ABSTRACT

Title of Dissertation:	VALUE-ADDING FACTORS IN COLD-PRESSED EDIBLE SEED OILS AND FLOURS.
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This study examined cold-pressed edible seed oil and flour from fruit and other plant sources for value-adding components and properties. The tested fruit seeds included black raspberry, red raspberry, boysenberry, marionberry, blueberry, cranberry, pinot noir grape, and chardonnay grape. The other seeds included onion, parsley, cardamom, mullein, roasted pumpkin, and milk thistle. The seed oils were examined for fatty acid profile, carotenoids, tocopherols, total phenolic content (TPC), oxygen radical absorbance capacity (ORAC), DPPH$^\bullet$ scavenging activity, oxidative stability index (OSI), peroxide value, refractive index, density, and color. The seed flours were tested for total fat, fatty acid profile, TPC, total anthocyanin content, chelating capacity, HT-29 cancer cell antiproliferation, ORAC, DPPH$^\bullet$, and hydroxyl radical scavenging.

All of the fruit seed oils contained significant levels of α-linolenic acid (18:3n-3) while none of the other seed oils contained a significant amount. The range of α-linolenic acid in the fruit seed oils was 19.6 to 32.4 g/100 g with red raspberry seed oil having the highest concentration. Zeaxanthin was the primary carotenoid in

all seed oils and comprised approximately 79% of the combined total. Pumpkin seed oil contained the highest concentration of β-carotene at 6.0 mg/kg, and onion contained the most α-tocopherol from 498-692 mg/kg. Parsley and cardamom seed oil extracts had very high ORAC values of 1,100 and 942 μmol trolox (TE) equivalents per g oil (TE μmol/g), respectively, and their OSI were not determinable under the experimental conditions.

Among the seed flours, cranberry was the only sample that contained significant α-linolenic acid having 30.7 g/100 g oil, while the parsley seed flour sample contained 86.2 g/100g oleic acid (18:1n-9) and was significantly higher than all others. Milk thistle and chardonnay grape seed flour extracts had ORAC values of 1131 and 1076 TE μmol/g flour, respectively, and both significantly inhibited the growth of HT-29 cells at 6 mg/mL media. The chardonnay sample killed all cells following 24 h of treatment. The obtained data suggests the potential value-adding use of these seed oils and flours as dietary sources of special fatty acids, carotenoids, tocopherols, antioxidants, and antiproliferative agents, for optimal human health.

VALUE-ADDING FACTORS IN COLD-PRESSED EDIBLE SEED OILS AND
FLOURS.

by

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Introduction

Fruit juice and wine are produced worldwide on an enormous scale, and seeds are a major byproduct from manufacturing. Leftover seeds are usually discarded because they are not considered to have much value. However, seeds contain energy, nutrients including vitamins and minerals, and other health beneficial components such as antioxidants. Recently, the grape industry has been marketing grape seed extract as an excellent source of antioxidants, but many wine and juice producers still dispose of seeds that may contain high concentrations of valuable beneficial components. The value-added components present in all seeds may include dietary essential fatty acids that have critical roles in human health. They also contain vitamins including the fat soluble vitamin E as α-tocopherol and the 7 other tocopherol vitamers; they contain the provitamin A, β-carotene, and other carotenoids. Seeds have demonstrated to have strong antioxidant activities demonstrating reducing activities greater than vitamin C and E.

In this study, cold-pressed fruit seeds including red raspberry, black raspberry, marionberry, blueberry, cranberry, pinot noir grape, and chardonnay grape, and other seed oils and flours including onion, roasted pumpkin, parsley, mullein, cardamom, and milk thistle were examined. The oils were examined for fatty acid profile, oxidative stability, peroxide value, antioxidant activities against DPPH$^•$, the peroxyl radical using the oxygen radical absorbing capacity (ORAC), and physical properties. The flours were tested for oil content, fatty acid profile, antioxidant activities using the ORAC assay, the hydroxyl radical, and DPPH$^•$, and antiproliferation effects

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against the HT-29 human colon cancer cell line. The results of these analyses may have a significant impact on the value of fruits and other plant foods not currently harvested for their seeds, and may increase the overall profit for related agricultural markets. The specific objectives of this research were:

1. To evaluate cold-pressed edible fruit seed oils for chemical compositions, antioxidant activities, and physical properties;

2. To evaluate edible fruit seed flours for chemical compositions, antioxidant activities, and anticancer activity;

3. To examine other edible seed oils for chemical compositions, antioxidant activities, and physical properties;

4. To investigate other edible seed flours for chemical compositions, antioxidant activities, and anticancer activity.

Chapter 1: Literature Review

Overview of Seeds

Many food crops are grown for their whole fruits and include wheat, corn, rice, safflower, soybean, rapeseed, peanut, sunflower, cotton, and canola. However, some food crops are only grown for specific parts of their fruit such as the mesocarp (flesh), and their seeds are discarded as waste byproducts. These types of crops are primarily harvested for juice and juice concentrate, wine, jam, soup, and salad production, and billions of tons of their seeds are discarded each year either as cheap animal feed or simply thrown away at a cost. Seed components can be separated into two parts: oil and flour. The flour remains following oil extraction, which is performed by many methods including solvent extraction and cold-pressing. Seeds have very different and complex chemical compositions that are nutritionally grouped as macronutrients, micronutrients, and other components. Other components include other phytochemicals such as phenolic antioxidants that have demonstrated potential beneficial health properties. Seeds oils also have other properties that include oxidative stability and color. These components and properties of seeds are valuable and need to be examined and reported which may ultimately lead to increased crop values and increase farm-gate profits for growers and processors.

Cold-Pressing

Oils are conventionally extracted from ground seed meal using organic solvents such as hexane with heat, followed by solvent evaporation. Cold-pressing is a method that does not use organic solvents or heat to extract seed oil. A typical cold-

press machine is screw driven and removes oil by applying grinding pressure to seeds. The residual remaining after extraction is the cake or flour. Another type of cold-pressing applies pressure directly to seeds in a barrel with slits down the side that allow oil to run out. Cold-pressing is believed to be a better technique for retaining beneficial value-added components in seed oils that might be lost by evaporation or chemically modified using conventional solvent extracting methods (*1*).

Potential Value-Adding Components in selected edible fruit seeds

Macronutrients

The macronutrients in cold-pressed fruit seeds include protein, carbohydrates, fats, and fiber. The oil from cold-pressed seeds mainly contains lipophilic compounds while the flour is comprised of both hydrophilic and hydrophobic compounds. Cold-pressed seed oils are predominantly triglycerides which are 3 fatty acids esterified to a glycerol backbone, while flours are comprised of a mixture of the macronutrients. Fatty acids are vital components of all human diets not simply as an energy source, but they are required by all cells of the body in the form of phospholipids for structural membrane integrity. They are energy dense compounds containing more than 2 times the energy per weight than proteins or carbohydrates, and are classified depending on their level of saturation. Fatty acids with zero double bonds are classified as saturated, one double bond are monounsaturated (MUFA), and more than one double bond are polyunsaturated (PUFA). Seed oils are comprised of fatty acids that differ in their carbon chain lengths; however, they are mainly comprised

fatty acids with 16, 18, and 20 carbons. Two fatty acids found in seed oils that are essential to human diets include linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3)

Linoleic acid is a PUFA with two double bonds at carbons 9 and 12 using the delta numbering system, and is classified as an omega-6 (ω6) fatty acid. Linolenic acid is also a PUFA with three double bonds at carbons 9, 12, and 15 and is classified as an ω3 fatty acid. These two fatty acids are essential to humans because they cannot be synthesized de novo, therefore, they must be obtained from exogenous sources. These essential fatty acids are required by the brain and nervous system and for normal growth and vision development in infants (*2, 3*). Deficiencies of essential fatty acids are characterized by dry flaky skin, decreased growth and wound healing, diarrhea, increased anemia, and an increased rate of infections (*4*). Aside from being nutritionally essential, linoleic acid has been considered a cholesterol lowering fatty acid for a number of years, and LDL is the primary cholesterol that it decreases, (*5-9*). Using oleic acid as a baseline, it has been estimated that increasing linoleic acid intake lowers cholesterol about half as much as saturated fatty acids increase it (*5*). Alpha-linolenic acid has also been shown to decrease LDL, but it does not seem to be as effective as linoleic acid (*6*). The cardiac protective effects of linolenic acid are not completely clear. However, animal studies have shown positive effects regarding cardiac arrhythmia, thrombosis, and inflammation which may correlate to its conversion to docosahexaenoic (DHA) (*6*).

Both linoleic and linolenic fatty acids are converted *in vivo* to longer chain fatty acids by desaturases and elongases and have related biological functions. Linoleic acid is converted to arachidonic acid (20:4n-6), which can be converted by

clycooxygenase and lipoxygenase to eicosanoids including prostaglandins (2-series) PGE_2 and $PGE_{2\alpha}$, prostacyclin PGI_2, thromboxane TXA_2, and leukotrienes (4-series) LTA_4, LTC_4, and LTE_4. These molecules act as local regulators, which unlike hormones, are not produced by a gland and do not travel in the blood to their site of action. These eicosanoids mediate pain sensitivity, inflammation, swelling, and platelet aggregation (*10*). Alpha-linolenic acid is converted to eicosapentaenoic acid (EPA) (20:5n-3) and further to DHA (22:6n-3). EPA is converted by cyclooxygenase and lipoxygenase to yield (3-series) prostanoids including PGE_3, PGI_3 and TXA_3, and (5-series) leukotrienes including LTB_5, LTC_5, and LTE_5 (*11*). These prostanoids from EHA have anti-inflammatory and anti-aggregative effects and may balance eicosanoid effects. DHA has been linked to many health benefits including reducing the risk of heart disease, cancers, hypertension, and autoimmune disorders (*12-17*)

Oleic acid (18:1n-9) is another important fatty acid found in seed oils. It is the primary fatty acid found in olive oil comprising 68-73% (*18*) but has also been seen out of this range in other studies on olive oil. Olive oil is the key component and predominating source of fat in the Mediterranean diet. The Mediterranean diet has several different variations, but is characterized by a high intake of fat. In some of the variations of the diet, over 40% of the total energy consumed is from fat, (the American Dietetic Association recommends no more than 30%), however, there is a negative correlation between fat consumed as olive oil and cardiovascular disease (CVD) compared to other parts of the world that do not consume olive oil in appreciable amounts (*19*). Diets high in oleic acid have been experimentally demonstrated to lower total cholesterol – primarily LDL cholesterol. Also, a meta-

analysis of several previous studied revealed that there was no significant difference between diets containing high levels of oleic acid and diets with high levels of linoleic acid in their effectiveness to lower total cholesterol and LDL when experimentally replaced for saturated fat in equal amounts (*20*)*.* Furthermore, LDL composed of high levels of oleic acid have shown a decreased susceptibility to oxidation compared to LDL containing high concentrations of PUFA (*21, 22*). In November 2004, the FDA published a qualified heath claim for olive oil stating that the consumption of 2 tablespoons of olive oil in place of saturated fat without increasing total calories can decrease one's overall risk for heart disease (*23*).

Micronutrients

Micronutrients found in seeds include vitamins, provitamins, and minerals. Fat soluble vitamins including vitamins E, K, and provitamin A are primarily found in the oil. The flour also contains the fat soluble vitamins at lower concentrations than in the oil, but also contains water soluble vitamins including the vitamin B complex if present in the seed.

Vitamin E is comprised of eight different vitamers that are further classified into 2 groups of 4 called tocopherols and tocotrienols. The tocopherols have saturated side chains and the tocotrienols are unsaturated with three conjugated double bonds on the side chain. The classes of both are differentiated by substitutions of methyl groups on the phenol ring, and are named α-, β-, δ-, and γ-tocopherol or tocotrienol. The 4 tocopherols have vitamin E activity while only α-tocotrienol demonstrates appreciable activity among the tocotrienols. Vitamin E primarily functions to maintain stability of membranes by preventing initial oxidation and stopping the

cascading oxidation of polyunsaturated fatty acids by lipid peroxyl radicals (*24*).

Vitamin A, as retinol, is not found in seed oils, however, precursors to vitamin A

including β-carotene and α-carotene, are prevalent. When hydrolyzed, β-carotene

provides 2 retinol molecules while α-, and λ-carotene can provide one.

Vitamin A performs many functions in the body. It is necessary for visual

light detection by binding with opsin in the rod cells of the eye, and acts as a hormone

by affecting gene expression in the nucleus. Retinoic acid receptors (RAR) and

retinoid receptors (RXR) are nuclear receptors that bind to retinoic acid and 9cis

retinoic acid. The latter is specific for 9cis retinoic acid. When bound to

corresponding retinoic acid species, RXR and RAR complexes are specific for DNA

sequences called retinoic acid response elements, and the complexes have been found

to affect numerous genes including those involved in cellular developmental

processes (*25*). Vitamin A is also required by immature epithelial cells in the lungs,

skin, trachea, and GI tract for normal development. Vitamin A, as retinol, is required

by both male and female reproductive systems; it is involved in bone development

and maintenance, and it is involved in normal immune function (*24*). Other

carotenoids also found in seed oils include lutein, cryptoxanthin, and zeaxanthin that

may possess antioxidant abilities.

Antioxidants

Antioxidants are chemicals that can react with radicals and prevent the

oxidation of other molecules (*26*). In order for a chemical to be defined as an

antioxidant, it must meet two conditions. When present at low concentrations it must

delay or prevent the oxidation of another compound, and the radical formed from the resultant reaction must be relatively stable and must not promote oxidation (*27*).

Theories for antioxidant protective mechanisms in living systems include the donation of electrons or hydrogen atoms to oxidized molecules such as fatty acids, phospholipids, and proteins, which stabilizes them, scavenging radical compounds and atoms such as $OH^\bullet$, $O_2^{-\bullet}$, and $ROO^\bullet$, which become oxidized instead of a 'good' molecule, and they may coordinate transition metals such as copper and iron, which would prevent the interaction of the metal with an oxidizable molecule, therefore, preventing oxidation (*28*).

Below are simplified examples of how phenolic antioxidant compounds can stop lipid oxidation chain reactions, hydroxyl radical and superoxide damage, and metal ion free radical damage by reducing oxidized molecules to less harmful molecules (*28*).

Lipid Oxidation	$ROO^\bullet$ + AntioxidantH	ROOH + Antioxidant$^\bullet$	
	$RO^\bullet$ + AntioxidantH	ROH + Antioxidant$^\bullet$	
Hydroxyl	$OH^\bullet$ + AntioxidantH	H_2O + Antioxidant$^\bullet$	
Superoxide	$O_2^{-\bullet}$ + AntioxidantH	O2H + Antioxidant$^\bullet$	
Ferric	Fe^{3+} + Antioxidant	Fe^{2+} + Antioxidant$^\bullet$	
Cupric	Cu^{2+} + Antioxidant	Cu^{+} + Antioxidant$^\bullet$	
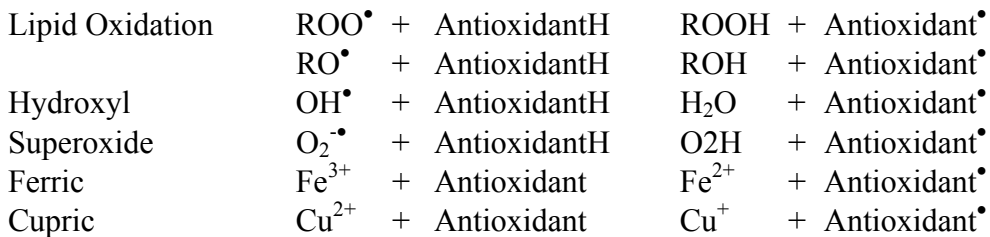
Antioxidants are very important dietary components. Nutrient antioxidants that are essential to the body include vitamin C and vitamin E. These vitamins protect the body from free radical damage among other functions. Antioxidant compounds found in edible seed oils and flours include the previously discussed vitamin E and carotenoids but also include phenolic and polyphenolic acids. Phenolic compounds have demonstrated to be powerful antioxidants. Individual phenolic compounds and foods containing phenolic compounds have shown strong abilities to significantly extend the presence of vitamin C when exposed to ascorbic acid oxidase (*29*) and

individual phenolic compounds have shown to be very effective free radical scavengers when compared to trolox, a water soluble vitamin E analog (*30*).

Many epidemiological investigations indicate that dietary antioxidants are associated with reducing the risk of chronic diseases including cardiovascular disease, cancer, and diabetes (*31-33*). It is claimed that the phenols in red wine protect against heart disease caused by LDL oxidation. Phenols and polyphenols are implicated in the 'French Paradox' where the population in Southern France has a very low rate of heart disease despite their high-fat diet and smoking habits relative to nations with similar habits that do not consume as much red wine (*30*). Grape seed extract (GSE) is a commercially available product that contains highly concentrated phenolic compounds and is sold over the counter in supplement form. Investigations have demonstrated that GSE is a more powerful radical scavenger than both vitamins C and E protecting against tobacco induced oxidative stress, skin damage from UV light, and human liver cells *in vitro* against cytotoxicity among other protective qualities (*34*).

Natural antioxidants including vitamin E and phenolic compounds are prevalent at significant levels in seed oils, and seed flours and may prevent lipid peroxidation as effective as commercially used synthetic antioxidants including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Growing consumer concerns regarding the possible long term detrimental effects of synthetic antioxidants are increasing the demand for natural antioxidants.

Potential Value-Adding Components Detected in Selected Edible Fruit Seeds

<u>Red Raspberry Seed</u>

Red raspberry (*Rubus ideaus*) is a member of a group of fruits called caneberries, and Oregon is their primary growing location in the United States. Other caneberries include red black raspberry, boysenberry, and marionberry. In 2004, worldwide production of raspberries was 500,000 metric tons (MT) and 30,000 MT were grown in the United States. United States red raspberry producers process approximately 99% of the berries and treat the remaining seeds as waste. Red raspberry seed has been shown to comprise 3.3 to 5.1% of the whole fresh weight of the fruit (*35*) and may contain up to 20% oil (*36*). Hypothetically, this means that 1.1 to 1.4 million liters of red raspberry oil could have been produced in 2004. Raspberry seed oil is used as an ingredient in cosmetic products for its anti-inflammatory properties. It has been shown to prevent gingivitis, eczema, rashes, and other skin lesions. It may also be used by the sunscreen industry because of its UV absorbing properties (*37*).

Red raspberry seed oil extracted by hexane and cold-pressing were examined for their fatty acid compositions and phytochemical composition (*37*). The oil contained 29.1% α-linolenic acid and 53% linoleic acid. The oil also had a total tocopherol content of 97 mg/100 g oil in the hexane-extracted red raspberry seed oil, and had 61 mg/100 g oil in the corresponding cold-pressed seed oil, both of which were greater than total tocopherol in safflower (57 mg/100 g oil) and grape seed (12 mg/100 g oil) oils. The difference in the tocopherol content between the hexane extracted and cold-pressed raspberry seed oils could possibly be explained by the

presence of non-lipid contaminants from cold-pressing that dilute tocopherols in the oil sample and better extraction of tocopherols by hexane (*37*). Another study of raspberry seed oil by Xu et al (*38*) investigated the chemical composition of 6 raspberry varieties. The fatty acid contents of the six seed oil samples were highly unsaturated, all over 95%. Linoleic acid was the primary fatty acid from 51.0-54.6%, linolenic acid was 27.8-35.6%, and oleic acid was 9.8-15.5% of the total fatty acids. Tocopherols were also analyzed and α-tocopherol was detected from 22.4-52.6 mg/100 g oil.

In 2006, Camire et al (*39*) investigated the anthocyanin content in raspberry powder along with 3 other fruit powders including blueberry, cranberry, and Concord grape. The concentration of anthocyanins in raspberry fruit powder was the lowest among the 4 tested fruits at 173.6 mg/100 g; however, it significantly increased the total phenolic and total anthocyanin content of white cornmeal cereal. Pantelidis et al, 2006 (*40*) investigated six samples of red raspberry including 2 Heritage, 2 Autumn Bliss, and 2 Fallgold varieties for total phenolic content (TPC) and total anthocyanin content (TAC) in the whole dried and fresh fruit. The TPC ranged from 1.1-to 2.5 mg gallic acid equivalents per g dry weight (GAE mg/g) in the Autumn Bliss variety, which had both the low and high values among the six varieties. The TAC were 48.2-49.1 mg/100 g fresh weight in the Heritage, 35.1-39.1 mg/100 g in the Autumn Bliss, and 1.3-3.4 mg/100 g in the Fallgold variety. The low anthocyanin values for the Fallgold variety were explained by their lighter skin color compared to the others (*40*).

Red raspberry has been examined *in vitro* for antiproliferation effects on human cancer cell lines (*35, 41*). Four varieties of raspberry including 'Anne,' 'Goldie,' 'Heritage', and 'Kiwigold' were analyzed for the ability to inhibit the growth of $HepG_2$ human liver cancer cells (*41*). Fresh raspberries were extracted using 80% acetone and directly added to media. The results showed that there was a dose dependent growth inhibition response from all samples. The Goldie variety had the most inhibitory activity showing the lowest effective dose (EC_{50}) among the 4 tested raspberries (*41*). In 2005, Juranic et al (*35*), tested water extracts from seeds of five varieties of raspberry for antiproliferative effects on human colon cancer LS174 cells. The seed extracts inhibited cell proliferation, and inhibition was correlated to ellagic acid concentration.

<u>Black Raspberry Seed</u>

Black raspberry (*Rubus occidentalis* L.), another caneberry, is grown primarily for its juice. In 2004, 1000 MT were harvested in the United States and 99.9% was processed. The seed oil from black raspberry has recently been shown to contain high levels of unsaturated fatty acids and has demonstrated strong antioxidant activity (*42*). The primary fatty acid in the seed oil was linoleic acid followed by α-linolenic acid at 53.5 and 31.2 g/100 g oil, respectively. The seed oil also demonstrated an oxygen radical absorbance capacity (ORAC) value of 52.3 micromoles trolox equivalent (TE) per g oil (TE μmol/g) (*42*).

The whole fruit from black raspberry has been examined and is important to document because seed compositions, including antioxidants, may be correlated to whole fruit compositions. In 2002, Wada and Ou (*43*) examined the ORAC, TPC, and

TAC of whole dried caneberries including black raspberry, evergreen blackberry, red raspberry, boysenberry, and marionberry. The whole dried black raspberry fruit had significantly higher values in all three tests. It had an ORAC value of 453 TE μmol/g; a TPC of 57.6 GAE mg/g, and a TAC of 3465 mg/100 g. Two other investigations on the juice of whole black raspberry have shown high levels of antioxidants compared to other fruits (44, 45). Black raspberry (cv Jewel) juice demonstrated higher ORAC, TPC, and TAC, than red raspberry, strawberry and blackberry cultivars on a fresh weight basis (44), and it was further reported to have stronger or comparable radical scavenging activities against hydroxyl radical, superoxide radical, hydrogen peroxide, and singlet oxygen (45).

Black raspberry has also been investigated for intracellular protein expression (46). Black raspberry extract was examined for effects on the expression of cellular transcription factors in mouse epidermal cells (JB6 C1 41). The results of their investigation showed that black raspberry extracts might impair signal transduction pathways leading to activation of AP-1 and NFkappaB, therefore, inhibiting tumor development (46). These data suggested the potential health benefits of consuming black raspberry and black raspberry-based products.

Boysenberry Seed

Boysenberry (*Rubus ursinus × idaeus*) production in 2004 was approximately 2,400 MT. Bushman et al, 2004 (42) recently examined boysenberry seed oil for fatty acid concentration and antioxidant activity. The seed oil demonstrated a high concentration of unsaturated fatty acids comprising 95.4% of the total. Linoleic acid was the predominating fatty acid at 59.1% followed by linolenic acid at 22.1%. The

oil also demonstrated an ORAC value of 56.7 TE μmol/g. In 2002, Wada and Ou (*43*) examined boysenberry fresh fruit for its antioxidant capacity, TPC, and TAC. The fruit was determined to have an ORAC value of 42 TE μmol/g, which was higher than that of red raspberry on a dry weight basis. It also had a TPC of 6.0 GAE mg/g and a TAC of 1.3 mg/100 g.

