (1989). Homogenized samples (100g) were sonicated with an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) for 25 min at 30°C under refluxing conditions with 100 mL of a mixture of acetone-water-acetic acid (70:29.5:0.5, v/v/v) .

The resulting slurries were centrifuged at 4000 x *g* (ICE Centra MS, International Equipment Co., Needham Heights, MA, USA) for 5 min and the supernatants were collected and extraction

was repeated two more times. Combined supernatants were evaporated under vacuum using a rotary evaporator (Buchi, Flawil, Switzerland) at 40 °C and lyophilized for 72 hours at - 46 °C and 34 X 10^{-3} mbar (Freezone, model 77530, Labconco Co., Kansas City, MO, USA). Residues of cranberry samples were air-dried for 12 hours and stored at -20 °C until used to extract bound phenolic compounds.

3.2.3. Extraction of free and esterified phenolic compounds

Free phenolic compounds and those liberated from soluble esters were extracted from the lyophilized crude phenolic extract (Chandrasekara & Shahidi, 2010). An aqueous suspension of extract (250 mg in 10 mL) was adjusted to pH 2 with 6 M HCl, and free phenolics (1:5, v/v) were extracted five times into diethyl ether and ethyl acetate (1:1, v/v). The free phenolic extract was evaporated to dryness under vacuum at room temperature. The water phase was neutralized to pH 7 with 2 M NaOH and then lyophilized. The resulting residue was dissolved in 10 mL of 2 M NaOH and hydrolyzed for 4 hours at room temperature under a nitrogen atmosphere. After acidification to pH 2 with 6 M HCl, phenolic acids released from soluble esters were extracted from the hydrolysates five times with diethyl ether and ethyl acetate (1:1, v/v) and evaporated to dryness under vacuum.

3.2.4. Extraction of bound phenolic compounds

The residue of the cranberry sample obtained after extraction of soluble phenolics was hydrolyzed with 2 M NaOH at room temperature for 4 hours with stirring under nitrogen. The resulting slurry was acidified to pH 2 with 6 M HCl and bound phenolic compounds were

extracted five times with diethyl ether and ethyl acetate (1:1, v/v) and subsequently desolventized to dryness at room temperature, using a rotary evaporator.

3.2.5. Determination of total phenolic content

Total phenol content was expressed as gallic acid equivalents (GAE; mg gallic acid eq/g of dried fruit weight) as described by Singleton and Rossi (1965) with slight modifications. Folin Ciocalteu's phenol reagent (0.5 mL) was added to centrifuge tubes containing 0.5mL of methanolic extracts. Contents were mixed thoroughly by vortexing and 1mL of sodium carbonate (75g/L) was added to each tube after 3 minutes. Finally, total volume was adjusted to 10mL with distilled water and mixed thoroughly. Tubes were then allowed to stand for 45 minutes at ambient temperature. Contents were centrifuged for 5min at 4000xg (ICE Centra M5, International Equipment Co., Needham Heights, MA, USA). Absorbance of the supernatant was read at 725nm using appropriate blanks for background subtraction. Total phenolic content in each extract was determined using a standard curve prepared for gallic acid.

3.2.6. Determination of total flavonoid content

Total flavonoid content was measured by the colourimetric method described by Kim *et al* (2003) with slight modifications. One millilitre of extracts were added to 10 mL volumetric flask containing 4 mL distilled water. Sodium nitrite (0.3 mL of 5% solution) was added the tube and allowed to react for 5 min. Then, 0.3 mL 10% $AlCl_3$ was added to the reaction mixtures and allowed to stand for 1 min. Finally, 2 mL of 1M NaOH solution was added and the total volume

was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured at 510 nm against a blank prepared in a similar manner by replacing the extract with distilled water. Total flavonoid content was calculated from a standard curve for catechin was expressed as micromoles of catechin equivalents (CE) per gram of dried cranberry.

3.2.7. Determination of total anthocyanin contents

The total anthocyanin content was determined by the pH differential method (Giusti and Wrolstad, 2001), which is a rapid and simple spectrophotometric method based on the anthocyanin structural transformation that occurs with a change in pH (colored at pH 1.0 and colorless at pH 4.5). Each cranberry extract (0.5 mL) was diluted with 2.5 mL of 0.025 M potassium chloride buffer, pH 1.0 and 0.4 M sodium acetate buffer, pH 4.5, separately. The diluted solutions were then left at room temperature for 15 min, and the absorbance of each dilution was read at 520 and 700 nm, respectively, against a blank cell filled with distilled water.

The anthocyanin content was calculated using the following equation:

Anthocyanins content as cyanidin-3-glucoside equivalents (mg / g of dry matter)

=A * MW * DF/ (€ X W)

Where A = absorbance (A_{520nm} - A_{700nm}) pH 1.0 – (A_{520nm} - A_{700nm}) pH 4.5, MW = molecular weight of cyanidin-3- glucoside (C_{15} H_{11} O_6, 449.2), DF =dilution factor, € = molar absorptivity (26,900), and W= sample weight (g).

3.2.8. Measurement of total antioxidant capacity using trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay is based on scavenging of 2, 2' azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$). A solution of $ABTS^{\bullet+}$ was prepared in 2.5 mM saline phosphate buffer (pH 7.4, 0.15M sodium chloride) (PBS) by mixing 2.5mM AAPH with 2.0 mM $ABTS^{\bullet+}$. The solution was heated for 20 min at 60°C, protected from light and stored in the dark at room temperature until used. The radical solution was used within 2 h as the absorbance of the radical itself decreases with time. Cranberry extracts were dissolved in PBS and diluted accordingly so that their absorbance fits in the range of values in the standard curve. For measuring antioxidant capacity, 0.04 mL of the sample solution was mixed with 1.96 mL of $ABTS^{\bullet+}$ solution. Absorbance of the above mixture was monitored at 734 nm over a six min period. The decrease in absorbance at 734 nm, for 6 min after the addition of a test compound, was used for calculating TEAC values. A standard curve was prepared by measuring the reduction in absorbance of $ABTS^{\bullet+}$ solution at different concentrations of trolox. Appropriate blank measurements (decrease in absorption at 734 nm due to solvent without any compound added) were made and the values recorded (Van den Berg *et al* 1999) as modified by Siriwardhana & Shahidi (2002).

TEAC values were determined as follows:

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

$$\Delta A_{trolox} = m \times [trolox]$$

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

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

3.2.9. DPPH radical scavenging capacity (DRSC) using electron paramagenetic resonance (EPR) spectrometry

The DRSC assay was carried out according to the method explained by Madhujith and Shahidi (2006) with slight modifications. Two millilitres of 0.3 mM solution of DPPH in methanol were added to 500µL of appropriately diluted free, esterified and bound phenolics extracts in methanol. The contents were mixed well, and were kept 10 min in dark. The mixture was then passed through the capillary tubing, which guides the sample through the sample cavity of a Bruker e-scan EPR spectrophotometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA, USA) and spectrum was recorded on Bruker E-scan food analyzer (Bruker Biospin Co). The parameters were set as follows: 5.02 x 10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep width, 3495.258 G centre field, 5.12 ms time constant, 9.795 GH_Z microwave frequency, 86.00 kH_Z modulation frequency, 1.86 G modulation amplitude. *DRSC* of the extracts was calculated using the following equation:

DPPH radical scavenging capacity % =

100 – [EPR signal intensity for the medium containing the additive / EPR signal intensity for the control medium] x 100.

The results were expressed as micromoles of trolox equivalents per gram of dried cranberry sample.

3.2.10. Determination of oxygen radical absorbance capacity (ORAC)

The $ORAC_{FL}$ determination was carried out using a Fluostar Optima plate reader (BMG Labtech, Durham, NC, USA) using fluorescein as the probe and AAPH as the radical generator. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), at a final reaction volume of 200 μL in a 96-well Costar 2650 black plate (Nepean, ON). Fluorescein dissolved in a phosphate buffer (120 μL; 64 nM, final concentration) was injected into each well using the first injector pump into the wells containing the extract (20 μL of cranberry extract). The mixture was incubated for 15 min at 37 °C in the built-in incubator, and subsequently APPH solution (60 μL; 29 mM final concentration), equilibrated at 37 °C, was rapidly injected into the wells. The plate was shaken for 4 s after each addition at a 4 mm shaking width. Fluorescence was recorded every minute for 60 min and the antioxidant activity of the extracts was calculated as trolox equivalents using a standard curve prepared with 1-10 μM (final concentration) control (trolox, buffer, fluorescein, and AAPH) and positive control (phosphate buffer and fluorescein) were used. Filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used (Madhujith & Shahidi, 2006).

3.2.11. Reducing power activity

The reducing power of extracts was determined by the method of Amarowicz *et al* (1995) and Oyaize (1986). Each phenolic extract (0.3–1.0 mg) was dissolved in 1.0 ml of ethanol and 2.5 ml of a 0.2 M phosphate buffer (pH 6.6) solution and 2.5 mL of a 1% (w/v) solution of potassium

ferricyanide [$K_3Fe(CN)_6$] was added for determination of reducing power. The mixture was incubated in a water bath at 50 °C for 20 min. 2.5 mL of a 10% (w/v) solution of trichloroacetic acid (TCA) were added and the mixture was subsequently centrifuged at 3010 x g for 10 min. After that, 2.5 mL of the supernatant was separated and 2.5 mL of distilled water and 0.5 mL of a 0.1% (w/v) solution of ferric chloride was added and mixed well. Absorbance of the reaction mixture was read spectrophotometrically at 700 nm. The results were expressed as micromoles of trolox equivalents (TE) per gram of dried sample.

3.2.12. Measurement of iron (II) chelation capacity

The ability of the phenolic extract to chelate iron (II) was assessed by the method of Liyana-Pathirana *et al* (2006) with slight modifications. A portion of the extract (0.2 mL) in ethanol was mixed with 0.02 mL of 2mM $FeCl_2$. The reaction was initiated by adding of 0.2 mL 5 mM Ferrozine, followed by vigorous shaking and allowed to stand for 10 min for colour development. The absorbance was measured spectrophotometrically at 562 nm. The results were expressed as micromoles of EDTA equivalents per gram of dried sample using EDTA standard curve.

3.2.13. Hydroxyl radical scavenging assay using EPR Spectroscopy

The hydroxyl radical scavenging capacity was determined according to the method explained by Chandrasekara and Shahidi (2011). Concentrations of 0.2 to 3 mg/mL cranberry extracts were

prepared by dissolved in deionized water. Then, extracts (0.1 mL) were mixed with 0.1 mL of H_2O_2, (10 mM) and 0.2 ml of DMPO (17.6 mM) and 0.1 mL of $FeSO_4$ (0.1 mM). After 3 min, the mixture was passed through the capillary tubing, which guides the sample through the sample cavity of a Bruker e-scan EPR spectrophotometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA, USA). The results were expressed as micromoles of catechin equivalents (CE) per gram of dried fruit weight. Deionized water was used as the control in place of the extract.

3.2.14. Determination of 2-thiobarbituric acid reactive substances (TBARS) in cooked ground pork model system

During lipid oxidation, malondialdehyde (MDA), a minor secondary oxidation product of fatty acids with 3 or more double bonds, is formed. MDA reacts with 2-thiobarbituric acid (TBA) to form a pink TBA-MDA complex that is measured spectrophotometrically at its absorption maximum of 532 nm (Shahidi & Zhong, 2007).

The effect of cranberry extracts in the inhibition of production of thiobarbituric acid reactive substances (TBARS) in cooked pork was determined (Wijeratne *et al* 2006). Ground pork was mixed with deionized water in Mason jars. Cranberry phenolic extracts (bound, free, esterified) and BHT were added separately to meat (20 g) and thoroughly mixed with a glass rod. A control sample was prepared without cranberry extract. Samples were cooked in a thermostated water bath at 80 ± 2ºC for 40 min while stirring every 5 min. Meat samples were mixed thoroughly, after cooling to room temperature and transferred into plastic bags, followed by storing in a refrigerator at 4ºC for 14 days. Samples were analyzed for TBARS on days 0, 4, 7 and 14 days.

A 2 g portion of each sample was weighed into a centrifuge tube to which 5 mL of a 10% (w/v) solution of trichloroacetic acid (TCA) were added and vortexed (Fisher Vortex Genie 2; Fisher Scientific, Nepean, ON) at high speed for 2 min. An aqueous solution (0.02 M) of 5 mL of TBA reagent was then added to each centrifuge tube, followed by further vortexing for 30 s. The samples were subsequently centrifuged at 3000x*g* for 10 min and the supernatants were filtered through a Whatman No. 3 filter paper. Filtrates were heated in a boiling water bath for 45 min, cooled to room temperature in cold water, and the absorbance of the resultant pink-coloured was measured. A standard curve was prepared using 1,1,3,3-tetramethoxypropane as a precursor of the malondialdehyde (MDA; 0, 1, 2, 3 and 6 ppm). The TBARS values were then calculated using the standard curve and expressed as milligrams MDA equivalents per kg of sample.

3.2.15. Supercoiled strand DNA scission by peroxyl radicals

The inhibition activity of the cranberry extracts against supercoiled DNA strand scission induced by peroxyl radical was evaluated according to the methods of Hiramoto *et al* (1996) and Chandrasekara and Shahidi (2011). Concentration of 10µg/mL of plasmid supercoiled DNA (pBR 322) was prepared by using 0.5 M phosphate buffered saline (PBS) pH 7.4.

The DNA (2 µL) and PBS (2 µL) was added to 2 µL of (0.05mg/ml) extract samples and extract was mixed well. Then, 4 µL of AAPH (7 mM) dissolved in PBS were added to each extract samples. The mixture was mixed well again and incubated at 37°C for 90 min. Upon completion of incubation, 2 µL of the loading dye (consisting of 0.025 g of bromophenol blue, 0.025g of

xylene cyanol, 5mL of glycerol and 5 mL of distilled water) were added to the extracts and loaded to a 0.7% (w/v) agarose gel. The gel was prepared in 40 mM Tris-acetic acid (7.25g/L) - EDTA buffer, 1 mM EDTA (0.36g/l), pH 8.5. Thereafter, 5 µL SYBR Safe were added to DNA gel, setting at 85 Volt for 75 min at 4 °C. DNA strands were visualized under ultraviolet light.

DNA retention (%) = (Supercoiled DNA content in sample/Supercoiled DNA content in control)*100

3.2.16. Effect of cranberry extracts on preventing cupric ion induced human low density lipoprotein (LDL) cholesterol peroxidation

The methods of Chandrasekara and Shahidi (2011) and Andreasen *et al* (2001) were used to measure inhibitory activities of cranberry extracts against human LDL cholesterol oxidation by measuring conjugated dienes (CD) produced in the system. Human LDL cholesterol (in PBS, pH 7.4, with 0.01% EDTA) was dialyzed with 10mM PBS (pH 7.4, 0.15 M NaCl) for 12h under nitrogen at 4°C, and EDTA-free LDL was subsequently diluted with PBS to obtain a 0.1 mg/ml. The diluted LDL cholesterol solution (0.8 mL) was mixed with 100 µL of extract (0.125 and 0.5 mg/mL) in an Eppendorf tube. Oxidation of LDL cholesterol was initiated by adding 0.1 ml of 100 µM $CuSO_4$ solution in distilled water. The mixture was incubated at 37°C for 24 h. The initial absorbance (t=0) was read at 234nm immediately after mixing and conjugated diene (CD) hydroperoxides formed were measured at 11, 22 and 24 h intervals. The corrected absorbance at 24 h against 0 h was employed to calculate the percentage inhibition of CD formation using the following equation.

$$\text{Percentage inhibition of CD formation} = \frac{(\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{sample}})}{(\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{native}})} \times 100$$

Where, $Abs_{oxidative}$ = absorbance of LDL mixture and distilled water with $CuSO_4$ only, Abs_{sample} = absorbance of LDL with extract and $CuSO_4$, and Abs_{native} = absorbance of LDL with distilled water.

3.2.17. Determination of major phenolic compounds by HPLC/ESI-MS/MS

The major phenolic compounds present in the cranberry samples were determined by HPLC, using an Agilent 1100 HPLC unit (Agilent Technologies, Palo Alto, CA, USA) with a UV-diode array detector (UV-DAD). A slightly modified version of the method outlined by Lin and Hanly (2007) was used. Genotypes of wild clone NL2 and pilgrim were used for HPLC analysis. Briefly, samples were prepared by dissolving 0.5 mL of free, esterified, and bound phenolic extracts of cranberry in 2.0 mL of 50% HPLC grade methanol and passing through a 0.45-μm syringe filter (purchased from Sigma-Aldrich Canada Ltd, Oakville, ON) before injection into a reverse phase C18 column (250 mm length, 4.6 mm i.d., 5 μm particle size, Sigma-Aldrich Canada Ltd.) with a guard column.

The column oven temperature was set at 25 °C. The mobile phase consisted of a combination of B (0.1% formic acid in acetonitrile) and D (0.1% formic acid in water). The gradient was as follows: 0 min, 100% D; 5 min, 10% B; 35 min, 15% B; 45 min, 40% B; 55 min, 100% B; 65 min, 100% D and then held for 10 min before returning to the initial conditions. The flow rate was 0.5 mL/min and the wavelengths of detection were 280, 306, 350 and 520 nm. HPLC of cranberry extracts was analyzed online by using a mass selective detector system (LC-MSD-Trap-SL, Agilent). In mass spectral analysis, ESI (electrospray ionization) at negative ion mode

was selected as it was able to provide detailed structural information through collision induced dissociation.

3.2.18. Statistical analysis

All experiments were carried out in triplicates and results were reported as mean ± standard deviation. The significance of differences among the values was determined at $p < 0.05$ using analysis of variance (ANOVA) followed by Turkey's multiple range tests using SPSS 16.0 for Windows (Snedecor & Cochran, 1980).

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Total phenolic content (TPC)

The Folin–Ciocalteu method is generally used for the measurement of TPC, although the reaction measures all constituents possessing hydroxyl groups and having reducing power (Huang *et al* 2005). The content of free phenolics in tested cranberry samples in the decreasing order was: pilgrim > cranberry wild clone NL2 > cranberry wild clone NL3 > cranberry wild clone PEI > cranberry wild clone NL1 > market mature > market immature (Table 4.1). The esterified phenolic compounds in the extracts of cranberry varieties followed a similar trend.

Pilgrim genotype contained the highest amount of free (15.60±1.02 mg GAE/g of dried fruit weight), esterified (12.88±0.84 mg GAE/ g of dried fruit weight) and bound (1.55±0.19 mg GAE/g of dried fruit weight) phenolics. Furthermore, the results indicated that the TPC in the free phenolic form of all genotypes contained the highest amount compared to the bound and esterified extracts, except in the market sample where esterified phenolic form were present in higher quantities than the free form. The percentage of free phenolics in the total phenolic content of pilgrim, cranberry wild clone NL2, cranberry wild clone NL3, cranberry wild clone PEI, cranberry wild clone NL1, market mature and market immature were 52, 58, 64, 68, 82, 44 and 36 %, respectively. It was also noted that TPC in the bound form had the lowest level ($\leq$ 9%) in all genotypes. There was no significant ($p>0.05$) difference between free phenolic content in cranberry wild clone NL3 and cranberry wild clone PEI (Table 4.1).

