ABSTRACT

Title of Dissertation:	CHEMICAL COMPOSITIONS OF SELECTED SEED FLOUR EXTRACTS AND THEIR POTENTIAL HEALTH BENEFICIAL PROPERTIES.
	Uyory Choe, Doctor of Philosophy, 2020
Dissertation directed by:	Professor Liangli (Lucy) Yu Department of Nutrition and Food Science

The rationale of the current dissertation research is to investigate health beneficial components and properties of blackberry, broccoli, carrot, cucumber, and milk thistle seed flours. This can lead to potential utilization of these seed flours in nutraceuticals and functional foods. Also, this can add value to oil manufacturers and the seed producers while reducing environmental contaminations.

Blackberry, broccoli, carrot, cucumber, and milk thistle seed flours were extracted with 50% acetone and evaluated for their phytochemical compositions along with their potential gut microbiota modulating, free radical scavenging, and anti-inflammatory and anti-proliferative capacities. Thirteen, nine, ten, and fifteen compounds were detected in the blackberry, broccoli, carrot, and milk thistle seed flour extracts, with sanguiin H6, glucoraphanin, kaempferol-3-O-rutinoside, and silychristin as the primary component of each, respectively. All five seed flour extracts enhanced total number of gut bacteria and altered the abundance of specific

bacterial phylum or genus in vitro. The blackberry seed flour extract had the greatest relative DPPH radical scavenging capacity and $ABTS^{\bullet+}$ scavenging capacity values of 362 and 267 μmol trolox equivalent (TE)/g, respectively. Milk thistle seed flour had the greatest oxygen radical absorbing capacity and hydroxyl radical (HO•) scavenging capacity values of 634 and 10420 μmol trolox equivalent (TE)/g, respectively. Also, all five seed flour extracts suppressed LPS induced IL-1β mRNA expressions in J774A.1 cells and the proliferation of LNCaP prostate cancer cell line. The results might be used to promote the value-added food utilization of these seed flours in improving human health.

CHEMICAL COMPOSITIONS OF SELECTED SEED FLOUR EXTRACTS AND THEIR POTENTIAL HEALTH BENEFICIAL PROPERTIES.

by

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Introduction

Vegetable, fruit, and herb seed flours are by-products from the manufacture of the seed oils. Investigation of their health beneficial components and properties can lead to potential utilization of these seed flours in nutraceuticals and functional foods and add value to oil manufacturers and the seed producers while reducing environmental contaminations.

Previous studies have shown that fruit, vegetable and spice seeds flours such as red raspberry, cranberry, grape, parsley, mullein, and milk thistle seed flours are rich in tocopherols, carotenoids, polyphenols, and possess free radical scavenging, anti-proliferative, and anti-inflammatory capacities.

Even though health beneficial fat-soluble components are extracted by oils, literature suggests that seed flours have potential health beneficial components and properties. For example, carrot and broccoli seed flours were reported to contain health-promoting phytochemicals such as polyphenols and glucosinolates.

In addition, with more and more interest in gut microbiota and human health, it is interesting whether and how blackberry, broccoli, carrot, cucumber, and milk thistle seeds and their components such as seed flours may alter gut microbiota.

The goal of this dissertation research is to investigate health-beneficial components and properties of blackberry, broccoli, carrot, cucumber, and milk thistle seed flours for potential utilization of these seed flours in nutraceuticals and functional foods. The specific objectives of this research are:

1. To identify chemical compositions of the selected cold-pressed seed flours.
2. To investigate the interaction between cold-pressed seed flours and gut microbiota.
3. To evaluate *in vitro* free radical scavenging capacities, anti-inflammatory properties, and anti-proliferative activities of the cold-pressed seed flours.

In the current research, thirteen, nine, ten, and fifteen compounds were detected in the blackberry, broccoli, carrot, and milk thistle seed flour extracts, with sanguiin H6, glucoraphanin, kaempferol-3-O-rutinoside, and silychristin as the primary component of each, respectively. All five seed flour extracts enhanced total number of gut bacteria and altered the abundance of specific bacterial phylum or genus *in vitro*. The blackberry seed flour extract had the greatest relative DPPH radical scavenging capacity and $ABTS^{\bullet+}$ scavenging capacity values of 362 and 267 μmol trolox equivalent (TE)/g, respectively. Milk thistle seed flour had the greatest oxygen radical absorbing capacity (ORAC) and hydroxyl radical (HO•) scavenging capacity values of 634 and 10420 μmol trolox equivalent (TE)/g, respectively. Also, all five seed flour extracts suppressed LPS induced IL-1β mRNA expressions in J774A.1 macrophage cells and the proliferation of LNCaP prostate cancer cells. The results might be used to promote the value-added food utilization of these seed flours in reducing the aging-associated chronic human diseases.

Chapter 1: Literature review

Liangli (Lucy) Yu, Uyory Choe, Yanfang Li, & Yaqiong Zhang, Oils from Fruit, Spice, and Herb Seeds.
In *Bailey's Industrial Oil and Fat Products* (7th ed.). **2020**, John Wiley & Sons, Ltd.
Yanbei Wu, Jiawei Wan, Uyory Choe, Quynhchi Pham, Norberta W. Schoene, Qiang He, Bin Li, Liangli Yu, & Thomas T. Y. Wang, Interaction Between Food and Gut Microbiota: Impact on Human Health.
Ann. Rev. Food Sci. T. **2019**, *10* (1), 389–408.

Specialty edible oils have been produced from fruit, vegetable, and spice seeds for value-added commercial production of the special oils rich in beneficial phytochemicals and/or special fatty acids such as 18:3n-3. Seed flours are by-products of oil production. Science-based development of value-added utilization of the seed flours may enhance the profitability of agricultural and food industries while reducing the potential environmental contamination. For example, in recent years, grape seed flours became commercially available and widely used for baking products. This commercialization and utilization were possible because of the recent study that has shown health beneficial effects such as reducing plasma cholesterol levels and abdominal fat content of grape seed flour. Similarly, other edible seed flours from fruit, vegetable, and spice may have potential health benefits. However, due to limited research, many seed flours are wasted at a cost. In this chapter, specialty oils and flours, and their potential health beneficial components and properties have been reviewed.