Marionberry Seed

Marionberry (*Rubus ursinus*) is also a member of the caneberry fruits, and in 2004, 12,860 MT were grown in the United States with 98.9% of the total processed. Marionberry seed oil has been analyzed for fatty acid profile and antioxidant activity (*42*). Similar to the other tested caneberry seed oils, the oil from marionberry contained a substantial level of unsaturated fatty acids including linoleic acid, linolenic acid and oleic acid at concentrations of 62.7, 15.2, and 15.1 g/100 g oil, respectively. The oil also had an ORAC value of 119.7 TE μmol/g, which was the highest among 5 tested caneberry species (*42*).

Marionberry fruit has previously been examined for antioxidant activity and phenolic contents (*43, 47*). In 2002, Wada and Ou (*43*) tested whole dry marionberry fruit for ORAC, TPC, and TAC. The dried fruit demonstrated an ORAC value of 215 TE μmol/g, a TPC of 44.8 GAE mg/g, and a TAC of 1192 mg/100 g (*43*). TPC and ascorbic acid contents of freeze-dried and air-dried marionberry grown conventionally or organically have also been evaluated (*47*). The TPC for freeze dried samples were approximately 380-590 mg GAE/100 g, and TPC for air dried samples were 350-490 mg GAE/100 g calculated as fresh weight for conventionally and

organically grown marionberries, respectively. Ascorbic acid was not detected in marionberries (*47*).

<u>Cranberry Seed</u>

Cranberry (*Vaccinium macrocarpon*) is grown in the United States at higher latitudes. In 2004, 400,000 MT were grown worldwide and 300,000 MT were grown in the United States. Cranberry seeds are mainly a byproduct of juice production, and cranberry seed oil is commercially available (*1, 48, 49*). Previous investigations on cranberry seeds have primarily examined the oil.

One previous study found that cranberry seed oil contained 35-40% linoleic acid and 30-35% α-linolenic acid, along with 20-25% oleic acid, a trace amount of arachidonic acid (20:4n-6), and possibly EPA (*48*). Another study reported 44.3% linoleic acid and 22.3% α-linolenic acid, along with 22.7% oleic acid; however, arachidonic acid and EPA were not detected in the cold-pressed cranberry seed oil (*1*). Significant levels of phytochemicals including β-sitosterol (1.3 g/kg oil), α-tocopherol (341 mg/kg oil) and γ-tocopherol at 110 mg/kg oil were detected (*48*), and significant antioxidant activities were also seen in cold-pressed cranberry seed oil extract (*50*). Cold-pressed cranberry seed oil extract directly reacted with and quenched DPPH and ABTS$^+$ radicals, and had a TPC of 1.6 mg GAE/g oil (*50*). Cranberry seed oil components have also shown potential to reduce the oxidation of human LDL that may help reduce the risk of heart disease (*50*). Additionally, cold-pressed cranberry seed oil demonstrated similar oxidative stability to commercial soybean and corn oils (*1*). In a recent study, Carmire et al. (*39*) examined whole dehydrated fruit powder from 4 fruits including cranberry, blueberry, Concord grape,

and raspberry for anthocyanin content. The cranberry powder had an anthocyanin content of 327.1 mg/100 g powder, which was the second highest amount behind the blueberry. In 2006, fresh cranberry was examined for total phenolic content, total anthocyanins, and effects on human MCF-7 breast cancer including cell proliferation, apoptosis, and cell cycle analysis (*51*). The fresh cranberry fruit was extracted with 80 % acetone, evaporated and brought back into 100% H_2O for analyses. The total phenolic content was determined to be 5.7 GAE mg/g fresh weight and total anthocyanins were determined to be 90 mg/100 g. MCF-7 proliferation was significantly reduced in a dose dependent manner from 5 to 30 mg/mL cranberry extracts. Apoptosis was observed to be 25 % higher than control at 50 mg/mL, and cell cycle was arrested in a dose dependently in the G_1 and G_2/M phases.

Cranberry seed may be an excellent dietary source of α-linolenic and linoleic acid, may be used to improve the dietary ratio of n-6/n-3 fatty acids, may provide a significant level of natural antioxidants including phenolic compounds and tocopherols, and may contain antiproliferative compounds

Blueberry Seed

Like cranberries, blueberries (*Vaccinium corymbosum*) also grow in the northern region of the United States, and in 2004, the United States produced 240,000 MT of blueberries. Blueberry seed oil data has not been published, but the fruit has shown powerful antioxidant activity and is rich in phenolic compounds, particularly anthocyanins (*52*). A recent study on blueberry powder has shown to contain anthocyanins at 465 mg/100 g powder (39) and was the highest compared to other fruit powders including cranberry, raspberry, and Concord grape. Antioxidant status

following consumption of freeze dried blueberry powder was examined in 8 middle-aged male subjects using serum ORAC and total antioxidant status (TAS) (*53*). The antioxidant status determined by both methods was significantly higher in subjects consuming blueberries after 1 and 4 hours of ingestion. Blueberries, rich in anthocyanins, may reduce the risk of many degenerative diseases due to their potent antioxidant capacity.

Blueberries have recently been investigated for antiproliferation effects against cancer cell lines *in vitro*. The antiproliferative effect on HepG2 liver cancer cells has recently been investigated (*54*). Blueberry was initially extracted using MeOH:acetone:H_2O:formic acid (40:40:20:0.1) and was further fractionated to flavonols, tannins, and anthocyanins. The blueberry anthocyanin extract was the most effective treatment inhibiting cell growth by 50% at 70-150 μg/ mL. Apoptosis was also significantly increased which may partially explain the growth inhibition. In another study by Schmidt et al, 2006 (*55*) blueberry extracts were determined to be more effective against the proliferation of androgen sensitive (LNCaP) human prostate cancer cells compared to the androgen insensitive (DU145) human prostate cancer cells by 58% compared to control. Commercially prepared edible cold-pressed blueberry seed oil and flour is now available.

<u>Grape Seed</u>

Grapes (*Vitis spp*.) are grown worldwide. In 2004, 66,000,000 MT were grown throughout the world, and 6,500,000 MT were harvested in the United States where approximately half were processed into wine. Grape seeds are byproducts from the manufacturing of grape products that include wine, juice, jam, and jelly.

Grape seeds have been examined for chemical composition and properties. Egyptian grown cabarina red grape seed oil has been studied (*56*). The oil was extracted from ground grape seed meal using hexane at room temperature. Linoleic acid was the predominant fatty acid comprising 62.1 g/100 g oil, and oleic acid was the next highest at 17.1 g/100 g oil (*56*). Significant levels of palmitic acid and stearic acid were also found. The fatty acid contents were consistent with previous and current observations of 41 grape varieties (*57-59*). Iodine value (IV), and peroxide value (PV) were also determined for grape seed oil (*56*). The IV was 130 g I/100 g oil, and the PV was 2.92 mequiv O_2/kg oil. Refractive index, specific gravity, saponification value, unsaponifiable matter %, and acid value were other characteristics examined (*56*).

Antioxidant activity and phenolic compounds in grape seeds have recently been studied. Two varieties of ground grape seeds including 'Black Beauty' and 'Sunbelt' extracted using MeOH:H_2O:formic acid (60:37:3) were examined for ORAC, TPC, and TAC (*60*). The ORAC values for the grape seed extracts were 1100 and 700 TE μmol/g, TPC were 51.3 and 95.3 GAE mg/g; the TAC were 65 and 232 mg/100 g for Black Beauty and Sunbelt varieties, respectively. Another study investigating dried Concord grape powder found it to contain TAC at 234.3 mg/100 g powder (*39*), and solid grape pomace demonstrated a TAC of 174-200 mg/ 100 g pomace (*61*).

Grape has recently been evaluated for antiproliferation against cancer cells *in vitro* (*54*). Fractions from muscadine grape extract were tested for antiproliferative effects on HepG2 liver cancer cells. The anthocyanin fraction of the grape was the

most effective at inhibiting cell growth by 50% at doses form 100-300 µg/mL

suggesting that grape may contribute to reducing the risk of liver cancer.

Onion Seed

In 2005, 57,400,277 MT of onions (*Allium cepa* L.) were harvested worldwide

with 3,353,136 MT of the total grown in the United States. One study of the nutrient

composition of onion seed examined total fat and fatty acid composition (*62*). The

seed was determined to contain approximately 23% crude fat which was comprised of

44.6% linoleic acid, 34.3% oleic acid, 9.1% palmitic acid, and 4.4% stearic acid.

Other fatty acids including α-linolenic acid and myristic acid were also detected at

concentrations below 1%. The total unsaturated fatty acids in the onion seed oil were

79.2% of the total.

Pumpkin Seed

In 2005, 19,816,731 MT of pumpkins, including gourds and squash

(*Curcubita spp.*), were grown worldwide, and 464,500 MT were produced in the

United States. Several studies have reported a wide range of fatty acid compositions

in pumpkin seeds, and the predominating fatty acid reported in the seeds has been

linoleic acid, however, it has not been the most prevalent in all studies. The range of

linoleic acid in pumpkin seed oil has varied from 4.9-58.9 % of total fatty acids (*63-
68*). In a study of pumpkin seed oil in by Spangenberg and Ogrinc (*67*), the

predominating fatty acid was palmitic acid at 49.2 %, and Glew et al, 2006 (*63*) found

oleic acid highest at 45.4% of the total fatty acids. Other phytochemical and

properties have also been detected in pumpkin seeds. Alpha tocopherol was detected

at 3.0 mg/100g oil from pumpkin seeds grown at 3 different locations (*64*). The oil

and flour of roasted pumpkin seed were found to contain TPC at 0.39 and 0.91 GAE

mg/g, respectively. The refractive index of different pumpkin seed oil samples has

been determined at 3 temperatures. The refractive index at 25° C was 1.4616 (*70*)**,** at

40° C was 1.4706 (*71*), and at 60° C it was 1.4615 (*68*).

Parsley Seed

Parsley seed (*Petroselinum crispum*) has been investigated for chemical

compositions including fatty acid profile. The oil from parsley seed primarily

contained oleic acid at 81.9% which was almost 7 times higher than linoleic acid

which was the second most prevalent fatty acid at 12.5% (*72*). In 2006, Hinneburg et

al. (*73*) tested whole parsley herb for TPC and its ability to quench DPPH radicals

using (DPPH$^{\bullet}_{ED-50}$). The herb was determined to contain TPC of 29.2 GAE mg/g and

was able to quench 50% of DPPH radicals at 12 mg/mL under the experimental

conditions.

Mullein Seed

Common mullein (*Verbascum Thapsus* L.) has been used as a medicinal plant

to treat coughs, congestion, and other respiratory problems. It has also been used as a

diuretic and to treat inflammation (*74*). Mullein has been minimally investigated for

chemical compositions and properties. In a 1978 study, de Pascual et al (*75*)

examined mullein seed oil for its fat content and fatty acid profile. Results showed

that the seeds contained 30% oil, and the oil was primarily comprised of linoleic acid

(73.6%), followed by oleic acid (7.1%), with 18% unidentified. The refractive index

of the oil determined at 20° C was 1.4725, and the iodine value was 132.2. In a recent

study by Petrichenko and Razumovskaja in 2003 (*76*), the fatty acid profile of

common mullein was examined and the results were similar to the previous study.

The primary fatty acids were linoleic acid at 77.1%, oleic acid at 10.5%, and palmitic

acid at 6.8%.

Cardamom Seed

The seed of cardamom (*Elettaria cardamomum*) is used as the spice

ingredient for food and is very popular in Indian cuisine. Limited studies have been

performed on the chemical components and properties cardamom. In 1995,

Chandrashekar et al (*77*) investigated the fatty acid profile of the seed from two

cardamom varieties, and the results were very similar between the two with little

variation. Oleic acid was the predominating fatty acid at 43.1-44.1% which was

followed by linoleic acid at 21.4-22.1%, palmitic acid at 20.8-21.2%, and linolenic

acid at 7.8%. The TPC and antioxidant activity against DPPH$^\bullet$ of cardamom seed

meal extracted using ultrapure H_2O have also been examined (*73*). The TPC of the

cardamom seed water soluble extract was 24.2 GAE mg/g and quenched 50% of

DPPH radicals at approximately 7.9 mg/mL under experimental conditions.

Milk Thistle Seed

Milk thistle (*Silybum marianum*) has been primarily used as an herbal dietary

supplement for the treatment and protection of the liver and has recently been used

clinically in Europe and Asia. The bioactive components in milk thistle are

antioxidant polyphenolic compounds called silymarins, and the predominating

silimarin is silibinin. Several prior studies on silymarins, primarily silibinin, have demonstrated these milk thistle components have antiproliferative effects on cancer cells from several different tissues and work at the molecular level (*78-93*). Antiproliferative effects on cancer cell lines have been demonstrated in prostate (*78-83*), bladder (*84-86*), colon (*87, 88*), liver (*89*), lung (*90-92*), and breast (*93*) by several mechanisms including cell cycle arrest, decreases in CDK and cyclins, and increasing apoptosis. However, one recent study on mammary carcinogenesis in a rat and mouse model demonstrated increases mammary tumors from silymarin treatment compared to control (*94*).

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Chapter 2: Fatty Acid Composition and Antioxidant Properties of Cold-Pressed Marionberry, Boysenberry, Red Raspberry, and Blueberry Seed Oils

Parry et al., *J. Agric. Food Chem.* **2005,** *53*, 566-573.

Abstract

Cold-pressed fruit seed oils from marionberry, boysenberry, red raspberry, and blueberry were evaluated for their fatty acid composition, carotenoid content, tocopherol profile, total phenolic contents (TPC), oxygen radical absorbance capacity (ORAC), DPPH• scavenging capacity, oxidative stability index (OSI), peroxide value and antioxidant properties. All of the fruit seed oils contained significant levels of α-linolenic acid ranging from 19.6 to 32.4 g per 100 g oil in the marionberry and red raspberry seed oils, respectively, and all had a low ratio of n-6 to n-3 fatty acids (1.64-3.99). The total carotenoids ranged from 12.5 to 30.0 μmoles per kg oil, and zeaxanthin was the major carotenoid compound in all tested fruit seed oils, along with β-carotene, lutein, and cryptoxanthin. Total tocopherol content was 260.6-2276.9 μmoles per kg oil, including α-, γ-, and δ-tocopherols. OSI values were 20.1, 20.3, and 44.8 hours for the marionberry, red raspberry, and boysenberry seed oils, respectively. The highest TPC was observed in the red raspberry seed oil at 2.0 gallic acid equivalents/g oil, while the highest ORAC value was in boysenberry seed oil extract (77.9 μmoles trolox equivalents per g oil). All tested fruit seed oils directly reacted and quenched DPPH radicals in a dose and time dependent manner. These data suggest that the tested cold-pressed fruit seed oils have potential in improving human nutrition.

Introduction

Edible seed oils have been extracted and used as food ingredients since ancient times. Today, many crops, including safflower, linseed, cotton, peanut, sunflower, corn, and soybean are grown exclusively, or in large part, for the oil in their seeds. As evidence linking health benefits to the consumption of vegetable oils continues to grow, many consumers prefer to use vegetable oils instead of animal fats, and plant oils with additional health beneficial phytochemicals are in high demand. Several phytochemicals detectable in edible seed oils may include, but are not limited to, tocopherols, carotenoids, phenolic and polyphenolic compounds, and essential fatty acids such as α-linolenic acid (18:3n-3). Alpha-Linolenic acid is an essential ω-3/n-3 fatty acid that cannot be synthesized in the human body and must be obtained through the diet. Alpha-linolenic acid may be converted to longer chain n-3 fatty acids that include EPA (20:5n-3) and DHA (22:6n-3) *in vivo* through enzymatic elongation and desaturation reactions. Both EPA and DHA are reported to provide potential health benefits in the prevention of cancer, heart disease, hypertension, and autoimmune disorders (*1-6*). Some recent studies have indicated that reducing the dietary ratio of n-6 to n-3 fatty acids might decrease the risk of heart disease and cancer (*4, 7*). The current dietary ratio of n-6 to n-3 fatty acids is approximately 10/1, and the recommended ratio has been estimated to be 4/1 (*8, 9*). As the demand for n-3 fatty acids increases, novel dietary sources of oils rich in n-3 fatty acids are in high demand for improving human nutrition.

Aside from essential fatty acids, edible seed oils may contain significant levels of other beneficial phytochemicals including tocopherols and carotenoids. In 2002,

Oomah et al reported a total vitamin E equivalent of 97 mg per 100 g raspberry seed oil that included α-, γ-, and δ-tocopherols at concentrations of 71, 272, and 17.4 mg/100 g oil, respectively (*10*). Significant levels of tocopherols have also observed in blackcurrant, and goldenberry seed oils (*11, 12*). Tocopherols and phenolic compounds are natural antioxidants that may reduce radical mediated cellular damage (*11, 13, 14*), and they may provide health benefits to consumers in addition to their common nutritional value and functional properties in food products.

Fruit seeds are a major byproduct from the manufacture of fruit juice. Recent studies have documented encouraging findings of beneficial components and physicochemical properties of fruit seed oils (*10-13, 15*). For example, cold-pressed black raspberry seed oil contained 35% α-linolenic acid and demonstrated significant antioxidant activities (*13*). Cranberry seed oil was a rich source of essential fatty acids, containing between 35-44 % linoleic acid (18:2n-6) and 23-35% α-linolenic acid (*15, 16*), and had significant levels of β-sitosterol, and α- and γ- tocopherols (*16*). Cranberry seed oil extract has also displayed significant radical scavenging activities against DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, protected protein from oxygen radical attack, and suppressed lipid oxidation in human LDL (*17*). These results suggest that fruit seed oils might serve as valuable edible oils for developing functional foods having desired health benefits. Further investigation on chemical compositions and properties of fruit seed oils is required to evaluate the potential of fruit seeds as quality sources of oil for food applications. The Cold-pressing procedure is a seed oil extraction process that does not involve chemicals or heat prior to or during the extraction (*15*);

therefore, the oil may retain more phytochemicals including natural antioxidants and are preferred by consumers.

Other important properties of edible oils include stability, which can be measured by oxidative stability index (OSI) and peroxide value (PV). The OSI procedure determines the ability of an oil to resist accelerated oxidation at high temperatures and increased airflow (*15*). The PV is the concentration of lipid peroxides present in the oil samples. Both OSI and PV are indicators of overall shelf life of edible oils.

The present study was conducted to evaluate selected commercial cold-pressed boysenberry (*Rubus* hybrid), marionberry (*Rubus* hybrid), red raspberry (*Rubus ideaus*), and blueberry (*Vaccinium corymbosum*) seed oils for their fatty acid composition, tocopherols, carotenoids, total phenolic content, antioxidant activities, oxidative stabilities, and physical properties. The information obtained from this study can be used to evaluate the potential use of these fruit seed oils as food products for improving human health and nutrition

Materials and Methods

<u>Materials</u>

Cold-pressed boysenberry, marionberry, red raspberry, and blueberry seed oils were donated by the Badger Oil Company (Spooner WI USA) and extracted upon arrival. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH·), gallic acid, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), were purchased from Sigma-Aldrich (St. Louis, MO); β-cyclodextrin (RMCD) was purchased from Cyclolab R & D Ltd. (Budapest, Hungary), and 2,2'-azobis (2-amino-propane) dihydrochloride

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(AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Wesson corn oil and Richfood soybean oil were purchased from a local grocery store. All other chemicals and solvents were of the highest commercial grade and used without further purification.

Extraction and Sample Preparation

Oils were extracted with 80% MeOH and 100% MeOH and evaluated for DPPH[•] scavenging activity, oxygen radical absorbance capacity (ORAC), and total phenolic contents (TPC). One gram of oil was extracted 3 times by 3 mL of solvent by vortexing for 5 min and centrifugation at 6000 rpm for 5 min. The supernatants were collected and combined then brought to 10 mL final volume of extraction solvent. Oil extractions were kept in the dark under N_2 until analyzed.

Fatty Acid Composition

Fatty acid methyl esters (FAME) were prepared using the previously described procedure (*18*). The FAME samples were analyzed by GC-FID and GC-MS for fatty acid compositions. GC-FID analysis was conducted using a Hewlett Packard 5890 Series II gas chromatograph equipped with a FID and an HP 7673A automatic injector (Agilent Technologies, Palo Alto, CA). The column was a fused silica CP 88 column (100 m × 0.25 mm i.d. with a 0.20 μm film thickness, Varian, Inc., Walnut Creek, CA), and hydrogen was the carrier gas at a flow rate of 1.0 mL/min. Oven temperature was maintained at 75ºC for 2 min, then 5 ºC/min to 175 ºC and held for 33 min, and 5 ºC/min to 225 ºC and held for 15 min. Measurements were taken in triplicate. GC-MS analyses were conducted using an Agilent 6890 Network GC

system equipped with Agilent 5973 Mass Selective Detector and an Agilent 7683 automatic injector (Agilent Technologies, Palo Alto, CA). The column was a fused silica CP 88 column (50 m × 0.25 mm i.d. with a 0.20 μm film thickness, Varian, Inc., Walnut Creek, CA). Helium was the carrier gas at a flow rate of 1.2 mL/min. Initial oven temperature was 75ºC and increased to 225ºC over 80 min. One measurement was taken from each oil sample for determination.

Carotenoid Profile

One mL of cold-pressed fruit seed oil was dissolved in 160 mL of methanol/tetrahydrofuran (1:1, v/v) and analyzed for carotenoid composition using a HPLC-DAD-ESI-MSMS (high performance liquid chromatography-diode-array-detector-electrospray ionization tandem mass spectrometry) method (*19, 20*). A TSQ Quantum tandem mass spectrometry (Thermo-Finnigan, San Jose, CA, USA) equipped with an ESI interface and Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). Analyses were conducted according to a previously described protocol with slight modification (*20*). HPLC separation was accomplished using a Zorbax SB C18 column (Agilent Technologies, Palo Alto, CA, USA), 50mm × 1.0 mm i.d. with a particle size of 3.5 μm, at room temperature. The carotenoids were eluted using a mobile phase of water (solvent A) and methanol:acetonitrile:iso-propanol (54:44:2) (solvent B). The gradient procedure was: 1) linear from 50% to 99% of solvent B with the flow rate increasing from 0.2 to 0.27 mL/min in the first 10 min, and 2) 99% of solvent B with the flow rate of 0.27 mL/min for 10 min. The column was re-equilibrated with 50 % solvent B for 10 minutes prior to the following injection. Detection was set at 440 nm. The TSQ

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Quantum was operated in the positive-ion mode under the following conditions: nitrogen (> 99.7%) was used for the sheath gas at 30 psi, and for auxiliary gas at 5 units. The temperature of the heated capillary was 300° C, and the spray voltage of ESI was set at 4.5 kV. Collision induced dissociation (CID) was achieved using argon as the collision gas at greater than 1.0 mTorr above the normal, and the applied collision offset energy was – 45 eV. Identification of carotenoids was accomplished by comparing the HPLC retention time and selected reactant monitoring (SRM) analysis of the sample peaks with the pure carotenoid compounds (*21*). The m/z was set for lutein and zeaxanthin from 568.6 (molecular ion) to 157.3 (major fragment), and m/z 552.6 to 145.3 and 536.6 to 119.3 for cryptoxanthin and β-carotene, respectively. Data was obtained with Xcalibur software system (Thermo-Finnigan, San Jose, CA, USA). The quantification of carotenoid compounds was determined by comparing the total ion counts to external standards of the individual compounds. All measurements were conducted in triplicate.

Tocopherol Composition

The fruit seed oils in methanol/tetrahydrofuran solutions prepared for carotenoid analyses were used to evaluate the α-, δ-, and γ-tocopherol compositions in the oils. Separation was performed by HPLC using a Zorbax SB C18 column (Agilent Technologies, Palo Alto, CA, USA), 30mm × 1.0 mm i.d. with 3.5 μm particle size at ambient temperature. The tocopherols were eluted with a gradient mobile phase of water (solvent A) and acetonitrile (solvent B). The procedure was performed as follows: 1) the gradient was linear from 80 to 99% of solvent B at 0.3 mL/min, and 2) 99% of solvent B was kept for 10 min. The column was re-

equilibrated for 10 minutes with 50% of solvent B before subsequent injections.

Tocopherols were identified by comparing the HPLC retention time and selected

reactant monitoring SRM analysis of the sample peaks with pure tocopherol

compounds. The m/z for α-tocopherol was set from 430.6 (molecular ion) to 165.3

(major fragment), and m/z 416.6 to 151.3 and 402.6 to 137.3 were set for γ-

tocopherol and δ-tocopherol, respectively. Quantification of tocopherols was

accomplished using total ion counts with external standards of the individual

tocopherol compounds, and triplicate measurements were analyzed.