In agreement with this finding, Sun *et al* (2002) reported that phenolics in soluble free form were higher than that of bound form in cranberry, strawberry, apple, red grape, peach, pear, grapefruit, banana and lemons. It is also important to highlight that phenolic acids in cranberry occur mainly in the bound form, esterified to sugars, and cell wall polysaccharides, mainly in the form of glycosides (Sanchez-Patan *et al* 2012; Sun *et al* 2002).

In general, the phenolic contents in all tested cranberry genotypes were higher than that in the market sample and those given in the existing literature (Sun *et al* 2002; Cote *et al* 2011). Furthermore, Kahkonen *et al* (2001) reported that the phenolic content of cranberry, raspberry and strawberry was lower than all five tested cranberry genotypes. Costantino *et al* (2001) quantified TPC to be 256 mg/100 g in fresh bilberry, and 221 mg/100 g in bilberry press residues. Grapes known as a rich source of polyphenols contained a TPC of 294 mg/100 g (Vinson *et al* 2001). The discrepancies in the results obtained in this study may vary due to their chemical nature, extraction method, and ratio of sample to solvent, extraction time, pH, and temperature (Chandrasekara & Shahidi, 2010). Zielinski *et al* (2000) reported that ultrasonic assisted extraction is a simple alternative to conventional extraction methods. Also they report that other compounds such as simple carbohydrates and/or amino acids may be present in the crude extracts and could interfere with determinations of TPC by the Folin-Ciocalteu assay, leading to discrepancies.

Table 4.1. Total contents of phenolics and flavonoids of cranberry fruit samples on a dry weight basis

Cranberry sample	Total phenolics (mg GAE/g dried fruit weight)			Total flavonoids (mg CE /g dried fruit weight)		
	Free	Esterified	Bound	Free	Esterified	Bound
Cranberry wild clone NL2	13.83±0.77[b]	8.59±0.44[b]	1.35±0.13[b]	7.34±0.19[b]	1.53±0.03[c]	1.20±0.03[b]
Pilgrim	15.60±1.02[a]	12.88±0.84[a]	1.55±0.19[a]	9.39±0.2[a]	9.18±0.03[a]	1.37±0.04[a]
Cranberry wild clone NL3	10.60±0.55[c]	4.76±0.24[c]	1.12±0.16 [c]	6.76±0.09[c]	2.20±0.03[b]	0.60±0.07[c]
Cranberry wild clone PEI	10.42±0.74[c]	4.04±0.58[c]	0.81±0.11[e]	7.62±0.09[b]	0.74±0.01[d]	0.21±0.04[e]
Cranberry wild clone NL1	8.99±0.74[d]	0.99±0.09[e]	0.94±0.05[d]	6.79±0.05[c]	0.39±0.01[e]	0.60±0.12[c]
Market-Immature	1.46±0.21[f]	2.16±0.25[d]	0.40±0.07[e]	0.31±0.05[d]	0.44±0.02[e]	0.37±0.01[d]
Market-Mature	2.65±0.32[e]	2.73±0.41[d]	0.70±0.07[d]	0.58±0.04[d]	0.47±0.02[e]	0.33±0.05[d]

[1]Data are expressed as means ± SD (n=3).Values with the same letter in the same column are not significantly different (p>0.05)

It is also of interest to indicate that TPC in all tested cranberry genotypes were higher compared to the European cranberry genotypes (Kraujalyte *et al* 2013). There is also evidence that the total amount of phenolics in cranberry varies among different varieties, cultivars, preharvest practices, environmental conditions, maturity stage at harvest, all of which control the accumulation of phenolic compounds by synthesizing different quantities and types of phenolics (Manganaris *et al* 2013).

4.2. Total flavonoid content (TFC)

Flavonoids perform a variety of functions in plants, including protection from UV radiation, defense against pathogens, pollinator attraction, pigmentation, and playing an essential role in reproduction (Koes *et al* 1994). They also exhibit a variety of biological activities in *in vitro* and *in vivo* studies, including antioxidative effects (Boyle *et al* 2000), reduction of cardiovascular disease and reduction of the risk of rheumatoid arthritis (Pattison *et al* 2004). Flavonoids are an important group of compounds present in large amounts in berries.

In this study, the pilgrim exhibited the highest total flavonoid content (19.94 mg CE/g of dried fruit weight), followed by cranberry wild clone NL2 (10.07 mg CE /g of dried fruit weight), cranberry wild clone NL3, cranberry wild clone PEI and cranberry wild clone NL1, respectively. It was also noted that the free phenolic fractions were present in highest flavonoid quantities in all varieties compared to the esterified and bound fractions (Table 4.1), except in the immature market sample where esterified phenolic fractions were present in higher quantities than the free form (Table 4.1). The free phenolic fraction of cranberry wild clone NL2, cranberry wild clone

PEI and cranberry wild clone NL3, cranberry wild clone NL1 did not show any significant ($p > 0.05$) difference among varieties.

In general, in all extracts the TFC in bound fraction were lower than their corresponding free and esterified fractions. The TFC in bound phenolic fraction of pilgrim had the highest level (1.37 mg of CE/g of dried fruit weight), whereas cranberry wild clone PEI showed the lowest (0.21 mg of CE/g of dried fruit weight). The TFC in the free fraction of pilgrim was highest compared to the free form of other genotypes. The TFC in bound fractions of all tested cranberry genotypes varied significantly ($p<0.05$) among varieties, except cranberry wild clone NL3, cranberry wild clone NL1 and immature market, mature market. In contrast to the results obtained in this study, Tuloi *et al* (2014) reported that TFC (47.5 mg CE/100g of fresh fruit) in cranberry were higher than the subjected genotypes.

As we mentioned above, the free phenolic fractions were present in highest flavonoid quantities in all tested cranberry varieties compared to the esterified and bound fractions (Table 4.1). In agreement with this finding, Meyers *et al* (2003) have reported bound fraction were lower in strawberry than their corresponding free and esterified fractions.

The TFC reported in this study for different cranberry genotypes were lower than those reported for other berries, namely strawberry, blueberry and raspberry (Lin & Tang 2007). Furthermore, the TFC of all tested cranberry genotypes were lower than those of four different raspberry genotypes (Liu *et al* 2002). The variety of berries may account for differences in the results obtained as well as possible variations in the extraction conditions, cultivars considered,

environmental conditions, and maturity stage at harvest (Manganaris *et al* 2013). To the best of our knowledge, this is the first time that TFC was determined in cranberry as free, esterified and bound forms.

4.3 Total anthocyanin contents

Total anthocyanins content in the whole cranberry extracts was determined as mg cyanidin-3-O-glucoside equivalents per gram of dry fruit weight. Anthocyanins content in different genotypes of cranberry were in the decreasing order of cranberry wild clone NL3 > cranberry wild clone NL2 > pilgrim > market mature > cranberry wild clone NL1 > cranberry wild clone PEI > market immature (Table 4.2).

The highest content of anthocyanins was present in cranberry wild clone NL3 (2.22±0.10 mg/g of dried fruit weight), while anthocyanin content in pilgrim genotype was 1.15±0.07 mg/ g of dried fruit weight. This result is in agreement with that of viskelis *at el* (2009) who reported that the total anthocyanin in overripe pilgrim was 1.37 mg/g of sample. There was also no significant difference ($p>0.05$) between cranberry wild clone NL1, pilgrim and cranberry wild clone PEI and the immature market sample. The color of cranberries is due to the presence of anthocyanins. Major anthocyanins in cranberry are galactosides and arabinosides of cyanidin and peonidin (macheix *et al* 1990; Neto, 2007). The amount of anthocyanins in cranberries depends on the degree of berry ripening and the peculiarities of cultivars, thus larger amounts of anthocyanins were found in overripe berries (viskelis *et al* 2009). Different types of anthocyanins were also seen due to ripeness and cultivar differences (Brown *et al* 2012; Sapers & Hargrave, 1987).

4.4. Reducing power (RP)

The reducing power shows the ability of a molecule or an extract to inhibit the radical chain reaction and can be considered an indicator of potential antioxidant activity of the extract. With this assay, the reduction of ferric to ferrous ion is measured spectrophotometrically and the intensity of Perl's Prussian blue is read, which depends on the reducing ability of the extract. The higher the absorbance, the greater is the reducing power (Miceli *et al* 2009). Reducing power of the free, esterified and bound phenolics of the extracts is shown in Table 4.3. The reducing power of genotypes followed a different trend compared to TPC and TFC with esterified phenolic of pilgrim showing the highest reducing power (1.12±0.02 mmoles trolox equivalent (TE) /g dried fruit weight), followed by cranberry wild clone NL2 (0.72±0.09 mmoles TE/g dried fruit weight), cranberry wild clone NL3, cranberry wild clone PEI, mature market sample and immature market sample, respectively, except in cranberry wild clone NL1, where free phenolic extract showed the highest reducing power compared to that of its bound and esterified forms.

The reducing power of the bound phenolic was comparable to those of the free and esterified phenolics that showed the lowest activity. Meantime, the bound phenolic extract of pilgrim had the highest reducing power (0.18 ± 0.01 mmoles TE /g dried fruit weight), whereas immature market sample showed the lowest (0.04 ± 0.00 mmoles TE /g dried fruit weight). According to reducing power activity of cranberry, esterified phenolic extracts are the dominant form of phenolics and contribute most to the antioxidant activity of cranberry followed by free and bound phenolics**.**

Compounds with high RP are capable of better donating electrons and thus reducing the oxidized intermediates in peroxidation process by acting as antioxidants. Reductants in the extracts reduce the ferric / ferricyanide complex to the ferrous form. Cranberry extracts tested in the present study exhibited a considerable RP, thereby acting as effective reductants. Reducing power activity of tested cranberry genotypes followed a similar trend that was associated with TPC and TFC**,** whereas, reducing power activity in tested cranberry samples in the decreasing order was: pilgrim > cranberry wild clone NL2 > cranberry wild clone NL3 > cranberry wild clone NL1 > market mature > cranberry wild clone PEI > market immature (Table 4.3).

In general, the RP in all tested cranberry genotypes was higher than those given in the existing literature (Celik *et al* 2008). Furthermore, the RP of all tested cranberry genotypes was higher than those reported for wild and cultivated red raspberry, blueberry, blackberry, red current, gooseberry and cornelian cherry (Cekic & Ozgen, 2010; Pantelidis *et al* 2007).

4.5. Ferrous ions chelating activity

Metals such as iron, copper, and nickel are essential minerals for normal physiology, but an excess of them may result in cellular injury by initiation of peroxidation in foods and biological systems. If it undergoes the Fenton reaction, this reduced metal may form highly reactive hydroxyl radicals and thereby contribute to oxidative stress. The resulting oxy radicals cause damage to cellular lipids, nucleic acids, proteins, carbohydrates and lead to cellular impairment.

Table 4.2. Total contents of anthocyanin in cranberry fruit extracts on a dry weight basis

Cranberry Sample	Anthocyanin Content (mg/g of dried fruit weight
Cranberry wild clone NL2	1.25 ± 0.11^{b}
Pilgrim	1.15 ± 0.07^{b}
Cranberry wild clone NL3	2.22 ± 0.10^{a}
Cranberry wild clone PEI	0.54 ± 0.05^{e}
Cranberry wild clone NL1	0.71 ± 0.04^{d}
Immature market	0.41 ± 0.02^{e}
Mature market	0.91 ± 0.04^{c}

Values with the same letter are not significantly different ($p>0.05$)

Chelation of free iron can prevent the formation of free radicals as well as preventing the impairment of vital organ function *in vivo.* Chelating agents, which bind prooxidant metals, are effective as secondary antioxidants because of the formation of a complex between them and the metal ions which renders the latter inactive so that they can no longer act as initiator of lipid oxidation (Shahidi & Zhong, 2007). Since ferrous ions are the most effective prooxidants in the food system, the good chelating effect would be beneficial and removal of free iron from circulation could be a promising approach to prevent oxidative stress-induced diseases (Ningappa *et al* 2008). The present study demonstrated significant chelating activity of bound, free and esterified phenolic extracts of cranberry genotypes against Fe^{2+} in all tested cranberry genotypes (Table 4.3 and Figure 4.1).

The chelating activity of bound phenolic extracts ranged from 0.32±0.00 to 5.16 ±0.34 μmol of EDTA (ethylenediaminetetraacetic acid) equivalents /g of cranberry on a dry weight basis. Bound phenolic extracts of cranberry wild clone NL2 had the highest ferrous ion chelating activity, whereas mature market sample showed the lowest. The results of this study indicate that phenolic extracts of pilgrim may serve better as a potential source of chelating agents. It contained bound phenolic extract of 3.88 ±0.19 μmol of EDTA equivalents /g of dry fruit weight, free phenolic extract of 1.27 ±0.06 μmol of EDTA eq /g of dried fruit weight and esterified extract of 4.27 ±0.71 μmol of EDTA eq /g of dried fruit weight. Esterified extracts of cranberry wild clone NL3 had the highest ferrous ion chelating activity (4.48 ±0.11 μmol of EDTA eq /g of dried fruit weight), whereas mature market sample showed the lowest (0.31 μmol of EDTA eq /g of dried fruit weight).

Table 4.3. Antioxidant capacity (reducing power and iron chelation) of cranberry fruit extracts on a dry weight basis

Cranberry sample	Reducing power (mmoles TE/g of dried fruit weight)			Iron chelating activity (μmoles EDTA e.q./ g dried fruit weight)		
	Free phenolics	Esterified phenolics	Bound phenolics	Free phenolics	Esterified phenolics	Bound phenolics
Cranberry wild clone NL2	0.39±0.00^{c}	0.72±0.09^{b}	0.12±0.00^{b}	1.13±0.02^{a}	2.12±0.07^{c}	5.16±0.34^{a}
Pilgrim	0.66±0.07^{a}	1.12±0.02^{a}	0.18±0.01^{a}	1.27±0.06^{a}	4.27±0.71^{a}	3.88±0.19^{b}
Cranberry wild clone NL3	0.34±0.09^{c}	0.66±0.00^{c}	0.12±0.01^{b}	1.2±0.12^{a}	4.48±0.11^{a}	2.51±0.00^{c}
Cranberry wild clone PEI	0.22±0.03^{d}	0.26±0.04^{d}	0.09±0.00^{c}	0.31±0.03^{b}	3.23±0.05^{b}	2.02±0.02^{d}
Cranberry wild clone NL1	0.49±0.00^{b}	0.15±0.01^{e}	0.11±0.00^{b}	0.03±0.00^{c}	1.42±0.01^{c}	2.53±0.04^{c}
Market-Immature	0.14±0.00^{e}	0.19±0.02^{e}	0.04±0.00^{d}	0.41±0.15^{b}	0.51±0.01^{d}	0.46±0.01^{e}
Market-Mature	0.21±0.00^{d}	0.23±0.03^{d}	0.11±0.01^{b}	0.32±0.11^{b}	0.31±0.00^{d}	0.32±0.00^{e}

[1]Data are expressed as means ± SD (n=3).Values with the same letter in the same column are not significantly different (p>0.05).

In this study, the total ferrous ion chelating activity of cranberry genotype extracts decreased in the order of pilgrim > cranberry wild clone NL2 > cranberry wild clone NL3 > cranberry wild clone PEI > cranberry wild clone NL1> immature market > mature market (Figure 4.1). No significant difference ($p>0.05$) existed between esterified phenolics of cranberry wild clone NL2, cranberry wild clone NL1 and pilgrim, cranberry wild clone NL3. Similarly, no such difference existed between free phenolics of cranberry wild clone NL2, pilgrim, cranberry wild clone NL3 and cranberry wild clone PEI, immature market, mature market (Table 4.3).

Pilgrim extract showed the strongest chelating capacity, which is 10 times greater than that detected in the mature market sample on a dried fruit weight basis. These results suggest the potential of all tested cranberry genotypes to prevent oxidative damage from free radical mediated oxidation. In general, the results of present study had lower values of metal chelation compared to the blueberry (Dastmalchi *et al* 2011). In addition to that, a similar trend was observed between metal ion chelation and TPC, TFC in all tested cranberry genotypes. This indicates that phenolics and flavonoid compounds are the major constituents in the fruits contributing to their metal ions chelation activity (Dastmalchi *et al* 2011).

4.6. Trolox equivalent antioxidant capacity (TEAC)

TEAC assays measure the ability of cranberry phenolic extracts to reduce *in vitro* formed radicals (Bohm & Schlesier, 2004). Table 4.4 and Figure 4.2 show the TEAC of free, esterified and bound phenolic extract of cranberry genotypes.

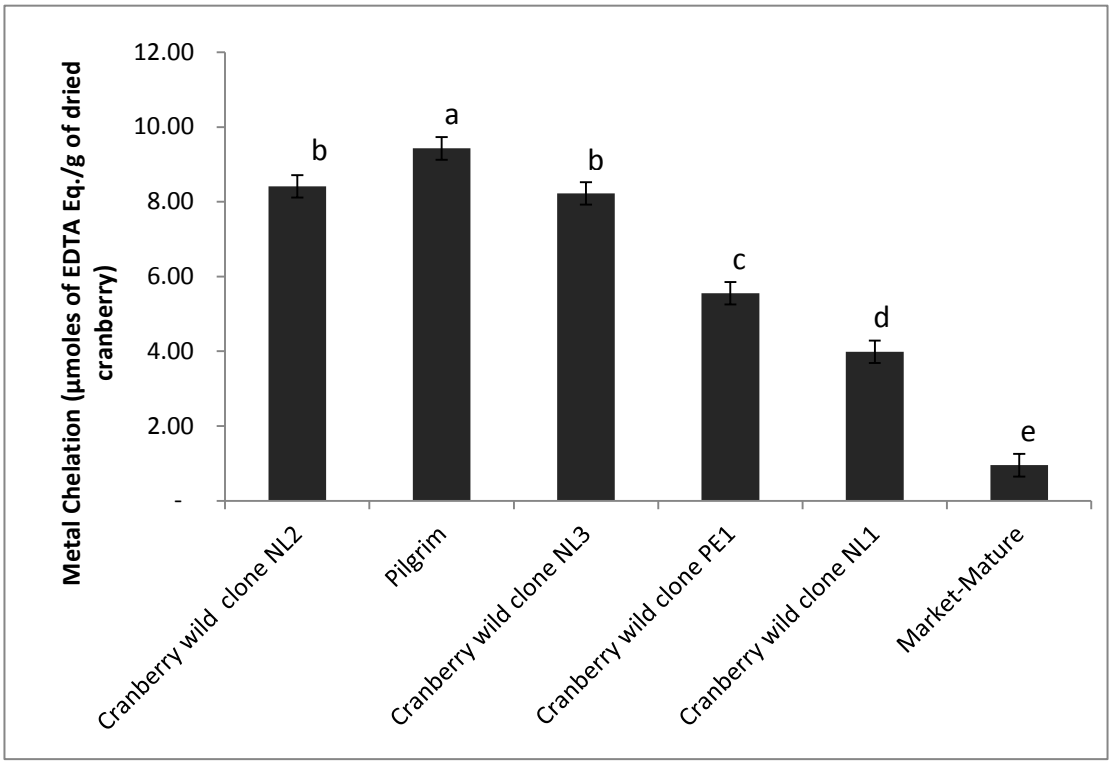


Figure 4.1. Total ferrous ion chelating activity of cranberry fruit samples on a dry weight basis

In general, samples with higher phenolic content were most effective as free radical scavengers. In this study, cranberry wild clone NL3 exhibited the highest total TEAC (979.66 µmoles trolox equivalents (TE) /g of dried fruit weight) and its esterified phenolic extract was responsible for 82% for total TEAC, followed by mature market sample (873.18 µmoles TE/g of dried fruit weight), pilgrim, cranberry wild clone NL2, cranberry wild clone PEI, immature market sample and cranberry wild clone NL1, respectively. The results indicated that esterified phenolic extract of cranberry wild clone NL3 had 5.4 times higher TEAC than that of its free phenolic form. However, mature market, pilgrim sample showed a similar quantity of free and esterified phenolics. Thus TEAC values of cranberry genotypes did not show any clear trend among free and esterified phenolic extracts. Meantime, bound phenolic extract of all genotypes showed the lowest TEAC values. The lowest value of 30.54 µmoles TE/g of dried fruit weight of bound phenolics was found in cranberry wild clone PEI (Table 4.4).