1.1 Specialty fats and oils are commercially needed

Edible seed oils have been used as food ingredients since ancient times (Parry, Su, Luther, Zhou, Yurawecz, Whittaker, et al., 2005). Today, edible seed oils are from crops including safflower, linseed, cotton, peanut, sunflower, corn, and soybean are grown exclusively, or in large part, for the oil in their seeds (Parry, Su, Luther, Zhou, Yurawecz, Whittaker, et al., 2005). 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.

1.2. Fruit, vegetable, and spice seed oils

Today, consumers' growing interest in improving their dietary nutrition is driving the development of novel seed oils with unique fatty acid profiles and other beneficial components including phytosterols and natural antioxidants. Fruit, vegetable, and spice seed oils are unique in their fatty acid profiles compared to crop seed oils. Also, some fruit, vegetable, or spice seed oils containing only small amounts of beneficial fatty acids but significant amounts of other valuable components such as antioxidants.

Fatty acid profiles of many fruit, vegetable, and spice seed oils have been reviewed (Yu, Choe, Li, & Zhang, 2020) and these seed oils are divided according to their primary or distinguishing fatty acid, including oleic, linoleic, α-linolenic, γ-linolenic and petroselinic acids. Fruit, vegetable, and spice are listed according to their primary fatty acid profile in Tables 1.1–1.5.

Table 1.1. Edible seed oils rich in α-linolenic acid (18:3n-3)

Seed oil	Species
Black raspberry	*Rubus occidentalis* L., cv Jewel
Red raspberry	*Rubus ideaus*
Boysenberry	*Rubus* hybrid
Marionberry	*Rubus hybrid*
Blackberry	*Rubus fruticosus*
Blueberry	*Vaccinium corymbosum*
Cranberry	*Vaccinum macrocarpon*
Kiwi	*Actinidia chinensis*
Sea buckthorn	*Hippophae rhamnoides* L.
Basil	*Ocimum sp.*
Hemp	*Cannabis sativa*
Chia	*Salvia hispanica*

List of seed oils are adapted from Bailey's Industrial Oil and Fat Products' chapter, Oils from Herbs, Spices, and Fruit Seeds (Yu, Choe, Li, & Zhang, 2020).

Alpha linolenic acid (18:3n-3) is an 18-carbon fatty acid with three double bonds at carbons 9, 12 and 15. It is an essential ω-3 fatty acid that is a required nutrient for human beings and can be obtained through diets from both plant and animal sources. Alpha linolenic acid can be converted by elongases and desaturases to other beneficial n-3 fatty acids such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), which are implicated in normal brain development,

normal vision, and a decreased risk of heart disease. Novel dietary sources of n-3 fatty acids are desired for those who do not consume adequate amounts of fish and/or fish-based food products rich in long chain n-3 fatty acids.

Black raspberry seed oil (*Rubus occidentalis* L., cv Jewel). Black raspberry is a member of the genus, *Rubus* from the Roseacea family, which is also known as caneberries. The majority of black raspberry crops are located in the Northwest region of the United States, predominantly in California and Oregon. The annual harvests for black raspberries in California and Oregon in 2016 were 6.4 million and 4.72 million pounds, respectively. Nearly 99.5% of the total crop goes into post-harvest production (https://quickstats.nass.usda.gov/results/3EC5AB7B-215F-31F8-9556-89BA1BA8E58B), and seeds are a major byproduct thereof.

The fatty acid profile of two cold-pressed black raspberry seed oils demonstrated high concentrations of both n-3 (ω-3) and total unsaturated fatty acids. The concentration of α-linolenic acid (18:3n-3) was 35% of total fats, and unsaturated fatty acids comprised 98-99%. Linoleic acid was the predominant fatty acid, however, the ratios of n-6 to n-3 fatty acids were very low at 1.6:1. The other measurable fatty acids included oleic (18:1n-9), and palmitic (16:0) acids. The overall fatty acid composition of black raspberry seed oil was very similar to red raspberry seed oil (Parry & Yu, 2004).

Red raspberry seed oil (*Rubus ideaus*). Red Raspberry is a production crop grown throughout the world. In 2014, the total worldwide annual production of raspberry was more than 0.6 million tons (http://www.fao.org/faostat/en/#data/QC/visualize). The majority of commercial raspberries are grown in Eastern Europe, followed by Northern and Western Europe, the United States, and Chile. Like black raspberries, red raspberries are also grown in the Northwest region of the United States, and productions from California and Washington in the year 2015 were 208.8 and 89.8 million pounds, respectively (https://quickstats.nass.usda.gov/results/EB52A5A9-D64B-3B8A-89B3-619A3E38DA57).

Red raspberry seed oils, extracted by either hexane (Oomah, Ladet, Godfrey, Liang, & Girard, 2000) or cold-pressing (Parry & Yu, 2004b), were examined for their fatty acid compositions. Both methods detected very similar fatty acid profiles and high concentrations of α-linolenic acid. The crude oil from the hexane extract contained 29.1% α-linolenic acid and the extra virgin cold-pressed seed oil had 32.4% α-linolenic acid. Both of these samples were also very comparable in fatty acid compositions compared to the black raspberry seed oil discussed above. In addition to its α-linolenic acid content, red raspberry seed oil may contain significant level of tocopherols, and other natural antioxidants (Oomah, Ladet, Godfrey, Liang, & Girard, 2000, Parry & Yu, 2004b). Total tocopherol was 97 and 61 mg/100 g oil in the hexane extracted and the cold-pressed oils, respectively (Oomah, Ladet, Godfrey, Liang, & Girard, 2000, Parry & Yu, 2004b), whereas the antioxidant activity, measured as the oxygen radical absorbing capacity (ORAC), was 48.8 μmoles trolox

equivalents per gram of oil (Parry & Yu, 2004b). Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, is a water-soluble vitamin E analog and widely used as a standard antioxidant compound.