Total Phenolic Contents (TPC)

The TPC of the cold-pressed fruit seed oils was determined using freshly

prepared Folin-Ciocalteu reagent (22). Briefly, the reaction mixture contained 50 μL

of oil extract, 250 μL of the Folin-Ciocalteu reagent and 0.75 mL of 20 % sodium

carbonate and 3 mL of pure water. Reactions were carried out for 2 h at ambient

temperature, and the absorbance was measured at 765 nm and used to calculate the

TPC of the oils using gallic acid as a standard. The Folin-Ciocalteu reagent was

prepared by refluxing of sodium molybdate, sodium tungstate, 85 % phosphoric acid,

and concentrated hydrochloric acid for 10 hours. This was followed by the addition of

lithium sulfate, and oxidation with a few drops of bromine (23). The solution was

filtered and used for the assay. Triplicate measurements were taken.

Oxidative Stability Index (OSI)

OSI was determined using a Rancimat instrument (Model 743; Metrohm Ltd.,

Herisau, Switzerland). Oil samples were exposed to accelerated oxidation conditions

using the following parameters. Four mL of oil was placed in a reaction vessel, temperature was set at 80 °C, and the airflow rate was 7 L/ h (*15, 22*). The OSI was measured as the hours for an oil sample to develop measurable rancidity by induction point determination. The OSI of the fruit seed oils were compared to commercial corn and soybean oils. OSI measurements were taken in triplicate.

Peroxide Value (PV)

Cold-pressed fruit seed oils were examined for PV using the FOX version II assay (*24*). The FOX reagent was prepared by dissolving 880 mg BHT, 98 mg ammonium sulfate, 76 mg of xylenol orange, and 100 mL of 250 mM H_2SO_4 in 900 mL HPLC grade methanol. The FOX reagent was used within 24 hours of preparation. The reaction was initiated by mixing 950 μL FOX reagent with 50 μL of blank, oil solution or peroxide standard solution. Absorbance was determined at 560 nm after 10 min of reaction at ambient temperature. The peroxide value PV was calculated using a standard curve prepared using t-butyl peroxide. Triplicate measurements were conducted.

Refractive Index and Density

The refractive index values of the cold-pressed boysenberry, marionberry, red raspberry, and blueberry seed oils were determined at 24 °C using the *AOCS Official and Tentative Methods* procedure Cc 7-25 (*25*) using an ABBE Refractometer (American Optical Corporation, Buffalo, NY). Specific density was determined at 24 °C against 4 °C pure water using the *AOCS Official and Tentative Methods* procedure To 1b-64 (*26*).

Oxygen Radical Absorbance Capacity (ORAC)

ORAC values were determined according to a protocol described by Huang et al. (*27, 28*). Fluorescein (FL) was used as the fluorescent probe. The final assay mixture contained 0.067 µM of FL, 60 mM of AAPH, 300 µL of oil extract or 50% acetone as reagent blank. Fluorescence was measured at 490-515 nm excitation-emission wavelengths and were determined and recorded every minute. Trolox was used as the standard. All measurements were conducted in triplicate.

DPPH Scavenging Activity

DPPH scavenging activity of the cold-pressed fruit seed oil extracts were determined according to the previously reported procedure (*22*). One mL of fresh 0.2 mM DPPH$^\bullet$ solution was added to 1 mL fruit seed oil extract at concentrations of 0, 6.7, 8.0, 10, 20, or 40 mg oil equivalents/ mL to start the radical-antioxidant reaction. Absorbance was measured at 517 nm against a blank of pure methanol at 0.5, 1, 3, 6, 10, 20, 50, 80, and 1440 min of the reaction, and was used to estimate the remaining radical percentages compared to control. After 10 minutes of reaction, absorbance readings were used to compare the DPPH$^\bullet$ scavenging capacities of individual oil extracts. Triplicate measurements were taken. Dose and time dependencies of fruit seed oil extract and DPPH$^\bullet$ reactions were demonstrated by plotting the percent of DPPH$^\bullet$ remaining against time for each concentration of the seed oil extract tested.

Statistical Analysis

Data were reported as mean ± standard deviation for triplicate determinations. ANOVA and least significant difference tests (SPSS for Windows, Version Rel.

10.0.5., 1999, SPSS Inc., Chicago, IL) were used to identify differences among means. A Pearson Correlation test was used to determine the correlations among means. Statistical significance was declared at $P < 0.05$.

Results

Fatty acid Profile

The primary fatty acids detected in the red raspberry, marionberry, boysenberry, and blueberry seed oils were palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2n-6), and α-linolenic (18:3n-3) acids (**Table 2.1**). All of the seed oils contained relatively high levels of total unsaturated fatty acids that ranged from 91.3 to 97.8% in the boysenberry and red raspberry seed oils, respectively. Linoleic acid was the most prevalent fatty acid in all seed oils and was highest in marionberry seed oil at 62.8 g per 100 g oil. All the fruit seed oils also contained significant concentrations of α-linolenic acid, and had low ratios of n-6 to n-3 fatty acids from 1.6:1 to 4:1 (**Table 2.1**). The red raspberry sample exhibited the lowest ratio of n-6/n-3 fatty acids, and the marionberry seed oil demonstrated the highest ratio among the four tested cold-pressed seed oils.

Table 2.1. Fatty acid (FA) composition of the tested fruit seed oils (g/100 g oil)*

FA	Red raspberry	Marionberry	Boysenberry	Blueberry
16:0	1.3 ± 1.8	3.3 ± 0.1	4.2 ± 0.3	5.7 ± 0.4
18:0	1.0 ± 1.4	3.1 ± 0.2	4.5 ± 0.4	2.8 ± 0.1
18:1	12.4 ± 0.6	15.1 ± 0.1	18.0 ± 0.3	22.9 ± 0.1
18:2n-6	53.0 ± 1.9	62.8 ± 0.1	53.8 ± 0.3	43.5 ± 0.1
18:3n-3	32.4 ± 0.7	15.8 ± 0.1	19.5 ± 0.1	25.1 ± 0.3
Sat[1]	2.3 ± 0.0	6.4 ± 0.0	8.7 ± 0.2	8.6 ± 0.3
PUFA[2]	85.5 ± 2.6	78.6 ± 0.0	73.3 ± 0.3	68.6 ± 0.3
n-6/n-3[3]	1.6	4.0	2.8	1.7

*Fatty acid composition was reported as mean $\pm$ SD (n = 3). [1]Sat represents total saturated fatty acids (g/100 g oil).[2] PUFA represents total polyunsaturated fatty acids (g/100 g oil).[3] n-6/n-3 represents the ratio of n-6 to n-3 fatty acids in the oil.

Carotenoid Composition

The investigated carotenoids in the fruit seed oils were β-carotene, cryptoxanthin, lutein, and zeaxanthin. Zeaxanthin is the pigment that gives corn its characteristic yellow color and was the predominating carotenoid in all tested seed oils. Zeaxanthin comprised a combined average over 75% of the total carotenoids (**Table 2.2**). Boysenberry seed oil had the highest concentration of total carotenoids and was significantly higher than all other seed oils in β-carotene, zeaxanthin, and lutein (**Table 2.2**). The red raspberry seed oil contained the highest concentration of cryptoxanthin among all tested berry seed oils.

Table 2.2. Carotenoid contents in the cold-pressed fruit seed oils*

	β-Carotene (µg/kg oil)	Lutein (µg/kg oil)	Zeaxanthin (mg/kg oil)	Cryptoxanthin (mg/kg oil)	Total Carotenoids (µmoles/kg oil)
Blueberry	1352.3b ± 4.4	60.6c ± 0.0	7.8c ± 0.06	1.5b ± 0.00	19.0
Red raspberry	82.2d ± 1.8	78.6b ± 0.5	5.1d ± 0.03	1.8a ± 0.01	12.5
Marionberry	442.7c ± 8.7	53.3d ± 0.1	11.9b ± 0.08	0.9c ± 0.01	23.4
Boysenberry	2405.2a ± 3.3	97.7a ± 1.0	13.6a ± 0.03	0.7d ± 0.03	30.0

*Carotenoid contents were reported in mean ± SD (n = 3). Different letters within each column represent significance difference ($P < 0.05$).

Table 2.3. Tocopherol contents in the cold-pressed fruit seed oils*

	α-Tocopherol (mg/kg oil)	γ –Tocopherol (mg/kg oil)	δ-Tocopherol (mg/kg oil)	Total Tocopherols (µmol/kg oil)
Blueberry	71.1b ± 1.1	33.6d ± 0.6	6.0d ± 0.0	260.6
Red Raspberry	150.9a ± 1.6	558.7b ± 7.4	178.9b ± 0.4	2135.4
Marionberry	28.4c ± 0.2	328.3c ± 2.2	50.0c ± 1.8	978.0
Boysenberry	20.8d ± 0.1	688.6a ± 5.9	232.0a ± 2.1	2276.9

*Tocopherol contents were reported as mean ± SD (n = 3). Different letters within each column represent significance difference ($P < 0.05$).

<u>Tocopherol Profile</u>

The blueberry, red raspberry, marionberry, and boysenberry seed oils all differed in their α-, γ-, δ-, and total tocopherols. The red raspberry seed oil demonstrated the highest concentration of α-tocopherol at 150.9 mg/kg oil, and was significantly higher than all other tested fruit seed oils ($P < 0.0001$) and had more than twice the concentration found in the next highest seed oil (**Table 2.3**).The boysenberry seed oil had the highest total tocopherols but the lowest α-tocopherol concentration. Boysenberry seed oil contained significantly higher concentrations of both γ- and δ-tocopherols compared to all other fruit seed oils ($P < 0.001$) (**Table 2.3**).

<u>Total Phenolic Contents (TPC)</u>

TPC was determined for the fruit seed oils extracted by 80% and 100% MeOH. The TPC of the 100% MeOH extracts ranged from 1.27-2.00 mg gallic acid equivalents/g oil (GAE mg/g) and were significantly higher than the 80% MeOH extracts that ranged 0.09-1.00 GAE mg/g (**Figure 2.1**). Among all 100% MeOH extracts, the red raspberry seed oil had the highest TPC, and was followed by 100% MeOH extracts of boysenberry, blueberry, and marionberry seed oils. However, the cold-pressed blueberry seed oil had the highest TPC among the 80% MeOH extracts (**Figure 2.1**), indicating that these fruit seed oils might differ in their phenolic compound compositions aside from the TPC.
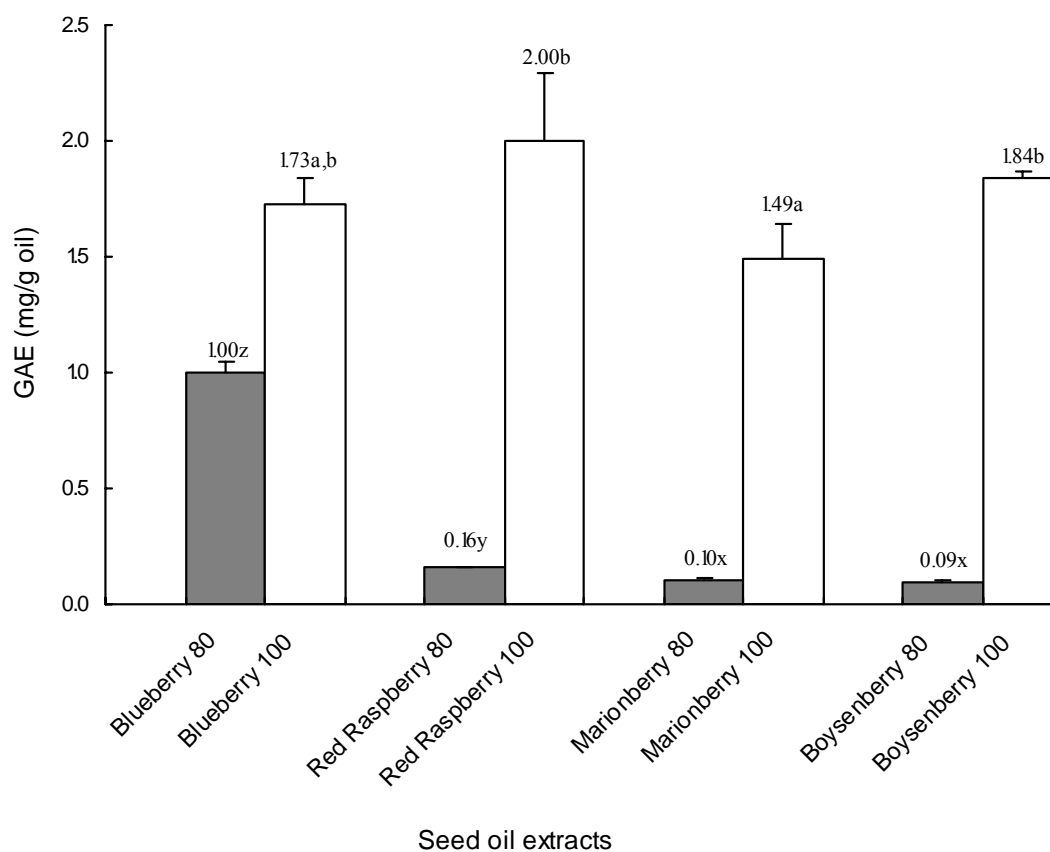
Figure 2.1. Total phenolic contents of the cold-pressed berry seed oils. Dark bars represent the 80% MeOH extraction and white bars represent the 100% MeOH extraction the cold-pressed fruit seed oils. GAE (mg/g oil) represents mg gallic acid equivalents per g. The vertical T-bars represent the standard deviation (n = 3) of each data point. Letters z, y, and x indicate significant differences among 80% MeOH extracts, and a, b, and c represent significant differences among 100% MeOH extracts ($P < 0.05$).

<u>Oxidative Stability Index (OSI)</u>

The OSI is a measurement of lipid peroxidation byproducts in oil or fat samples determined by induction time. Higher OSI values are indicative of better

oxidative stability or longer shelf-life. The OSI values of the fruit seed oils were measured and compared to commercial corn and soybean oils (**Table 2.4**). The cold-pressed boysenberry seed oil demonstrated the highest OSI value of 44.8 h, and was comparable but shorter than that the commercial corn and soybean oils that had OSI values 66 and 47 h, respectively. OSI values of the marionberry and red raspberry seed oil were 20.1 and 22.5 hours, respectively. OSI values and percent of α-linolenic acid were significantly correlated in the fruit seed oil samples ($r = -0.892$, $P = 0.017$).

Table 2.4. OSI, PV, RI, and density of the studied cold-pressed fruit seed oils*

	OSI (hours)	PV (meq O-OH/kg)	RI (24 °C)	Density (g/mL)
Blueberry	NA	41.4a ± 2.73	1.4783	NA
Red raspberry	20.3e ± 0.50	46.5a,b ± 1.59	1.4788	0.929
Marionberry	20.1e ± 0.35	85.2d ± 0.96	1.4774	0.934
Boysenberry	44.8c ± 0.83	41.3a ± 1.41	1.4758	0.948
Corn oil	66.0a ± 0.42	47.5a,b ± 0.55	NA	0.932
Soybean oil	46.8b ± 0.38	NA	NA	NA

*OSI: oxidative stability index. PV: peroxide value. RI: refractive index. Data were reported in mean ± SD (n = 3). PV was expressed as milli equivalents of t-butyl peroxide per kg oil (meq O-OH/kg). Different letters within columns represent significance difference ($P < 0.05$, n = 3). NA: not available.

Peroxide Value (PV)

The peroxide value (PV) is the total number of lipid peroxides present in an oil sample, and a higher PV is associated with a higher concentration of existing

peroxides. The tested cold-pressed fruit seed oils had a PV range of 41.3 – 85.2 milli equivalents of *t*-butyl peroxide per kg oil (mequiv O-OH/kg) (**Table 2.4**). The cold-pressed marionberry seed oil had the highest PV of 85.2 mequiv O-OH/kg, and the boysenberry oil sample had the lowest PV of 41.3 mequiv O-OH/ kg (**Table 2.4**). The commercial corn oil had a PV of 47.5 mequiv O-OH/kg, under the same experimental conditions.

Refractive Index and Density

The refractive index and density of the cold-pressed fruit seed oils are shown in **Table 2.4**.

Oxygen Radical Absorbance Capacity (ORAC)

ORAC values of the cold-pressed fruit seed oils are shown **Figure 2.2**. All ORAC values of the fruit seed oil samples extracted using 100% MeOH were higher than the ORAC values of the corresponding samples extracted using 80% MeOH. The 100 % MeOH extract of the boysenberry seed oil had an ORAC value of 77.9 μmol trolox equivalents per gram oil (TE μmol/g) which was significantly higher than all others samples from both extracting solvents. The 80% MeOH extract of the blueberry seed oil was significantly higher than all other samples extracted using 80% MeOH (**Figure 2.2**). These results suggests that the majority of the antioxidants present in the cold-pressed fruit seed oils were less polar compounds, and 100% MeOH is a preferred extracting solvent for ORAC measurements of fruit seed oils.
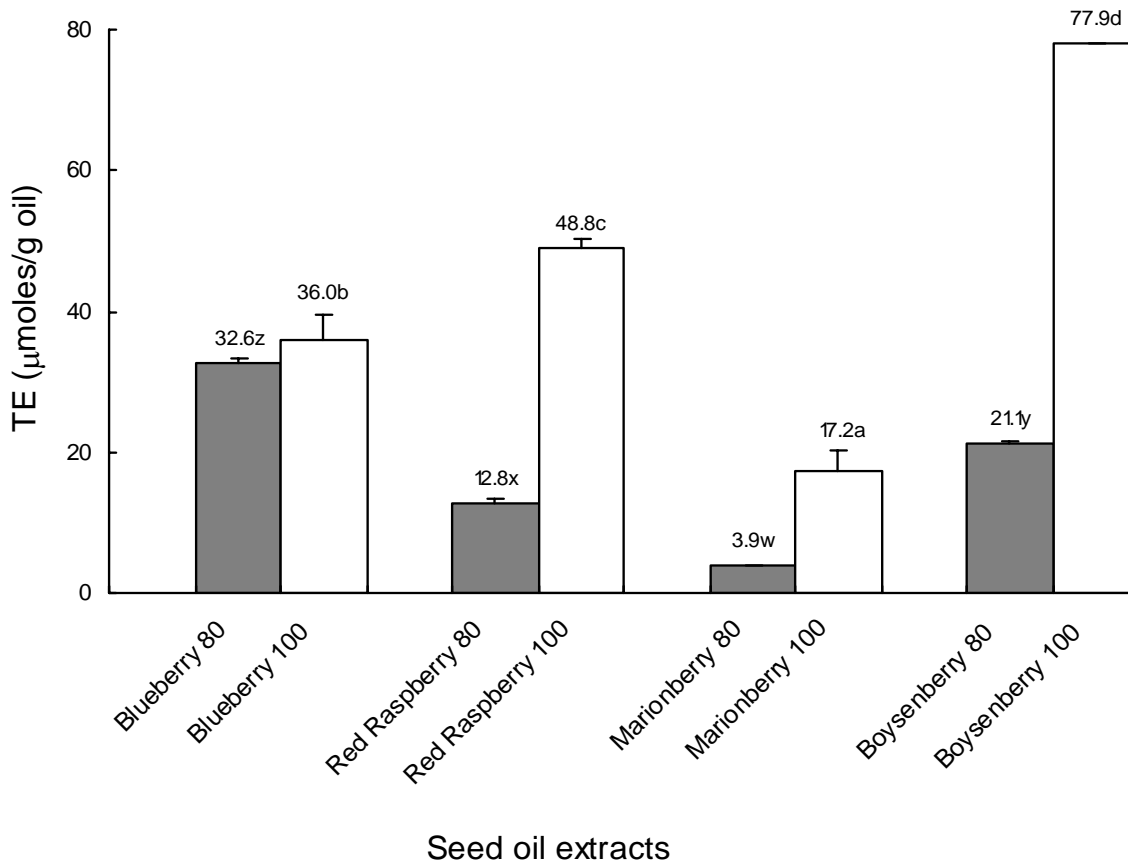
Figure 2.2. ORAC values of the cold-pressed fruit seed oils.

Dark bars represent 80% MeOH extracts, white bars represent the 100% MeOH extracts. TE stands for micromoles of trolox equivalent per g oil. The vertical T-bars represent the standard deviation (n = 3) of each data point. Letters z, y, x, and w indicate significant differences among 80% MeOH extracts, and a, b, c, and d represent significant differences among 100% MeOH extracts ($p < 0.05$).

DPPH Scavenging Activity

The DPPH$^{\bullet}$ scavenging activities of the fruit seed oils significantly differed (**Figure 2.3**). In agreement with ORAC measurements, the 100% MeOH extracts of the individual fruit seed oils exhibited greater DPPH$^{\bullet}$ scavenging activities than the

corresponding 80% MeOH seed oil extracts (**Figure 2.3**). The 100% MeOH extract of

the boysenberry seed oil had the greatest DPPH$^\bullet$ scavenging activity and was

followed by, red raspberry, blueberry, and marionberry seed oils. Additionally, the

DPPH$^\bullet$ scavenging activity of the cold-pressed fruit seed oils was found to be both

time and dose dependent. Time and dose effects of the 100% MeOH extract of the

blueberry seed oil is shown in **Figure 2.4A**, and time and dose effects of the 80%

MeOH extract of the raspberry seed oil is presented in **Figure 2.4B**.
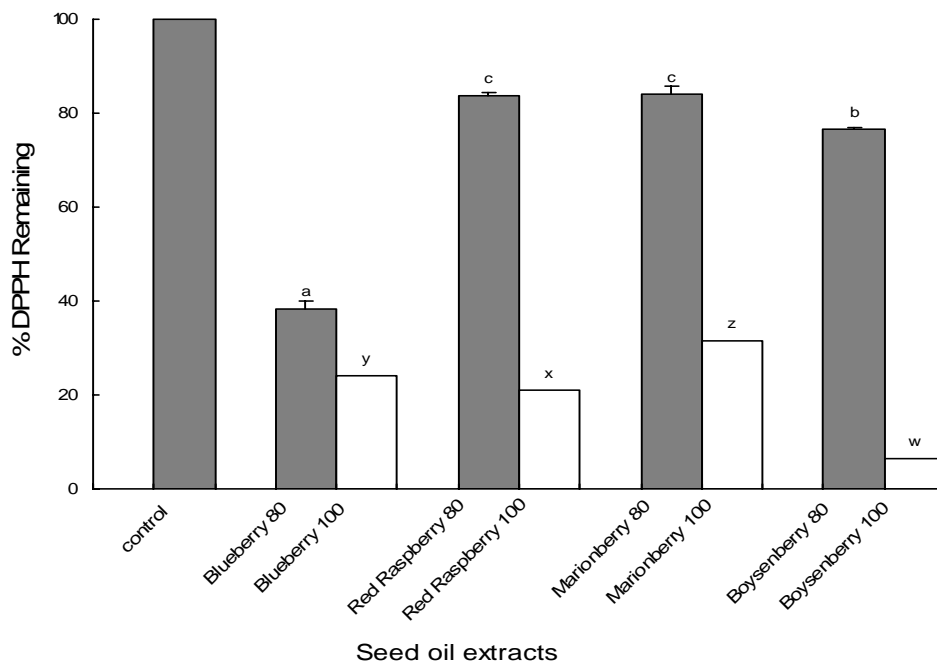
Figure 2.3. DPPH radical scavenging properties of the cold-pressed fruit seed oils. Dark bars represent 80% MeOH extracts and white bars represent 100% MeOH extracts. Initial DPPH$^\bullet$ concentration was 100 μM, and the seed oil extracts were 40 mg oil equivalent per mL. T-bars represent standard deviation (n = 3). Bars with different letters are significantly different $P < 0.05$.