TEAC method is useful in screening antioxidants, but antioxidant effectiveness must also be studied by other methods because their activity in foods is dependent on a number of factors, including polarity, solubility, metal-chelating capacity and the system used for their evaluation. Others have measured the TEAC activity of berries. According to Tulio *et al* (2014), TEAC of cranberry extract ranged from 13.04 to 13.78 µmoles TE/100g of fresh weight and a study by Seeram *et al* (2008) showed that TEAC of cranberry juice ranged from 6.7 to 14.8 µmoles TE/mL. Furthermore, TEAC of bilberry seeds ranged from 5.8 to 84.4 µmoles of TE/g of berry seed press residues and those for black currant were 67.2 to 74.7 µmoles of TE/g of berry seed press residues, respectively (Helbig *et al* 2008). In general, the results of the present study showed higher total TEAC compared to the reported values.

The total TEAC values of all tested cranberry genotypes did not show any clear trend which is similar to that of TPC and TFC of samples. Thus, samples with higher phenolic and flavonoid contents did not exhibit higher antioxidant activity. In agreement with this finding, some studies have reported a lower TPC for berries exhibiting a higher total equivalent antioxidant capacity (Helbig *et al* 2008). This demonstrates that the contents of total phenolics and flavonoids may not sufficiently explain the observed antioxidant activity of fruit and plant phenolic extracts, which are mixtures of different compounds with variable activities in the test samples. Also the TEAC value of an extract is the product of a sum of antioxidant compounds that will depend on the solvent used to extract them from the test matrix (Schlesier *et al* 2002).

4.7. Analysis of oxygen radical absorbance capacity (ORAC)

As shown in Table 4.4, the contribution of esterified phenolic of cranberry extracts towards the total ORAC was higher than the free and bound phenolic counterparts. Esterified phenolics of cranberry wild clone NL3 exhibited the highest ORAC followed by cranberry wild clone NL2, pilgrim, mature market sample, immature market sample, cranberry wild clone PEI and cranberry wild clone NL1. There were no significant differences ($p>0.05$) among esterified phenolic extract of cranberry wild clone NL2, pilgrim, cranberry wild clone NL3 and immature market sample.

Table 4.4. Antioxidant capacity (TEAC and ORAC) of cranberry fruit extracts on a dry weight basis

Cranberry sample	TEAC (μmoles TE/g of dried fruit weight)			ORAC (mmoles TE/g dried fruit weight)		
	Free phenolics	Esterified phenolics	Bound phenolics	Free phenolics	Esterified phenolics	Bound phenolics
Cranberry wild clone NL2	243.46±3.01^{d}	368.08±3.90^{c}	43.91±1.21^{b}	6.36±0.77^{b}	9.94±0.21^{a}	1.02±0.09^{c}
Pilgrim	364.33±1.39^{b}	361.45±2.75^{c}	36.17±2.01^{c}	6.63±0.23^{b}	9.62±0.79^{a}	4.44±0.18^{a}
Cranberry wild clone NL3	147.11±2.09^{e}	801.69±7.03^{a}	30.80±0.92^{d}	4.38±0.52^{d}	10.05±0.5^{a}	2.02±0.16^{b}
Cranberry wild clone PEI	322.54±2.05^{c}	221.71±1.92^{d}	30.54±0.89^{d}	8.14±0.59^{a}	4.87±0.49^{c}	0.93±0.04^{c}
Cranberry wild clone NL1	176.41±1.99^{f}	62.88±2.94^{f}	31.45±0.75^{d}	6.73±0.29^{b}	3.69±0.53^{c}	2.62±0.17^{b}
Market-Immature	95.48±3.01^{g}	157.67±2.01^{e}	41.85±2.01^{b}	5.72±0.46^{c}	8.72±0.36^{a}	2.71±0.26^{b}
Market-Mature	391.94±2.33^{a}	421.72±4.23^{b}	59.52±1.05^{a}	6.91±0.39^{b}	8.93±0.06^{a}	2.23±0.36^{b}

[1]Data are expressed as means ± SD (n=3).Values with the same letter in the same column are not significantly different (p>0.05)

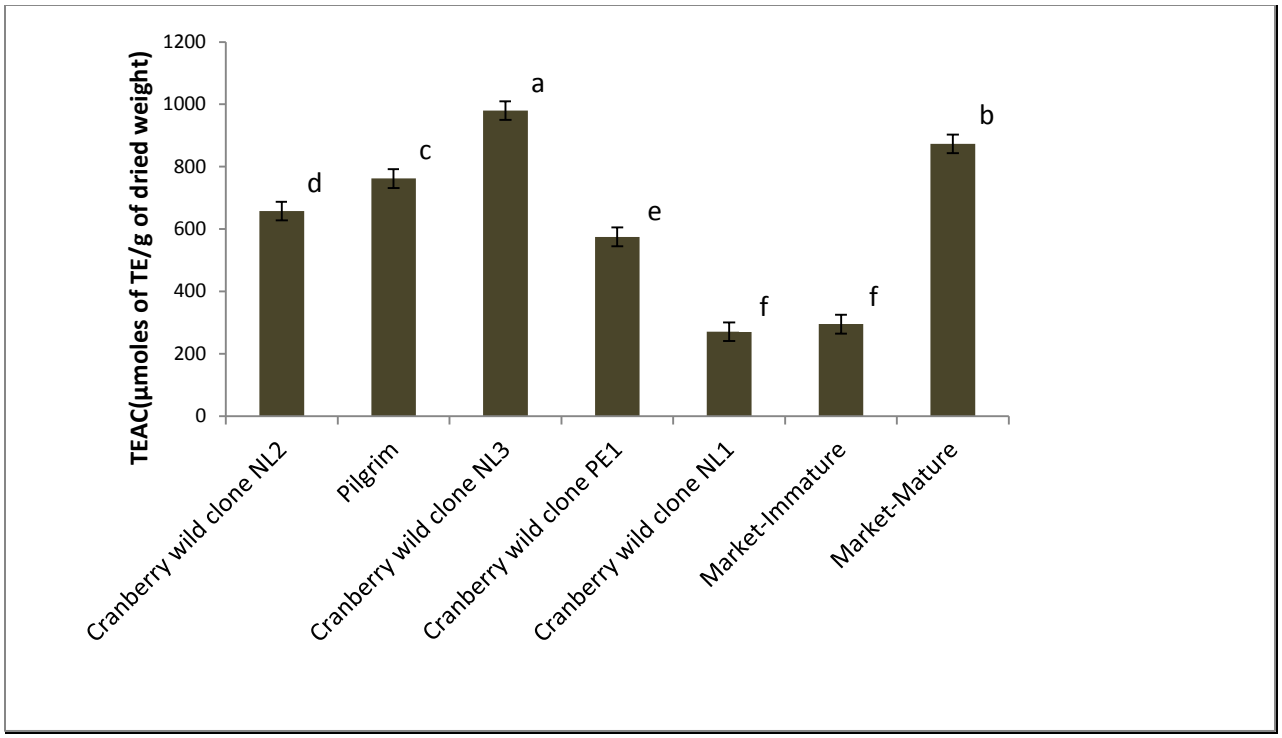


Figure 4.2. Total trolox equivalent antioxidant capacities (TEAC) of cranberry fruit samples on a dry weight basis

Free phenolics of cranberry wild clone PEI extracts showed the highest peroxyl radical scavenging activity, followed by mature market sample, cranberry wild clone NL1, pilgrim, cranberry wild clone NL2, immature market sample and cranberry wild clone NL3. Bound phenolic extracts of pilgrim showed the highest peroxyl radical scavenging activity, followed by immature market sample, cranberry wild clone NL1, mature market sample, cranberry wild clone NL3, cranberry wild clone NL2, and cranberry wild clone PEI.

The ORAC assay is among the standard assays accepted for measuring the antioxidative activity of botanicals, herbs, and nutraceuticals. It has been widely employed for the assessment of the free radical scavenging capacity of human plasma, proteins, DNA, pure antioxidants, and plant/food extracts (Madhujith & Shahidi, 2007). ORAC assay is based on the inhibition of the peroxyl radical-induced oxidation initiated by thermal decomposition of azo compounds such as 2,2´-azinobis [3-ethylbenzthiazoline-6-sulphonic acid] (AAPH) and ORAC is the only assay that combines both inhibition time and degree of inhibition into a single quantity (Prior *et al* 2005). In ORAC, fluorescein is employed as the probe resulting in the loss of fluorescence, which is detected with a fluorometer (Shahidi & Zhong, 2007). The ORAC assay measures the radical chain breaking ability of antioxidants by monitoring the inhibition of peroxyl radical, and the fluorescence decay indicates its reaction with peroxyl radical. In the presence of antioxidative compounds fluorescein decay is inhibited, and the intensity can be measured at 485 nm excitation and 525 nm emission wavelength.

On a dried fruit weight basis, the pilgrim had significantly higher ORAC values than the other genotypes. In this study, ORAC values of esterified, free and bound phenolics in all tested cranberry genotypes were in the range of 10.05-3.69, 8.14-4.38 and 4.44-0.93 mmoles of TE/g of dried fruit weight, respectively (Table 4.4). The total ORAC for cranberry genotypes were in the order of pilgrim > mature market > cranberry wild clone NL2 > immature market > cranberry wild clone NL3 > cranberry wild clone PEI > cranberry wild clone NL1 (Figure 4.3). This trend is very similar to those of TPC and TFC of the samples, except for the market samples. Thus, samples with higher total phenolic contents exhibited higher antioxidant activity. These results suggest that the antioxidant activity of fruits is derived mainly from the contribution of their phenolic compounds. In agreement with this trend, previous research has shown a linear relationship between total phenolic content and ORAC in various berry crops (Wang *et al* 2000; Parry *et al* 2006), suggesting that the phenolic compounds contribute to their oxygen radical absorbing capacities and the total phenolic content is a better indicator for oxygen radical absorbing components in berries. However, little information is available on the contribution of individual phenolic compounds to total antioxidant activity in berry crops (Zheng *et al* 2003) and also ORAC value of all tested cranberry genotypes had the highest activity compared to the reported values for cranberry, blackberry, raspberry and strawberry (Wang *et al* 2000; Zheng *et al* 2003; Parry *et al* 2006).

4.8. DPPH radical scavenging capacity (DRSC) using electron paramagnetic resonance (EPR)

The DRSC for cranberry genotypes were in the order of pilgrim > cranberry wild clone NL2 > cranberry wild clone NL3 > Immature market > cranberry wild clone PEI > mature market > cranberry wild clone NL1 (Table 4.5). DRSC of all genotypes were mainly contributed by their

esterified phenolics, except in cranberry wild clone NL1, where the free phenolic extracts displayed the highest activity.

There were no significant differences ($p>0.05$) among esterified phenolic extract of cranberry wild clone NL2, pilgrim, cranberry wild clone NL3, cranberry wild clone PEI, mature market and immature market sample. It also indicated that the bound phenolic extracts in all genotypes had the lowest DPPH radical scavenging activity as compared to their free and esterified counterparts, except in immature market sample, where the free phenolic forms and had a lower activity. It is also interesting that the esterified phenolic extracts contributed more than 78% toward the total DRSC activity except for cranberry wild clone NL1, where its contribution was only 29%.

According to Tulio *et al* (2014), the DRSC activity of cranberry phenolic extracts ranged from 6.13 to 6.28 µmoles TE/100g of fresh weight. However, this study presents higher total DRSC values compared to those reported. This trend is also very similar to the TPC and TFC of samples, indicating that samples with higher phenolic content exhibit higher antioxidant activity. However, cranberry wild clone NL1 had a higher total phenolic content than market samples (immature and mature) which showed a lower DRSC, because of the existing differences in the chemical composition contributing to scavenging activity. Shahidi and Naczk (2004) and Velioglu *et al* (1998) reported that the antioxidant activity of a given food or food product depends on the chemical nature of its constituents and, not always their quantities, as the efficacy of compounds present varies considerably.

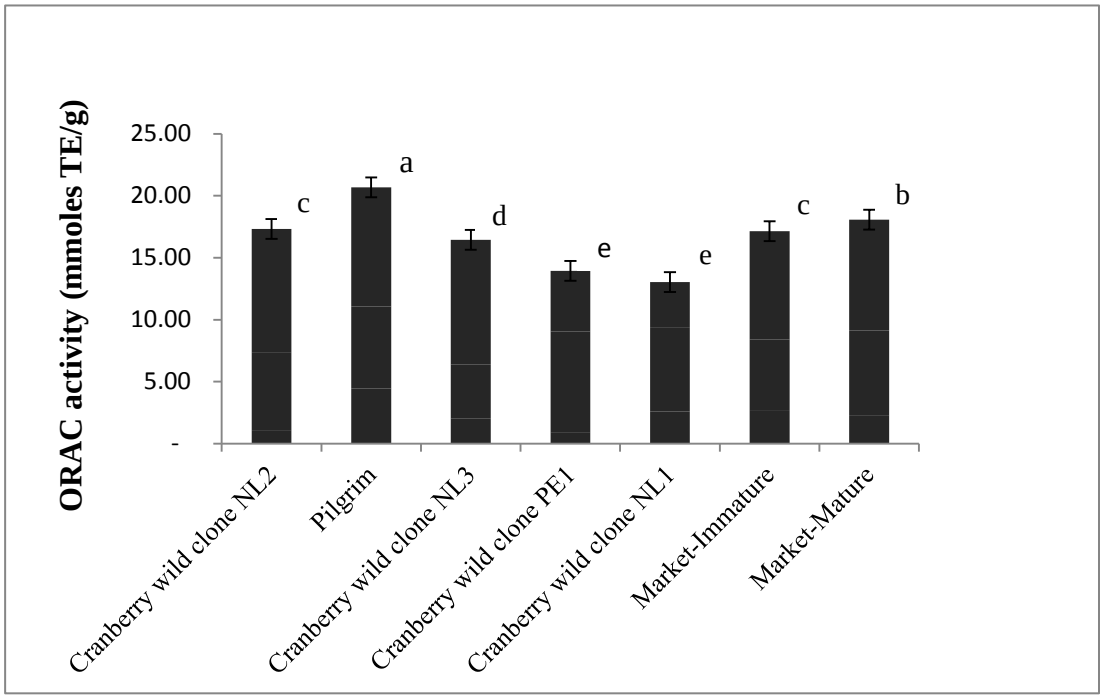


Figure 4.3. Total oxygen radical absorbance capacity (ORAC) of cranberry fruit samples on a dry weight basis

Table 4.5. DPPH radical scavenging activity and hydroxyl radical scavenging capacity of phenolics in cranberry fruit extracts on a dry weight basis

Cranberry sample	DPPH radical scavenging activity (μmoles TE/g of dried fruit weight)			Hydroxyl radical scavenging capacity (μmoles CE/g of dried fruit weight)		
	Free phenolics	Esterified phenolics	Bound phenolics	Free phenolics	Esterified phenolics	Bound phenolics
Cranberry wild clone NL2	165.39±3.31[a]	949.54±7.15[a]	98.58±2.15[a]	197±0.34[b]	251.89±3.82[a]	29.74±1.15[d]
Pilgrim	154.21±2.95[a]	1095.91±6.11[a]	82.41±1.55[b]	183.85±0.51[b]	198.91±1.75[c]	183.24±3.03[a]
Cranberry wild clone NL3	161.99±0.71[a]	948.97±1.15[a]	68.79±4.11[c]	251.50±2.47[a]	229.17±1.99[b]	124.18±2.2[b]
Cranberry wild clone PEI	164.05±4.86[a]	861.94±8.78[a]	53.82±2.93[c]	249.90±4.05[a]	205.84±0.15[c]	72.87±1.17[c]
Cranberry wild clone NL1	163.75±5.83[a]	93.56±1.16[b]	62.95±2.73[c]	200.38±1.15[b]	155.84±3.11[d]	80.98±0.93[c]
Market-Immature	94.64±1.03[b]	916.57±6.35[a]	109.32±3.32[a]	75.82±3.15[c]	24.96±0.35[f]	64.81±1.05[c]
Market-Mature	84.43±1.52[b]	743.47±4.19[a]	80.44±1.01[b]	80.40±1.45[c]	79.88±0.77[e]	16.42±0.55[e]

[1]Data are expressed as means ± SD (n=3).Values with the same letter in the same column are not significantly different (p>0.05)

4.9. Hydroxyl radical scavenging capacity using EPR spectroscopy

Hydroxyl radical scavenging activity of free, esterified and bound phenolics were 75.82-251.50, 24.96-251.89 and 16.42-183.24 μmoles CE/g dry fruit weight, respectively (Table 4.5). The main contribution for all genotypes was from their free phenolics, except in cranberry wild clone NL2 and pilgrim, where the esterified phenolic extracts showed the highest values. This trend is fairly different from that for DPPH radical scavenging activity, possibly due to the existing differences in their scavenging power for the two radicals. In addition the bound phenolics in all genotypes showed the lowest hydroxyl radical scavenging activity as compared to their free and esterified forms, except for the immature market sample. As shown in Table 4.5, cranberry wild clone NL3 had the highest total hydroxyl radical scavenging activity (604.85 μmoles CE/g dried fruit weight) whereas immature market sample had the lowest.

The hydroxyl radical is a biologically relevant radical species that can cause severe damage to biomolecules. Quantitative measurement of hydroxyl radicals has been a challenging task due to the lack of a controllable hydroxyl radical source. In the presence of low concentrations of Fe (II), H_2O_2 is converted to hydroxyl radical via Fenton reaction. Many other transition metal ions such as Cu (II), Ti (II), Cr (II), and Co (II) also react with H_2O_2 in a similar manner to generate hydroxyl radicals (Goldstein *et al* 1993).

Ou *et al* (2002) have evaluated a number of phenolic compounds for their hydroxyl radical scavenging capacity and ORAC and revealed that the former is consistently lower than ORAC among the tested compounds. This pattern was true for most foods tested by Ou *et al* (2002). A

similar trend was observed for all tested cranberry genotypes as well. Ou *et al* (2002) observed that phenolic compounds with metal chelation potential showed higher hydroxyl radical scavenging values, whereas the compounds with poor metal chelation activity displayed lower values. Phenolics act as metal chelators, thereby blocking the reaction sites for H_2O_2. This coordination leads to reduced concentration of Fe (II) which effectively reduces the generation of hydroxyl radicals.