Boysenberry seed oil (*Rubus* hybrid). Like the other caneberries (black raspberry, red raspberry, marionberry), boysenberry also prefers the growing conditions found in the Northwest region of the U.S. However, aside from Oregon, boysenberry is also grown in Northern California as a production crop. In 2015 and 2016 the total boysenberry productions in the U.S. were 2.46 and 2.16 million tons, respectively (https://quickstats.nass.usda.gov/results/96D83112-5015-3F26-8C8E-34FE7B6D477B).

Also, like the other cold-pressed caneberry seed oils, boysenberry seed oil had a high percentage (19.5%) of n-3 α-linolenic acid and a low n-6 to n-3 ratio of 2.8:1. Total unsaturated fatty acids constituted over 91% of the seed oil and PUFA were very high at 73.3%, but stearic, palmitic, and total saturated fatty acids were higher than all other caneberry seed oils. Interestingly, the boysenberry seed oil demonstrated the best antioxidative potential using the oxygen radical scavenging capacity (ORAC) test compared to the blueberry, black raspberry, and red raspberry seed oils, which are known to be potent antioxidants (Parry & Yu, 2004b).

Marionberry seed oil (*Rubus hybrid*). Marionberry is a blackberry hybrid. It is another member of the caneberry family and is also grown in the Northwest U.S.,

specifically, Oregon. The production in 2015 was 22.4 million pounds and in 2016 was 29.3 million pounds (https://quickstats.nass.usda.gov/results/F78E5A17-BCD3-343B-ACFD-DA8D5A86EE64). Marionberry comprises almost one half of the total caneberry production in Oregon.

In 2004, Parry and Yu (Parry & Yu, 2004b) examined the chemical composition and physicochemical properties of the cold-pressed marionberry seed oil. The oil was shown to contain a relatively high percentage of n-3 fatty acid in the form of α-linolenic acid (15.7%). This amount was lower than that of other caneberry seed oils including black raspberry, red raspberry, and boysenberry seed oils, tested under the same conditions. The n-6 to n-3 fatty acid ratio was 4:1, which was the highest among the tested caneberry group.

Blackberry seed oil (*Rubus fruticosus*). Blackberry grows in Europe, Northwestern Africa, Central Asia, and North and South America. Mexico is the leading producer of blackberries and exports blackberries to North America and Europe during off-season. In the U.S., Oregon is the leading commercial blackberry producer and produced 58.3 million pounds in 2016 (https://quickstats.nass.usda.gov/results/7D7AEF61-1B66-3534-A2A2-DD8D9D861A63). Blackberries contain numerous large seeds and those seeds are not preferred by consumers. However, blackberry seeds contain oil (30-31%), protein (21-22%), carbohydrate (43-44%) and phenolics (127-143 gallic acid equivalents mg/100 g oil). Also, blackberry seed oils are rich in linolenic and linoleic acids.

Blackberry seed oils contain linoleic (61.2-63.7 g/100 g oil) followed by linoleic (13.8-17.6 g/100 g oil), oleic (14.7-19.2 g/100 g oil) and palmitic (3.7-4.7 g/100 g oil) acids (Hoed, Clercq, Echim, Andjelkovic, Leber, Dewettinck, et al., 2009). In addition to fatty acid contents, blackberry seed oils contain sterols including cholesterol (0.2-0.3% of total sterol), brassicasterol (0.01-0.02% of total sterol), campesterol (2.5-2.8% of total sterol), stigmasterol (1.7-2.1% of total sterol) and Δ7-stigmastenol (0.2% of total sterol) (Gecgel, Velioglu, & Velioglu, 2011).

Blueberry seed oil (*Vaccinium corymbosum*). Blueberries are grown in temperate climates throughout the world; however, the largest producers are the United States and Canada. In 2015 and 2016, the U.S. harvested 566 and 593 million pounds, respectively (https://quickstats.nass.usda.gov/results/E9AE06AC-B9D2-3EAB-ACE9-99B7EFC50692).

The cold-pressed blueberry seed oil was investigated by Parry et al, 2004 (Parry & Yu, 2004b) and was rich in n-3 fatty acids. Alpha linolenic acid was the sole source of the n-3 and comprised 25.1% of the total fatty acids. The ratio of n-6 to n-3 fatty acids was 1.7:1. Linoleic acid (18:2n-6) was the most prevalent fatty acid in the blueberry seed oil followed by α-linolenic, oleic, palmitic (16:0) and stearic (18:0) acids. The blueberry seed oil also showed a significantly higher antioxidant capacity compared to marionberry, black raspberry, cranberry and pumpkin seed oils using the oxygen radical absorbance capacity (ORAC) test. In summary, blueberry seed oil may serve as an excellent dietary source of n-3 fatty acids and natural antioxidants.

Cranberry seed oil (*Vaccinum macrocarpon*). The North American cranberry, *Vaccinum macrocarpon*, is best adapted to grow at higher latitudes and in bog terrains. It is grown for production in Wisconsin, Maine, New Jersey, Oregon, and Washington in the United States, and it is grown for production in British Columbia and Quebec in Canada. Cranberries are also grown in Europe but are a different species of *Vaccinum*. The total production in the U.S. for the year 2017 was 9 million barrels (https://quickstats.nass.usda.gov/results/F2F40756-E570-3FF7-BF71-94D810B9248F).

Several studies have confirmed that the seed oil from the North American variety of cranberry contains significant levels of α-linolenic acid. In a U.S. patent, Heeg and Lager (Heeg & Lager, 2002) reported the α-linolenic acid content of cranberry seed oil to be between 30 and 35% of total fatty acids. In 2003, Parker and others (Parker, Adams, Zhou, Harris, & Yu, 2003) found 22.3% α-linolenic acid in the cold-pressed cranberry seed oil, and in 2004, Parry and others (Parry & Yu, 2004b) determined the oil to contain 32.0% α-linolenic acid from two different lots of the seed oil. The ratios of n-6 to n-3 fatty acids in all were low from 1.2:1 to 2:1. Also, all of the studies documented similar ratios among the rest of the common fatty acids found in cranberry seed oil including, in order of higher amount present: linoleic, oleic, palmitic, stearic, and eicosadienoic acids (20:2). In addition to α-linolenic acid, cranberry seed oil is rich in natural antioxidants (Yu, Zhou, & Parry,

2005). These antioxidants may directly react with free radicals and prevent lipid oxidation in human low-density lipoprotein.