A. Blueberry (100% MeOH)
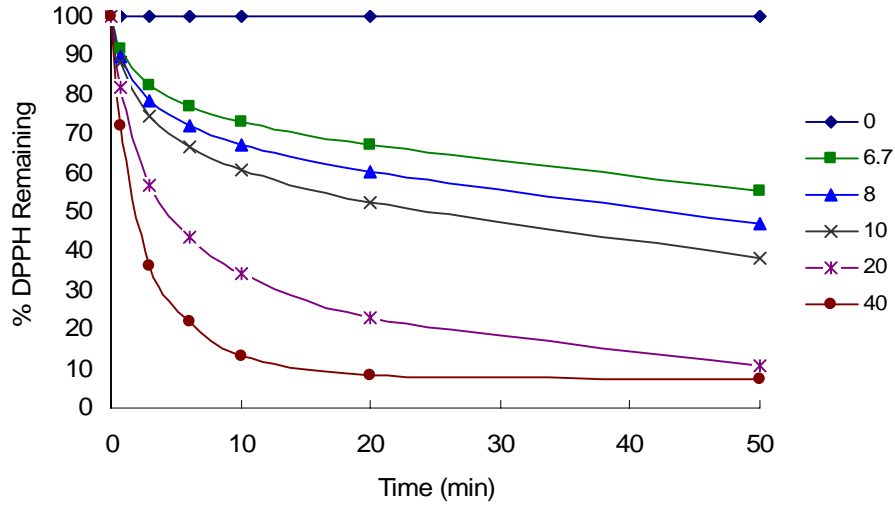
B. Raspberry (80% MeOH)
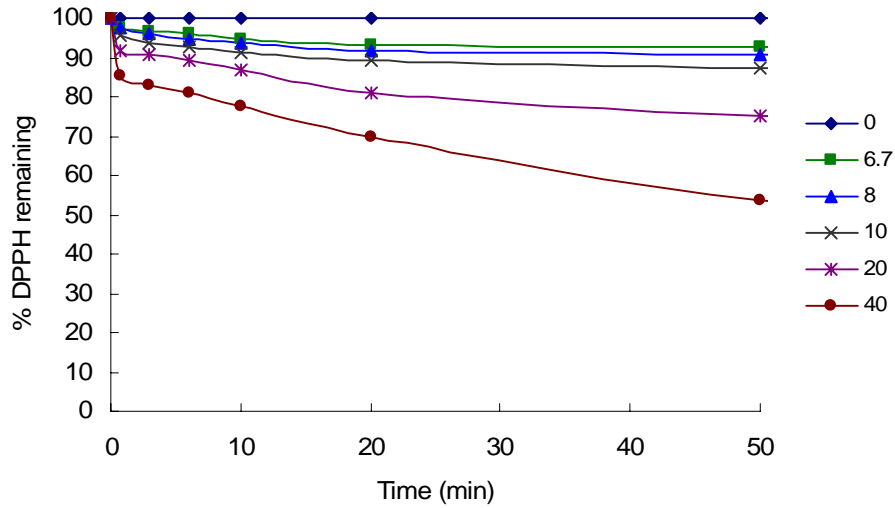
Figure 2.4. Dose and time effects of the oil antioxidants-DPPH• reactions. 4A) represents the dose-time effects of 100% MeOH extract of blueberry seed oil and DPPH• reactions, 4B) represents the dose-time effects of 80% MeOH extract of raspberry seed oil and DPPH• reactions. 0, 6.7, 8, 10, 20, and 40 represent the final concentrations in mg oil equivalents per mL reaction mixture. The initial DPPH• radical concentration was 100 µM in all reaction mixtures.

Discussion

Fruit seeds are a major byproduct from fruit processing and are typically discarded as waste. The generation of novel uses of seeds such as specialty edible fruit seed oils rich in bioactive food ingredients can add farm-gate value to the fruit and increase profits for production and processing industries, while improving human nutrition and health. The determination of the bioactive components is critical to promote the utilization and the production of these specialty fruit seed oils as nutraceuticals in functional food and cosmetic products. Edible seed oils may contain health promoting compounds including α-linolenic acid (18:3n-3), tocopherols, carotenoids, and other natural antioxidants, which have been linked to several beneficial effects.

The current study showed that cold-pressed boysenberry, marionberry, red raspberry, and blueberry seed oils contained significant levels of α-linolenic acid, an ω-3 (n-3) essential fatty acid. The α-linolenic content in the cold-pressed red raspberry seed oil was 32.4%, which is comparable to the α-linolenic content of 35.2-35.3% previously detected in cold-pressed black raspberry seed oil (*13*), and higher than the content of 22.3%, 29.1% and 19.3% observed in cold-pressed cranberry, raspberry (*Rubus idaeus* L.) and hemp seed oils, respectively (*10, 15*). This indicates that the cold-pressed red raspberry seed oil is an excellent dietary source for α-linolenic acid, and the cold-pressed blueberry, marionberry, and boysenberry seed oils may also significantly contribute to dietary α-linolenic acid containing 15.8-25.1% α-linolenic acid. Additionally, linoleic acid (18:2n-6), the ω-6 (n-6) essential fatty acid, was the predominating fatty acid in all of the tested seed oils, ranging from

43.5% in the blueberry seed oil to 62.8% in the marionberry seed oil. Linoleic and α-linolenic acid are essential fatty acids that cannot be synthesized by humans and must be obtained through the diet. This study demonstrated that the cold-pressed boysenberry, marionberry, red raspberry, and blueberry seed oils are rich dietary sources for essential fatty acids.

Recently, evidence has been mounting indicating that reducing the intake ratio of n-6 to n-3 fatty acids may be beneficial in preventing the development of several health problems including cancer, bone health, and cardiovascular disease (*4, 29 - 32*). In this study, the cold-pressed boysenberry, marionberry, red raspberry, and blueberry seed oils had very low ratios of n-6 to n-3 fatty acids and were similar to cranberry, hemp and black raspberry seed oils previously (*10, 13, 15*), suggesting the possible utilization of these fruit seed oils in reduction of the dietary intake of n-6 to n-3 fatty acids.

Concentrations of carotenoids and tocopherols were significant in the cold-pressed fruit seed oils, and all were significantly different. Boysenberry seed oil contained the highest total carotenoids (30 μmol/ kg oil) and β-carotene (2405 μg/kg oil or 2280 μg/L oil). This β-carotene content was much higher than that found in peanut (130 μg/L oil), soybean (280 μg/L oil), and corn (1200 μg/L oil) oils investigated by Cabrini and others (*33*). Cold-pressed boysenberry seed oil also had the highest total tocopherols at 2276.9 μmol/ kg oil, but had the lowest concentration of α-tocopherol. The primary tocopherol in the red raspberry seed oil was the γ isomer at approximately 63% of total tocopherols. This was in agreement with observation by Oomah et al. (*10*) that detected α-, γ-, and δ-tocopherols in both cold-

pressed and hexane extracted red raspberry seed oils, and γ -tocopherol was approximately 75 % of the total tocopherols. Interestingly, the present study detected the ratio of α- and δ-tocopherols at 0.83 to 1, which was very different from the previously reported ratios of 6.5 to 1 or 4.1 to 1 for cold-pressed and hexane extracted red raspberry seed oils, respectively (10). This difference may be reflective of the influence of growing conditions and variation among raspberry genotype on the phytochemical production. Alterations in phytochemical compositions have been noted among genotype, growing condition, and the interaction between genotype and environmental conditions (22). All of the cold-pressed fruit seed oils had lower concentrations of α-tocopherol compared to tested commercial extra virgin olive, peanut, corn, and sunflower seed oils that had a range of 174-578 mg/L, but the values were similar to that of 89 mg/L in the soybean oil (33).

Boysenberry seed oil extract exhibited the strongest scavenging activity against both DPPH and peroxyl radicals among the 100% MeOH fruit seed oil extracts, and was followed by that of red raspberry, blueberry, and marionberry, respectively. The blueberry seed oil extract had the highest radical scavenging capacity against both tested radicals among the 80% MeOH extracts, and the boysenberry extract had the lowest radical scavenging capacity. These differences may be due to individual solvent systems having different extraction efficiencies for different antioxidant compounds. The effects of extraction solvent on antioxidant activity have been observed and discussed in details in our previous study of wheat antioxidants (34).

In conclusion, the present study indicates that cold-pressed boysenberry, marionberry, red raspberry, and blueberry seed oils might serve as excellent dietary sources for α-linolenic acid, essential fatty acids, carotenoids, and tocopherols. This study also showed that the tested berry seed oils contain significant levels of natural antioxidants. The use of these fruit seed oils in food and cosmetic products may enhance the profitability of fruit production and processing industries.

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Chapter 3: Chemical Compositions, Antioxidant Capacities, and Antiproliferative Activities of Selected Fruit Seed Flours

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Abstract

Seed flours from cold-pressed black raspberry, red raspberry, blueberry, and cranberry, pinot noir grape, and chardonnay grape were examined for their total fat content, fatty acid composition, total phenolic content (TPC), total anthocyanin content (TAC), radical scavenging capacities against the peroxyl (ORAC) and stable DPPH radicals, chelating capacity against Fe^{2+}, and antiproliferative activities against the HT-29 human colon cancer cell line. Significant levels of fat were detected in the fruit seed flours and their fatty acid profiles may differ from their respective seed oils. Cranberry seed flour had the highest concentration of α-linolenic acid (30.9 g/100 g fat) and the lowest n-6 to n-3 fatty acid ratio (1.2/1). The ORAC value of the chardonnay seed flour was 1076.4 μmol trolox equivalents (TE)/g flour, and its TPC was 186.3 mg gallic acid equivalents (GAE)/g flour. These values were 3 to 12 times higher than the other tested fruit seed flours. Furthermore, ORAC values were significantly correlated to TPC under the experimental conditions ($P < 0.05$). These fruit seed flours also differed in their TAC values and Fe^{2+} chelating capacities. In addition, black raspberry, cranberry, and chardonnay grape seed flour extracts were evaluated for their antiproliferative effects on HT-29 colon cancer cells. All three tested seed flour extracts significantly inhibited HT-29 cell proliferation. The data from this study suggest that these fruit seed flours have the potential for developing

value-added uses as dietary sources of natural antioxidants and antiproliferative agents for benefiting human health.

Introduction

Growing scientific evidence suggests several food components may reduce the risk of several chronic diseases including heart disease, cancer, and autoimmune afflictions and improve general human health (*1-5*). Some of these components include but are not limited to ω-3 fatty acids, vitamins, carotenoids, dietary fibers, and natural antioxidants such as anthocyanins and other phenolic acids (*4-9*). Dietary antioxidants are believed to play a crucial role in human health by preventing important biological molecules such as DNA, proteins, and membrane lipids from oxidative damage (*10-11*). Novel food ingredients rich in natural antioxidants and other beneficial factors are now in high demand for improving food quality and optimizing human health.

Fruit seeds are byproducts of fruit processing, and seed flour is the major byproduct from seed oil production. Our recent study demonstrated that black raspberry seed flour obtained from a cold-pressing procedure contained a significant level of antioxidants (*8*). The black raspberry seed flour was extracted with 50% acetone at ambient temperature and with 100% ethanol using Soxhlet. Both extracts were able to directly react with and quench DPPH and $ABTS^+$ radicals, and they contained significant levels of phenolic compounds (*8*). Additionally, the black raspberry flour contained 5.3% oil by weight and α-linolenic acid (18:3n3) comprised approximately 33 g/100 g of the total fatty acids in the oil (*8*). Alpha-linolenic acid is the precursor for the longer chain n-3 polyunsaturated fatty acids, EPA (20:5n-3) and

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DHA (22:6n-3). EPA and DHA may reduce the risk of heart disease, cancer, hypertension, and autoimmune disorders (*12-17*). These data suggest the potential to develop novel uses of fruit seed flours as food ingredients rich in beneficial food factors for improving human diets, and enhance the profitability of fruit production and processing industries. Additional research is required to investigate fruit seed flours for their of health beneficial factors to promote their value-added utilization as beneficial food ingredients.

The present study investigated the seed flours of black raspberry (*Rubus occidentalis* L., cv Jewel), red raspberry (*Rubus ideaus*), blueberry (*Vaccinium corymbosum*), pinot noir grape (*Vitis vinifera*), and chardonnay grape (*Vitis vinifera*) and cranberry (*Vaccinium macrocarpon*) for their total phenolic and total anthocyanin contents, total fat and fatty acid profile, free radical scavenging activities, chelating capacity, and antiproliferative activity against HT-29 human colon cancer cells. The data obtained from this study will be used to promote the potential utilization of these edible fruit seed flours in food products for improving human health.

Materials and Methods

<u>Materials</u>

Flours from black raspberry, red raspberry, blueberry, cranberry, pinot noir grape and chardonnay grape seeds were provided by Badger Oil Company (Spooner WI USA). These fruit seed flours were the solid cakes from the cold-pressing process. Potassium chloride, sodium acetate, gallic acid, 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^\bullet$), ethylenediaminetetraacetate (EDTA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were purchased from Sigma-Aldrich

(St. Louis, MO); 2,2'-azobis (2-amino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA), and β-cyclodextrin (RMCD) was purchased from Cyclolab R & D Ltd. (Budapest, Hungary). Disposable cell culture ware was purchased from Corning Glass Works (Corning, NY). McCoy's 5A Medium Modified with L-glutamine, antibiotic/antimycotic, and fetal bovine serum (FBS), 0.25 % trypsin with 0.9 mM EDTA were purchased from Invitrogen (Carlsbad, CA). HT-29 human colon cancer cells were purchased from American Type Culture Collection (Rockville, MD). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Extraction and Sample Preparation

One g of seed flour was extracted with 10 mL of 50 % acetone at ambient temperature. The extracts were examined for total phenolic content (TPC), chelating capacity, oxygen radical absorbance capacity (ORAC), DPPH$^\bullet$ scavenging capacity, and cancer cell growth inhibition (*8*). Flours were extracted for estimating the total anthocyanin concentration (TAC) with acidic methanol containing 2% 12M HCl (v/v). For cell growth inhibition, the 50% acetone solvent was evaporated, and the solid residue was quantitatively re-dissolved in DMSO to obtain a final concentration of 1 g flour equivalents per 1 mL of DMSO. All extracts were kept in the dark under N_2 before analyses.

Total Phenolic Content (TPC)

Folicin-Ciocalteu (FC) reagent was used to determine the TPC of the fruit seed flour extracts following a laboratory procedure previously described (*18*). The

reaction mixture contained 250 μL of fresh FC reagent, 750 μL of 20% Na_2CO_3, 50 μL of the fruit seed flour extract or standard, and 3 mL of pure H_2O. Absorbance was determined at 765 nm following two hours of reaction at ambient temperature and used to calculate the TPC of the seed flours using gallic acid as the standard. The FC reagent was freshly prepared by refluxing a mixture of sodium tungstate, sodium molybdate, 85% phosphoric acid, and concentrated HCl for 10 hours. This was followed by a reaction with lithium sulfate, and oxidation using a few drops of bromine. The resulting solution was then filtered and used for the assay.

Total Anthocyanin Content (TAC)

TAC was determined by a pH differential method (*19-21*). Anthocyanins undergo a reversible structural conversion from pH 1 to pH 4.5, and consequently, absorb light at 510 nm at pH 1 but negligibly at pH 4.5. Degraded polymeric anthocyanins absorb light at pH 1 and pH 4.5, therefore, are not measured in this experiment. Absorbance was measured at 700 nm to correct for haze. Extracts were added to two buffer systems at the same dilution. The first buffer system was 0.025 M potassium chloride pH 1.0, and the second buffer system was 0.4 M sodium acetate pH 4.5. After adding the sample extract, both buffer systems were equilibrated for 15 minutes then absorptions were read at 510 and 700 nm. The calculated absorption was determined by the equation: $A = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}$ and the TAC in the testing solution was calculated as mg cyanidin-3-glucoside equivalents (CGE mg/100 g): $(A \times 449.2 \times \text{Dilution Factor} \times 1000)/(\varepsilon \times 1)$, with the $\varepsilon = 26{,}900$. The molecular weight of cyanidin-3-glucoside is 449.2g/mol.

<u>Fatty Acid Composition</u>

Oils were extracted from the flour samples using a Soxhlet apparatus, and hexane was the solvent. Fatty acid methyl esters (FAME) were prepared from the oil extracts according to the previously described method using HCl in MeOH following saponification (*22*). Fatty acid compositions were analyzed using a Shimadzu GC-2010 with a FID and a Shimadzu AOC-20i autosampler (Shimadzu, Columbia, MD). A Supelco 2380 column, 30 m × 0.25 mm i.d. with a 0.20 μm film thickness (Supelco Inc., Bellefonte, PA) was used to separate FAME. Helium was the carrier gas at a flow rate of 0.8 mL/min. Injection volume was 1 μL at a split ratio of 10/1. Time and temperature ramps began at 142 ºC increasing 6 ºC/min to 184 ºC, held for 3 min, and then increased 6 ºC/min to 244 ºC (*23*). Identification of the individual FAME was determined by comparing GC retention time with that of the authorized pure individual commercial compounds.

<u>Oxygen Radical Absorbing Capacity (ORAC)</u>

ORAC was determined using the previously described protocol (*24, 25*). Fluorescein was the fluorescent probe. The final assay mixture contained 60 mM AAPH, 0.067 μM of fluorescein, 300 μL of flour extract, standard, or 50% acetone for the reagent blank. Fluorescence was recorded every minute, and the area under the curve of fluorescence *vs.* time plot was calculated and compared against a standard curve prepared using trolox. ORAC values were expressed as μmol trolox equivalent (TE) per g of the fruit seed flour. Triplicate measurements were conducted.

DPPH$^\bullet$ Scavenging Activity

DPPH (2,2-diphenyl-1-picryhydrazyl) radical scavenging capacities of the cold-pressed seed flour extracts were analyzed using a previously described procedure (*26*). One mL of freshly prepared 200 μM DPPH$^\bullet$-50 % acetone solution was mixed with equal volume of seed flour extract to start the radical-antioxidant reaction. Absorbance was measured at 517 nm against a blank of 50% acetone and used to estimate the remaining radical levels according to a standard curve. The seed flour extracts were tested for their effective dose ($ED_{50\text{-DPPH}}$) concentrations at 20 minutes of reaction. The $ED_{50\text{-DPPH}}$ is the concentration of extract needed to quench 50% of the DPPH radicals under experimental conditions at a predetermined time. Time and dose effects of extracts and DPPH$^\bullet$ reactions were demonstrated by plotting the percent of DPPH$^\bullet$ remaining against time for each concentration of the seed flour extract tested.

Chelating Capacity

Chelating capacity against iron II was measured using the 2,2;-bipyridyl competition assay (*27*). The reaction assay contained 30 μL 1.8 mM $FeSO_4$, 400 μL of standard or sample solution, 200 μL of pH 7.4 Tris-HCl buffer, 50 μL 0.1% 2,2'-bipyridal in 0.2 M HCl, and 200 μL 7% RMCD in 50% acetone. Absorbance was read at 522 nm. EDTA was used as the standard, and measurements were conducted in triplicate.

HT-29 Cancer Cell Proliferation Inhibition

HT-29 human colorectal adenocarcinoma cell line characterized by Fogh (*28*) were cultured in T-150 flasks in Mcoy's 5A media containing 10% FBS and 1% antibiotic/antimycotic. Cells were incubated at 37 °C in a humidified atmosphere at 5% CO_2 (*29, 30*).

Cell proliferation was determined following a modified procedure using 12-well plates (*30*). Cells were seeded at 2.5, 3.0 and 5.0×10^4 per well for the black raspberry, cranberry, and chardonnay grape seed flour extracts, respectively. Cells were incubated for 24 h in growth media at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were then treated with media containing the DMSO solution of the fruit seed flour extracts at 3 and 6 mg flour equivalents per mL culture media for all tested fruit seed flour extracts. Control cells were treated with same volume of DMSO. All treatments were changed daily, and live cells were counted on day 1 through day 4 of treatment.

Statistical Analysis

Data were reported as mean ± standard deviation (n = 3). Analysis of variance and least significant difference tests (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) were performed to identify differences among means. A Pearson Correlation test was conducted to determine the correlations among means. Statistical significance was declared at $P < 0.05$.

Results and Discussion

Total Phenolic Content (TPC)

The TPC values of the fruit seed flours ranged from 14.5 to 186.3 mg gallic acid equivalents per g flour (GAE mg/g) (**Table 3.1**). The chardonnay grape seed flour had the highest TPC among all tested fruit seed flours, and pinot noir grape seed flour had the second highest value at 55.5 GAE mg/g. The TPC values of these two grape seed flours were higher or comparable to that of 29.9-57.1 GAE mg/g seed detected in 10 muscadine grape varieties (*31*). Black raspberry seed flour had a TPC value of 41.2 GAE mg/g (**Table 3.1**) and was comparable to that of 45.6 GAE mg/g previously reported for black raspberry seed flour (*8*). Furthermore, the TPC values of red raspberry and black raspberry seed flours were lower than the TPC in respective whole dried fruits (*32*). The whole dried fruits of red raspberry and black raspberry fruits had TPC values of 36.9 and 57.6 GAE mg/g and the TPC values of the red raspberry and black raspberry seed flours were 25.1 and 41.2 GAE mg/g, respectively (**Table 3.1**). The tested cranberry seed flour had a TPC value of 14.6 GAE mg/g (**Table 3.1**). These data suggest that these fruit seeds may potentially serve as natural sources of dietary phenolic compounds. Additionally, TPC values of the fruit seed flours were significantly correlated to their ORAC values (r = 0.992, *P* < 0.01), suggesting that phenolic compounds contribute to their oxygen radical absorbing capacities.

Total Anthocyanin Contents (TAC)

The highest TAC of 61.3 mg cyanidin 3-glucoside equivalents (CGE) per 100 g of flour (CGE mg/100 g) was detected in the black raspberry seed flour, and no

anthocyanin was detected in the red raspberry flour under the experimental conditions (**Table 3.1**). The TAC of the black raspberry seed flour from this study was lower than that found in the whole dried black raspberry fruit at the level of 3465 CGE mg/100 g (*32*). This TAC value is higher than that detected in fresh whole raspberry fruits examined by Liu and others on a per weight basis (*33*). Liu et al. reported that four varieties of whole fresh raspberry had a TAC range of 0.17-57.6 CGE mg/100 g (*33*). The present study also found significant levels of TAC in the cranberry, blueberry, and chardonnay grape seed flours at a ranging from 6.9 to 7.4 CGE mg/100 g (**Table 3.1**). The cranberry seed flour TAC value of 13.8 CGE mg/100 g was lower but comparable to that of 19.8 to 65.6 CGE mg/100 g reported for ten different cultivars of fresh cranberry (*34*). Blueberry seed flour had a TAC value of 7.4 CGE mg/100 g and was much lower than TAC observed in the 87 highbush blueberry cultivars that ranged from 890-3310 CGE mg/100 g on a fresh weight basis (*4*). Additionally, the TAC values of grape seed flours from this study were lower than that of 68.5-150.7 CGE mg/100 g reported for four varieties of grapes on a fresh weight basis (*35*). These data indicate that anthocyanins may not be concentrated in fruit seeds, although black raspberry seed may contain a significant level of anthocyanins.

Table 3.1. Phytochemical compositions of the cold-pressed edible seed flours*

	TPC (GAE mg/g flour)	TAC (CGE mg/100 g flour)	Total Fat (g/100 g flour)
Black Raspberry	41.2c ± 1.20	61.3a ± 5.85	NA
Red Raspberry1	25.1d ± 2.26	0.0e ± 0.00	4.6
Red Raspberry2	NA	NA	5.4
Blueberry1	15.8e ± 0.63	7.4c ± 0.35	3.7
Blueberry2	NA	NA	2.8
Cranberry	14.6f ± 0.04	13.8b ± 1.39	6.8
Pinot Noir Grape	55.5b ± 11.23	0.28d ± 0.10	1.2
Chardonnay Grape	186.3a ± 5.13	6.85c ± 0.29	5.3

*Red raspberry1 and 2, and Blueberry1 and 2 are two different samples from the same variety. TPC is the total phenolic content in the respective fruit seed flours and is measured as gallic acid equivalents (GAE) milligrams per g flour (GAE mg/g flour). TAC is the total anthocyanin content and is measured as mg cyanidin-3-glucoside equivalents (CGE) per 100 g seed flour (CGE mg/100 g flour). Values in columns with different letters are significantly different ($P < 0.05$).