4.10. Determination of 2-thiobarbituric acid reactive substances (TBARS) in cooked pork model system

In the present work the production of TBARS, the secondary products of lipid oxidation, in cooked comminuted pork was measured and expressed as percentage of inhibition of TBARS formation. The 2-thiobarbituric acid method is widely used for measuring lipid peroxidation in muscle foods due to its simplicity and rapid nature (Chandrasekara & Shahidi, 2012).

The TBARS values of antioxidant-treated pork samples stored at 4ºC over 7 days are shown in Figure 4.4. The soluble phenolic extracts and BHT were added at 200 ppm. The extracts were effective in inhibiting the oxidation of cooked pork in comparison with the control, which showed the highest TBARS value at the end of the 7 days of storage period. BHT was used as a

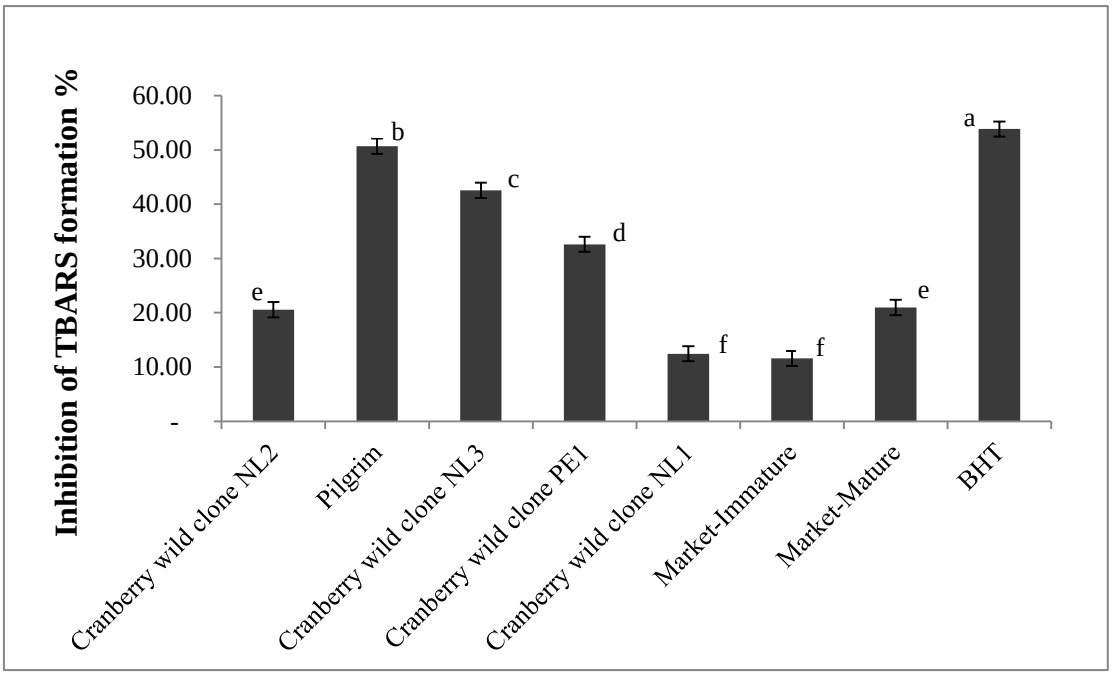


Figure 4.4. Thiobarbituric acid reactive substances (TBARS) in cooked pork model system of dried cranberry on a dry weight basis after 7 days of storage at refrigerated temperature

According to Figure 4.4, the percentage inhibition of TBARS formation in the BHT treated sample was 53.84 % on day 7 of storage. Pilgrim was highly effective in inhibiting oxidation in a cooked pork model system. The samples arranged in the order of their effectiveness in inhibiting the formation of TBARS (%) and reported as malondialdehyde (MDA) equivalents(%) were in the order of pilgrim (48.67%) > cranberry wild clone NL3 (42.55%) > cranberry wild clone PEI (32.60%) > mature market (20.96%) > cranberry wild clone NL2 (20.54%) > cranberry wild clone NL1 (12.44%) > immature market (11.58%) (Figure 4.4).

Pilgrim was highly effective in inhibiting oxidation in a cooked pork model system and immature market extracts showed lowest protective effect against lipid oxidation (Figure 4.4). In comminuted pork model system, iron released from haem moieties act as catalyst of lipid oxidation. It has been shown that the rate of iron release from porphyrin ring depends on time, intensity and temperature of cooking (Frankel *et al* 2000). Free non-haem iron catalyzes the oxidation of polyunsaturated fatty acyl components. It indicated that phenolic extract of all five tested cranberry genotypes may act as effective chelators of free iron ions as well as scavengers of peroxyl radicals formed during initiation and propagation steps in lipid oxidation. Phenolics of pilgrim displayed effective ferrous ion chelating activities whereas immature market sample showed a weak activity.

The inhibition of formation of TBARS for all tested cranberry genotypes was similar to that of total phenolic contents (TPC) and total flavonoid contents (TFC) of the samples except cranberry wild clone NL2 genotype. Thus, samples with higher phenolic contents inhibited lipid oxidation and rancidity development. In agreement with this trend, extracts enriched in phenolic acids,

anthocyanins, flavonols and proanthocyanidins showed the greatest inhibitory effect on lipid oxidation in cooked pork (Lee *et al* 2006).

4.11. Effect of cranberry extracts on preventing cupric ion-induced human low density lipoprotein (LDL) cholesterol peroxidation

Natural antioxidants from dietary sources that can inhibit LDL cholesterol oxidation are of great importance in the prevention of cardiovascular diseases. It has been shown that the uptake of oxidized LDL by macrophages and smooth muscle cells leads to the formation of fatty streaks or vascular lesions which further accumulate lipids (Decker *et al* 2001). Flavonoids, exhibit strong antioxidant potential, and intake of flavonoid-rich foods has been related to decreased morbidity and mortality from heart disease (Ruel *et al* 2005).

Free radical scavenging activity or metal ion chelation properties of phenolic compounds inhibits LDL cholesterol oxidation. In addition, phenolic compounds can protect endogenous antioxidants such as tocopherols, beta-carotene, lycopene and ubiquinol that act against oxidation of LDL cholesterol molecules, or inhibit enzymes such as xanthine oxidase involved in the initiation of oxidation or cell mediated LDL cholesterol oxidation (Chandrasekara & Shahidi, 2011).

In this study, the protective activity of phenolic extracts of cranberry for chelating cupric ions and thus reducing metal catalyzed oxidation of LDL cholesterol was demonstrated. It was noted that at the beginning the rate of conjugated diene (CD) formation was slowed down as LDL cholesterol molecules contain antioxidant compounds. The rapid oxidation started after the

depletion of endogenous antioxidants of LDL cholesterol molecules. Figure 4.5 shows the inhibitory activities of cranberry extracts at a concentration of 0.5 mg/mL against human LDL cholesterol oxidation induced by cupric ion.

The ability of phenolic compounds to inhibit copper ion-mediated LDL cholesterol oxidation may be attributed to their capacity to remove cupric ions from the medium (Decker *et al* 2001). It was noted that cranberry wild clone NL3 had a low LDL cholesterol oxidation inhibition, accounting for 15.09±0.23%, whereas immature market and mature market sample exhibited high inhibitory activities against LDL cholesterol oxidation, accounting for 39.33±0.05 and 35.63±0.04%, respectively, after 24 hours of incubation (Figure 4.5 and Table 4.6). This study also showed that market samples (immature and mature) had the highest inhibition of LDL cholesterol oxidation compared to cranberry wild clone NL2, pilgrim and cranberry wild clone NL3. However, this is lower than that of the control trolox, where inhibition of LDL cholesterol oxidation was 46±0.12%. The results also showed that a significant difference ($p>0.05$) existed between immature market and mature market extracts.

The cranberry wild clone NL3 genotypes and immature market sample inhibited LDL cholesterol oxidation by 15 and 39%, respectively. However, cranberry wild clone NL3 genotype had 4 times higher TPC than immature market. Therefore, the present results suggest that irrespective of the TPC, differences in phenolic composition of cranberry extracts play a major role in the observed effects. It has been reported that anthocyanins derived from grape juices, wines, and berries are the major compounds contributing to the *in vitro* antioxidant activity, thus preventing LDL oxidation (Tesissers *et al* 1996; Abuja *et al* 1998).

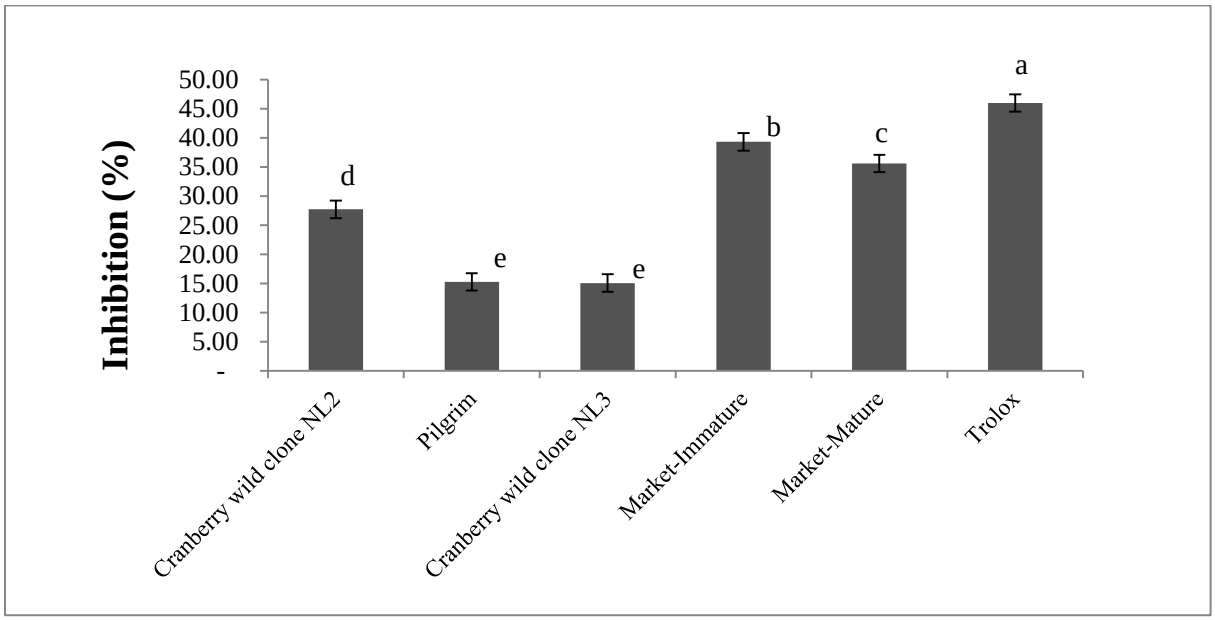


Figure 4.5. Inhibition of cupric ion-induced human low density lipoprotein (LDL) cholesterol oxidation by cranberries

Table 4.6. Effect of cranberry extracts on preventing cupric ion induced human low density lipoprotein (LDL) peroxidation after 24 hours

Cranberry Sample	Inhibition %
Cranberry wild clone NL2	28±0.03[d]
Pilgrim	15±0.02[e]
Cranberry wild clone NL3	15±0.05[e]
Immature market	39±0.05[b]
Mature market	36±0.04[c]
Trolox	46±0.12[a]

Values with the same letter are not significantly different ($p>0.05$)

4.12. Supercoiled strand DNA scission by peroxyl radicals

Oxidants produced as by-products of mitochondrial electron transport and products from lipid peroxidation that escape the numerous antioxidant defense systems can cause damage to cellular macromolecules, including DNA, and such damages can lead to mutation and cancer initiation. Oxidation damage of DNA results in a wide range of scission products, which include strand breaks and sister formation, chromatid exchange, DNA-DNA, and DNA-protein cross-links as well as base modification (Ames & Shigenaga, 1993). DNA damage is often measured as single strand-breaks, double strand-breaks, or chromosomal aberrations (Breimer, 1990). In the present study, free, esterified and bound phenolics of wild clone NL2, pilgrim, wild clone NL3, as well as immature and mature market sample extracts were evaluated for their capacity in inhibiting peroxyl radical-induced DNA supercoiled strand scission. Figures 4.6A and 4.6B show the activity of cranberry extracts for inhibiting peroxyl radical-induced DNA supercoiled strand scission.

DNA molecules are easily attacked by free radicals that induce base modification and strand scission; they lead to mutagenesis and possibly cancer. Thus, the effectiveness of the extracts to prevent scission of the DNA strands is a reflection of their positive effects against many diseases in biological systems. Peroxyl radicals are known to exert oxidative damage in biological systems due to their comparatively long half-life and thus greater affinity to diffuse into biological fluids in cells (Hu *et al* 2001). Soluble extracts from different cranberry extracts were dissolved in PBS at a concentration of 0.05 mg/mL before mixing them with DNA. Figure 4.7 shows the percentage of supercoiled DNA strands retained after incubation with peroxyl radicals generated by 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH). Soluble esterified

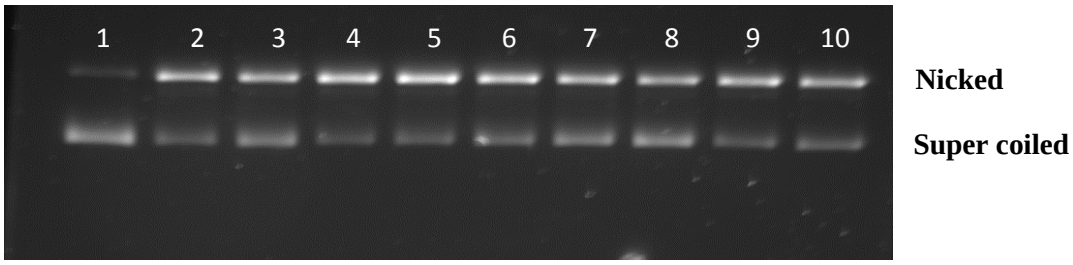


Figure 4.6 A. Effect of cranberry phenolic extracts in peroxyl radical treated DNA system.

Lane 1: Blank (DNA only); Lane 2: Control (DNA and AAPH); Lane 3: bound of wild clone NL2 extract; Lane 4: free of wild clone NL2 extract; Lane 5: esterified of wild clone NL2 extract; Lane 6: bound of pilgrim extract; Lane 7: free of pilgrim extract; Lane 8: esterified of pilgrim extract; Lane 9: bound of wild clone NL3 extract, Lane 10: free of wild clone NL3 extract,

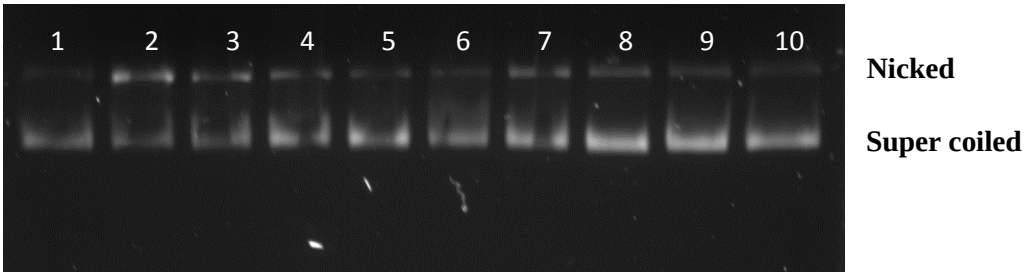


Figure 4.6 B. Effect of cranberry phenolic extracts in peroxyl radical treated DNA system

Lane 1: Blank (DNA only); Lane 2: Control (DNA and AAPH); Lane 3: esterified of wild clone NL3 extract; Lane 4: bound of immature market extract; Lane 5: free of immature market extract; Lane 6: esterified of immature market extract; Lane 7: bound of mature market extract; Lane 8: free of mature market extract; Lane 9: esterified of mature market extract, Lane 10: Blank (DNA only).

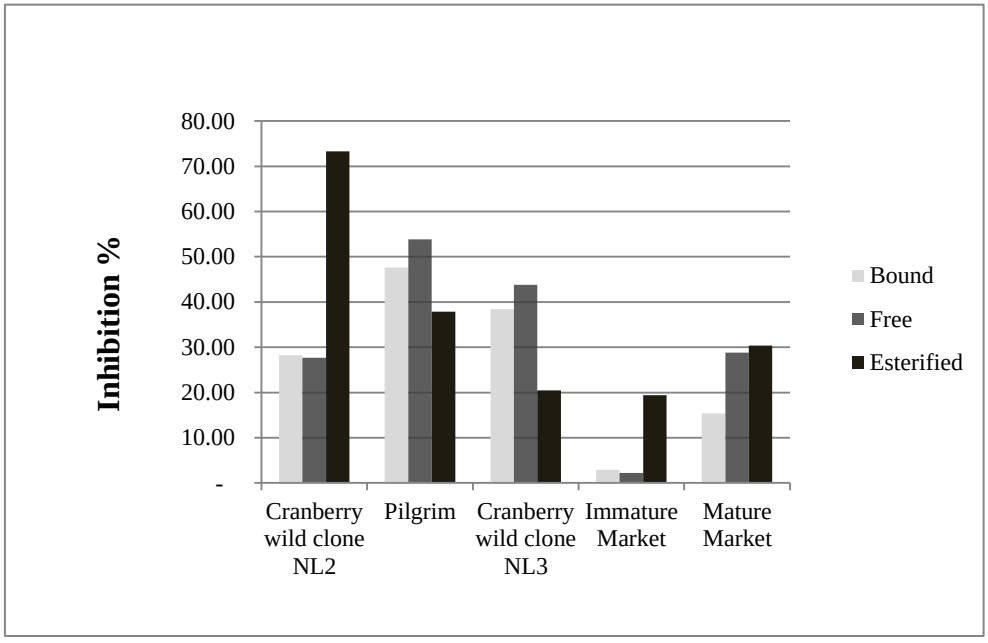


Figure 4.7. Inhibition percentage of supercoiled pBR 322 plasmid DNA scission in peroxyl radical-mediated systems with each phenolic extracts from different cranberry samples

phenolic extracts from cranberry wild clone NL2 were most effective in showing DNA strand scission inhibition of 73.33% followed by free phenolic extract of pilgrim (53.84%) and bound phenolic extract of pilgrim (47.63%), while extracts of bound and free phenolics of immature market sample showed a low activity of approximately 2.5%.

The cranberry extracts showed good strand scission inhibiting activity. In the absence of any antioxidant, it may be expected that the peroxyl radical abstracts a hydrogen atom from the nearby DNA to generate a new DNA radical, which initiates a free radical chain reaction resulting in the cleavage of the DNA molecule. However, in the presence of antioxidants, this chain reaction was terminated by abstracting a hydrogen atom from the antioxidant molecule (Hu & Kitts, 2000).

Densitometric scanning and analysis of the gels used in the supercoiled DNA assay to study the ability of the different treatments on protecting the supercoiled DNA against AAPH induced oxidative nicking revealed that DNA treated with esterified phenolic extract of cranberry wild clone NL2 genotype retained 73.33% of the supercoiled DNA (Figure 4.7). This was followed by free phenolic extract of pilgrim genotype, which protected 53.84% of the supercoiled DNA from oxidative damage. Cranberry genotypes of cranberry wild clone NL2, pilgrim and cranberry wild clone NL3 were more effective in retaining the supercoiled DNA compared with the cranberry market samples.