Kiwi seed oil (*Actinidia chinensis*). Kiwi (*Actinidia chinensis*) or Chinese gooseberry is an edible berry species native to China. The origin of *Actinidia chinensis* is known as the northern Yangtze river valley. *Actinidia chinensis* is growing in the entire southeast of China. It has a greenish-brown skin and bright green flesh. The fruit has a soft texture and sweet with a unique flavor. It is a commercial crop in several countries, such as New Zealand, Italy, Chile, and Greece. The world's production of kiwi in 2014 was over 3.4 million metric tons and approximately 53% of that was grown in China. The U.S. production in 2016 was 28,300 tons (https://quickstats.nass.usda.gov/results/DF6F36E1-6348-3D13-97B7-C22BB6E2F528). Kiwi seed oil is rich in sterols including campesterol (1.9-2.1%), stigmasterol (2.4%), β-sitosterol (58.5-59.7%), Δ5-avenasterol (13.1%), Δ7-sitosterol (9.5-9.7%), and Δ7-avenasterol (13.4-14.2%). Also, kiwi seed oil is rich in tocopherols and tocotrienols, with γ-tocopherol as the primary tocopherol compound at a level of 9.5-11.1 mg/kg. The γ-tocotrienol (21.1-22.1 mg/kg) is the primary component and followed by δ-tocotrienol (2.5-2.7 mg/kg). In addition to tocopherols and tocotrienols, kiwi seed oil is rich in α-linolenic acid (58.4%) and total unsaturated fatty acids (76.01%). The predominant fatty acid in the oil is α-linolenic acid (58.4 g/100 g oil), which is followed by linoleic (17.5 g/100 g oil), oleic (14.6 g/100 g oil), palmitic (6.0 g/100 g oil), and stearic (3.1 g/100 g oil) acids. Lauric, arachidic, and

myristic acids are minor constituents (Hoed, Clercq, Echim, Andjelkovic, Leber, Dewettinck, et al., 2009).

Sea buckthorn (*Hippophae rhamnoides* L.) seed oil. Sea buckthorn is native to Asia and Europe. It is a hardy plant that is also being considered as a major commercial crop in Canada. It has been used in Tibetan, Mongolian and Chinese traditional medicine for more than 1000 years and has demonstrated many beneficial health attributes (Yang & Kallio, 2001). The fruit has a good flavor and is very nutritious. The whole berries contain a higher concentration of vitamin C than strawberry, kiwi, orange, and tomato, and the fruit also contains a higher concentration of vitamin E than wheat embryo, safflower, maize, and soybean (Janick, 1999).

In 2001, Yang and Kallio (Yang & Kallio, 2001) investigated the lipid compositions of two subspecies of *Hippophae rhamnoides* L. The subspecies were *H. rhamnoides* L. '*sinensis*' and *H. rhamnoides* L. '*rhamnoides*'. Twelve samples of *sinensis* and 9 samples of *rhamnoides* were grown at different locations in China and Finland, respectively. All the seed oils had relatively high percentages of α-linolenic acid, γ-linolenic acid (18:3n-6), and oleic acid (18:1n-9). These seed oil samples also had an n-6 to n-3 fatty acid ratio under 2:1. Other constituent fatty acids included palmitic, stearic and vaccinic (18:1n-7) acids. Kallio and others (Kallio, Yang, Peippo, Tahvonen, & Pan, 2002) examined the fatty acid composition of the subspecies *sinensis*, and *mongolica* of *Hippophae rhamnoides* L. Both displayed fatty acid profiles very similar to those found in the previous study.

Basil seed oil (*Ocimum sp.*). Basil is a popular herb grown throughout the world and is an ingredient in many recipes. There are more than 50 species, but sweet basil, *Ocimum basilicum*, is the most common variety (Angers, Morales, & Simon, 1996).

In 1996, Angers and others (Angers, Morales, & Simon, 1996) investigated the fatty acid composition of the seed oils of 4 species of basil including *Ocimum basilicum*, *Ocimum canum*, *Ocimum gratissimum*, and *Ocimum sanctum.* Also, four different varieties of *Ocimum basilicum* were tested. All samples were compared to flaxseed oil and had similar fatty acid profiles in regard to α-linolenic, palmitic, and stearic acids. The flaxseed oil had 52% α-linolenic acid, and the basil seed oils had 57.4 to 62.5% α-linolenic acid. The ratio of n-6 to n-3 fatty acids of the flaxseed oil was 1:3.2, and the basil seed oils were 1:2.7 to 1:3.4.

Hemp seed oil (*Cannabis sativa*). Hemp is an ancient crop that is still cultivated by many societies and has many different uses. The fiber from hemp has been used to make rope, paper, and clothing; hemp is used for medicinal purposes, and its seed oil is commercially available (Oomah, Busson, Godfrey, & Drover, 2002).

Recent investigations of hemp seed oil (Parker, Adams, Zhou, Harris, & Yu, 2003; Pierce & Brooks, 2000; Oomah, Busson, Godfrey, & Drover, 2002) showed similar findings in fatty acid compositions. The n-3 fatty acid, α-linolenic acid, was determined to constitute between 15.1 and 19.4% of total fat. Gamma linolenic acid (18:3n-6) was also detected in two of the studies and comprised up to 3.6% of total fatty acids (Pierce & Brooks, 2000; Oomah, Busson, Godfrey, & Drover, 2002). The

most prevalent fatty acid was linoleic in all of the studies, which was between 53.4 and 60.0% of total fatty acids and was followed by α-linolenic, oleic, palmitic, γ-linolenic, and stearic acids. Eicosadienoic, arachidic (20:0), and behenic (22:0) acids were also detected in small quantities. Furthermore, Aladić and others (Aladić, Jarni, Barbir, Vidović, Vladić, Bilić, et al., 2015) investigated the influence of extraction conditions on concentrations of tocopherols and fatty acids in hemp seed oil. Different extraction conditions significantly altered the concentrations of different tocopherols, while the fatty acid profiles were slightly changed. The concentration of tocopherol was significantly increased using supercritical CO_2 extraction and the amount of α-tocopherol in supercritical extracts ranged from 37.09 to 110.61 mg L^{-1}, while γ-tocopherol content ranged from 95.4 to 181.6 mg L^{-1}, depending on the applied conditions (Aladić, Jarni, Barbir, Vidović, Vladić, Bilić, et al., 2015).