Fatty Acid Composition

Cranberry seed flour had the highest oil content among the samples containing 6.8 g/100 g flour (**Table 3.1**). The cranberry seed flour also had the highest concentration of α-linolenic acid (18:3n-3) containing more than 30 g/100 g oil and had a very low n-6 to n-3 fatty acid ratio at approximately 1.2:1 (**Table 3.2**). Previously examined black raspberry seed flour also demonstrated a high level of α-linolenic acid at 33.2 g/100g oil and had a low n-6 to n-3 ratio of 1.6:1 (*8*). The chardonnay grape seed flour had the highest linoleic acid (18:2n-6) content of approximately 66 g/100 g oil and was followed by cranberry seed flour at 39.9 g/100 g oil. All of the tested seed flours had relatively high concentrations of oleic acid (18:1n-9) containing 19.2-46.1 g/100 g oil (**Table 3.2**). The linoleic acid concentration in the pinot noir grape seed flour was determined to be 13 g/100 g oil and was notably lower than reported grape seed oils that have demonstrated linoleic acid from 50.1 to 77.8 g/100 g oil (*36*). In this study, the oil from red raspberry seed flour contained total saturated fatty acids from 47-51 g/100 g oil, and the oil from blueberry seed flour contained total saturated fatty acids from 51-59 g/100 g oil (**Table 3.2**). These saturated fatty acid contents were different to those reported in their respective seed oils containing 2.3 and 8.6 g/100 g oil in red raspberry and blueberry seed oils, respectively (*23*). Cranberry and chardonnay grape seed flours had fatty acid profiles similar to their previously reported respective seed oils (**Table 3.2**) (*37-39*). These results from this study demonstrate that fruit seed flours may contain significant levels of oils, and they may have different fatty acid compositions compared to their respective seed oils.

Table 3.2. Fatty acid (FA) profiles of the studied cold-pressed seed flours (g/100 g oil)*

FA	Raspberry1	Raspberry2	Blueberry1	Blueberry2	Cranberry	Pinot Noir	Chardonnay
12:0	Nd	Nd	1.3 ± 0.24	1.8 ± 0.12	t	0.8 ± 0.16	nd
14:0	1.5 ± 1.64	0.3 ± 0.01	0.1 ± 0.00	0.2 ± 0.00	t	0.4 ± 0.01	t
16:0	26.0 ± 0.58	28.0 ± 0.23	26.3 ± 0.95	29.9 ± 0.31	5.4 ± 0.01	35.0 ± 0.05	7.8 ± 1.07
16:1	2.0 ± 0.11	2.0 ± 0.15	0.4 ± 0.16	0.4 ± 0.08	0.1 ± 0.00	0.5 ± 0.01	0.2 ± 0.02
18:0	16.0 ± 0.23	18.8 ± 0.22	20.5 ± 0.53	25.7 ± 0.22	1.3 ± 0.00	2.7 ± 0.05	4.3 ± 0.57
18:1	34.1 ± 0.72	37.0 ± 0.02	46.1 ± 1.53	40.1 ± 0.53	25.1 ± 0.04	32.2 ± 0.05	19.2 ± 1.94
18:2	13.2 ± 0.23	8.0 ± 0.08	2.3 ± 0.50	0.1 ± 0.17	36.9 ± 0.05	13.0 ± 0.03	65.9 ± 3.95
18:3	1.9 ± 0.18	0.9 ± 0.13	nd	Nd	30.9 ± 0.02	nd	1.8 ± 0.25
20:0	3.5 ± 0.06	3.8 ± 0.22	2.8 ± 2.33	1.5 ± 0.11	0.1 ± 0.01	1.3 ± 0.02	0.2 ± 0.03
20:1	1.8 ± 0.19	1.3 ± 0.11	0.3 ± 0.00	0.3 ± 0.00	0.2 ± 0.00	1.0 ± 0.02	0.5 ± 0.06
SFA	47.0	50.9	51.0	59.0	6.8	53.2	12.4
MUFA	37.9	40.3	46.7	40.8	25.4	33.7	19.8
PUFA	15.1	8.8	2.3	0.2	67.8	13.0	67.7
n-6/n3	6.94	8.89	NA	NA	1.19	NA	36.61

*Data were expressed as mean ± SD (n = 3). Raspberry1 and Raspberry2 are 2 different samples of the same variety of red raspberry. Blueberry1 and 2 are different samples of the same variety. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, and n-6/n-3 is the ratio of n-6 to n-3 fatty acids. nd: not detected. t: trace.

Oxygen Radical Absorbance Capacity (ORAC)

All tested fruit seed flours displayed significant oxygen radical absorbing capacities having ORAC values of 110.5-1076 TE μmol/g (**Table 3.3**). Chardonnay grape seed flour had the highest ORAC among all tested fruit seed flours on a per weight basis. The chardonnay grape seed flour had an ORAC value that was more than 3 times higher than the pinot noir grape flour and almost 10 times higher than the cranberry seed flour (**Table 3.3**). The red raspberry and black raspberry seed flours were determined to have ORAC values of 275.5 and 296.2 TE μmol/g, respectively (**Table 3.3**) which were comparable to but contradictory to ORAC values of 171 and 453 TE μmol/g reported for whole dried red raspberry and black raspberry, respectively (*32*). Also noted in this study was that the ORAC values of cranberry and blueberry seed flours were 110.5 and 152.9 TE μmol/g, respectively (**Table 3.3**), and were higher than the ORAC determined in fresh cranberry and blueberry fruits (*4, 6*). In 2003, Zheng et al. reported ORAC values of 18.5 and 28.9 TE μmol/g for whole fresh cranberry and blueberry, respectively (*6*), and another study found ORAC values in the range of 4.6-31.1TE μmol/g fresh fruit for blueberry cultivars (*4*). These data suggest that fruit seed flours are excellent dietary sources for oxygen radical reducing components.

DPPH$^\bullet$ Scavenging Activity

The chardonnay seed flour contained the highest amount of DPPH$^\bullet$ scavenging agents and had an $ED_{50\text{-DPPH}}$ value of 39 μg flour equivalents/mL (**Table 3.3**). The $ED_{50\text{-DPPH}}$ is the concentration of a substance that will reduce the amount of

DPPH$^\bullet$ to half of the original concentration under the experimental conditions. The ED$_{50\text{-DPPH}}$ of the chardonnay seed flour was 4 times lower than that of pinot noir grape seed flour and 32 times lower than the cranberry seed flour (**Table 3.3**). This study used 50% acetone for antioxidant extraction because our previous study showed that 50% acetone extract quenched 14.9% more DPPH radicals compared to a 100% ethanol extract of black raspberry seed flour (*8*). Also, all tested fruit seed flour extracts demonstrated time and dose effects in their reactions with DPPH$^\bullet$, and the reactions of the pinot noir grape and red raspberry seed flour extracts are shown in **Figure 3.1**. Fruit seed flour extracts with stronger DPPH radical scavenging capacity also had higher ORAC values, but the correlation was not significant.

Table 3.3. Antioxidant activities of cold-pressed edible seed flours

	[a]ORAC (TE µmol/g)	[b]ED$_{50\text{-DPPH}}$ (µg flour eq/mL)	[c]Chelating Capacity (EDTA eq mg/g)
Black Raspberry	296.2b ± 42.0	200	3.6ab ± 0.20
Red Raspberry1	275.5b ± 32.4	510	3.9a ± 0.07
Blueberry1	152.9c ± 27.3	670	1.9d ± 0.24
Cranberry	110.5c ± 22.0	1260	2.1d ± 0.02
Pinot Noir Grape	312.8b ± 16.5	160	2.6c ± 0.17
Chardonnay Grape	1076a ± 72.9	39	3.3b ± 0.14

[a]ORAC is expressed as µmol trolox equivalent (TE) per g seed flour (TE µmol/g).

[b]ED$_{50\text{-DPPH}}$ is µg flour equivalents per mL reaction mixture (µg flour eq/mL) to decrease the concentration of DPPH$^\bullet$ to half of the initial concentration at 20 min of reaction. [c]Chelating Capacity is expressed as EDTA equivalent mg per g flour (EDTA eq mg/g). Values in the same column with different letters are significantly different ($P < 0.05$).

A. Pinot Noir
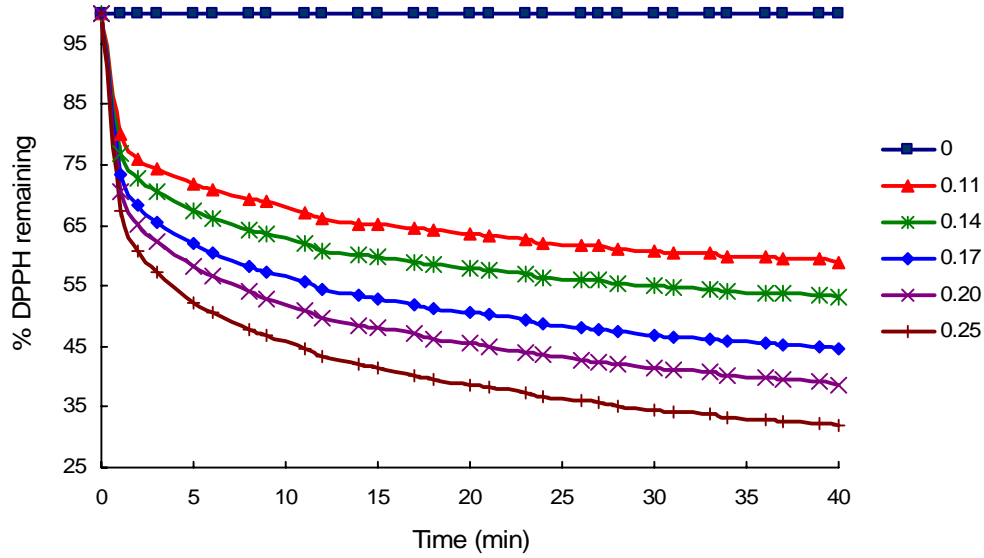
B. Red Raspberry
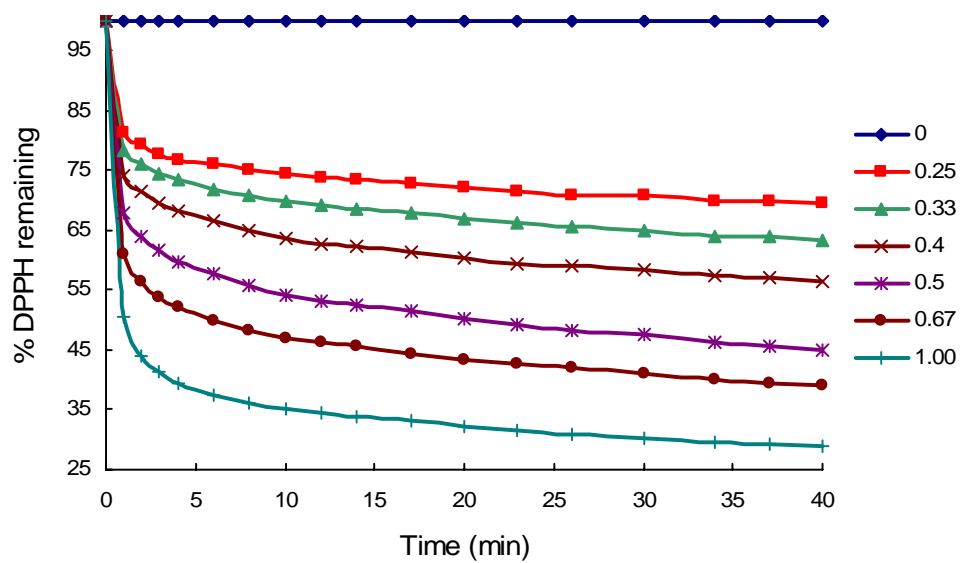
Figure 3.1. Kinetic and dose effects of fruit seed flour antioxidants-DPPH$^{\bullet}$ reactions. Flours were extracted using 50% acetone. Representative numbers are mg seed flour equivalents per mL in initial reaction mixture; Figure A) Reactions with pinot noir grape seed flour extract and Figure B) Reactions with red raspberry seed flour extract.

<u>Chelating Capacity</u>

All fruit seed flour extracts displayed significant chelating capacities against

Fe^{2+}. The values ranged from 1.9 to 3.9 EDTA equivalents mg/g flour (**Table 3.3**).

The red raspberry seed flour extract had the highest chelating capacity but was not

significantly higher than the black raspberry seed flour extract. The ability of these

fruit seed flours to coordinate metals may protect against metal ion induced free

radical oxidation. Transition metals may act as catalysts to generate the first few

radicals that initiate oxidative chain reactions in food and biological systems. It needs

to be pointed out that metal chelating properties of fruit seed flours may also interfere

with essential mineral absorption.

<u>HT-29 Cell Proliferation Inhibition</u>

Chardonnay grape, black raspberry, and cranberry seed flour extracts in

DMSO were evaluated for their potential antiproliferative activities at 3 and 6 mg

seed flour equivalents per mL media. The chardonnay grape seed flour extract was

lethal to all living HT-29 cells at both treatment levels following 24 hours of exposure

(**Figure 3.2**), whereas the black raspberry and cranberry seed flour extracts dose-

dependently suppressed cell proliferation under the same experimental conditions

(**Figure 3.2**). Both black raspberry and cranberry seed flour extracts also inhibited

HT-29 cell proliferation in a dose-dependent manner over a four day treatment period

(**Figure 3.3**). These results indicate that these fruit seed flours contain different levels

of antiproliferative components. In order to evaluate their potential utilization in

cancer prevention, additional research is required to evaluate the antiproliferative

activities of these fruit seed flour components on other cancer cells and normal cells,

investigate the underlying mechanisms, and characterize the chemical structures contributing to antiproliferative activity

In 2004, more than 66 million metric tones (MMT) of grapes were harvested globally (http://faostat.fao.org/faostat/), and the US forecast for 2005 production was 6.5 MMT with more than half of that projected for wine and juice production (http://www.nass.usda.gov). The total 2004 worldwide cranberry production was 394,394 metric tones (MT) (http://faostat.fao.org/faostat/), and over 70% was grown in the US (http://www.nass.usda.gov). From the 70% grown in the US, 99.9% of the cranberries were processed. Additionally, worldwide raspberry production in 2004 was 461,485 MT (http://faostat.fao.org/faostat/). Red raspberry and black raspberry 2004 productions in the United States were 30,455 and 1,000 MT, respectively, and over 99.9 % of each was processed (http://www.nass.usda.gov). Fruit seeds are byproducts from fruit processing and may be used to obtain oils with special fatty acid compositions or other health beneficial components (*23*). Fruit seed flours are byproducts of fruit seed oil production, and are treated as low value wastes. Characterizing bioactive components in fruit seed flours and demonstrating their potential beneficial properties may lead to value-added utilization of these fruit seed flours which may enhance the profitability of the fruit production and processing industries and fruit seed oil manufacturing. The results from the current research suggest that these fruit seed flours may serve as dietary sources of natural antioxidants and contain antiproliferative compounds. Additional research is required to investigate the effects of food formulation, processing and storage on the availability of these beneficial components and properties, as well as the chemical

and biochemical mechanisms relating to their antioxidant and antiproliferative properties in order to promote their utilization in food and dietary supplemental products for health promotion and disease prevention.
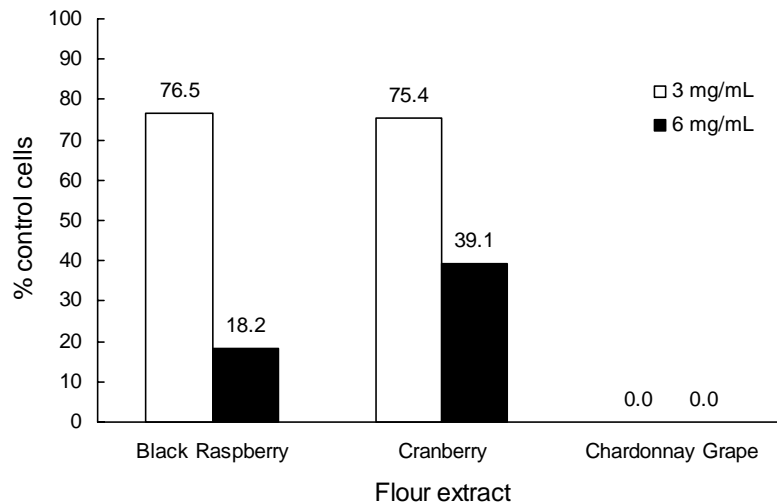
Figure 3.2. Antiproliferation of HT-29 colon cancer cells treated with fruit seed extracts. Antiproliferative effects of the fruit seed flour extracts were expressed as % control cells after exposure to treatment for 24 hours. Black raspberry, cranberry, and chardonnay grape stand for the DMSO solutions of black raspberry, cranberry, and chardonnay grape seed flours prepared from the respective 50% acetone extracts, respectively. The final concentrations of for all fruit seed flour extracts were 3 and 6 mg flour equivalents/mL in the initial culture media.

A. Black Raspberry
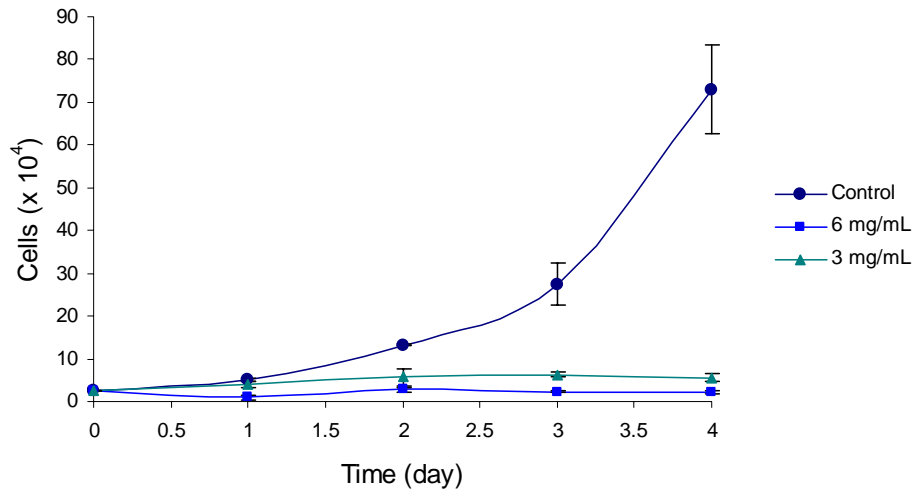
B. Cranberry
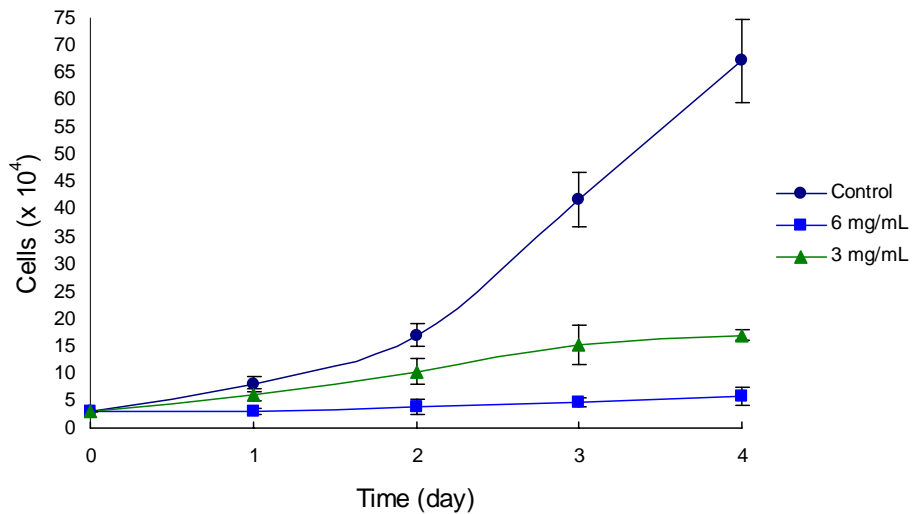
Figure 3.3. Dose and time effects of the fruit seed flour extracts on HT-29 cell growth. DMSO solutions of black raspberry and cranberry seed flours prepared from the respective 50% acetone extracts. The final concentrations were 3 and 6 mg flour equivalents/mL in the initial culture media. 3A) Effect of black raspberry seed flour extract and 3B), Effect of cranberry seed flour extract.

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Chapter 4: Characterization of Cold-Pressed Onion, Parsley, Cardamom, Mullein, Roasted Pumpkin, and Milk Thistle Seed Oils

Abstract

Cold-pressed onion, parsley, cardamom, mullein, roasted pumpkin, and milk thistle seed oils were analyzed for their fatty acid composition, tocopherol content, carotenoid profile, total phenolic content (TPC), oxidative stability index (OSI), color, physical properties, and radical scavenging activities using the oxygen radical absorbance capacity (ORAC) assay and the stable DPPH radical. Parsley seed oil had the highest oleic acid content of 81 g/100 g total fatty acids and the lowest saturated fat among all tested oils. Roasted pumpkin seed oil contained the highest concentration of zeaxanthin, β-carotene, p-cryptoxanthin, lutein, and total carotenoids at 28.5, 6.0, 4.9, 0.3 mg/kg oil, and 71 μmol/kg oil, respectively. The onion seed oil contained the highest α-tocopherol and total tocopherols under the experimental conditions. One of the parsley seed oils exhibited the highest ORAC value of 1098 μmol trolox equivalents per g oil and had the strongest DPPH$^\bullet$ scavenging capacity; however, ORAC values were not significantly correlated to DPPH$^\bullet$ scavenging capacity among all samples. The highest TPC was detected in one of the onion seed oils at 3.4 mg gallic acid equivalents per g oil and OSI values were 13.3, 16.9-31.4, 47.8, and 61.7 h for the milk thistle, onion, mullein, and roasted pumpkin seed oils, respectively. These data suggest that these seed oils may serve as dietary sources of special fatty acids, carotenoids, tocopherols, phenolic compounds, and natural antioxidants.

Introduction

Edible seed oils are a group of very important food ingredients. Novel specialty seed oils rich in health beneficial factors are in high demand because of consumers' interest in disease prevention and health promotion through improving diets. These beneficial factors include special fatty acid compositions such as high monounsaturated or n-3 fatty acids, carotenoids, tocopherols, and antioxidative phenolic compounds (*1-4*). Several edible oils from herb, spice, and fruit seeds have been shown to contain special fatty acid profiles (*4*). For example, American ginseng seed oil contained approximately 87% oleic acid, and basil seed oil had 57-63% α-linolenic acid (18:3n-3), the essential n-3 fatty acid (*4*). Cold-pressed edible seed oils may be preferred by consumers since the cold-pressing procedure involves neither heat nor chemicals, and may retain more beneficial phytochemicals. Previous studies showed that cold-pressed carrot seed oil had about 82% oleic acid (18:1n9), which has been associated with lowering the risk of cardiovascular disease (*5*). Also noted was that cold-pressed edible hemp and fruit seed oils contain significant levels of α-linolenic acid (18:3n-3), which may be converted to longer chain n-3 polyunsaturated fatty acids, EPA (20:5n-3) and DHA (22:6n-3), *in vivo* through enzymatic elongation and desaturation. EPA and DHA are reported to potentially reduce the risk of heart disease, cancer, hypertension, and autoimmune disorders (*6-8*). These data suggest the possibility of developing novel edible seed oils having special fatty acid compositions for improving human nutrition.

Previous studies have also shown that cold-pressed edible seed oils may contain significant levels of carotenoids and tocopherols (*2*). For instance, cold-

pressed boysenberry seed oil had a total carotenoid concentration of 30 μmol/kg oil

(*2*), and cold-pressed blueberry, red raspberry, marionberry, and boysenberry seed

oils contained significant levels of α-, γ-, and δ-tocopherols at ranges of 21-151, 33-

689, and 6-232 mg/kg oil, respectively (*2*). Tocopherols have also been detected in

raspberry, blackcurrant, and goldenberry seed oils (*3, 9, 10*). Carotenoids and

tocopherols are well recognized for their potential health benefits, and the

characterization of carotenoid and tocopherol profiles in cold-pressed edible seed oils

may provide a scientific basis to promote their consumption for improving human

nutrition.

Additionally, significant levels of phenolic components have previously been

detected in cold-pressed edible seed oils (*2*). Phenolic compounds have demonstrated

powerful antioxidative activities and may reduce free radical mediated cellular

damage (*2, 3, 11-13*). Several cold-pressed edible seed oils have been studied for their

antioxidant activities (*2, 14*). In 2005, Parry et al. (*2*) found that cold-pressed

blueberry, red raspberry, marionberry, and boysenberry seed oil extracts had

considerable free radical scavenging abilities against peroxyl and DPPH radicals.