In this assay, cranberry genotype of pilgrim had the highest DNA protective functions (Table 4.7). Cranberry wild clone NL2 and pilgrim extracts with high phenolics content showed better

Table 4.7. Total inhibition percentage of DNA strand scission induced by peroxyl radicals with each cranberry genotypes

Cranberry Samples	Inhibition %
Cranberry wild clone NL2	43.11±0.09[a]
Pilgrim	46.44±0.12[a]
Cranberry wild clone NL3	34.24±0.05[b]
Immature market	8.18±0.05[d]
Mature market	24.84±0.04[c]

Values with the same letter, in the same row or column, are not significantly different ($p > 0.05$).

protection compared to the others (Table 4.7), indicating that protection was directly proportional to the concentration of total phenolics present.

The peroxide radical in the AAPH-induced DNA damage is generated by Fenton reaction, which involves iron as a metal prooxidant (Hiramoto *et al* 2003). Phenolics are powerful electrophiles because of their delocalization abilities and therefore can act as strong chelators of transition metals by forming coordinate bonds (Hider *et al* 2001; Yang *et al* 2001; Bors & Michel, 2002). It is therefore possible that cranberry extracts protect the DNA from oxidative damage by chelating iron and making it unavailable to generate peroxyl radicals (Hider *et al* 2001; Yang *et al* 2001).

4.13.1. Identification of major phenolic compounds of cranberry genotypes by using HPLC/ESI-MS/MS

The mass spectral data and identification results for peaks in the representative chromatograms of different phenolic fractions of cranberry are shown in Table 4.8 and displayed in Figures 4.8, 4.9 and 4.10. The identity of free, esterified, and bound phenolic compounds in cranberry wild clone NL2 and pilgrim genotypes were determined using HPLC-DAD and HPLC-MS analyses and by comparison of their retention times and mass spectral data with those of the available standards and reports in the literature (Figures 4.8, 4.9 & 4.10). The predominant phenolic acids and flavonoids present in cranberry samples that were identified and quantified (mg/100g dried fruit weight), as listed in Tables 4.9 and 4.10. The HPLC retention times of the phenolic acids, namely gallic, chlorogenic, caffeic, *p*-coumaric, sinapic and ferluic acids were 14.59, 27.61 33.58, 46.62, 48.63, 49.44 min, respectively, and also retention times of the flavonoids such as

catechin, epicatechin and quercetin were 26.34, 36.92, 54.98 min, respectively. Phytochemicals in cranberry fruit belong to a diverse group that includes 4 classes of flavonoids (flavonols, anthocyanins, proanthocyanidins and flavan-3-ols), hydroxybenzoic acids, hydroxycinnamic acids and triterpenoids. Phenolic acids (chlorogenic, caffeic, *p*-coumaric and ferulic acids) predominated in the cranberry samples. According to Velioglu *et al* (2006), based on HPLC/UV data of the European cranberry, besides chlorogenic acid, as the main phenolic, several other compounds, namely catechin, epicatechin, and cyanidin-3-glucoside were present.

A total of 50 different phenolic compounds, including flavonoids, and hydrobenzoic acids, as well as hydroxycinnamic acids were monitored by HPLC. Among these compounds only 29 phenolic compounds were detected in the cranberry samples. Results from the identification and quantification of the free, esterified and bound phenolic fractions confirmed compounds presented in Tables 4.9 and 4.10. Several hydroxybenzoic acids, namely gallic, protocatechuic, synaptic, syringic, chlorogenic, caffeic, *P*-coumaric and ferulic acids were identified in cranberry by comparison of their retention times (RT) and UV and MS spectral data with those of the available standards (Table 4.8 and Figures 4.8, 4.9). The MS spectra generated for hydroxybenzoic acids showed loss of CO_2 giving $[M\text{-}H\text{-}44]^-$ as a characteristic ion. Thus, ions at m/z 125, 109, 179 and 153, were detected for gallic, protocatechuic, sinapic and syringic acids, respectively (Table 4.8). Several compounds belonging to different classes of flavonoids, namely flavan-3-ol (monomers, and dimers), flavonols and their glycosides, flavones and flavanonol were identified in cranberry phenolic extracts (Table 4.8 and Figures 4.8, 4.9, 4.10). In general flavonoids are conjugated with sugars and occur as glycosides of *O*- or *C*- forms, but they also exist as free aglycones (Stobiecki, 2000).

Chromatogram - A

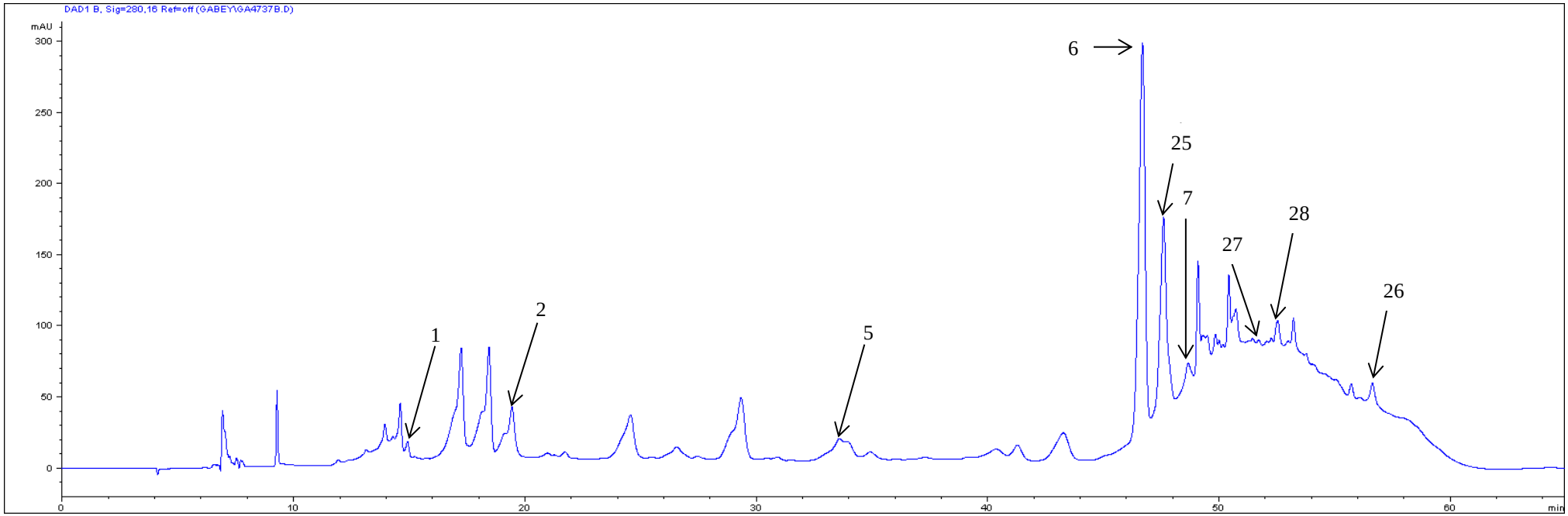


Figure 4.8. Representative HPLC chromatogram of bound phenolic fraction of wild clone NL2. Choice of phenolic type and varieties was based on displaying a maximum number of peaks representing phenolic compounds.

Chromatogram - B

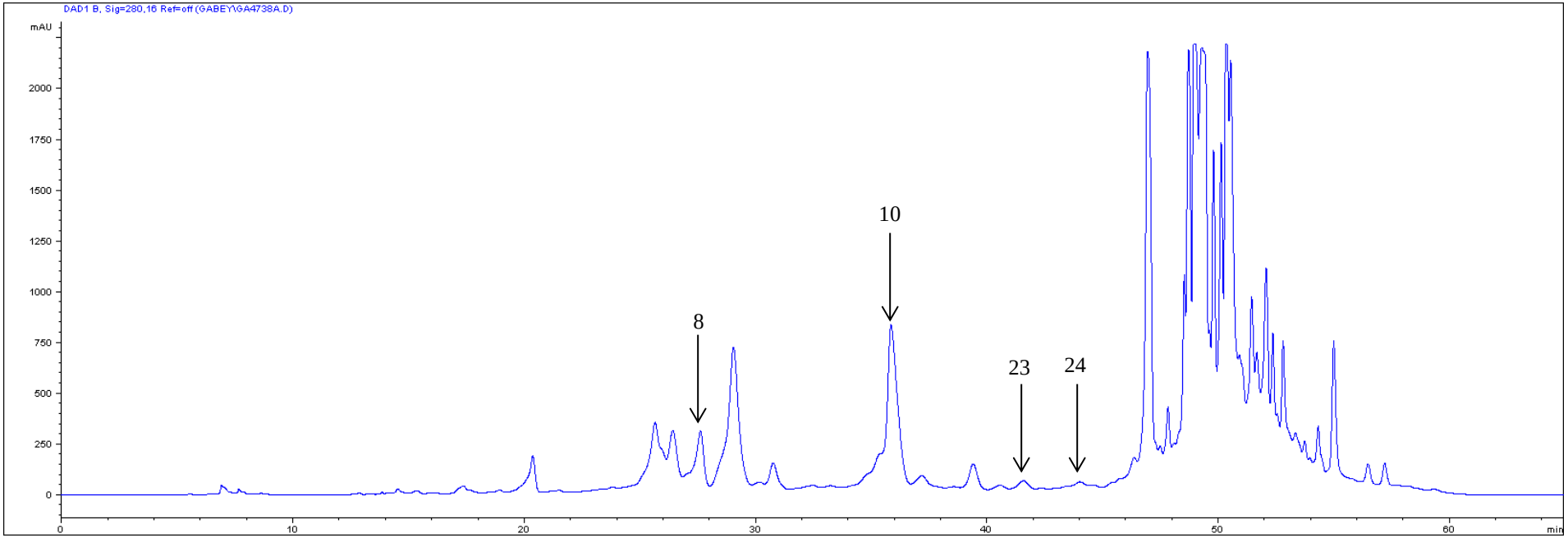


Figure 4.9. Representative HPLC chromatogram of free phenolic fraction of wild clone NL2. Choice of phenolic type and varieties was based on displaying a maximum number of peaks representing phenolic compounds.

Chromatogram - C

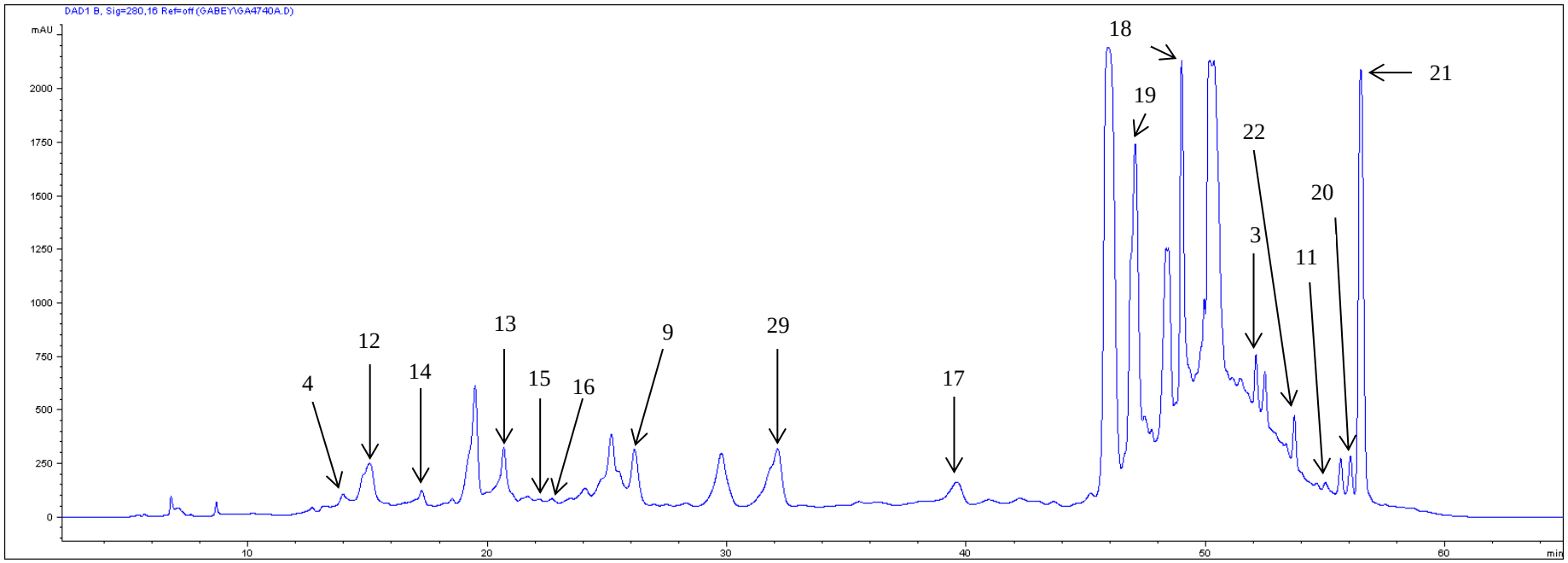


Figure 4.10. Representative HPLC chromatogram of esterified phenolic fraction of wild clone NL2. Choice of phenolic type and varieties was based on displaying a maximum number of peaks representing phenolic compounds.

4.12.2. Quantification of phenolic compounds in wild clone NL2 and pilgrim genotypes

The contents of phenolic compounds as quantified by HPLC are presented in Table 4.10. The results showed that the content and type of phenolic compounds varied depending on the cranberry genotype as well as phenolic fraction considered. However, as expected, the cranberry varieties tested showed essentially the same phenolic compounds in each fraction though the contents differed between the two tested varieties which confirm the fact that phenolic content is influenced by the variety. The highest flavonoid level was detected in the free phenolic fractions of the tested cranberry verities (Table 4.10). As observed in previous studies, and confirmed in this study, soluble phenolic fraction, composed of free and esterified fractions, of two tested cranberry varieties showed higher TPC and antioxidant activity than that of insoluble bound phenolics in several *in vitro* systems (Chandrasekara & Shahidi, 2011).

Phenolic acids

Phenolic acids contribute to the characteristic and unique flavour of berries (Vattem *et al* 2005). This family of compounds includes derivatives of hydroxycinnamic acids (HCA) and hydroxybenzoic acids (HBA). They both have very similar molecular structures composed of a backbone phenol ring, although HCA have an additional ethylenic group between the aromatic ring and the carboxylic acid group. Compounds classified in these two families of acid derivatives may otherwise differ by the number and position of hydroxyl and methoxyl groups attached to the phenol ring.

Table 4.8. Individual phenolic compounds identified in wild clone NL2

Peak No		Molecular Weight (g/moles)	$[MH]^-$ (m/z)	ESI negative fragments (m/z)
Hydrobenzoic and Hydrocinnamic Acids				
1	Gallic	170	169	125
2	Protocatechuic	154	153	109
3	Sinapic	224	223	179
4	Syringic	198	197	153
5	Caffeic	180	179	135,167
6	*p*-coumaric	164	163	119,139
7	Ferulic	194	193	135
8	Chlorogenic	354	353	191
Flavonoids				
9	(+)-Catechin	290	289	245
10	(-)-Epicatechin	290	289	245
11	Quercetin	302	301	121,179
12	Kaempherol hexoside	448	447	257,285,327,401
13	Luteolin 7-*O*-glucoside	448	447	121,177,285,313,381
14	Cyanidin 3-*O*-arabinoside	419	417	285,287
15	Proanthocyanidin dimer B- type	578	577	287,289,407,425
16	Proanthocyanidin dimer A- type	576	575	285,289,407,423
17	Quercetin 3-*O*-rhamnoside	449	448	303
18	Proanthocyanin trimer A-type	864	863	449,559,693,711,737
19	Cyanidin -3-*O*-galactoside	449	448	285,287
20	Myricertin 3-*O*-arabinoside	451	450	317
21	Myricertin 5-*O*-galactoside	479	477	257,262,298,317,355
22	Proanthocyanidin A- type	592	591	285,303,421,451,465, 573
23	Procyanidin tetramer A-type	1152	1151	449,693,737,863,981
24	Procyanidin trimer	866	865	739,713,577,575,451,407,289,287
25	Quercertin 3-*O*-galactoside	464	463	301, 271, 255, 151
26	Proanthocyanidin B- type	594	593	556,456,449,423,303,289, 285
27	Myricetin 3-O-galactoside	479	478	257,262,298,355
28	Proanthocyanidin dimer	574	575	283,289,323,421,529, 555
29	Proanthocyanidin trimer	866	865	739,713,577,575,451,407,289, 287

Table 4.9. Individual phenolic compounds identified in the extracts of wild clone NL2 and pilgrim quantified using HPLC in negative ionization mode

Peak No	Phenolic Compounds	Molecular Weight	$[MH]^-$ (m/z)	ESI negative fragments (m/z)	Wild clone NL2			Pilgrim		
					Bound	Free	Esterified	Bound	Free	Esterified
Hydrobenzoic and Hydrocinnamic Acids										
1	Gallic	170	169	125	D	ND	ND	D	ND	D
2	Protocatechuic	154	153	109	D	D	D	D	ND	D
3	Synapic	224	223	179	ND	D	D	ND	ND	D
4	Syringic	198	197	153	D	ND	D	D	ND	D
5	Caffeic	180	179	135,167	D	D	D	D	ND	D
6	*p*-coumaric	164	163	119,139	D	D	D	D	ND	D
7	Ferulic	194	193	135	D	D	D	D	D	D
8	Chlorogenic	354	353	191	D	D	D	ND	D	D
Flavonoids										
9	(+)-Catechin	290	289	245	D	D	D	D	D	D
10	(-)-Epicatechin	290	289	245	D	D	D	D	D	D
11	Quercetin	302	301	121,179	D	D	D	D	D	D
12	Kaempherol hexoside	448	447	257,285,327,401	D	ND	D	D	D	ND
13	Luteolin 7-*O*-glucoside	448	447	121,177,285,313,381	ND	ND	D	ND	ND	ND
14	Cyanidin 3-*O*-arabinoside	419	417	285,287	D	D	D	D	D	ND
15	Proanthocyanidin dimer B- type	578	577	287,289,407,425	D	D	D	D	D	D
16	Proanthocyanidin dimer A- type	576	575	285,289,407,423	D	D	D	D	D	D
17	Quercetin 3-*O*-rhamnoside	449	448	303	ND	ND	D	ND	ND	ND
18	Proanthocyanin trimer A-type	864	863	449,559,693,711,737	ND	D	D	ND	D	D
19	Cyanadin -3-*O*- galactoside	449	448	285,287	ND	ND	D	ND	D	ND
20	Myricetin 3-*O*-arabinoside	451	450	317	D	D	D	D	D	ND
21	Myricetin 5-*O*-galactoside	479	477	257,262,298,317,355	ND	ND	D	ND	D	ND
22	Proanthocyanidin A- type	592	591	285,303,421,451,465, 573	ND	D	D	D	D	D
23	Procyanidin tetramer A-type	1152	1151	449,693,737,863,981	ND	D	ND	ND	D	D
24	Procyanidin trimer	866	865	739,713,577,575,451,407,289, 287	ND	D	ND	ND	D	D
25	Quercertin 3-*O*-galactoside	464	436	301, 271, 255, 151	D	D	ND	ND	ND	ND
26	Proanthocyanidin B- type	594	593	556,456,449,423,303,289, 285	D	D	ND	ND	ND	D
27	Myricetin 3-*O*-galactoside	479	478	257,262,298,355	D	ND	ND	D	ND	ND
28	Proanthocyanidin dimer	574	575	283,289,323,421,529, 555	D	ND	ND	D	ND	ND
29	Proanthocyanidin trimer	866	865	739,713,577,575,451,407,289, 287	ND	ND	D	ND	ND	D