Chia seed oil (*Salvia hispanica*). Chia is an annual herbaceous plant native to Mexico and Guatemala. Recently, chia has been cultivated for commercial purposes in Argentina, Colombia, Ecuador, Peru, Bolivia, Paraguay, and Australia. Chia seeds have been consumed in beverage or bakery products.

Grzegorz and others investigated the fatty acid composition of the chia seed oils obtained by classical solvent extraction, supercritical fluid extraction with CO_2 and screw-cold-pressing and screw-hot-pressing (Dąbrowski, Konopka, & Czaplicki, 2018). The concentrations of α-linolenic and linoleic acids in chia seed oils obtained with different method were about 60-62.6 and 19.5-21 g/100 g oil, respectively.

Earlier in 2012 and 2010, Ciftci and others (Ciftci, Przybylski, & Rudzińska, 2012) and Ayerza (Ayerza, 2010) reported a ratio of α-linolenic acid in the range of 61.1 to 66.7 g/100 g oil. In addition to its α-linolenic acid content, chia seed oil may contain significant level of β-sitosterol, tocopherols, and other natural antioxidants such as phenolic compounds (Dąbrowski, Konopka, & Czaplicki, 2018; Álvarez-Chávez, Valdivia-López, Aburto-Juárez, & Tecante, 2008). β-Sitosterol is the primary sterol with a level of 2813.3 (hot pressing) to 3497.7 mg/kg (hexane extraction) (Dąbrowski, Konopka, & Czaplicki, 2018). The total tocopherol ranged from 498.1 (hot pressing) to 739.4 mg/kg (SC-CO_2 90 °C) (Dąbrowski, Konopka, & Czaplicki, 2018). In addition, the highest level of phenolic content was 172 mg/kg for oil extracted using acetone (Dąbrowski, Konopka, & Czaplicki, 2018).

Table 1.2. Edible seed oils rich in γ-linolenic acid (18:3n-6)

Seed oil	Species
Blackcurrant	*Ribes nigrum* and other *Ribes* species
Evening primrose	*Oenothera*

List of seed oils are adapted from Bailey's Industrial Oil and Fat Products' chapter, Oils from Herbs, Spices, and Fruit Seeds (Yu, Choe, Li, & Zhang, 2020).

Gamma-linolenic acid (18:3n-6) is an important unsaturated fatty acid. It is the precursor for biosynthesis of arachidonic acid that is a precursor for prostaglandin formation. Recently, γ-linolenic acid has been recognized for its potential health benefits in prevention and treatment of cardiovascular disorders, premenstrual

syndrome, atopic eczema, rheumatic arthritis, and alcoholism (Yaniv, Ranen, Levy, & Palevitch, 1989; Ratnayake, Matthews, & Ackman, 1989).

Blackcurrant seed oil (*Ribes nigrum*) and other *Ribes* species. Blackcurrant is cultivated for the production of its berries. Blackcurrant is rich in ascorbic acid and exhibited high levels of antioxidant activity (Lister, Wilson, Sutton, & Morrison, 2002). Blackcurrant is mainly consumed in the form of juice and the seeds are the byproduct of juice production. Blackcurrant seed oil was analyzed for fatty acid composition, tocopherols, and their prostaglandin E_2 production reduction potential (Wu, Meydani, Leka, Nightingale, Handelman, Blumberg, et al., 1999; Goffman & Galletti, 2001; Castillo, Dobson, Brennan, & Gordon, 2002). Blackcurrant seed oil is an excellent dietary source of both γ-linolenic (18:3n-6) and α-linolenic (18:3n-3) acids. Gamma-linolenic acid constituted 12-25% of the total fatty acids, while α-linolenic acid comprised the other 10-13%. The fatty acid composition was dependent on genotype and growing conditions. The seed oil also had significant levels of tocopherols (Goffman & Galletti, 2001). The total tocopherol content was 1.2-2.5 mg/g oil, with a mean value of 1.7 mg/g oil for 10 oil samples. The major tocopherol in the blackcurrant seed oil was γ-tocopherol, but β-tocopherol was not detected in the blackcurrant seed oil. In 1999, Wu and others (Wu, Meydani, Leka, Nightingale, Handelman, Blumberg, et al., 1999) investigated the effect of dietary supplementation with blackcurrant seed oil on the immune response of healthy elderly subjects. The seed oil moderately enhanced immune function through reducing the production of

prostaglandin E_2, suggesting that blackcurrant seed oil may have potential in preventing cancer, cardiovascular disease, and other health problems.

Other Ribes species, including *R. grossularia* (red-black gooseberries), *R. grossularia* (yellow gooseberries), *R. nigrum* (blackcurrants) *R. rubrum* (red currants) and *R. nigrum* × *R. hirtellum* (jostaberries), were also examined for γ-linolenic acid concentration and tocopherol content in the seed oils. Among the tested samples, blackcurrant seed oil had greatest level of γ-linolenic acid, and all three-species currant had total concentration of tocopherols over 1.0 mg/g oil (Goffman & Galletti, 2001).