Boysenberry seed oil extract had an oxygen radical scavenging capacity (ORAC) of

77.9 μmol trolox equivalent (TE) per g oil (TE μmol/g oil) (*2*). Cranberry seed oil has

demonstrated significant radical scavenging activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ and

also suppressed human LDL oxidation (*14*). It is well accepted that antioxidants may

prevent important cellular components such as DNA, membrane lipids, and proteins

from oxidative damage, and suppress the pathology of cardiovascular disease, cancer,

and other aging associated health problems. Edible oils rich in natural antioxidants may help to reduce the risk of chronic diseases.

In the present study, cold-pressed onion (*Allium cepa* L.), parsley (*Petroselinum crispum*), cardamom (*Elettaria cardamomum*), mullein (*Verbascum thapsus*), roasted pumpkin (*Curcubita pepo* L.), and milk thistle (*Silybum marianum*) seed oils were investigated for their fatty acid profile, carotenoid and tocopherol composition, total phenolic content, antioxidant activities, oxidative stability, color, and physical properties. The oxidative stability, color, and physical properties are important characteristics related to the utilization of these oils as food. The obtained data will be used to promote the potential utilization of these oils in food products for improving human nutrition and health.

Materials and Methods

<u>Materials</u>

Cold-pressed onion, parsley, cardamom, mullein, roasted pumpkin 'Triple Treat' variety, and milk thistle seed oils were obtained from Badger Oil Company (Spooner, WI). All oil samples were fresh and extractions were obtained immediately after arrival. Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) were purchased from Sigma-Aldrich (St. Louis, MO); β-cyclodextrin (RMCD) was purchased from Cyclolab R & D Ltd. (Budapest, Hungary), and 2,2'-azobis (2-amino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA).

All other chemicals and solvents were of highest commercial grade and used without further purification.

Extracting and Testing Sample Preparation

Extractions were performed using 100% MeOH. One gram of each cold-pressed edible seed oils was extracted with 3 mL of MeOH by vortexing at ambient temperature, centrifuging, and obtaining the MeOH fraction 3 times. The 3 MeOH-oil fractions were combined, and the final volume of each extract testing solution was brought to 10 mL by the addition of 1 mL MeOH (*2*). The solutions were kept in the dark under nitrogen until further analysis.

Fatty Acid Composition

Fatty acid methyl esters (FAME) were prepared from oils according to the previously described method (*2*). The FAME were analyzed by GC-FID for fatty acid profiles. Analysis was conducted using a Shimadzu GC-2010 with a FID and a Shimadzu AOC-20i autosampler (Shimadzu, Columbia, MD). A Supelco 2380 column, 30 m × 0.25 mm i.d. with a 0.20 μm film thickness (Supelco Inc., Bellefonte, PA), was used to separate FAME. Helium was the carrier gas at a flow rate of 0.8 mL/min. Injection volume was 1 μL at a split ratio of 10/1. Initial oven temperature was set at 142 ºC and increased 6 ºC/min to 184 ºC, held for 3 min, and then increased 6 ºC/min to 244 ºC. Identification of the individual FAME was accomplished by comparing GC retention time with that of the authorized pure individual commercial compounds.

Carotenoid Composition

Concentrations of β-carotene, lutein, cryptoxanthin and zeaxanthin were measured following the previously described method (*2, 15, 16*). Briefly, 1 mL of cold-pressed seed oil was dissolved in 160 mL of methanol/tetrahydrofuran (1:1, v/v) and analyzed for carotenoid composition using HPLC-DAD-ESI-MSMS (high performance liquid chromatography-diode array detector-electron spray ionization-tandem mass spectrometry). A TSQ quantum tandem mass spectrometer (Thermo-Finnigan, San Jose, CA) equipped with an ESI interface and an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a Zorbax SB C18 column, 50 mm × 1.0 mm i.d. with a 3.5-μm particle size (Agilent Technologies, Palo Alto, CA, USA), was used to separate the carotenoids. Identification of the individual compounds was accomplished by comparing HPLC retention time and selected reactant monitoring (SRM) analyses of the sample peaks with that of the authorized pure individual commercial compounds. Quantifications of the carotenoid compounds were conducted using the total ion counts with an external standard. Data were obtained using Xcalibur software system (Thermo-Finnigan, San Jose, CA, USA). Measurements were conducted in triplicate.

Tocopherol Profile

The methanol/tetrahydrofuran solution in methanol/tetrahydrofuran (1:1, v/v) prepared for carotenoid composition were also used to quantify α-, γ-, and δ-tocopherol concentrations by the previously described method (*2*). HPLC with a Zorbax SB C18 column, 30 mm × 1.0 mm i.d with a 3.5-μm particle size (Agilent Technologies, Palo Alto, CA, USA), was used to separate the tocopherol compounds.

Individual tocopherols were identified by peak retention time and selected reactant monitoring with those of the pure commercial tocopherols. Quantification was determined using the total ion counts of external standards of the individual compounds. Triplicate measurements were performed.

<u>Total Phenolic Content (TPC)</u>

The Folin-Ciocalteu (FC) reagent was used to determine the TPC of the cold-pressed seed oils following procedure previously described by Yu et al. (*17*). In Brief, the reaction mixture contained 250 µL of fresh FC reagent, 750 µL of 20% Na_2CO_3, and 3 mL of pure H_2O to which 50 µL of oil extract or standard was added to start the reaction. Absorbance was determined at 765 nm following 2 h of reaction at ambient temperature and was then used to calculate the TPC of the oil samples. Gallic acid was used as the standard. The FC reagent was freshly prepared by refluxing a mixture of sodium tungstate, sodium molybdate, 85% phosphoric acid, and concentrated HCl for 10 h. This was followed by reaction with lithium sulfate, and oxidation with a few drops of bromine (*18*). The solution was then filtered and ready for assay. Triplicate measurements were taken.

<u>Oxygen Radical Absorbance Capacity (ORAC)</u>

ORAC was determined using the previously described protocol (*17, 18*). Fluorescein was used as the fluorescent probe. The complete assay solution contained 0.067 µM of fluorescein, 60 mM of AAPH, 300 µL of oil extract or MeOH for the reagent blank. The fluorescence of an assay mixture was recorded every minute, and

the area under the curve of fluorescence *vs.* time was calculated and compared against a standard curve prepared with trolox.

DPPH$^\bullet$ Scavenging Activity

The DPPH$^\bullet$ scavenging capacities of the cold-pressed seed oil extracts were analyzed following a previously described procedure using the stable DPPH radical (*19*). A freshly prepared DPPH$^\bullet$-MeOH solution was mixed with seed oil extracts at concentrations of 10, 12.5, 16.7, and 25 mg oil equivalents/mL to start the radical-antioxidant reaction. The final concentration was 100 μM for DPPH$^\bullet$ and the final reaction volume was 2.0 mL. Absorbance was read at 517 nm, measured against a blank of pure methanol at 0.67, 3, 6, 10, 20, 50, 80, and 1440 min of the reaction, and used to estimate the percentage of remaining DPPH radicals. Also, the DPPH$^\bullet$ scavenging capacities of the individual oil extracts were compared at 40 mg oil equivalents/mL following 10 min of reaction. Comparisons were made using absorbance readings at 517 nm. The time and dose dependencies of cold-pressed seed oil extracts and DPPH$^\bullet$ reactions were demonstrated by plotting the percent of DPPH$^\bullet$ remaining at timed intervals for each dose of the seed oil extract tested.

Oxidative Stability Index (OSI)

The OSI of each cold-pressed edible seed oils was examined using a Rancimat instrument (Model 743; Metrohm Ltd., Herisau, Switzerland). To obtain the OSI, 4 mL of oil was placed in an oxidation reaction vessel heated to 80 °C with an airflow rate of 7 L/ h (*1*). The OSI was determined as the time for an oil sample to develop a measurable rancidity indicated by induction time. The OSI of the cold-pressed seed

oils were compared to commercial corn and soybean oils under the same experimental conditions.

Determination of Refractive Index and Density

The refractive indices of the cold-pressed seed oils were analyzed at 24 °C according to the *AOCS Official and Tentative Methods* procedure Cc 7-25 (*20*) using an ABBE Refractometer (American Optical Corporation, Buffalo, NY). The specific densities were measured at 24 °C against pure H_2O at 4 °C according to the *AOCS Official and Tentative Methods* procedure: To 1b-64 (*21*).

Color

Oil colors were evaluated using a HunterLab ColorFlex spectrophotometer (Hunter Associates Laboratory, Inc., Reston, VA). Fifteen mL of each oil was added to a sample cup, and color values were obtained using D65/10° (daylight 65 illuminant/10° observer) setting.

Statistical Analysis

Data were reported as mean ± standard deviation (n = 3). Analysis of variance and least significant difference tests (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) were performed to identify differences among means, while a Pearson Correlation test was employed to determine the correlations among means. Statistical significance was declared at $P < 0.05$.

Results and Discussion

<u>Fatty Acid Composition</u>

The cold-pressed parsley seed oils contained over 92% unsaturated fatty acids, and was predominantly oleic acid (18:1n-9) at a level of 81 g per 100 g total fatty acids (**Table 4.1**). The ratio of oleic to linoleic acids was approximately 7.4:1, which was a little higher than that of 6.6:1 previously reported for parsley seed oil by Gunstone (*22*) which had an oleic acid concentration of 81.9%. The concentrations of oleic acid in the parsley seed oils were significantly higher than the concentrations commonly found in olive oil, which are normally in a range between 68-73% (*23*). It has recently been observed that hamsters fed diets rich in oleic acid had reduced atherosclerotic development compared to hamsters fed diets rich in linolenic acid, and they also had reduced aortic accumulation of oxidized LDL, which may be positively associated with the formation of fatty streaks, the earliest identifiable lesions of atherosclerosis (*24*). The FDA has recently approved a qualified health claim for olive oil relating its potential ability to reduce the risk of coronary heart disease (*25*), and it is believed that the high concentration of MUFA may contribute to the beneficial effects (*26*). These results suggest that parsley seed oil may be an excellent source for consumers who prefer a diet rich in MUFA.

None of the cold-pressed seed oils contained significant levels of α-linolenic acid (18:3n-3), the essential n-3 fatty acid. Onion, mullein, and milk thistle seed oils had high PUFA contents (**Table 4.1**), and linoleic acid (18:2n-6) was the primary fatty acid. The biomechanical functions of PUFA are currently under extensive research including their influence/impact on cellular signaling and membrane

structure, gene expression and prostaglandin biosynthesis, and endocrine, nervous, and immune systems mediation (*27*). The cold-pressed onion seed oil had 64-65% linoleic acid which is much higher than the 45% linoleic acid found in Indian onion seed oil (*28*). The oleic acid concentration in the onion seed oil was 25-26%, which is lower than that of 34% reported by Rao (*28*). Also noted was that the level of palmitic acid (16:0) was higher but the stearic acid (18:0) was lower than previously reported in onion seed oil. The primary fatty acids in the cold-pressed roasted pumpkin seed oil were linoleic, oleic, palmitic and stearic acids at levels of 47.2, 36.3, 8.9, and 6.4%, respectively. These four fatty acids were also the primary fatty acids reported in roasted and un-roasted pumpkin seed oils that had concentrations of 54.6, 27.6, 5.4, and 12.4% for linoleic, oleic, palmitic and stearic acids, respectively (*29*). The differences in the fatty acid compositions may be partially due to variety and growing conditions of the pumpkin seeds in the two studies. Additionally, the total unsaturated fatty acid content was 69.2, 90.5, 84.2, and 86.1% in the cold-pressed cardamom, mullein, roasted pumpkin, and milk thistle seed oils, respectively, and the ratios of oleic to linoleic acid were 0.4, 3.2, 0.2, 0.8, and 0.4 for the onion, cardamom, mullein, roasted pumpkin, and milk thistle seed oils, respectively (**Table 4.1**).

Table 4.1. Fatty acid (FA) profiles of the studied cold-pressed seed oils (g/100 g oil)[*]

FA	Onion1	Onion2	Parsley1	Parsley2	Cardamom	Mullein	Pumpkin	Milk Thistle
14:0	tr	nd	tr	tr	1.5 ± 0.05	tr	0.1 ± 0.01	0.1 ± 0.00
16:0	6.4 ± 0.01	7.1 ± 0.01	3.1 ± 0.01	3.1 ± 0.00	26.4 ± 0.13	6.0 ± 0.02	8.9 ± 0.01	8.9 ± 0.02
16:1	0.2 ± 0.01	0.2 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	1.6 ± 0.02	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.01
18:0	2.4 ± 0.00	1.8 ± 0.00	4.2 ± 0.01	4.2 ± 0.02	2.3 ± 0.04	2.7 ± 0.01	6.4 ± 0.01	4.8 ± 0.01
18:1	24.8 ± 0.02	26.0 ± 0.01	81.0 ± 0.04	80.9 ± 0.03	49.2 ± 0.22	16.1 ± 0.02	36.3 ± 0.01	23.8 ± 0.03
18:2	65.2 ± 0.03	64.0 ± 0.02	11.0 ± 0.01	11.0 ± 0.02	15.2 ± 0.04	73.1 ± 0.04	47.2 ± 0.01	60.8 ± 0.03
18:3	0.1 ± 0.03	0.3 ± 0.02	0.5 ± 0.01	0.5 ± 0.02	2.7 ± 0.01	1.0 ± 0.00	0.2 ± 0.01	0.2 ± 0.03
20:0	0.3 ± 0.00	0.3 ± 0.00	0.1 ± 0.00	0.1 ± 0.01	0.4 ± 0.02	0.7 ± 0.00	0.5 ± 0.01	nd
20:1	0.4 ± 0.01	0.4 ± 0.02	nd	nd	0.5 ± 0.02	0.2 ± 0.00	0.4 ± 0.01	1.2 ± 0.06
SAT^z	9.3	9.3	7.4	7.5	30.8	9.4	15.9	13.8
MUFA^y	25.4	26.6	81.1	81.0	51.3	16.4	36.7	25.2
PUFA^x	65.3	64.2	11.5	11.6	17.9	74.2	47.4	61.1

[*]Fatty acid composition was reported as mean $\pm$ standard deviation (n = 3). tr: trace. nd: not detected. ^z SAT represents total saturated fatty acids (g/100 g oil). ^y MUFA represents total monounsaturated fatty acids (g/100 g oil). ^x PUFA represents total polyunsaturated fatty acids (g/100 g oil).

Carotenoids and Tocopherols

Significant levels of carotenoids (**Table 4.2**) and tocopherols (**Table 4.3**) were detected in the cold-pressed seed oils. Zeaxanthin was the primary carotenoid compound present in all oils, although its level varied by almost 3000 fold among the samples. The cold-pressed roasted pumpkin seed oil had the highest total carotenoid concentration followed by the parsley and mullein seed oils. Their levels were comparable to those observed in cold-pressed red raspberry, blueberry, marionberry and boysenberry seed oils (12.5-30.0 μmoles/kg) reported by Parry et al. (*2*). The roasted pumpkin seed oil also had the highest β-carotene content of 5958 μg/kg or 5481 μg/L among all the tested oil samples and was much higher than that previously observed in cold-pressed boysenberry (2405.2 μg/kg), blueberry (1352 μg/kg), marionberry (442.7 μg/kg), and red raspberry (82.2 μg/kg) seed oils (*2*). It was also much higher than corn (1200 μg/L), soybean (280 μg/L), and peanut (130 μg/L) oils (*30*). These data suggest that the cold-pressed roasted pumpkin, parsley and mullein seed oils may serve as dietary sources of carotenoids, primarily zeaxanthin.

Concentrations of α-, γ-, δ-, and total tocopherols from the cold-pressed oils are shown in **Table 4.3**. The two onion seed oils contained significantly higher amounts of α-tocopherol ($P < 0.0001$) and more than double the total tocopherols found in the other tested seed oils. The concentrations of α-tocopherol at 498 and 682 mg/kg onion seed oil are equal to 460 and 634 mg/L, respectively. These levels are higher or comparable to commercial extra virgin olive oil, peanut, corn, and sunflower seed oils (174-578 mg/L), and higher than soybean oil (89 mg/L) (*30*), but

much lower compared to wheat germ oil (1330 mg /kg oil) (*31*). The values are also higher than those detected in cold-pressed blueberry, red raspberry, marionberry and boysenberry seed oils (21-151 mg/kg oil) (*2*), and comparable to hexane extracted and cold-pressed red raspberry seed oil that contained 710 and 460 mg/kg oil, respectively (*9*). The onion seed oil also had the highest total tocopherol content of 1.8-2.0 mmol/kg oil, which was comparable to cold-pressed red-raspberry and boysenberry seed oils (2.1 and 2.3 mmol/kg), respectively (*2*). Interestingly, the studied seed oils differed in their tocopherol isomer compositions (**Table 4.3**). The cold-pressed parsley seed oils had the highest ratios of α- to γ-tocopherols at 7.4:1 and 10.7:1, while mullein and roasted pumpkin seed oils had much lower ratios of α- to γ-tocopherols which were approximately 1:8. The range of α- to γ-tocopherols in the cardamom, onion, and milk thistle seed oils ranged from 2.4 to 4.5:1. Mullein seed oil contained highest level of δ-tocopherol among all tested seed oils while parsley and cardamom seed oils demonstrated the lowest δ-tocopherol contents containing less than 2 mg per kg oil. In summary, the cold-pressed onion seed oil from this study is the preferred dietary source for total, α-, and γ- tocopherols with significant level of δ-tocopherol. The cold-pressed mullein and roasted pumpkin seed oils may serve as dietary sources for γ-tocopherol, and the mullein seed oil may also provide dietary δ-tocopherol.

Table 4.2. Carotenoid contents in the cold-pressed seed oils[*]

	β-Carotene (µg/kg)	Lutein (µg/kg)	Zeaxanthin (mg/kg)	Cryptoxanthin (mg/kg)	Total Carotenoids (µmol/kg)
Onion1	nd	17.3e ± 0.3	1.74d ± 0.014	0.51f ± 0.001	4.01
Onion2	nd	17.9e ± 0.2	1.22f ± 0.004	0.75e ± 0.007	3.52
Parsley1	783.8d ± 4.2	216.4b ± 2.4	20.40b ± 0.128	1.43b ± 0.019	40.28
Parsley2	989.1c ± 12.5	207.1c ± 1.8	20.55b ± 0.104	1.20d ± 0.019	40.49
Cardamom	nd	nd	0.03g ± 0.000	nd	0.05
Mullein	1121.0b ± 8.7	62.1d ± 1.0	0.01c ± 0.000	1.30c ± 0.025	15.80
Pumpkin	5957.6a ± 108.2	270.1a ± 1.1	28.52a ± 0.508	4.91a ± 0.008	70.59
Milk thistle	nd	nd	1.312e ± 0.001	nd	2.30

[*]Carotenoid content of each cold-pressed seed oils was reported as mean ± SD (n = 3). Different letters within columns represent significant difference ($P < 0.05$). nd: not detected.

Table 4.3. Tocopherol contents in the cold-pressed seed oils[*]

	α-Tocopherol (mg/kg)	γ-Tocopherol (mg/kg)	δ-Tocopherol (mg/kg)	Total Tocopherols (μmol/kg)
Onion1	681.9a ± 18.4	219.2a ± 4.6	28.6b ± 0.1	1973.8
Onion2	498.1b ± 27.2	156.3b ± 4.0	23.0c ± 0.0	1762.6
Parsley1	29.5d ± 0.3	2.8f ± 0.2	0.9h ± 0.0	77.6
Parsley2	29.9d ± 0.3	4.0e ± 0.0	1.2g ± 0.0	80.6
Cardamom	10.4f ± 0.0	4.3d ± 0.1	1.6f ± 0.0	38.4
Mullein	27.1e ± 0.2	213.3a ± 1.9	76.2a ± 0.2	759.4
Pumpkin	26.8e ± 0.9	216.3a ± 2.4	19.2d ± 0.0	625.6
Milk thistle	156.3c ± 0.9	35.1c ± 0.4	7.0e ± 0.0	464.2

[*]Tocopherol contents were reported as mean ± SD (n = 3). Different letters within columns represent significant difference ($P < 0.05$).

Total Phenolic Content (TPC and Antioxidant Activities)

The TPC of the tested cold-pressed seed oils ranged from 0.98 to 3.35 mg gallic acid equivalent (GAE) per g oil (GAE mg/g) (**Figure 4.1**). The TPC values of the milk thistle, onion, and cardamom seed oils were higher than those detected in cold-pressed boysenberry, blueberry, red raspberry, and marionberry seed oils (1.5-2.0 GAE mg/g) (*2*). The TPC values of the cold-pressed seed oils from this study were much higher than the TPC values of cold-pressed black raspberry seed oils (0.04-0.09 GAE mg/g) (*11*). This difference may be explained by the extraction solvents used in the two studies. Methanol used in the current study is a preferred solvent for oil antioxidant extraction compared to 50% acetone or 70-80% methanol due to its better solubility of lipophilic antioxidants. Significant differences in TPC were observed between the two onion and parsley seed oils ($P < 0.05$), indicating the

possible influence of seed quality and processing conditions. It is well accepted that

genotype, growing conditions such as soil and temperature, interaction between

genotype and growing conditions, post-harvest treatments including the mechanical

grinding during oil extraction, and storage conditions may significantly alter the

chemical composition of selected botanical materials. Additional research is required

to fully understand and explain why the differences in the TPC values between the

two onion seed oils and parsley seed oils exists; however, the different TPC values

may partially explain the relative radical scavenging capacities and oxidative

stabilities of these cold-pressed edible seed oils.
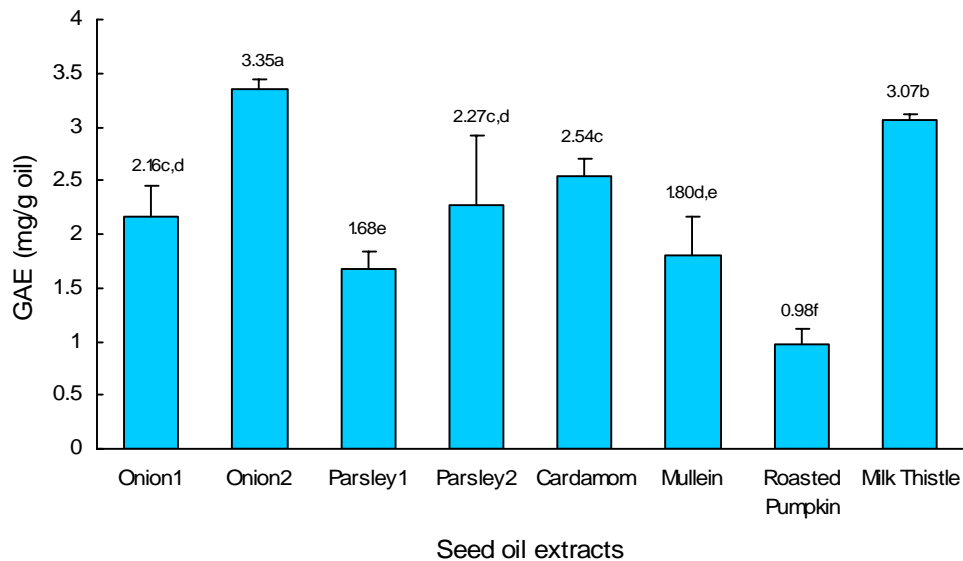

Figure 4.1. Total phenolic contents of the cold-pressed seed oils. GAE denotes

gallic acid equivalents. T-bars represent the standard deviation (n = 3). Different

letters indicate significant difference ($P < 0.05$).

ORAC is a widely accepted measurement of free radical scavenging activity. The ORAC values of the cold-pressed seed oil extracts are shown in **Figures 4.2a and 4.2b**. Parsley, cardamom and milk thistle seed oils demonstrated ORAC values over 100 μmol trolox equivalent (TE) per g oil (TE μmol/g) , whereas the mullein, onion and roasted pumpkin seed oils had ORAC values less than 30 TE μmol/g. Parsley seed oil exhibited the highest ORAC value and was approximately 1000 times higher than the roasted pumpkin seed oil. The ORAC values from the cold-pressed seed oils from this study were higher than cold-pressed boysenberry, red raspberry, blueberry and marionberry seed oils (78, 49, 36, and 17 TE μmol/g oil, respectively) (*2*). The ORAC values were also higher than wheat grain (51 TE μmol/g) (*18*), and wheat bran (107-136 TE μmol/g) (*18, 32*), common dried vegetables (19-154 TE μmol/g) (*34*), and dry fruits (13-154 TE μmol/g) (*34, 35*). These data suggest that the cold-pressed parsley, cardamom, milk thistle seed oils contain high concentrations of peroxyl radical scavenging components.