*D - Detected, ND - Not detected

Table: 4.10: Content of prominent compound (mg/100g dried cranberry sample) in the extracts of wild clone NL2 and pilgrim quantified using HPLC in negative ionization mode

Peak No	Phenolic Compounds	Wild clone NL2			Pilgrim		
		Bound	Free	Esterified	Bound	Free	Esterified
Hydrobenzoic and Hydrocinnamic Acids							
1	Gallic	4±0.10^{a}	-	-	3±0.90^{a}	-	-
2	Protocatechuic	-	-	-	24±0.91^{a}	-	24±2.19^{a}
3	Sinapic	-	-	59±1.67^{a}	-	-	0.05±0.00^{b}
4	Syringic	-	-	-	3±0.87^{a}	-	-
5	Caffeic	7±0.02^{c}	5±0.91^{c}	79±3.12^{b}		-	123±4.98^{a}
6	*p*-coumaric	26±0.10^{b}	2±0.16^{c}	245±1.66^{a}	30±0.23^{b}	-	5±1.16^{c}
7	Ferulic	4±0.09^{b}	-	39±2.12^{a}	-	-	39±2.22^{a}
8	Chlorogenic	-	31±1.41^{b}	-	-	47±1.76^{a}	6±0.90^{c}
Flavonoids							
9	(+)-Catechin	5±0.24^{d}	66±0.44^{b}	119±5.33^{a}	2±0.61^{d}	34±2.64^{c}	73±3.15^{b}
10	(-)-Epicatechin	5±0.34^{e}	874±42.11^{b}	76±1.65^{d}	30±3.23^{e}	1798±56.29^{a}	171±5.41^{c}
11	Quercetin	-	112±4.92^{b}	8±0.11^{d}	-	37±2.98^{c}	157±13.46^{a}
12	Kaempherol hexoside	5±0.10^{c}	-	23±1.12^{b}	7±0.51^{c}	121±9.78^{a}	-
13	Luteolin 7-*O*-glucoside	-	-	3±0.60^{a}	-	-	-
14	Cyanidin 3-*O*-arabinoside	3±0.09^{c}	43±0.19^{b}	4±0.72^{c}	2±0.10^{c}	84±9.34^{a}	-
15	Proanthocyanidin dimer B- type	2±0.00^{c}	336±16^{a}	7±0.40^{c}	-	338±11.12^{a}	24±1.87^{b}
16	Proanthocyanidin dimer A- type	2±0.00^{d}	74±2.30^{b}	35±1.98^{c}	4±0.87^{d}	134±2.54^{a}	-
17	Quercetin 3-*O*-rhamnoside	-	-	343±7.18^{a}	-	-	-
18	Proanthocyanin trimer A-type	-	442±7.32^{a}	196±8.23^{b}	-	459±32.31^{a}	22±6.75^{c}
19	Cyanadin -3-*O*-galactoside	-	-	7±0.18^{b}	-	82±4.15^{a}	-
20	Myricertin 3-*O*-arabinoside	8±0.08^{c}	150±6.75^{b}	273±6.53^{a}	-	166±2.79^{b}	-
21	Myricetin 5-*O*-galactoside	-	-	316±3.75^{a}	-	51±1.79^{b}	-
22	Proanthocyanidin A- type	-	5±0.09^{a}	6±0.92^{a}	-	3±0.19^{b}	-
23	Procyanidin tetramer A-type	-	15±1.31^{a}	-	-	16±1.11^{a}	-
24	Procyanidin trimer	-	3±0.67^{b}	-	-	16±2.44^{a}	-
25	Quercertin 3-*O*-galactoside	54±2.12^{b}	126±7.12^{a}	-	-	-	-
26	Proanthocyanidin B- type	10±0.87^{b}	18±1.12^{a}	-	-	-	-
27	Myricetin 3-*O*-galactoside	2±0.11^{a}	-	-	-	-	-
28	Proanthocyanidin dimer	6±0.34^{b}	-	-	16±0.37^{a}	-	-
29	Proanthocyanidin trimer	-	-	18±1.12^{a}	-		15±1.89^{a}

*HBA and HCA were quantified with the calibration curve of corresponding standard at 280nm, Flavonoids were quantified with the calibration curve of catechin at 280nm, Epicatechin was quantified with the calibration curve of epicatechin at 280nm

The presence of HCA derivatives in plant foods is more frequent than that of HBA derivatives (Macheix & Fleuriet, 1998). HCA derivatives are the most widely distributed group of phenolic compounds in plant foods (Shahidi & Naczk, 1995).

HCA derivatives reported in cranberry include ferulic acid, *p*-coumaric acid, coumaroyl glucose, feruloyl glucose, glycosylated sinapic acid, caffeoyl glucose, and diglucoside of caffeoyl glucose (Macheix *et al* 1990; Hakkinen *et al* 1999). These compounds rarely occur in the free form; they are instead associated with other types of compounds such as arabinoxylans or cellulose in the cell walls or may be glycosylated to simple sugars or occur as soluble esters. In this work, in agreement with previous research findings, the soluble phenolics that included both the free and esterified fractions, was present at a higher concentration, as reflected in TPC and antioxidant activity, than that of the insoluble bound phenolics for the two tested cultivars as shown in several *in vitro* systems.

Ferulic, *p*-coumaric, caffeic, and sinapic acids are often found combined with sugars by means of glycosidic linkage, or with organic acids such as quinic acid (Escarpa & Gonzalez, 2001). The concentration of HBA derivatives is generally low in food of plant origin, the exception being for the majority of berries, whose content of protocatechuic, ellagic, and gallic acids is very high (Shahidi & Naczk, 1995).

Among HCA and HBA, *p*-coumaric acid (from 2±0.16 to 245±1.16 mg of *p*-coumaric acid/100g of dried fruit weight) was the most abundant compound followed by caffeic acid (from 5±0.91 to 123±4.98 mg of caffeic acid /100g of dried fruit weight), ferulic acid (from 4±0.09 to 39±2.22

mg of ferulic acid/100g of dried cranberry) and chlorogenic acid (from 6±0.90 to 47±1.76 mg of chlorogenic acid/100g of dried fruit weight) in cranberry wild clone NL2 and pilgrim genotypes (Table 4.9). HBA and HCA were quantified with the calibration curve of corresponding standard at 280nm. Phenolic acids present in a medium concentration range included gallic, protocatechuic, synapic, and syringic acids. These results are in accordance with previous studies that found gallic, chlorogenic, *p*-hydroxybenzoic, ferulic and *p*-coumaric acids were predominant in cranberry pomace (Zheng & Shetty, 2000). According to Velioglu *et al* (2006), The European cranberry bush fruits contained 2037 mg kg^{-1} chlorogenic acid. This is more than 5 times higher than our results and those of other researchers (Chen *et al* 2001). Chlorogenic acid concentration may be varied among cranberry cultivars, stage of maturity, extraction method and climate factors (light, temperature) (Bohm, 1998).

Flavonols

Flavonols are found in abundance in Ericaceae fruits such as cranberry, blueberry, and bilberry (Robards & Antolovich, 1997). They are known to be concentrated mainly in the skin of fruits (Hawker *et al* 1972). On a weight basis, cranberry is one of the leading fruit sources of flavonols. Approximately 75% of cranberry flavonols consist of quercetin glycosides (quercetin 3-*O*-galactoside), although other flavonols have also been reported in the fruit at lower concentrations; these were quercetin, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinopyranoside, quercetin 3-*O*-arabinofuranoside, quercetin 3-*O*-rhamnoside, myricetin, and myricetin 3-*O*-galactoside (Macheix *et al* 1990; Hakkinen *et al* 1999; Zheng & Wang, 2003; Chen & Zuo, 2007; Lin & Harnly, 2007; Neto, 2007).

In this study, we were able to identify and quantify four major flavonols in wild clone NL2 and pilgrim genotypes; these were quercetin 3-*O*-rhamnoside (343±7.18 mg of CE/100g of dried fruit weight), myricertin 3-*O*-arabinoside, quercertin 3-*O*-galactoside and myricetin 3-*O*-galactoside (Table 4.10). Flavonols were quantified with the calibration curve of catechin at 280nm, except for epicatechin, which was quantified with its own calibration curve at 280nm. Myricertin 3-*O*-arabinoside (166±2.79 mg of CE/100g of dried fruit weight) was only found in pilgrim genotype. In agreement with this study, Chen and Zuo, (2007) also identified two major flavonol glycosides, quercetin galactoside and quercetin arabinoside, in American cranberry fruits. The chromatograms obtained for HPLC analysis are illustrated in Figure 4.8.

Catechin and epicatechin are important constituents of fruits, and their presence has been reported in cranberry (Macheix *et al* 1990). Quantification of catechin and epicatechin is shown in Table 4.10 and the latter was predominant in cranberry wild clone NL2 and pilgrim genotypes, mainly in the free phenolic form, occurring at 874±42.11 mg/100g of dried wild clone NL2 and at 1798±56.29 mg /100g of dried piligrim genotype. Meanwhile, catechin was present at corresponding values of 66±0.44 and 34±2.64 mg/100g, respectively. Velioglu *et al* (2006) identified catechin and epicatechin in European cranberry bush with a catechin content of 290.4 mgL^{-1}. According to Maatta-Riihinen *et al* (2005), catechin and epicatechin contents in cranberry were 417 and 447 µg/mg of dried fraction, respectively. These values were lower than those of this study. The concentrations of catechin and epicatechin may vary depending on the cultivar, stage of maturity, extraction method and climate factors (light, temperature), as well as the storage time and presence of other substances (Naczk & Shahidi, 2004).

Anthocyanins

Anthocyanins are generally found in fruits and more specifically in red, purple, and blue berries (Nijveldt *et al* 2001; Higdon, 2007). Their concentrations in food tend to increase as fruits ripen in response to climatic factors (light, temperature) (Bohm, 1998). The colour of anthocyanins is due to their chromophore units and may be influenced by some constituents of the plant cells (Brouillard *et al* 1997). Anthocyanins consist of an anthocyanidin molecule bound to one or more sugar moieties (Robards & Antolovich, 1997). Glycosylation of anthocyanidins almost always occurs at the C_3 position with glucose, arabinose, and galactose being the most common sugar moieties (Strack & Wray, 1994; Robards & Antolovich, 1997). Anthocyanins with sugars at both the C_3 and C_5 positions and 3, 7-diglycosides do also occur as they are considered more stable than C_3-*O*-glycosylanthocyanins (Strack & Wray, 1994; Bohm, 1998).

Anthocyanins are stored in an organized aqueous medium in the cell vacuoles, where there is a slightly acidic environment and is rich in inorganic ions. Polyphenols are essential for transformation of these pigments that enable the formation of molecular complexes and subsequent colour changes and stabilization (Brouillard & Dangles, 1993). Several studies have suggested that the anthocyanin contents and their corresponding antioxidant activities contribute to the protective effect of fruits and vegetables against degenerative and chronic diseases (Heinonen *et al* 1998; Record *et al* 2001). The numbers of different anthocyanins were seen due to ripeness and cultivar differences (Brown *et al* 2012; Sapers & Hargrave, 1987; Vvedenskaya & Vorsa, 2004) and some acylated anthocyanins being present in lowbush blueberry (Prior *et al* 2001).

Results from the identification and quantification of anthocyanins in cranberry fruits are presented in Tables 4.9 and 4.10. Anthocyanins were quantified with the calibration curve of catechin at 280nm. The major anthocyanins detected in cranberry wild clone NL2 and pilgrim genotype were cyaniding-3-*O*-arabinoside and cyanadin-3-*O*-galactoside. Cyanidin-3-*O*-arabinoside and cyanadin-3-*O*-galactoside were predominant in cranberry wild clone NL2 and pilgrim genotype, respectively. This agrees with the results reported by Grace *et al* (2013). Commercial cranberry contained six anthocyanins, which were galactosides, glucosides, and arabinosides of both cyanidin and peonidin and wild Alaskan lowbush cranberry displayed only cyanidin glycosides as the dominant anthocyanin, with non-detectable levels of peonidins. However, macheix *et al* (1990) and Neto (2007) have reported that the major anthocyanins in cranberry are galactosides and arabinosides of cyanidin and peonidin. In the HPLC analysis, anthocyanins are quantified mainly in free extracts of wild clone NL2 and pilgrim. Anthocyanins in bound and esterified extracts were found in trace amount. Therefore could not quantify. In agreement with this, Brown *et al* (2012) has not been reported peonidin-3-*O*-glucoside in their samples, which might be due to a lower detection limit from the method used.

In this study, during the fractionation of bound, the residue of the cranberry sample obtained after extraction of soluble phenolics was hydrolyzed with NaOH at room temperature for 4 hours and the water phase was neutralized to pH 7 with NaOH during the fractionation of esterified extracts. According to da Costa *et al* (1998), anthocyanins can be found in different chemical forms which depend on the pH of the solution. At pH 1, the red coloured flavylium cation is the predominant species. At pH between 2 and 4, the quinoidal blue coloured species dominate while at pH between 5 and 6 only two colourless species can be observed, which are carbinol

pseudobase. At pH values higher than 7, the anthocyanins are degraded into simple phenolic acids (Castaneda-Ovando *et al* 2009); hence absence of anthocyanins in the bound phenlics examined in this work.

Proanthocyanidins

Flavan-3-ols in cranberry occur as monomers, or in the oligomeric and polymeric forms (called proanthocyanidins; PACs). The contents of PACs in cranberry vary according to the nature of the interflavan linkage,constitutive units, and degree of polymerization (DP). Proanthocyanins were quantified with the calibration curve of catechin at 280nm.

According to the nature of the interflavan linkage, both A- and B-type PACs were found in cranberry. B-type PACs are those in which monomeric units are linked through the C_4 position of the upper unit and the C_6 or C_8 positions of the lower unit, whereas A-type PACs contain an additional ether type bond between the C_2 position of the upper unit and the hydroxyl group at C_7 or C_5 of the lower unit ($C_2{-}O{-}C_7$ or $C_2{-}O{-}C_5$). It has been estimated that A-type PACs account for 65% of total PACs in cranberry (Sanchez-Patan *et al* 2012). In agreement with this study, A-type PACs dimers and trimers were more abundant than B-type PACs dimers and trimers in wild clone NL2 and pilgrim genotypes (Table 4.10). Grace *et al* (2014) showed a similar trend, whereas commercial cranberry had higher percentages of A-type dimers and trimers (23.2 and 12.1%, respectively) than B-type analogues (9.2 and 3.6%, respectively).

According to Naczk and Shahidi (2006), cranberries serve as a good source of anthocyanins, flavonol glycosides, proanthocyanidins and phenolic acids. Sinapic, caffeic and *p*-coumaric acids are the major bound phenolic acids while *p*-coumaric, 2,4-dihydroxybenzoic and vanillic acids are the predominant free phenolic acids. The most abundant anthocyanins in American cranberries are 3-*O*-galactosides and 3-*O*-arabinosides of cyanidin and peonidin, while 3-*O*-glucosides of cyanidin and peonidin are dominant in European cranberries. Furthermore, polymeric proanthocyanidins comprised 63% of total proanthocyanidins in cranberries.

CHAPTER 5

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

5.1 Summary

Cranberry is an excellent source of beneficial functional ingredients including antioxidant polyphenols. This study compared five different cranberry varieties and two market samples with respect to their total phenolic content, total flavonoid content and antioxidant activity in order to demonstrate their potential as a source of natural antioxidants and associated potential health benefits. Their antioxidant potential and efficacy in a food and biological model systems was further investigated. The phenolic constituents of cranberry were fractionated into their respective free, esterified and bound forms to provide a complete picture of their phenolic composition. The content of free phenolics in tested cranberry varieties was in the decreasing order of pilgrim > cranberry wild clone NL2 > cranberry wild clone NL3 > cranberry wild clone PEI > cranberry wild clone NL1 > market mature > market immature. A similar trend was also followed by the esterified forms of phenolic compounds in the cranberry samples tested. Phenolics were predominantly present in the free form in all the examined cranberry varieties, except the market samples. Examination of the total free, esterified and bound phenolics indicated that pilgrim contained the highest amount of free (311.94 mg GAE/g of dried fruit weight), esterified (257.57 mg GAE/g of dried fruit weight) and bound (30.95 mg GAE/g of dried fruit weight) phenolics among all varieties analyzed.

The content of total flavonoids in tested cranberry varieties was in the decreasing order of pilgrim > cranberry wild clone NL2 > cranberry wild clone NL3 > cranberry wild clone PEI > cranberry wild clone NL1 > market mature > market immature. The flavonoids were predominantly present in the free form in the all tested cranberry varieties, while the contribution of bound form toward total flavonoid content was lower than that of the esterified form for all tested cranberry genotypes.

With respect to the antioxidant activity tests carried out for five cranberry varieties, oxygen radical absorbance capacity (ORAC), DPPH radical scavenging capacity (DRSC) and metal chelation, samples which had the highest phenolic and/or flavonoid content were most effective as free radical scavengers. Pilgrim showed the highest ORAC, DRSC and radical scavenging activity, followed by wild clone NL2, wild clone NL3, wild clone PEI and wild clone NL1. Wild clone NL3 showed the highest trolox equivalent antioxidant capacity (TEAC) and hydroxyl radical scavenging capacity.

The extracts were also effective in inhibiting the oxidation of comminuted pork in comparison with the control which showed the highest TBARS values at the end of a 7-day storage period. The samples arranged in the order of their effectiveness in inhibiting the formation of TBARS and reported as malondialdehyde (MDA) equivalents (%) were in the order of BHA (53.84%) > pilgrim (48.67%) > cranberry wild clone NL3 (42.55%) > cranberry wild clone PEI (32.60%) > mature market (20.96%) > cranberry wild clone NL2 (20.54%) > cranberry wild clone NL1 (12.44%) > immature market (11.58%). In a biological model system, pilgrim was most effective

in inhibiting DNA strand scission by 46.44%, followed by wild clone NL2 (43.11%) and wild clone NL3 (34.24%), while immature market sample exhibited low activity of around 8%.

The HPLC analysis of samples examined showed that gallic, caffeic, p-coumaric, ferulic and chlogenic acids were the most abundant phenolic acids in wild clone NL2. Catechin, epicatechin, proanthocyanidin dimer B- type, proanthocyanidin dimer A- type and myricertin 3-O-arabinoside were predominant flavonoids in the free form in wild clone NL2 and pilgrim; epicatechin being the most abundant. The highest content of anthocyanins was found in the pilgrim variety.

5.2 CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

The study clearly demonstrated that wild clone NL2 serve as promising sources of natural antioxidants for the further development. Although, the control Pilgrim showed the highest potential as an antioxidant. Thus, this issue must be considered in association with other potential benefits that might be rendered through other mechanisms independent of antioxidant potential.

Cranberry varieties are rich in a number of phytochemicals that display antioxidative properties. The study reported here has clearly established that cranberry serves as a promising source of natural antioxidants for the development of nutraceuticals or value-added products. Two of the varieties exhibited excellent characteristics in terms of their antioxidant potential compared to those in the market and other tested materials.