Evening primrose seed oil (*Oenothera spp.*). Evening primrose belongs to the Onagraceae family and produces a large number of highly fertile seeds. The roots of evening primrose are used in human diet, while its bark, leaves, and essential oil are used for medicinal purposes (http://botanical.com/botanical/mgmh/p/primro70.html) (Yaniv, Ranen, Levy, & Palevitch, 1989; Hudson, 1984). Evening primrose seed oil is a natural source of γ-linolenic acid (18:3n-6). Hudson (Hudson, 1984) evaluated 192 evening primrose (*Oenothera* spp.) seed oil samples for their fatty acid compositions. The normal range of γ-linolenic acid concentration was 8-14% and the extreme range was 2-20% of total fatty acids, with a median of 10.4% (Hudson, 1984). Linoleic acid normally accounted for 65-80% of total fatty acids and the median was 73%, which was as high as that of any known vegetable oil. Other studies showed that common evening primrose (*Oenothera biennis*) seed oils contained 4.9-9.24% of γ-linolenic

acid, along with 64-73.88% linoleic acid (Ratnayake, Matthews, & Ackman, 1989; Rodrigues, Costa, Almeida, Cruz, Ferreira, Vilhena, et al., 2015; Ghoreishi & Bataghva, 2011). In addition, the growing conditions were found to alter the γ-linolenic acid content in the seed oil. The concentration of γ-linolenic acid ranged from 5.5-9.6% of total fatty acids (Yaniv, Ranen, Levy, & Palevitch, 1989). Ratnayake (Ratnayake, Matthews, & Ackman, 1989) reported that evening primrose seed oil from Canada contained 64.3% of linoleic acid and 4.9% of γ-linolenic acid. These previous studies indicate that evening primrose (*Oenothera* spp.) seed oil contains significant level of natural γ-linolenic and oleic acids, and linoleic acid is the predominant fatty acid.

Table 1.3. Edible seed oils rich in linoleic acid (18:2n-6)

Seed oil	Species
Watermelon	*Citrullus vulgaris*
Melon	*Cucumis melo*
Melon	*Colocynthis citrullus L.*
Goldenberry	*Physalis peruviana* L.
Grape	*Vitis spp*.
Rose fruit	*Rosa canina* L.
Paprika	*Capsicum annuum*
Apple	*Malus genus*
Red pepper	*Capsicum sp.*
Onion	*Allium cepa*
Black cumin	*Nigella sativa* L.
Plantago L.	*Plantaginaceae*
Passion fruit	*Passiflora edulis*
Guava	*Psidium guajava*
Dragon fruit	*Hylocereus spp.*
Strawberry	*Frafaria F. × ananassa*
Sweet lemon	*Citrus limetta*
Lemon	*Citrus limon*
Grapefruit	*Citrus paradise*
Orange	*Citrus sinensis*
Mandarin orange	*Citrus reticulata*
Citron	*Citrus medica L.*
Kumquat	*Citrus japonica*
Tomato	*Solanum Lycopersicum*

List of seed oils are adapted from Bailey's Industrial Oil and Fat Products' chapter, Oils from Herbs, Spices, and Fruit Seeds (Yu, Choe, Li, & Zhang, 2020).

Linoleic acid (18:2n-6) is an essential fatty acid, which must be obtained through diets. Linoleic acid rich seed oils include watermelon, melon (*Cucumis melo and Colocynthis citrullus*), goldenberry, grape, rose fruit, paprika, apple, red pepper, onion, black cumin, plantago, passion fruit, guava, dragon fruit, strawberry, sweet lemon, lemon, grapefruit, orange, mandarin orange, citron, kumquat, and tomato (Table 1.3).

Watermelon seed oil (*Citrullus vulgaris*). Watermelon is taxonomically classified as a member of the Curcubitaceae family, which is also known as the gourd family. Other gourds include pumpkins, cucumbers, squash and other melons. It prefers warm climate growing conditions and is produced worldwide where conditions permit. Over 1,200 varieties have been cultivated and about 250 varieties are grown in North America, (http://www.watermelon.org/index.asp?a=dsp&htype=about&pid=39). The total US production of watermelons in 2014 was 3.2 billion pounds. In the US, the top four watermelon producing states are Texas, Florida, Georgia and California, and which account for approximately 44 percent of US total productions. (http://www.agmrc.org/commodities-products/vegetables/watermelon/). Watermelon seeds are consumed as snack food worldwide, and are used to prepare edible oil in some countries.

Watermelon seed oil was prepared and evaluated for its physicochemical properties (El-Adawy & Taha, 2001; El-Adawy & Taha, 2001b). The seed oil consisted of 60% linoleic acid (18:2n-6) and 78.4% total unsaturated fatty acids. The

predominant fatty acid in the oil was linoleic acid, which was followed by oleic, palmitic and stearic acids. Linolenic, palmitoleic, and myristic acids were minor constituents. The refractive index, acid value, free fatty acids, and peroxide value of watermelon seed oil were determined to be 1.4696, 2.82 (mg KOH/g oil), 3.40 (mequiv O_2/kg oil), and 1.41 (% as oleic acid), respectively. The saponification value of watermelon seed oil was 201 (mg KOH/g oil) and its iodine value was 115 (g I/100 g oil), which was significantly higher than pumpkin seed oil at 109 (g I/100 g oil) (El-Adawy & Taha, 2001; El-Adawy & Taha, 2001b).

Melon seed oil (*Cucumis melo*). Melon is a member of the Cucurbitaceae family, and grows best in tropical regions. The pulp of the fruit has pleasant flavor and taste, and the seeds are generally treated as waste, however, medicinal effects have been reported for the seeds (Melo, Narain, & Bora, 2000; Lazos, 1986). Hexane-extracted seed oil of *Cucumis melo* hybrid AF-522 was determined to contain 64 g of linoleic acid per 100 g of total fatty acids (Melo, Narain, & Bora, 2000). Significant amount of oleic, palmitic, and stearic acids were also detected in the melon seed oil. The specific gravity (at 28 °C), refractive index (at 28 °C), and iodine value (g I/100 g oil) of the seed oil were 0.9000, 1.4820, and 112, respectively, under the experimental conditions (Melo, Narain, & Bora, 2000). Earlier in 1986, Lazos (Lazos, 1986) extracted the oil from *Cucumis melo* seeds and examined its physicochemical properties (Lazos, 1986). Linoleic acid was the primary fatty acid and accounted for 64.6% of the total (w/w), along with 20.1% oleic acid, and 14.7% total saturated fatty

acids. Iodine value (g I/100 g oil) and refractive index (at 40 °C) of the seed oil were 124.5 and 1.4662, respectively.