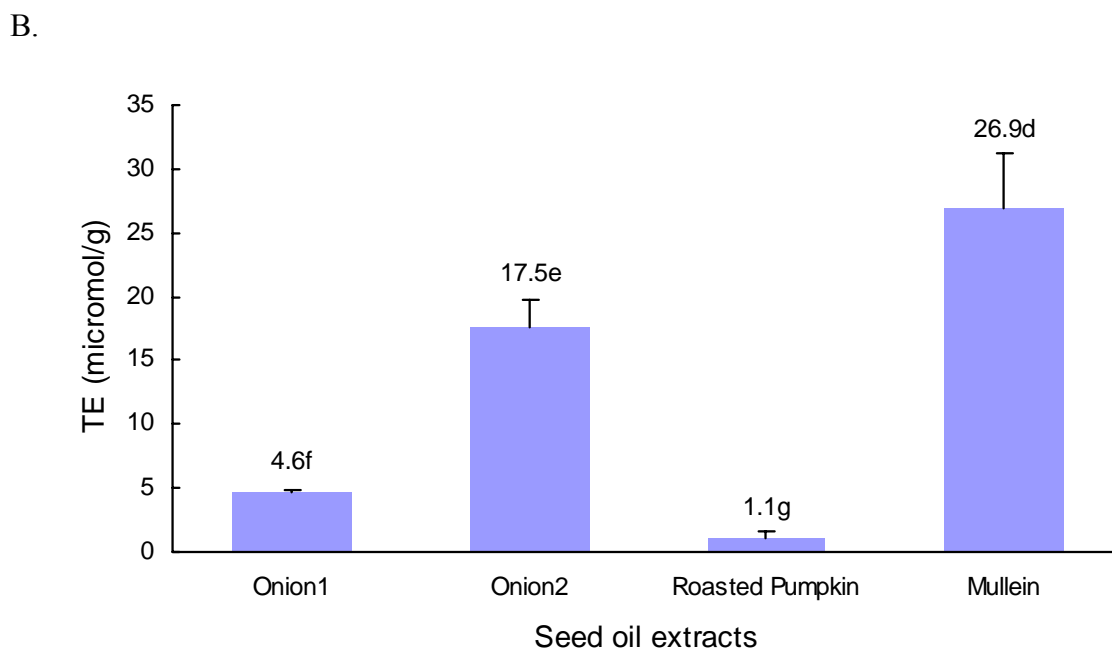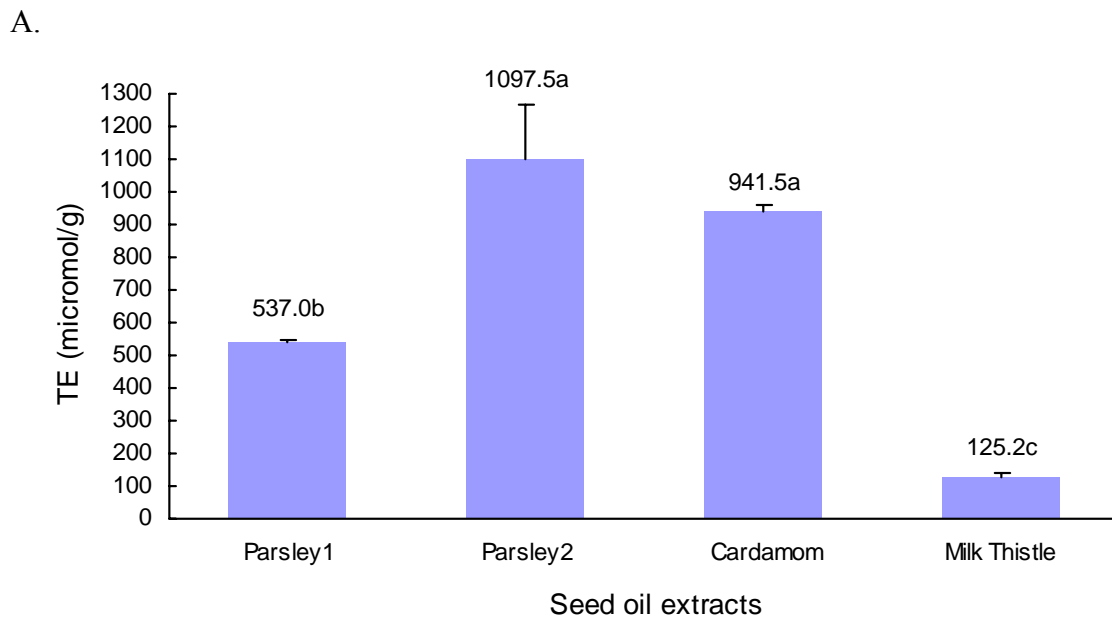A.

B.

Figure 4.2. ORAC values of the cold-pressed seed oils. TE (micromol/g) denotes

μmol of trolox equivalents per g oil. T-bars represent the standard deviation (n = 3).

Different letters indicate significant difference ($P < 0.05$).

<u>DPPH• Scavenging Capacity</u>

The cold-pressed seed oils differed in their DPPH• scavenging abilities, although all directly reacted with and quenched the free DPPH• in the reaction mixtures (**Figure 4.3**). Parsley seed oils exhibited the strongest DPPH• scavenging capacity, and quenched 87-91% radicals in the reaction mixtures after 10 minutes (**Figure 4.3**).The onion seed oil extract had the next strongest DPPH• scavenging activity followed by the cardamom, roasted pumpkin, and milk thistle seed oil extracts. Also, all of the tested cold-pressed seed oils reacted with DPPH• in a dose and time dependent manner. The time and dose effects of the onion1 and roasted pumpkin seed oil extracts against DPPH• are shown in **Figure 4.4A** and **Figure 4.4B**, respectively. In contrast to the previous observations by Parry et al. (*2*), it was noted in the present study that a high ORAC value of a seed oil extract might not guarantee a higher DPPH• scavenging capacity. For instance, the onion seed oil extracts had the second strongest DPPH• scavenging capacity, but the second lowest ORAC among the tested seed oils (**Figure 4.2**). This observation indicates potential influence(s) of the free radical system on antioxidant activity estimation, because different chemical mechanisms may be involved in the individual radical-antioxidant reactions. In the ORAC assay, antioxidants compete against the fluorescent probe for peroxyl radicals generated from the radical initiator (AAPH) and the prevention of fluorescence decrease is measured and used to calculate the relative radical scavenging activity of a potential antioxidant sample. Chemicals capable of directly interacting with the fluorescent probe and radical initiator may alter the ORAC values in either direction. On the other hand, DPPH radical scavenging capacity assay measures the reduction

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of the radical in the assay mixture. In addition to the radical scavenging agents, color

background from the potential antioxidant chemicals or the products generated from

the antioxidant-radical reaction may alter the DPPH$^\bullet$ scavenging capacity estimation

by influencing absorbance readings. It is also well accepted that antioxidant

compounds with different chemical structures may have different activities against

different free radicals due to their electronic and steric interactions. To fully

understand why the estimated antioxidant activities may vary among different radical

systems, it may be necessary to identify the individual compounds that are

significantly involved in the different antioxidant/radical reactions.
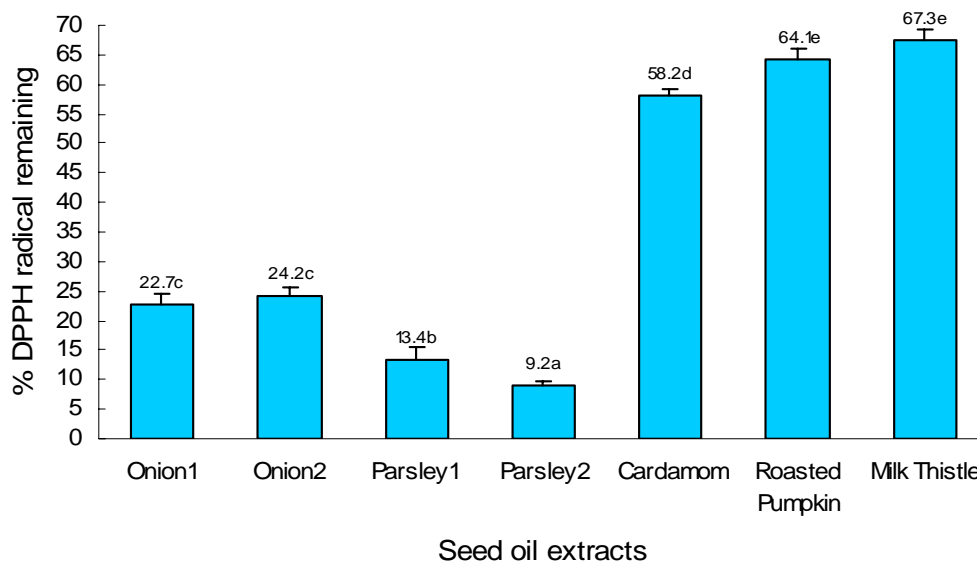
Figure 4.3. DPPH radical scavenging properties of the cold-pressed seed oils. The

initial DPPH$^\bullet$ radical concentration was 100 µM in all reaction mixtures, while the

final concentration of the cold-pressed seed oil extracts was 40 mg oil equivalent per

mL. Measurements were taken at 10 min of reaction. T-bars represent the standard

deviation (n = 3). Different letters represent significant difference ($P < 0.05$)

A. Onion seed oil
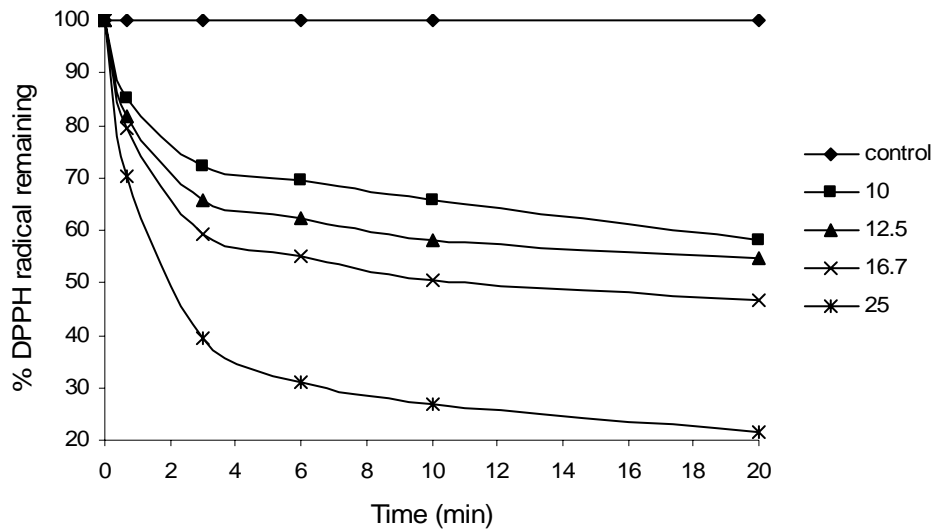
B. Roasted pumpkin seed oil

Figure 4.4. Kinetic and dose effects of oil extract-DPPH• reactions. Control, 10, 12.5, 16.7, and 25 represent mg seed oil equivalents per mL in initial reaction mixture extracted with 100% MeOH. 4A) Reactions with onion seed oil extract, B) Reactions with roasted pumpkin seed oil extract.

Oxidative Stability Index (OSI) and Physicochemical Properties

The OSI determines the oxidative stability of an oil or fat sample, and higher OSI values are associated with longer shelf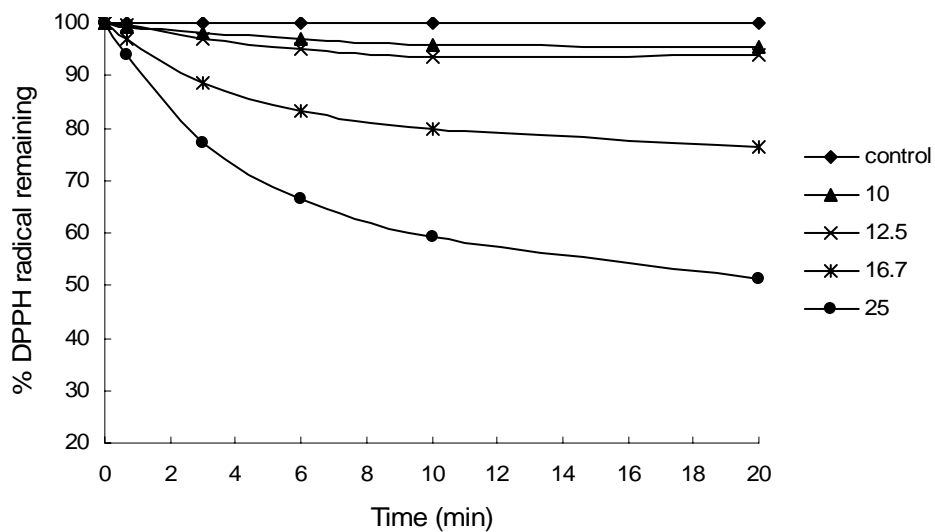 life. The OSI values for the cold-pressed parsley and cardamom seed oils were not measurable under the experimental conditions possibly due to very high levels of volatile components (**Table 4.4**). The other tested cold-pressed seed oils differed in their OSI values. The cold-pressed roasted pumpkin seed oil demonstrated the highest measurable OSI value of 61.7 h, and was followed by the cold-pressed mullein seed oil (**Table 4.4**). The OSI value of the roasted pumpkin seed oil was comparable to the OSI value of commercial corn oil, whereas the OSI value of the mullein seed oil was similar to that of commercial soybean oil (**Table 4.4**). The cold-pressed milk thistle seed oil had the lowest OSI value of 13.3 h. Interestingly, the OSI value of onion1 oil was nearly double the value of onion2 oil (**Table 4.4**) possibly indicating differences of seed quality on oil OSI.

Refractive index and density of the cold-pressed seed oils are listed in (**Table 4.4**). The refractive index values ranged from $1.4335_D{}^{25}$ to $1.4862_D{}^{25}$ for the milk thistle and parsley2 samples, respectively. The two onion seed oils or the two parsley seed oils exhibited the same or close refractive index values. The density of the cold-pressed seed oil samples ranged 0.920-0.985 g/mL.

Color is another characteristic that is important for determining visual acceptance of oil. The Hunter L-, a-, and b- values of the cold-pressed seed oils are shown in **Table 4.5**. The 'L' value is the lightness of a sample from 0 to 100 with 100 being pure white. The 'a' value describes red (+) to green (-). The 'b' value represents

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yellow (+) to blue (-). Zero values for 'a' and 'b' values represent gray. The tested seed oils differed in their colors. The cold-pressed milk thistle seed oil was the lightest within the group and had the most yellowness.

In summary, cold-pressed parsley seed oil contained 81% oleic acid making it an excellent choice for consumers desiring a dietary oil rich in monounsaturated fat. Cold-pressed parsley seed oil is also rich in free radical scavenging agents, and may serve as a dietary source of natural antioxidants for health promotion and disease prevention. Cold-pressed onion seed oil is rich in α- and total tocopherols, whereas cold-pressed roasted pumpkin seed oil contained 71 µmoles total carotenoid and 21 mg zeaxanthin per kg of oil. In addition, cold-pressed onion, milk thistle, parsley, cardamom, mullein, and roasted pumpkin seed oils contain significant levels of total phenolic compounds. These data suggest the potential food application of these oils in improving human nutrition.

Table 4.4. Oxidative stability index, refractive index, and density of the cold-pressed seed oils[*]

	OSI (h)	Refractive Index (nD25)	Density (g/mL)
Onion1	31.4c ± 1.41	1.4752	0.930
Onion2	16.9d ± 0.37	1.4752	0.923
Parsley1	> 369.4	1.4858	0.985
Parsley2	> 148.4	1.4862	0.981
Cardamom	> 63.5	1.4666	0.954
Mullein	47.8b ± 1.1	1.4753	0.933
Pumpkin	61.7a ± 2.1	1.4721	0.920
Milk thistle	13.3e ± 0.3	1.4335	0.921
Soybean oil	46.8 ± 0.4	NA	NA
Corn oil	66.0 ± 0.4	NA	NA

[*]Data were reported as mean ± SD (n = 3). Different letters within columns represent significant difference ($P < 0.05$). NA: not available.

Table 4.5. HunterLab color measurements of the cold-pressed seed oils [*]

	L value	a value	b value
Onion1	3.50c ± 0.02	-1.38b ± 0.43	3.90c ± 0.26
Onion2	3.17d ± 0.07	-1.26bc ± 0.37	2.96d ± 0.12
Parsley1	1.96e ± 0.08	2.88a ± 0.22	1.92e ± 0.15
Parsley2	1.22f ± 0.04	2.70a ± 0.25	1.44f ± 0.14
Cardamom	6.03b ± 0.15	-0.63c ± 0.27	7.44b ± 0.31
Mullein	2.07e ± 0.05	2.30a ± 0.16	3.05d ± 0.12
Pumpkin	0.70g ± 0.11	2.60a ± 0.16	0.78g ± 0.10
Milk thistle	10.64a ± 0.19	-1.73b ± 0.85	11.60a ± 0.32

[*]Data were reported as mean ± SD (n = 3). Color measurement parameters: D65/ 10° illuminant/ observer, 'L' measure of the lightness increasing from 0 (dark) to 100 (light), 'a' measure of red (+) to green (-), 'b' measure of yellow (+) to blue (-).

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Chapter 5: Chemical Composition, Antioxidant Properties, and Antiproliferative Activity of Pumpkin, Parsley, Mullein, Cardamom, and Milk Thistle Seed Flours

Abstract

Cold-pressed seed flours from pumpkin, parsley, mullein, cardamom, and milk thistle were tested for total fat, fatty acid profile, total phenolic content (TPC), radical scavenging activities using the oxygen radical absorbance capacity (ORAC), the hydroxyl radical scavenging capacity (HOSC) and the relative DPPH$^\bullet$ scavenging capacity (RDSC) assays, and antiproliferative activities against the HT-29 human colon cancer cell line. The cold-pressed parsley seed flour contained a very high concentration of total fat at 17.6 g/100 g flour which consisted primarily of oleic acid at 86.2 g/100 g oil. Saturated fatty acids were found in high concentrations in all seed flours except that of the parsley. The mullein seed flours contained 39.0-62.1 g saturated fat per 100 g oil. All seed flours demonstrated significant TPC and antioxidant activities. Milk thistle seed flour had the highest TPC of 25.0 mg gallic acid equivalent per g flour (GAE mg/g) followed by that of parsley at 8.1 GAE mg/g. Milk thistle seed flour extract also had significantly higher antioxidant activities compared to the other extracts in all tested radical systems and all values for milk thistle were at least 2.5 times higher than the second highest value. The milk thistle extract had an ORAC value of 1131 μmol trolox equivalents (TE) per g flour (TE μmol/g), a HOSC value of 893 TE μmol/g, and an RDSC value of 61 TE μmol/g. Also, ORAC, HOSC, and TPC values were all significantly correlated ($P < 0.01$).

The milk thistle seed flour significantly inhibited the growth of HT-29 colon cancer

These data suggest that these seed flours may serve to significantly enhance food

products with natural sources of antioxidants, and components in milk thistle seed

flour need to be further investigated further for mechanisms explaining

antiproliferative activity.

Introduction

The results of numerous studies have demonstrated evidence that certain food

components may reduce the risk of chronic diseases and improve human health.

Novel food ingredients rich in beneficial components including natural antioxidants

are in high demand for improving food quality and optimizing human health. Seeds

are byproducts from processing, and seed flour is the primary byproduct from seed oil

production. Seed flour has been shown to contain many health beneficial components

such as special fatty acids, natural antioxidants, vitamins, and other properties.

Previous studies have demonstrated that fruit seeds contain dietary essential fatty

acids including α-linolenic acid (18:3n-3). Alpha-linolenic acid may be converted *in*

vivo to eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA). EPA and

DHA which may have protective effects against several chronic diseases including

heart disease, stroke, and cancer(*1-4*). Recently, cold-pressed black raspberry (*5*) and

cranberry (*6*) seed flours, were examined for their fatty acid profiles and α-linolenic

acid was determined at 33.2 and 30.9 g/100 g oil, respectively. Seed flours may also

contain significant natural antioxidants. Seven fruit seed flours extracted using 50%

acetone demonstrated significant radical scavenging activities using DPPH$^\bullet$ and

ORAC assays (*6*) and the tested fruit flour extracts also had strong antiproliferative activities against HT-29 human cancer cell line with chardonnay grape seed flour completely killing all cells after 24 h (*6*). Other important components found in seeds include tocopherols and carotenoids, including α-tocopherol and the provitamin A, β-carotene (*7*). These data suggest that seed flours rich in health beneficial factors may be used as novel food ingredients for improving human diets, and also enhancing profits of food production and processing industries. Continuing research is required to investigate seed flours for their contents of health beneficial factors to promote their value-added utilization as healthy food ingredients.

The purpose of the current study was to investigate the selected cold-pressed seed flours including roasted pumpkin (*Curcubita, pepo* L. 'Triple Treat'), parsley (*Petroselinum crispum*), mullein (*Verbascum thapsus*), cardamom (*Elettaria cardamomum*), and milk thistle (*Silybum marianum*) for total fat content, fatty acid profile, total phenolic content (TPC), antioxidant activities using the oxygen radical absorbance capacity (ORAC) assay, hydroxyl radical scavenging capacity (HOSC) assay, relative DPPH$^\bullet$ scavenging capacity (RDSC) assay, and antiproliferative effects against HT-29 human colon cancer cells.

Materials and Methods

<u>Materials</u>

Cold-pressed roasted pumpkin, parsley, mullein, cardamom, and milk thistle seed flours were obtained from Botanic Oil Innovations, Inc., Spooner WI. Gallic

acid, 2,2- ethylenediaminetetraacetate (EDTA), Diphenyl-1-picrylhydrazyl radical (DPPH•), sodium acetate, potassium chloride, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid were purchased from Sigma-Aldrich (St. Louis, MO). Randomly methylated β-cyclodextrin (RMCD) was purchased from Cyclolab R & D Ltd. (Budapest, Hungary), and 2,2'-azobis (2-amino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA). Disposable cell culture ware was purchased from Corning Glass Works (Corning, NY). McCoy's 5A Medium Modified with L-glutamine, antibiotic/antimycotic, fetal bovine serum (FBS), 0.25 % trypsin with 0.9 mM EDTA were purchased from Invitrogen (Carlsbad, CA), and HT-29 human colorectal adenocarcinoma cancer cells were purchased from American Type Culture Collection (Rockville, MD). ATPlite 1step Luminescence ATP Detection Assay System was purchased from PerkinElmer Life and Analytical Sciences, Boston, MA. All other chemicals and solvents were of the highest commercial grade and used without further purification.

Extractions

The cold-pressed seed flours were extracted for total fat content and fatty acid profiles using a Soxhlet apparatus and hexane as the extracting solvent. Flours were extracted with 50% acetone at 10 mL per g flour for total phenolic content TPC, oxygen radical absorbance capacity ORAC, HOSC, and RDSC. For cancer cell growth inhibition, the 50% acetone extracting solvent was evaporated and the residual solids were re-suspended in 50% DMSO at a final concentration of 1 g flour equivalent per 2 mL 50% DMSO. All samples were stored in the dark under nitrogen

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until analyzed.

Total Phenolic Content (TPC)

TPC of the seed flour extracts was determined using Folin and Ciocalteu's (FC) reagent following the previously described method (8). The FC reagent was freshly prepared, and the final reaction mixture contained 250 µL FC reagent, 750 µL 20% Na_2CO_3, 50 µL of seed flour extract or standard, and 3 mL H_2O. Absorbance was determined at 765 nm following two hours of reaction at ambient temperature. Gallic acid was used as the standard. Measurements were taken in triplicate.