However, *in vivo* animal and human clinical studies are still needed to demonstrate the benefit of cranberry-based diets, especially for the tested genotypes and to confirm safe use of such products, as such or as functional food ingredients. Absorption, accessibility and metabolism of phenolics involved should also be studied. Similarly, the studies reported here also demonstrated that cranberries contained a number of antioxidant compounds which can effectively scavenge various reactive oxygen species / free radicals under *in vitro* conditions. The broad range of activities of the extracts suggests that multiple mechanisms are responsible for the antioxidant activity of cranberry extracts and clearly indicates their potential application as food ingredients or specialty chemicals. For the first time, it extensively examined all three forms of phenolics, namely free, esterified and bound phenolics along with their contribution to antioxidant activity and clearly highlighted the importance of including the bound phenolics in the analysis and

reporting of total phenolics content. The qualitative/quantitative analysis of the extracts for phenolic acids showed the presence of gallic, chlorogenic, caffeic, *p*-coumaric and ferulic acids in cranberry, consistent with the earlier reports. However, further *in vivo* studies are needed to demonstrate the absorption and metabolism of cranberry phenolics.

In addition, to *in vivo* studies, application of cranberry products and phenolics as antioxidant food preservatives should be considered. Through the results in this study we showed that cranberries have a wide range of phytochemical components, which are responsible for the antioxidant properties observed *in vitro.* Confirmation of these potential health benefits, through mechanistic and clinical studies, may lead to further development of cranberry-based nutraceuticals and pharmaceuticals for preventive and therapeutic purposess.

REFERENCES

Abuja, P. M., Murkovic, M., & Werner, P. (1998). Antioxidant and prooxidant activities of eldberry (*Sambuscus nigra*) extract in low-density lipoprotein oxidation. *Journal of Agriculture and Food Chemistry, 46,* 4091-4096.

Acheson, R. M., & Williams, D. R. R. (1983). Does consumption of fruits and vegetables protect against stroke, *Lancet, 1,* 1191 - 1193.

Ames, B.N., Shigenagaand, M.K., & Hagen, T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging, *Proceedings of the National Academy of Sciences USA*, 90**,** 7915-7922.

Andreasen, M. F., Landbo, A. K., Christensen, L. P., Hansen, A., & Meyer, A. S., (2001). Antioxidant effects of phenolic rye (*Secale cereale L.*) extracts monomeric hydroxycinnamates, and ferulic acid dihydrodimers on human low density lipoprotein. *Journal of Agriculture and Food Chemistry*, *49,* 4090 - 4096.

Andriantsitohaina, R., Andriambeloson, E., & Stoclet, J. C. (1999). Mechanism of Endothelial Nitric Oxide-Dependent Vasorelaxation Induced by Wine Polyphenols in Rat Thoracic Aorta, *Journal of Cardiovascular Pharmacology, 33 (2*), 248-254.

Aron, P. M., & Kennedy, J. A. (2008). Flavan-3-ols: Nature, occurrence and biological activity, *Molecular Nutrition & Food Research, 52,* 79 – 104.

Baidez, A.G., Gomez. P., Del Rio, J.A., & Ortuno, A. (2007). Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. *Journal of Agricultural and Food Chemistry , 55,* 3373 - 3377.

Bao, J.S., Cai, Y.Z., Sun, M., Wang, G.Y., & Corke, H. (2005). Anthocyanins, flavonols, and free radical scavenging activity of Chinese bayberry (*Myrica rubra*) extracts and their color properties and stability. *Journal of Agricultural and Food Chemistry, 53,* 2327 - 2332.

Beatriz, A., Acosta-Estrada, Janet, A., Gutierrez-Uribe, Sergio,O., & Serna-Saldívar. (2014). Bound phenolics in foods, a review, *Food Chemistry, 152,* 46-55.

Beer, C., Myers, R.A., Sorenson, J.H., & Bucci, L.R. (2004). Comprehensive comparison of the antioxidant activity of fruits and vegetables based on typical serving sizes from common methods. *Current Topics in Nutraceutical Research, 2,* 227-250.

Bodet, C., Grenier, D., Chandad, F., Ofek, I., Steinberg, D., & Weiss, E. I. (2008). Potential oral health benefits of cranberry, *Critical Reviews in Food Science and Nutrition, 48(7),* 672-680.

Bohm, V., & Schlesier, K. (2004). Methods to Evaluate the Antioxidant Activity, *Production Practices and Quality Assessment of Food Crops,* 55 - 71.

Bohm, B. A. (1998). Introduction to flavonoids, Department of Botany, University of British Columbia, Vancouver, Canada. pp. 503 - 514.

Bomser, J., Madhavi, D. L., Singletary, K., & Smith, M. A. (1996). *In vitro* anticancer activity of fruit extracts from *Vaccinium* species. *Planta Medica, 62,* 212-216.

Bors, W., & Michel, C. (2002). Chemistry of the antioxidant effect of polyphenols. *Annals of the New York Academy of Sciences, 957,* 57 - 69.

Boyle, S.P., Dobson, V.L., Duthie, S.J., Kyle, J.A.M., & Collins, A.R. (2000). Absorption and DNA protective effects of flavonoid glycosides from an onion meal, *European Journal of Nutrition, 39(5),* 213 - 223.

Brouillard, R., Chassaing, S., & Fougerousse, A. (1997). Why are grape/fresh wine anthocyanins so simple and why is it that red wine color lasts so long?, *Phytochemistry, 64(7),* 1179 - 1186.

Brown, P. N., Murch, S. J., & Shipley, P. (2012). Phytochemical diversity of cranberry (*Vaccinium macrocarpon* Aiton) cultivars by anthocyanin determination and metabolomics profiling with chemometric analysis. *Journal of Agricultural and Food chemistry, 55,* 157-164.

Cai, Y.Z., Luo, Q., Sun, M., & Corke, H. (2004).Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Science, 74,* 2157 - 2184.

Cai, Y.Z., Sun, M., Xing, J., Luo, Q., & Corke, H. (2006). Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sciences, 78,* 2872 - 2888.

Cao, G., Booth, S. L., Sadowski, J. A., & Prior, R. L. (1998). Increase in human plasma antioxidant capacity after consumption of controlled diets high in fruits and vegetables. *American Journal of Clinical Nutrition, 68,* 1081 - 1087.

Cao, G., Sofic, E., & Prior, R. L. (1997). Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships, *Free Radical Biology & Medicine, 22,* 749 - 760.

Castaneda-Ovando, A., Pacheco-Hernandez, M. L., Paez-Hernandez, M. E., Rodriguez, J. A., & Galan-Vidal, C. A. (1998). Chemical studies of anthocyanins: A review, *Food Chemistry 4(113),* 859-871.

Cekic, C., & Ozgen, M. (2010). Comparison of antioxidant capacity and phytochemical properties of wild and cultivated red raspberries (*Rubus idaeus* L.), *Journal of Food Composition and Analysis*, 23, 540 - 544.

Celik, H., Ozgen, M., Serce, S., & Kaya, C. (2008). Phytochemical accumulation and antioxidant capacity at four maturity stages of cranberry fruit, *Scientia Horticulturae, 117,* 345 - 348.

Chandrasekara, A., & Shahidi, F. (2010). Content of Insoluble Bound Phenolics in Millets and Their Contribution to Antioxidant Capacity, *Journal of Agricultural and Food Chemistry, 58(11),* 6706 - 6714.

Chandrasekara, A., & Shahidi. F.(2011). Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLCDAD-ESI-MS. *Journal of Functional Foods, 3(3),* 144 - 158.

Chandrasekara, A., & Shahidi, F. (2012). Effect of processing on the antioxidant activity of millet grains, *Food Chemistry, 133(1),* 1 - 9.

Chen, H.Y., & Zuo, Y. (2007). Identification of flavonol glycosides in American cranberry fruit, *Food Chemistry, 101,* 1357 - 1364.

Conforti, F., Menichini, F., Formisano, C., Rigano, D., Senatore, F., & Arnold, N.A. (2009). Comparative chemical composition, free radical-scavenging and cytotoxic properties of essential oils of six Stachys species from different regions of the Mediterranean Area. *Food Chemistry, 116,* 898-905.

Costantino, L., Albasini, A., Rastelli, G., & Benvenuti, S. (1992). Activity of polyphenolic crude extracts as scavengers of superoxide radicals and inhibitors of xanthine oxidase. *Planta Medica, 58(4),* 342 - 344.

Cote, J., Caillet, S., Doyon G., Dussault, D., Salmieri, S, Lorenzo, G., Sylvain, J, F., & Lacroix, M,. (2011). Effects of juice processing on cranberry antioxidant properties. *Food Research International, 44,* 2907 - 2914.

Cote, J., Caillet, S., Doyon, G., Sylvain, J. F., & Lacroix, M. (2010). Bioactive compounds in cranberries and their biological properties. *Critical Reviews in Food Science and Nutrition, 50,* 666 - 679.

Crozier, A., Yokota, T., Jaganath, I.B., Marks, S., Saltmarsh, M., & Clifford, M. N. (2006). Secondary metabolites as dietary components in plant-based foods and beverages, in Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet, ed. by Crozier A, Clifford MN and Ashihara H. Blackwell, Oxford, pp. 208 - 302.

da Costa, C.T., Horton, D., & Margolis, S. A. (2006). Analysis of anthocyanins in foods by liquid chromatography, liquid chromatography–mass spectrometry and capillary electrophoresis, *Journal of Chromatography A, 881 (1-2),* pp. 403-410.

Dangles, O., Saito, N., & Brouillard, R. (1993). Anthocyanin intramolecular copigment effect, *Phytochemistry, 34(1-3),* 119 - 124.

Dastmalchi, K., Flores, G., Petrova, V., Edward J., & Kennelly, E. J. (2011). Edible Neotropical Blueberries: Antioxidant and Compositional Fingerprint Analysis, *Journal of Agricultural and Food Chemistry, 59,* 3020 - 3026.

Dawidowicz, A. L., & Olszowy, M. (2012). Mechanism change in estimating of antioxidant activity of phenolic compounds, *Talanta, 97(15),* 312 - 317.

Deshpande, S.S. (1985). Investigation of dry beans *Phaseolus vulgaris* L.: microstructure, processing and antinutrients, Ph.D. thesis, University of Illinois, Champaign, IL.

Escarpa, A., & Gonzalez, M. C. (2001). An Overview of Analytical Chemistry of Phenolic Compounds in Foods, *Critical Reviews in Analytical Chemistry, 31(2),* 57 -139.

Ferguson, L. R., Shuo-tun, Z., & Harris, P. J. (2005). Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29. *Molecular Nutrition and Food Research, 49,* 585 - 693.

Foo, L.Y., Lu, Y. R., Howell, A. B., & Vorsa, N. (2000b). A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated *Escherichia coli*. *Journal of Natural Products, 63,* 1225 - 1228.

Fowler, Z. L., & Koffas, M. A. G. (2009). Biosynthesis and biotechnological production of flavanones: current state and perspectives, *Applied Microbiology and Biotechnology, 83,* 799 - 808.

Frankel, E.N., Huang, S.-W., Aeschbach, R. & Prior, E. (1996). Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion. *Journal of Agricultural and Food and Chemistry, 44*, 131 - 135.

Fresco, P., Borges, F., Diniz, C., & Marques, M. P. M. (2006). New insights on the anticancer properties of dietary polyphenols. *Medicinal Research Reviews, 26,* 747 - 766.

Gaikwad, P., Barik, A., Priyadarsini, K.I., & Rao B.S.M. (2010). Antioxidant activities of phenols in different solvents using DPPH assay*, Research on Chemical Intermediates, 36,* 1065 - 1072.

Guay, D. R. P. (2009). Cranberry and Urinary Tract Infections, *Drugs, 69,* 775 - 807.

Gharras, H. E. (2009). Polyphenols: food sources, properties and applications – a review, *International Journal of Food Science and Technology, 44*, 2512 - 2518.

Girard, K.K., & Sinha, N. (2006). Cranberry, blueberry, currant, and gooseberry. In: Handbook of Fruits and Fruit Processing. pp. 369 - 390. Hui, Y.H., Ed., Blackwell Publishing, Ames, IA.

Giusti, M.M. & Wrolstad, R.E. (2001). Unit F1.2: anthocyanins. Characterization and measurement with UV-visible spectroscopy. In: Wrolstad, RE, editor. Current protocols in

food analytical chemistry. New York: John Wiley & Sons. pp. **1.2.1-1.2.13**.

Goldstein, S., Meyerstein, D., & Czapski, G. (1993). The Fenton reagents. *Free Radical Biology & Medicine, 15,* 435 - 445.

Gupta. K. (2003). Emerging antibiotic resistance in urinary tract pathogens. *Infectious Disease Clinics of North America, 17,* 243 - 259.

Habash, M. B., van der Mei, H. C., Busscher, H. J., & Reid, G. (1982). Adsorption of urinary components influences the zeta poten tial of uropathogen surfaces, *Colloids and Surfaces A , 19,* 13 - 17.

Hakkinen, S., Heinonen, M., Karenlampi, S., Mykkanen, H., Ruuskanen, J., & Torronen, R. (1999). Screening of selected flavonoids and phenolic acids in 19 berries. *Food Research Inenational, 32,* 345 - 353.

Hall, C. (2001). Sources of natural antioxidants: oilseeds, nuts, cereals, legumes, animal products and microbial sources. In J. Pokorny, N. Yahishlivera, & M. Gordon (Eds.), Antioxidants in food: Practical applications (pp. 159–209). Cambridge, England: Woodhead Publishing.

Halliwell, B. & Gutteridge, J.M.C. (1999). *Free* Radical *Biology and Medicine*, 3rd edition, (p- 936). Oxford University Press, Oxford.

Halliwell, B., Aeschbach,R., Loliger, J., & Aruoma, O.I. (1995). *Food and Chemical Toxicology, 33,* 601 - 617.

Ham, S.S., Kim, S.H., Moon, S.Y., Chung, M.J., Cui, C.B., & Han, E.K. (2009). Antimutagenic effects of subfractions of Chaga mushroom (*Inonotus obliquus*) extract. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 672,* 55-59.

Han, X.Z., Shen, T., &Lou, H.X. (2007). Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences, 8,* 950 - 988.

Harborne, J.B., & Williams, C.A. (2000). Advances in flavonoid research since 1992. *Phytochemistry, 55,* 481 - 504.

Heima, K.E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships, *The Journal of Nutritional Biochemistry, 13,* 572 - 584.

Helbig, D., Bohm, V., Wagner, A., Schubert, R. & Jahreis, G. (2008). Berry seed press residues and their valuable ingredients with special regard to black currant seed press residues, *Food Chemistry, 111,* 1043 - 1049.

Hider, R. C., Liu, Z., & Khodr, H.H. (2001). Metal chelation of polyphenols. *Methods Enzymology, 335,* 190 - 203.

Hiramoto, K., Yasuhara, Y., Sako, K., Aoki, K., & Kikugawa, K. (2003). Suppression of freeradical-induced DNA strand breaks by linoleic acid and low density lipoprotein *in vitro*. *Biological & Pharmaceutical Bulletin, 26,* 1129 - 1134.

Howell, A. B. (2007). Bioactive compounds in cranberries and their role in prevention of urinary tract infections, *Molecular Nutrition & Food Research, 51*, 732 - 737.

Howell, A. B., Botto, H., Combescure, C., Blanc-Potard, A. B., Gausa, L., & Matsumoto, T. (2010). Dosage effect on uropathogenic *Escherichia coli* anti-adhesion activity in urine following consumption of cranberry powder standardized for proanthocyanidin content: a multicentric randomized double blind study. BMC. *Infectious Diseases, 10,* 94.

Huang, D. Ou. B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53,* 1841 - 1856.

Huang, W-Y., Cai, Y-Z., & Zhang, Y. (2009). Natural Phenolic Compounds From Medicinal Herbs and Dietary Plants: Potential Use for Cancer Prevention, *Nutrition and Cancer, 62(1),* 1 - 20.

Huang, W.Y., Cai, Y.Z., Xing, J., Corke, H., & Sun, M. (2007). A potential antioxidant resource: endophytic fungi isolated from traditional Chinese medicinal plants. *Economic Botany, 61,* 14 - 30.

Jaganath, I. B., & Crozier, A. (2010). Dietary flavonoids and phenolic compounds, Plant Phenolics and Human Health, Biochemistry, Nutrition and Pharmacology, Edited by Cesar G. Fraga. John Wiley & Sons Ltd., Hoboken, NJ, pp. 1 - 38.

Jimenez, J.P., Serrano, J., Tabernero, M., Arranz, S., Diaz-Rubio, M.E., & Garcia-Diz, L. (2008). Effects of grape antioxidant dietary fiber in cardiovascular disease risk factors. *Nutrition, 24,* 646 - 653.

Kahkonen, M. P., Hopia, A. I., & Heinonen, M. (2001). Berry phenolics and their antioxidant activity, *Journal of Agriculture and Food Chemistry, 49,* 4076 - 4082.

Kandil, F. E., Smith, M. A. L., Rogers, R. B., Pepin, M. F., Song, L. L., & Pezzuto, J. M. (2002). Composition of a chemopreventive proanthocyanidin-rich fraction from cranberry fruits responsible for the inhibition of 12-O-tetradecanoyl phorbol 13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity. *Journal of Agricultural and Food Chemistry, 50,* 1063 - 1069.

Karadag, A., Ozcelik B., & Saner S. (2009). Review of methods to determine antioxidant capacities**,** *Food Analytical Methods, 2,* 41 - 60.

Karakaya, S., & Kavas, A. (1999). Antimutagenic activities of some foods. *Journal of Agricultural Food Chemistry, 79,* 237 - 242.

Kim, G. N., Shin, J. G., & Jang, H. D. (2009). Antioxidant and antidiabetic activity of Dangyuja (Citrus grandis Osbeck) extract treated with *Aspergillus saitoi*. *Food Chemistry, 117,* 35 - 41.

Kiokiasa, S., Varzakasb, T., & Oreopouloua, V. (2008). *In vitro* Activity of Vitamins, Flavonoids, and Natural Phenolic Antioxidants against the Oxidative Deterioration of Oil-Based Systems, *Critical Reviews in Food Science and Nutrition, 48(1),* 78 - 93.

Koes, E. R., Quattrocchio, F., & Mol, J.N.M. (1994). The flavonoid biosynthetic pathway in plants: function and evolution, *BioEssays,* researchgate.net.

Komes, D., Belscak-Cvitanovic, A., Horzic, D., Gordana Rusak, G., Likic, S. & Berendik, M. (2011). Phenolic Composition and Antioxidant Properties of Some Traditionally Used Medicinal Plants Affected by the Extraction Time and Hydrolysis. *Phytochemistry Educationtion, 22,* 172 - 180.

Kraujalyte, V., Venskutonis, P. R., Pukalskas, A., Cesoniene, L., & Daubaras , R. (2013). Antioxidant properties and polyphenolic compositions of fruits from different European cranberrybush (*Viburnum opulus* L.) genotypes, *Food Chemistry, 141,* 3695 - 3702.

Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., & Hilpert, K.F. (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *American Journal of Medicine, 113,* 71 - 88.