Melon seed oil (*Colocynthis citrullus L.*). *Colocynthis citrullus* L (melon) is a tropical vine that is native to West Africa. The fruit flesh is bitter and inedible; the edible part of the fruit is the seeds. Nwokolo and Sim (Nwokolo & Sim, 1987) examined the fatty acid composition of *Colocynthis citrullus* seed oil and found that it contained a relatively high percentage of linoleic acid that accounted for 57.7% of total fatty acids (Nwokolo & Sim, 1987). Oleic acid was the second major fatty acid (14.5%) but the percentage in total fatty acids was much lower than that of linoleic acid. The seed oil contained about 25.3% saturated fatty acids. Moussata and Akoh (Moussata & Akoh, 1998) also reported a similar fatty acid profile of *Colocynthis citrullus* L. seed oil. The primary fatty acid was linoleic acid contributing 65.4% of total fat. The other significant fatty acids included oleic (13.5%), palmitic (12.1%) and stearic (9.0%) acids.

Goldenberry (*Physalis peruviana* L.). Goldenberry, (*Physalis peruviana* L.), also known as cape gooseberry, is a perennial plant native to the Andes. It is also cultivated in the United States, South Africa, East Africa, India, New Zealand, Australia, and Great Britain (McCain, 1993). Goldenberry has a pleasant flavor that is similar to tomatoes and is eaten in many ways including in salads, cooked dishes, chocolate covered desserts, jams, preserves, and natural snacks (Ramadan & Mörsel,

2003). The fruit is an excellent source of vitamins A and C as well as minerals. Goldenberry seed oil was prepared by extracting lyophilized ground seed meal with chloroform-methanol and was characterized for fatty acid composition (Ramadan & Mörsel, 2003). Linoleic acid was the predominant fatty acid and constituted 76.1% of the total fat. Combined mono-unsaturated fatty acids were 12.2%, linolenic acid was 0.33%, and total polyunsaturated fatty acids were 76.1%. These data suggest that goldenberry seed oil may serve as an excellent dietary source for linoleic acid, the essential n-6 fatty acid, and may be a good choice for consumers seeking a greater intake of total unsaturated fatty acids.

The fat-soluble vitamins E and K, carotene, and phytosterols were also detected in the goldenberry seed oil (Ramadan & Mörsel, 2003). Total tocopherols were 29.7 mg/g oil, including 0.9 mg α-, 11.3 mg β-, 9.1 mg γ-, and 8.4 mg δ-tocopherols. The total vitamin K content was 0.12 mg/g oil, and the β-carotene concentration was 1.30 mg/g oil. In addition, significant levels of phytosterols were also detected. The major phytosterol in the goldenberry seed oil was campesterol having a concentration of 6.5 mg/g oil. Other phytosterols including ergosterol, stigmasterol, lanosterol, β-sitosterol, Δ5-avenosterol, and Δ7-avenosterol were also detected in the seed oil.

Grape seed oil (*Vitis spp*.) World grape production was 67 million metric tons in 2012 (http://www.fao.org/faostat/en/#data/QC/visualize). In the United States, the total 7.5 million tons were produced in 2017. Among the States, California is the

leading producer of grape and produced 6.6 million tons in 2017 which is 88% of U.S. annual production (https://quickstats.nass.usda.gov/results/69223626-0540-3B24-AE57-2215561FD658). Grape seeds are byproducts from the manufacturing of grape juice, jam, jelly and wine. In 1998, Abou Rayan and others (Abou Rayan, Abdel-Nabey, Abou Samaha, & Mohamed, 1998) investigated the characteristics and composition of Egyptian grown Cabarina red grape seed oil. Crude grape seed oil was extracted with hexane at room temperature. Linoleic acid was the major fatty acid detected and comprised more than 50% of the total fatty acids (Abou Rayan, Abdel-Nabey, Abou Samaha, & Mohamed, 1998). Oleic acid is the second major fatty acid of the seed oil, along with significant levels of palmitic and stearic acids. This finding is consistent with a previous observation in which linoleic acid accounted for 62% of the total fatty acids in grape seed oil (Massanet, Montiel, Pando, & Rodriguez, 1986). Iodine value (IV), and peroxide value (PV) were also determined according to the methods described in AOCS, 1983. The measured IV was 130 g I/100 g oil, and the PV was determined to be 2.92 mequiv O_2/kg oil. Other characteristics determined were refractive index, specific gravity, saponification value, unsaponifiable matter%, and acid value.

Rose fruit seed oil (*Rosa canina* L.). Rose, *Rosa canina* L., also known as dogberry or hop fruit, is in the Rosaceae family. The fruit of this particular species of rose is generally used to prepare a stew. The seeds from *Rosa canina* L. were investigated for their chemical composition and nutritional values for medicinal purposes. Seed

oils were prepared from fruits grown at three locations in Turkey, and evaluated for their fatty acid composition (Özcan, 2002). Linoleic acid was the primary fatty acid, which ranged from 48.6% to 54.4% of total fatty acids, followed by α-linolenic acid (16.4-18.4%) and oleic acid (14.7%- 18.4%). The seed oil contained approximately 85% total unsaturated fatty acids indicating that *Rosa canina* L. seed oil may be an excellent source for unsaturated and essential fatty acids.