Fatty Acid Composition

Fatty acid methyl esters were prepared from the hexane extracted oils using the previously described method (9). Fatty acid compositions were analyzed by a Shimadzu GC-2010 with a FID and a Shimadzu AOC-20i autosampler (Shimadzu, Columbia, MD). A Supelco 2380 column, 30 m × 0.25 mm i.d. with a 0.20 µm film thickness (Supelco Inc., Bellefonte, PA), was used with helium as the carrier gas at a flow rate of 0.8 mL/min. Injection volume was 1 µL at a split ratio of 10/1. Time and temperature ramps began with an initial oven temperature of 142 ºC increasing 6 ºC/min to 184 ºC, held for 3 min, and then increased 6 ºC/min to 244 ºC (7). Fatty acids were determined by comparing GC retention time with that of the authorized pure individual commercial compounds.

Oxygen Radical Absorbance Capacity (ORAC)

ORAC values for the 50 % acetone seed flours were examined using a Victor[3]

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multilabel plate reader (PerkinElmer, Turku, Finland) following a method previously described (10). Fluorescein was used as the fluorescent probe. The final assay mixture contained 0.067 μM fluorescein, 60 mM AAPH, 300 μL reagent blank, standard, or flour extract. Fluorescence measurements were recorded at 485 nm excitation 515 nm emission every minute, and the area under the curve of fluorescence *vs.* time plot was calculated and compared against a standard curve prepared with trolox. ORAC value was expressed as μmol trolox equivalents (TE) per g seed flour (TE μmol/g). Experiments were conducted in triplicate.

Hydroxyl Radical Scavenging Capacity (HOSC)

Hydroxyl radical scavenging activities were investigated using the high throughput method described by Moore et al, 2006 (11). Analyses were conducted in black polystyrene 96 well plates (FluroNunc) with a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). Fluorescence measurements were determined at 485 nm excitation and 535 nm emission. Hydroxyl radicals in the reaction are generated by a Fenton-like reaction using H_2O_2 and Fe^{3+}, and trolox was used to prepare the standard curve. The assay contained 170 μL 9.28 x 10^{-8} M fluorescein in 75 mM sodium phosphate buffer at pH 7.4, 30 μL blank, standard or extract, 40 μL 0.1990 M H_2O_2, and 3.43 mM $FeCl_3$. All tests were conducted in triplicate.

Relative DPPH$^\bullet$ Scavenging Capacity (RDSC)

The RDSC of the seed flour extracts were obtained using the high throughput assay described by Cheng et al, 2006 (12). A Victor^3 multilabel plate reader

(PerkinElmer, Turku, Finland) was used for assay determination using 96 well plates. The reaction mixtures contained 100 µL 0.2 M DPPH$^\bullet$ and 100 µL standards, control, blank, or sample. Absorbance readings were determined at 515 nm. The standard curve was derived from the area under the curve from different concentrations of trolox. Trolox equivalents (TE) were obtained by area under the curve for the sample. Measurements were conducted in triplicate.

HT-29 Cancer Cell Proliferation

HT-29 human adenocarcinoma colorectal cells characterized by Fogh (*13*) were propagated in T-150 flasks. Cell culture media included Mcoy's 5A media with 10 % FBS, and 1% antibiotic/antimycotic. Flasks were incubated at 37 °C in a humidified atmosphere at 5% CO_2 (*6*).

Cell proliferation was investigated using an ATP luminescence kit. The test is based on the assumption that ATP concentration is linearly correlated to cell number. Relative cell numbers are determine by the ATP dependent reaction with luciferin by the luciferase enzyme that produces light upon ATP hydrolysis (*14, 15*). Cells were added to 96 well plates at 2,500 cells per well then incubated for 24 hours prior to treatment. Cells were treated with control or the seed flour extracts in 50% DMSO at 3 and 6 mg flour equivalents per mL media final concentration. All wells contained concentrations of 6 µL DMSO vehicle per mL. Luminescence measurements were taken every 24 h for 4 days. All tests were conducted in triplicate.

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Data was analyzed using SPSS (SPSS for Windows, Version Rel. 10.0.5.,
1999, SPSS Inc., Chicago, IL). Data were reported as mean ± standard deviation (n =
3). Analysis of variance and Tukey's post hoc analysis were used to determine
differences among means. Pearson Correlation Coefficient was used to determine
correlations among means. Significance was declared at $P < 0.05$.

Results and Discussion

Total Phenolic Content (TPC)

TPC was investigated because phenolic compounds found in edible seed
flours have previously demonstrated powerful antioxidant activities and may be major
contributors to the overall antioxidant activities of foods. The TPC of the tested seed
flours were in a range from 1.65 to 25.02 mg gallic acid equivalents (GAE) per g
flour (GAE mg/g) (**Table 5.1**). The milk thistle seed flour had the highest TPC value
and was more than 3 times higher than the parsley which had the next highest value
of 8.11 GAE mg/g flour. The TPC of the two mullein samples were significantly
different from each other with mullein1 having 4.76 GAE mg/g compared to mullein2
that contained 4.14 GAE mg/g **Table 5.1**.

TPC has recently been examined in pumpkin, cardamom and parsley. In 2006,
Kosinska and Karamacexamined the TPC of roasted pumpkin seed flour and found it
contained 0.91 GAE mg/g flour (*16*). The TPC of the water soluble seed extract of
cardamom contained 24.2 GAE mg/g, and the whole parsley herb contained 29.2

GAE mg/g (*17*).

<u>Fatty Acid Composition</u>

The total fat content of the flours extracted using Soxhlet with hexane solvent was highest in the parsley seed flour at 17.6 g fat/100 g flour and was lowest in the cardamom seed flour that contained 0.7 g fat/100 g flour (**Table 5.1**).

All of the tested seed flour fats had relatively high levels of oleic acid that ranged from 36.5 to 86.2 g/100 g oil from the mullein2 and parsley seed flours, respectively, and the fatty acid content from the parsley seed flour was highly unsaturated at 96.1% of the total fatty acids (**Table 5.2**). Saturated fatty acid profiles of all other seed flours were relatively high and were primarily composed of stearic and palmitic acids. The mullein2, cardamom, and milk thistle seed flour oils had saturated fatty acid contents of 62.1, 56.2, and 54.8 g/100 g oil, respectively. The fatty acid profiles of the two mullein seed flour samples were significantly different between palmitic acid, stearic acid, oleic acid, and particularly, linoleic acid. Mullein1 had approximately 28.8 times more linoleic acid on a per weight basis compared to the mullein2 (**Table 5.2**).

A previous investigation of the fatty acid composition of parsley seed oil detected 81.9 % oleic acid and approximately 95% unsaturated fatty acids (*18*), which was similar to the current investigation. Pumpkin seeds fatty acid compositions have been investigated intensively and have reported a wide range of fatty acid compositions. The predominating fatty acid reported in the pumpkin seeds has been linoleic acid; however not in all studies. The range of linoleic acid in pumpkin seed

oil has varied greatly from 4.9 to 58.9% of total fatty acids (*19-24*). In a study by

Spangenberg and Ogrinc (*23*), the predominating fatty acid in pumpkin seed was

palmitic acid at 49.2%, while Glew et al, 2006 (*19*) found oleic acid highest in

pumpkin comprising 45.4% of the total fatty acids. Mullein seed has recently been

investigated for fatty acid composition (*25*). The resulting fatty acid profiles of

mullein seed were very different compared to the seed flours from our study. Their

investigation found the fatty acid composition of mullein seed to primarily consist of

linoleic acid at 77.1%, oleic acid at 10.5%, and palmitic acid at 6.8%. A 1995 study

of cardamom investigated the fatty acid profiles of two varieties (*26*). The fatty acid

compositions between the two varieties were very similar with little variation. Oleic

acid was the most prevalent at 43.1-44.1% followed by linoleic acid at 21.4-22.1%,

palmitic acid at 20.8-21.2%, and linolenic acid at 7.8%. Differences in fatty acid

profiles among same species of plant is commonly reported which may be partially

explained by genetics, harvest time, and growing conditions – primarily temperature.

Table 5.1. Phytochemical compositions of tested edible seed flours*

	TPC (GAE mg/g)	Total Fat (g/100g)
Pumpkin	$1.65e \pm 0.014$	12.3
Parsley	$8.11b \pm 0.226$	17.6
Mullein1	$4.76c \pm 0.014$	12.4
Mullein2	$4.14d \pm 0.060$	11.0
Cardamom	$1.91e \pm 0.088$	0.7
Milk Thistle	$25.02a \pm 0.585$	7.5

*Mullein1 and Mullein2 are 2 different samples from the same variety. TPC is the total phenolic content in the respective fruit seed flours and is measured as mg of gallic acid equivalents (GAE) in mg per gram flour (GAE mg/g). Total fat is expressed as g fat per 100 g flour (g/100g). Values in columns with different letters are significantly different ($P < 0.05$).

Table 5.2. Fatty acid (FA) profiles of the cold-pressed seed flours (g/100 g oil)*

FA	Pumpkin	Parsley	Mullein1	Mullein2	Cardamom	Milk Thistle
12:0	0.2 ± 0.00	t	nd	nd	nd	nd
14:0	0.2 ± 0.06	nd	t	0.1 ± 0.02	1.0 ± 0.08	0.5 ± 0.41
16:0	23.4 ± 0.01	2.8 ± 0.00	21.0 ± 0.02	28.1 ± 0.05	47.6 ± 0.10	27.4 ± 0.11
16:1	0.1 ± 0.01	0.1 ± 0.00	0.4 ± 0.01	0.3 ± 0.04	1.5 ± 0.02	0.2 ± 0.04
18:0	20.7 ± 0.02	1.0 ± 0.01	15.6 ± 0.05	30.8 ± 0.01	6.6 ± 0.04	17.7 ± 0.08
18:1	53.1 ± 0.04	86.2 ± 0.01	42.5 ± 0.07	36.5 ± 0.06	40.6 ± 0.02	37.6 ± 0.15
18:2	2.3 ± 0.06	9.4 ± 0.00	17.3 ± 0.02	0.6 ± 0.02	0.7 ± 0.02	4.6 ± 0.02
18:3	nd	0.4 ± 0.01	nd	nd	nd	nd
20:0	nd	0.1 ± 0.00	2.3 ± 0.04	3.1 ± 0.02	1.0 ± 0.04	9.3 ± 0.02
20:1	nd	t	1.0 ± 0.02	0.5 ± 0.03	0.9 ± 0.02	2.8 ± 0.23
SFA	44.5	3.9	39.0	62.1	56.2	54.8
MUFA	53.2	86.3	43.8	37.3	43.1	40.6
PUFA	2.3	9.8	17.3	0.6	0.7	4.6

*Data were expressed as mean ± SD (n = 3). Mullein1 and Mullein2 are two different samples of the same variety. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. nd: not detected. t: trace.

Oxygen Radical Absorbing Capacity (ORAC)

Antioxidant activities of the seed flour extracts were estimated using the ORAC assay. All of the seed flours demonstrated significant radical scavenging activities. The ORAC values of the flours ranged from 35.3 to 1130.7 TE μmol/g from the cardamom and milk thistle seed flours, respectively (**Table 5.3**). The ORAC values of the two extracts of mullein seed flour were significantly different from each other. Mullein1 had a higher ORAC value of 127.3 compared to 98.2 for Mullein2

(**Table 5.3).**

Table 5.3. Antioxidant activities of selected cold-pressed edible seed flours

	ORAC (TE μmol/g)	HOSC (TE μmol/g)	RDSC (TE μmol/g)
Pumpkin	37.6e ± 0.43	22.2d ± 2.07	2.2e ± 0.02
Parsley	390.0 b± 15.90	311.5b ± 20.97	18.1d ± 1.47
Mullein1	127.3c ± 10.09	74.3c ± 3.64	24.0b ± 1.29
Mullein2	98.2d ± 4.07	75.3c ± 3.44	21.2c ± 1.32
Cardamom	35.3e ± 2.08	22.6d ± 1.95	19.5cd ± 0.63
Milk Thistle	1130.7a ± 31.33	893.0a ± 62.42	61.1a ± 0.30

[a]ORAC is the oxygen radical absorbance capacity; HOSC is the hydroxyl radical scavenging capacity, and RDSC is the relative DPPH• scavenging capacity. All are expressed as trolox equivalents (TE) μmol per g seed flour (TE μmol/g). Values in the same column with different letters are significantly different ($P < 0.05$).

Hydroxyl Radical Scavenging Capacity (HOSC)

HOSC values were determined against a standard curve prepared with trolox. Milk thistle seed flour had the highest HOSC at 893.0 TE μmol/g and was followed by parsley, mullein, cardamom, and pumpkin. HOSC values were significantly correlated to both ORAC and TPC but not RSDC. (**Table 5.3).**

Relative DPPH Scavenging Capacity (RDSC)

Antioxidant activity against DPPH• was also evaluated against a trolox

standard curve and reported in trolox equivalents (TE) μmol per g flour (TE μmol/g). The milk thistle seed flour extract had an RDSC value of 61.1 TE μmol/g, and similar to the other antioxidant tests, had a significantly higher antioxidant activity compared to all other samples (**Table 5.3**). The mullein1 sample had a significantly higher RDSC value than mullein2, which was consistent with the results of the ORAC and TPC assays, but a difference was not seen against the hydroxyl radical.

HT-29 Cell Proliferation

Effects of cancer cell growth were determined for all of the seed flour extracts at 3 and 6 mg/ mL media final concentration. The results at 6 mg/ mL following 48 h of treatment are shown in **Figure 5.1** and significant results over 3 days of treatment are shown in **Figure 5.2**. The parsley and milk thistle seed flour extracts significantly inhibited cell growth compared to control, and the milk thistle extract demonstrated the strongest antiproliferative effects. The milk thistle flour extract was also dose dependent throughout the 4 day experiment. Differences in morphology of the HT-29 cells following 4 days of treatment as control and milk thistle seed flour extract at 6 mg per mL media are depicted in **Figure 5.3**. The granulated appearance and blebbing of the plasma membrane of the milk thistle treated cells are indicative of cell death. The results for the milk thistle seed flour on cancer cell growth inhibition are consistent with previous investigations on specific bioactive components in milk thistle. The bioactive components in milk thistle are antioxidant polyphenolic compounds called silymarins, and the predominating silimarin is silibinin. Several prior studies have demonstrated these milk thistle components to have

antiproliferative effects in several different cancer cell lines at the molecular level (*27-42*). Antiproliferative effects of milk thistle seed components have been demonstrated in prostate (*27-32*), bladder (*33-35*); colon (*36, 37*), liver (*38-41*), and breast (*42*) cancer cell models by several mechanisms including cell cycle arrest, decreases in CDK and cyclins, and increasing apoptosis. However, one recent study on mammary carcinogenesis in rat and mouse models demonstrated increases of mammary tumors from silymarin treatment compared to control (*43*). It was interesting to note that the pumpkin seed flour extract had a positive proliferative effect and was significantly higher than control on day 3 and day 4.
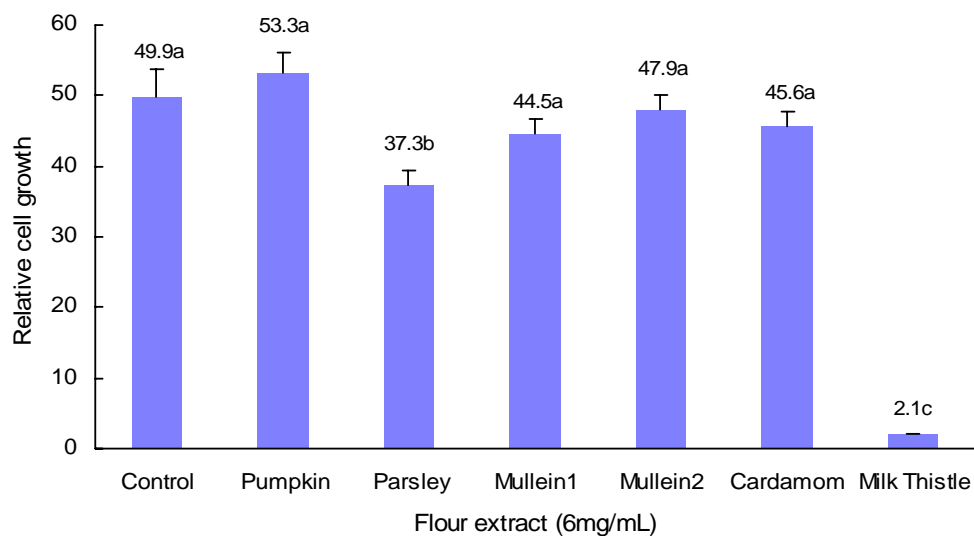

Figure 5.1. Relative growth of HT-29 cells . Cells were treated with 6 mg equivalent/mL seed flour extract and counted after 48 h of treatment. Relative cell growth was determined by ATP luminescence which is directly correlated to cell number.
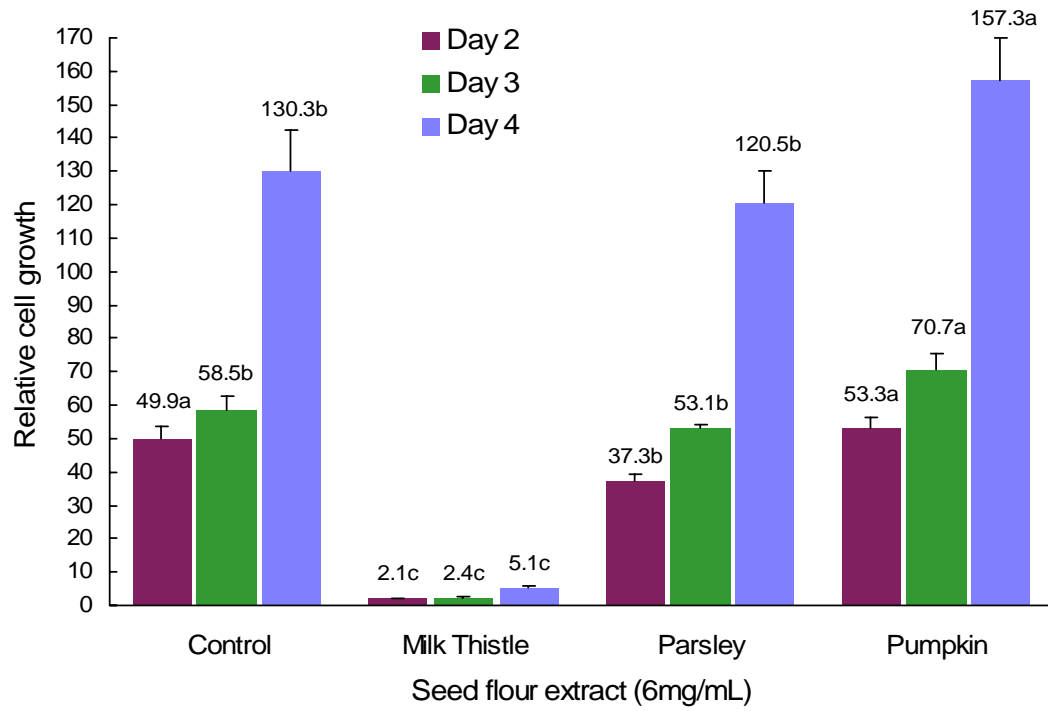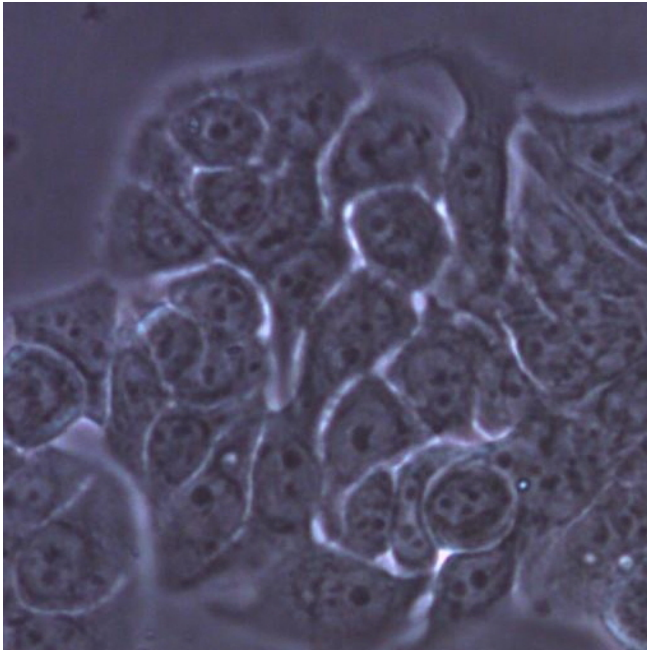
133

Figure 5.2. Relative growth of HT-29 cells. Cells were treated with 6 mg

equivalent/mL seed flour equivalents and counted on day 2 through day 4 of

treatment.

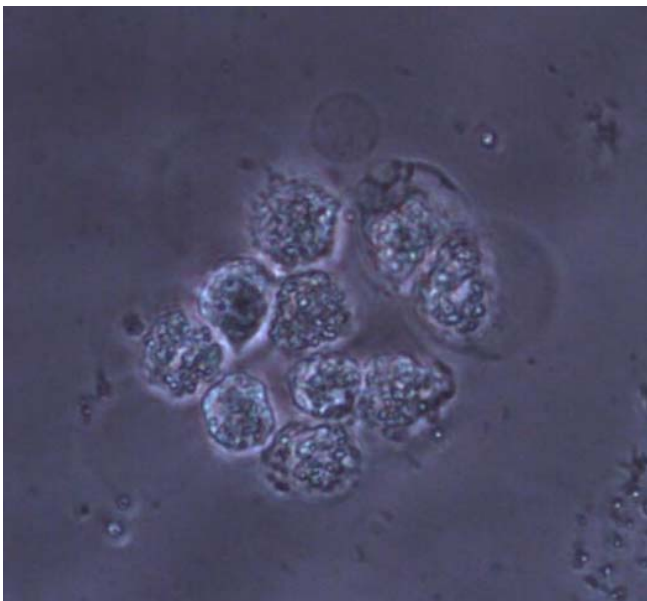**A. Control**

B. Milk Thistle

Figure 5.3. HT-29 cells (200x) following 4 days of treatment. Figure A) Control cells and Figure B) Cells treated with 6 mg/mL milk thistle seed flour extract. All wells contained 6 μL DMSO per mL media.

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Summary

In many food production operations, seeds are generally treated as waste byproducts. The primary objectives of these studies were to investigate several seeds for health beneficial components and properties that may significantly increase their value with the goals of improving human nutrition and increasing revenue for growers and producers through the dissemination of our findings.

The current investigations discovered several significant value-adding components and properties in cold-pressed seed oils and flours. The tested fruit seed oils contained significant levels of α-linolenic acid and may be used to decrease the ratio of dietary n-6 to n-3 fatty acids. Several seed oils and flours exhibited very strong antioxidant activities such as milk thistle seed oil and flour, chardonnay grape seed flour, and parsley seed oil, while pumpkin and onion seed oils contained very high levels of carotenoids and tocopherols, respectively. Also, Black raspberry, cranberry, chardonnay grape, and milk thistle seed flours significantly inhibited the proliferation of HT-29 colon cancer cells *in vitro*.

Future investigations of these seeds should include studies proposed to explain differences between compositions and properties of seed oils and flours from the same plant variety. The differences determined in these studies included compositional profiles, antioxidant properties, and physical properties which may be explained by genetic variation, time of harvest, and growing conditions such as temperature, sunlight exposure, UV exposure, water, humidity, and pollutants among other possible influences. The determination of the mechanisms behind the antiproliferation of HT-29 cancer cells from the black raspberry, cranberry, milk

thistle, and chardonnay grape seed flours including apoptosis and cell cycle analysis should be investigated. Quantitative determination of phenolic and flavonoid compositions of the seed flours should be studied, and the oils and flours should be examined as food ingredients in final functional food products for components, properties, and consumer acceptability.

These investigations had both strengths and weaknesses. Some of the strengths include that a large variety of seed oils and flours were examined and significant differences in compositions and properties were observed. Sound experiments and experimental techniques were employed and result variations were low. Also, several different antioxidant tests were performed that either restricted or reinforced antioxidant claims for seed oil and flour samples. The weaknesses of this study included the uncertainty that corresponding oil and flour samples were from the same cold-press extraction or even the same harvest, and not all tested seeds had corresponding oils and flours. Sample size was small (n = 3); however, experimental time and cost are major issues. Increasing the sample size by 1 would consequently increase the time and cost of most of the performed experiments by 25%.