Kristinova, V., Mozuraityte, R., Storrø, I., & Rustad, T. (2009). Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants. *Journal of Agricultural and Food Chemistry, 57,* 10377 - 10385.

Krygier, K., Sosulski, F.W., & Hogg, L.J. (1982). Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *Journal of Agricultural and Food Chemistry, 30,* 330 - 334.

Kuhnau, J. (1976). The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Review of Nutrition and Dietetics Home, 24,* 117 - 191.

Lee, C., Reed, D., & Richards, P. M. (2006). Ability of various polyphenolic classes from cranberry to inhibit lipid oxidation in mechanically separated turkey and cooked ground pork, *Journal of Muscle Foods, 17,* 248 - 266.

Liyana-Pathirana, M. C., & Shahidi, F. (2006). Importance of Insoluble-Bound Phenolics to Antioxidant Properties of Wheat, *Journal of Agricultural and Food Chemistry, 54,* 1256 - 1264.

Lin, J. Y., & Tand, C.Y. (2007). Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation, *Food Chemistry, 101*,140 - 147.

Liu, M., Li, Q. X., Webber, C., Lee, Y. C., Brown, J., & Liu, H. R. (2002). Antioxidant and Antiproliferative Activities of Raspberries, *Journal of Agriculture and Food Chemistry, 50,* 2926 - 2930.

Maatta-Riihinen, R.K., Kahkonen, P.M., torronen, R. A., heinonen, M.I. (2005). Catechins and Procyanidins in Berries of *Vaccinium* Species and Their Antioxidant Activity. Journal of Agriculture and Food Chemistry, 53, 8485-8491.

Macheix, J.J., Fleuriet, A., & Billot, J. (1990). Fruit Phenolics. CRC Press, Boca Raton, FL: CRC Press.

Macheix, J.J., & Fleuriet, A. (1998). Phenolic acids in fruits. In: Flavonoids in Health and Disease. pp. 35-60. Rice-Evans, C.A. and Packer, L., Eds., Marcel Dekker, New York, NY.

Madhujith, T., Izydorczyk, M., & Shahidi, F. (2006). Antioxidant properties of pearl edbarley fractions. *Journal of Agricultural and Food Chemistry, 54*, 3283 - 3289.

Madhujith, T., & Shahidi, F. (2006). Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *Journal of Agricultural Food Chemistry, 54,* 8048 - 8057.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: food sources and bioavailability, *American Society for Clinical Nutrition, 79,* 727 - 747.

Manganaris, A. G., Goulas, V., Vicente, R. A., & Terry, A. L. (2013). Berry antioxidants: small fruits providing large benefits, *Journal of the Science of Food and Agriculture, 94,* 825 - 833.

McKay, D. L., Chen, C. Y. O., Zampariello, C. A., & Blumberg, J. B. (2015). Flavonoids and phenolic acids from cranberry juice are bioavailable and bioactive in healthy older adults, *Food Chemistry, 168,* 233 - 240.

Meyers, K. J., Watkins, B. C., Pritts, P. M., & Liu, H.R. (2003). Antioxidant and antiproliferative activities of strawberries, *Journal of Agriculture and Food Chemistry, 51,* 6887 - 6892.

Michiels, J.A., Kevers, C. Pincemail, J., Defraigne, J.O., & Dommes, J. (2012). Extraction conditions can greatly influence antioxidant capacity assays in plant food matrices. *Food Chemistry, 130,* 986 - 993.

Miller, G. J., & Quakenbush, F. W. (1957). Antioxidant activity of phenols as related to effects of substituent groups, *Journal of the American Oil Chemists' Society, 34,* 404 - 407.

Millic, B. L., Diljas, S. M., & Canadanovic-Brunet J. M. (1998). Antioxidative activity of phenolic compounds on the metal-ion breakdown of lipid peroxidation system, *Food Chemistry, 61,* 443 - 447.

Naczk, M., & Shahidi, F. (1991). A critical evaluation of quantification methods of rapeseed tannins, presented at 8th International Rapeseed Congress, 9-11 July, Saskatoon, SK, Canada.

Naczk, M., & Shahidi, F. (1989). The effect of methanol-ammonia-water treatment on the content of phenolic acids of canola, *Food Chemistry, 31,* 159 - 164.

Naczk, M., & Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis, 41,* 1523 - 1542.

Nakama, T., Yamada, M., Osawa, T., & Kawakishi, S. (1993). Suppression of active oxygen induced cytotoxicity of flavonoids. *Biochemical Pharmacology, 45,* 265 - 267.

Neto, C. (2007). Cranberry and blueberry: Evidence for protective effects against cancer and vascular diseases. *Molecular Nutrition & Food Research, 51,* 652 - 664.

Neto, C.C. (2007). Cranberry and its phytochemicals: A review of *in vitro* anticancer studies. *Journal of Nutrition, 137,* 186S - 193S.

Nigam, P. S. (2009). Production of bioactive secondary metabolites. In: Nigam, PS, Pandey, A., editors. Biotechnology for agro-industrial residues utilization. first ed. Netherlands: Springer, 129 - 145.

Nijveldt, J, R., Nood, V. E., Hoorn, V. E.D., Boelens, G. P., Norren, V. K., & Leeuwen, V. A. P (2001). Flavonoids: a review of probable mechanisms of action and potential applications, *American Society for Clinical Nutrition, 74(4),* 418 - 425.

Nile, S. H., & Park S. W. (2014). Edible berries: Bioactive components and their effect on human health, *Nutrition, 30,* 134 - 144.

Ningappa, M. B., Dinesha, R., & Srinivas, L. (2008). Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (*Murraya koenigii* L.) extracts. *Food Chemistry, 106* , 720 - 728.

Ou, B., Hampsch-Woodill, M., Flanagan, J., Deemer, E. K., Prior, R. L., & Huang, D. (2002). Novel fluorometric assay for hydroxyl radical prevention capacity using fluoroscein as the probe. *Journal of Agriculture and Food Chemistry, 50,* 2772 - 2777.

Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., & Spiegelhalder, B. (2000). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer, 36,* 1235 - 1247.

Pantelidis, G.E., Vasilakakis, M., Manganaris, G. A., & Diamantidis, G. (2007). Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries, *Food Chemistry, 102,* 777 - 783.

Parry, J., Su, L., Luther, M., Zhou, K., Peter, Y.M., & Whittaker, P. (2005). Fatty acid composition and antioxidant properties of cold-pressed marionberry, boysenberry, red raspberry, and blueberry seed oils. *Journal of Agricultural and Food Chemistry*, *53,* 566 - 573.

Parry, J., Su, L., Moore, J., Cheng, Z., Luther, M., Rao, N. J., Wang, J., & Yu, L. L. (2006). Chemical Compositions, Antioxidant Capacities, and Antiproliferative Activities of Selected Fruit Seed Flours. *Journal of Agricultural and Food Chemistry, 54,* 3773 - 3778.

Pattison, J. D., Harrison, A. R., & Symmons, P.M.D. (2004). The role of diet in susceptibility to rheumatoid arthritis: a systematic review, *The Journal of Rheumatology, 31(7),* 1310 - 1319.

Pedersen, C. B., Kyle, J., Jenkinson, A. M., Gardner, P. T., McPhail, D.B., & Duthie, G. G. (2000). Effects of blueberry and cranberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. *European Journal of Clinical Nutrition, 54,* 405 - 408.

Pellegrini, N., Colombi, B., Salvatore, S., Brenna, O.V., Galaverna, G., Del Rio, D., Bianchi, M., Bennett, R.N., & Brighenti, F. (2007). Evaluation of antioxidant capacity of some fruit and vegetable foods: Efficiency of extraction of a sequence of solvents. *Journal of the Science of Food Agriculture, 871,* 103 - 111.

Porter, M. L., Krueger, C. G., Wiebe, D. A., Cunningham, D. G., & Reed, J. D. (2001). Cranberry proanthocyanidins associate with low-density lipoprotein and inhibit in vitro Cu^{2+} -induced oxidation, *Journal of the Science of Food and Agriculture, 81,* 1306 - 1313.

Reed, J. D. (2002). Cranberry flavonoids, atherosclerosis and cardiovascular health, *Critical Reviews in Food Science and Nutrition, 42,* 301 - 316.

Rekhy, R., & McConchie, R. (2014). Research review: Promoting consumption of fruit and vegetables for better health. Have campaigns delivered on the goals? , *Appetite, 79,* 113 - 123.

Ren, W.Y., Qiao, Z.H., Wang, H.W., Zhu, L., & Zhang, L. (2000). Flavonoids: promising anticancer agents. *Medicinal Research Reviews, 23,* 519 - 534.

Rice-Evans, C.A., Miller, N.J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20,* 933 - 956.

Robards, K., & Antolovich, M. (1997). Analytical chemistry of fruit bioflavonoids: A review. *Analyst, 2,* 11R - 34R.

Roy, S., Khanna, S., Alessio, H. M., Vider, J., Bagchi, D., & Bagchi, M.(2002). Anti-angiogenic property of edible berries. *Free Radical Research, 36,* 1023 - 1031.

Saanchez-Pataan, F., Bartolome, B., Martín-Alvarez, P.J., Anderson, M., Howell, A., & Monagas, M. (2012). Comprehensive Assessment of the Quality of Commercial Cranberry Products. Phenolic Characterization and in Vitro Bioactivity, *Journal of Agriculture and Food Chemistry, 60,* 3396 - 3408.

Sampietro, D.A., & Vattuone, M.A. (2006). Sugarcane straw and its phytochemicals as growth regulators of weed and crop plants. *Plant Growth Regulation, 48,* 21 - 27.

Schlesier, K., Harwat, M., Bohm, V., & Bitsch, R. (2002). Assessment of antioxidant activity by using different in vitro methods. *Free Radical Research, 36(2),* 177 - 187.

Seeram, N.P. Berries. In: Heber. D., Blackburn, G., Go, V.L.W., & Milner, J. (2006). Nutritional oncology. 2nd edition. London, UK, Academic Press, pp. 615 - 625.

Shahidi, F. (2000). Antioxidants in food and food antioxidants, *Molecular Nutrition, 44, 158* - 163.

Shahidi, F. (2000). Antioxidants in food and food antioxidants, *Nahrung, 44,* 158 - 163.

Shahidi, F., & Naczk, M. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A, 1054,* 95 - 111.

Shahidi, F., & Hong, C. (1991). Evaluation of malonaldehyde as a marker of oxidative rancidity in meat products. *Journal of Food Biochemistry, 15,* 97 - 105.

Shahidi, F., & Ho, C-T. (2007). Antioxidant measurement and applications: an overview. In Antioxidant measurement and applications. Shahidi, F. and Ho, C. T. Eds. ACS Symposium Series 956, American Chemical Society, Washington, DC. pp. 2 - 7.

Shahidi, F., & Naczk, M. (1995). Food Phenolics: Sources, Chemistry, Effects, Applications. Technomic Publishing Company, Lancaster, PA.

Shahidi, F., Rubin, L.J., & Wood, D.F. (1987). Control of lipid oxidation in cooked ground pork with antioxidants and lipid dinitrosyl ferrohemochrome. *Journal of Food Science, 52,* 45 - 74.

Shahidi, F., Wanasundara, J.P.K.P.D. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition, 32,* 67 - 103.

Shahidi, F., Wanasundara, U., Amarowicz, R., & Naczk, M. (1995). Natural antioxidants from canola meal. In Proceedings of 8th rapeseed international congress, pp. 873 - 875. Saskatoon, SK, Canada.

Shahidi, F., & Zhong, Y. (2005). Lipid Oxidation: measurement methods. In Bailey's Industrial Oil and Fat Products, vol 1. Shahidi, F. Ed. John Wiley & Sons Ltd., Hoboken, NJ, pp. 491 - 512.

Shahidi, F., & Zhong, Y. (2007). Measurement of antioxidant activity in food and biological systems. In Antioxidant measurement and applications. Shahidi, F. and Ho, C. T. Eds. ACS Symposium series 956, American Chemical Society, Washington, DC, pp. 36 - 66.

Shan, B., Cai, Y.Z., Sun, M., & Corke, H. (2005). Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *Journal of Agricultural and Food Chemistry, 53,* 7749 - 7759.

Simonetti, P., Pietta, P., & Testolin, G. (1997). Polyphenol content and total antioxidant potential of selected Italian wines. *Journal of Agricultural and Food Chemistry, 45,* 1152 - 1155.

Siriwardhana, W. K. S., & Shahidi, F. (2002). Antiradical activity of extracts of almond and its by-products, *Journal of the American Oil Chemists' Society, 79(9),* 903 - 908.

Siu, G. M., & Draper, H. H. (1978). A survey of the malonaldehyde content of retail meats and fish. *Journal of Food Science, 43,* 1147 - 1149.

Stagos, D., Kazantzoglou, G., Theofanidou, D., Kakalopoulou, G., & Magiatis, P. (2006). Activity of grape extracts from Greek varieties of *Vitis vinifera* against mutagenicity induced by bleomycin and hydrogen peroxide in *Salmonella typhimurium* strain TA102. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 609,* 165 - 175.

Stobiecki, M. (2000). Review: Application of mass spectrometry for identification and structural studies of flavonoid glycosides, *Phytochemistry, 54(3),* 237 - 256.

Sun, J., Chu, Y., Wu, X., & Liu, R. H. (2002). Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry, 50,* 7449 - 7454.

Surh, Y.J. (2003). Cancer chemoprevention with dietary phytochemicals. *Nature Reviews Cancer, 3,* 768 -780.

Tesissers, R. L., Frankel, E. N., Waterhouse, A. L., Peleg, H., & German, J. B.(1996). Inhibition of *in vitro* human LDL oxidation by phenolic antioxidants from grapes and wines, *Journal of the Science of Food and Agriculture, 70*, 55 - 61.

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients, 2,* 1231-1246.

Tulio, A. Z., Jablonski, J. E., Jacksona, L. S., Changa, C., Edirisinghe, I., & Burton-Freeman, B. (2014). Phenolic composition, antioxidant properties, and endothelial cell function of red and white cranberry fruits. *Food Chemistry, 157,* 540 - 552.

Tunga, Y-T., Wub, J-H., Kuoc, Y-H., & Chang, S-T. (2007). Antioxidant activities of natural phenolic compounds from Acacia confusa bark, *Bioresource Technology, 98,* 1120 - 1123.

Vasileioua, I., Katsargyris, A., Theocharis, S., Giaginis, C. (2013). Current clinical status on the preventive effects of cranberry consumption against urinary tract infections, *Nutrition Research, 33(8),* 595 - 607.

Vattem, D. A., Ghaedian, R., & Shetty, K. (2005). Enhancing health benefits of berries through phenolic antioxidant enrichment: Focus on cranberry. *Asia Pacific Journal of Clinical Nutrition 14,* 120 - 30.

Velioglu, Y.S., Mazza, G., Gao, L., & Oomah, B.D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *Journal of Agricultural and Food Chemistry, 46*, 4113 - 4117.

Velioglu,Y. S., Ekici, L., & Poyrazoglu, E. S. (2006). Phenolic composition of European cranberrybush (*Viburnum opulus* L.) berries and astringency removal of its commercial juice. *International Journal of Food Science and Technology, 41*, 1011 - 1015.

Verlangieri, A. J., Kapeghian, J. C., el-Dean, S., & Bush, M. (1985). Fruit and vegetable consumption and cardiovascular mortality. *Medical Hypothesis, 16,* 7 - 15.

Vinson, J. A., Su, X., Zubik, L., & Bose, P. (2001). Phenol antioxidant quantity and quality in foods: Fruits. *Journal of Agricultural and Food Chemistry, 49,* 5315 - 5321.

Viskelis, P., Rubinskiene, M., Jasutiene, I., Sarkinas, A., Daubaras, R. & Cesoniene, L. (2009). Anthocyanins, Antioxidative, and Antimicrobial Properties of American Cranberry (*Vaccinium macrocarpon* Ait.) and their Press Cakes. *Journal of Food Science, 74(2),* **157-161.**

Waladkhani, A. R., & Clemens, M. R. (1998). Effect of dietary phytochemicals on cancer development. *International Journal of Molecular Mededecine, 1,* 747 - 753.

Wang, Y.S., & Lin, H. (2000). Antioxidant Activity in Fruits and Leaves of Blackberry, Raspberry, and Strawberry Varies with Cultivar and Developmental Stage, *Journal of Agriculture and Food Chemistry, 48,* 140 - 146.

Weiss, E. I., Kozlovsky, A., Steinberg, D., & Lev-Dor, R. (2004). A high molecular mass cranberry constituent reduces mutan streptococci level in saliva and inhibits in vitro adhesion to hydroxyapatite. *FEMS Microbiology Letters, 232(1),* 89 - 92.

Wijeratne, S.S.K., Amaraowicz, R., & Shahidi, F. (2006). Antioxidant activity of almonds and their by-products in food model systems. *Journal of the American Oil Chemists' Society, 83,* 223 - 230.

Xu, G., Ye, X., Liu, D., Ma, Y., & Chen, J. (2008). Composition and distribution of Phenolicacids in Ponkan (*Citrus poonensis* Hort. ex Tanaka) and Huyou (*Citrus paradisi* Macf.Changshanhuyou) during maturity. *Journal of Food Composition and Analysis, 21,* 382 - 389.

Yan, X., Murphy, B. T., Hammond, G. B., Vinson, J. A., & Neto, C. C. (2002). Antioxidant activities and antitumor screening of extracts from cranberry fruit (*Vaccinium macrocarpon*). *Journal of Agricultural and Food Chemistry, 50,* 5844 - 5849.

Yang, C.S., Landau, J.M., Huang, M.T., & Newmark, H.L. (2001). Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annual Review of Nutrition, 21,* 381 - 406.

Yu, L. L., Zhou, K. L., & Parry, J. (2005). Antioxidant properties of cold-pressed black caraway, carrot, cranberry, and hemp seed oils, *Food Chemistry, 91,* 723 - 729.

Zhang, H.Y., Yang, D.P., & Tang, G.Y. (2006). Multipotent antioxidants: from screening to design, *Drug Discovery Today, 11,* 749 - 754.

Zhang, L., Ma, J., Pan, K., Go, V. L., Chen, J., & You, W. C. (2005). Efficacy of cranberry juice on *Helicobacter pylori* infection: a double-blind, randomized placebocontrolled trial. *Helicobacter, 10(2),* 139 - 145.

Zheng, W., & Wang, S.Y. (2003). Oxygen Radical Absorbing Capacity of Phenolics in Blueberries, Cranberries, Chokeberries, and Lingonberries, *Journal of Agriculture and Food Chemistry, 51,* 502-509.

Zheng, Z., & Shetty, K. (2000). Solid-state bioconversion of phenolics from cranberry pomace and role of *Lentinus edodes* ß-glucosidase. *Journal of Agriculture and Food Chemistry, 48,* 895 - 900.

Zhong, Y., & Shahidi, F. (2011). Antioxidant Behavior in Bulk Oil: Limitations of Polar Paradox Theory, *Journal of Agricultural and Food Chemistry, 60,* 4 - 6.

Ziebland, J., & Roe, Y. (2002). Effects of Fruit and Vegetable Consumption on Plasma Antioxidant Concentrations and Blood Pressure. *The Lancet, 359,* 1969 - 197.

Zielinski, H., Kozlowska, H. (2000). Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *Journal of Agricultural and Food Chemistry.48,*2008-2016.