Paprika seed oil (*Capsicum annuum*). Paprika (*Capsicum annuum*) is a commonly used flavor enhancer, and following production, the seeds are treated as waste. Paprika seed oil has been evaluated for their physicochemical properties (El-Adawy & Taha, 2001; El-Adawy & Taha, 2001b; Domokos, Bernáth, & Perédi, 1993). Paprika seed oil contained more than 82% of total unsaturated fatty acids with polyunsaturated fatty acids comprising 67.8% of the total fatty acids (El-Adawy & Taha, 2001; El-Adawy & Taha, 2001b). Oleic acid was the second major fatty acid at approximately 15% of the total. This fatty acid profile was consistent with a previous observation by Domokos and others (Domokos, Bernáth, & Perédi, 1993) on the fatty acid profile of Hungarian paprika seed oil. Linoleic acid comprised of 74.4% of the total fat, whereas oleic and palmitic acids made up 9.8 and 11.2% of the total fat, respectively (Domokos, Bernáth, & Perédi, 1993). The paprika seed oil was determined to contain 870 mg/kg oil total tocopherols, 380 mg/kg oil carotenoids, and 0.92% sterols (Domokos, Bernáth, & Perédi, 1993).

Apple seed oil (*Malus genus*). In 2017, the US production of apples was 104.4 billion pounds. New York, Michigan and California produced 12 billion, 800 million and 230 million pounds, respectively (https://quickstats.nass.usda.gov/results/E68753E1-7308-385A-8C7D-00ED3AFA254D). Apple seeds are a byproduct of apple processing. In 1971, Morice and others (Morice, Shorland, & Williams, 1971) investigated the seed oils from Granny Smith, Sturmer and Dougherty varieties of apple, and compared the results with the seed oils prepared from other apple varieties. The results showed similarities in the fatty acid profiles among the varieties. Oleic acid (24-42% of total fatty acids) and linoleic acid (48-64% of total fatty acids) consisted of 85-95% of the total fatty acids in all tested samples (Morice, Shorland, & Williams, 1971). The investigators also examined other physicochemical properties of apple seed oils. The Granny Smith apple seed oil had an iodine value (IV) of 127 g I/100 g oil; the Sturmer had an IV of 122.4 g I/100 g oil, and the Dougherty's IV was 119 g I/100 g oil. Apple seed oils may be useful as dietary sources for linoleic and oleic acids.

Red pepper seed oil (*Capsicum sp.*). Chili peppers, *Capsicum sp.* are members of the Solanacaea family. They originated in South America but are now grown worldwide. They are eaten in many dishes throughout the world and provide the feeling of 'hotness' mounth feeling. There is a very large range of hotness among the *Capsicum* species and that depends upon their concentration of capsaicin. Some selected peppers ranging from lowest in concentration of capsaicin include bell, Anaheim,

jalapeno, Hungarian wax, serrano, cayenne, and habanero. Red pepper seeds are byproducts from the production of red pepper powder. For centuries in South America, peppers have been used to treat such ailments as gastrointestinal disorders, and it is also thought to benefit those suffering from circulatory diseases. Capsaicin, the spicy chemical in peppers, has also been shown to be a potent anti-inflammatory *in vivo* (Sancho, Lucena, Macho, Calzado, Blanco-Molina, Minassi, et al., 2002).

The roasted red pepper seed oil contained an extremely high concentration of linoleic acid at approximately 74% and a high total unsaturated fat level (Jung, Bock, Baik, Lee, & Lee, 1999). The fatty acid profile was very similar to that of both goldenberry seed (*Physalis peruviana* L.) and safflower oils (Sancho, Lucena, Macho, Calzado, Blanco-Molina, Minassi, et al., 2002). The iodine value of roasted red pepper seed oil was determined to be 137 g I/100 g oil.

Onion seed oil (*Allium cepa*). Onion seeds contained about 23.6% crude fat. The onion seed oil contained 44.6% linoleic acid and 34.3% oleic acid (Rao, 1994). The total unsaturated fatty acids consisted 79% of the oil. A greater concentration of linoleic acid was determined in the cold-pressed onion seed oil obtained from Botanical Oil Co. (Spooner, WI). Linoleic acid accounted for 63.7% of total fatty acids, and oleic acid ranged 26.7-30.1%. The total unsaturated fatty acids were about 90% (Parry & Yu, 2004b). In summary, onion seed oil may serve as a dietary source of essential n-6 fatty acid and oleic acid.

Black cumin seed oil (*Nigella sativa* L.). Black cumin (*Nigella sativa*) is an annual spicy herb. It has been used for many years as a food preservative and a traditional medicine for protection against and a therapeutic remedy against a number of health disorders (Salem & Hossain, 2000). Black cumin seed and its oil have also been used for medicinal purposes (Ramadan & Mörsel, 2002). According to Ramadan and Mörsel (Ramadan & Mörsel, 2002), the seed contained about 28 ~ 35% oil. Black cumin seed oil contained a relatively high level of unsaturated fatty acids (~ 84% of the total fatty acids). The major fatty acid in the seed oil was linoleic acid, which accounted for about 57% of the total fatty acids, followed by oleic acid from 23.9-24.1%, along with small amount of palmitic and stearic acids. However, the iodine value of the seed oil was only 48.4 g I/100 g oil, which is much lower than the expected value relating to the level of unsaturation.

Plantago L. seed oil (*Plantaginaceae*). Plantago L. (*Plantaginaceae*) is widely distributed and there are more than 270 species around the world. The history of consuming the whole plants and seeds of *Plantago major, Plantago asiatica, and Plantago depressa* in foods and traditional medicine started from ancient years. In addition, the seeds of several species have also been consumed as snacks or added in cakes and breads. Recently, different species of *Plantago* seeds have been reported to have many healthy properties such as antioxidant, anti-inflammatory activities (Zhou, Lu, Niu, Liu, Zhang, Gao, et al., 2013). In 2015, Gong et al. examined hydroxyl, peroxyl, and DPPH radical scavenging capacities of *Plantago asiatic* L. seeds.

Plantago asiatic L. seeds exhibited a strong scavenging capacity against DPPH radical with a relative DPPH radical scavenging capacity (RDSC) value of 60.5 μmol Trolox equivalents/g. Also, *Plantago asiatic* L. seeds had ORAC and HOSC values of 32.65 and 21.6 μmol TE/g, respectively (Gong, Zhang, Niu, Chen, Liu, Alaxi, et al., 2015). The plantago seed oils were abundant in linoleic acid ranging from 41.2-60.8 g/100 g oil. The other two major fatty acids were oleic acid (14.2-27.1 g/100 g oil) and linolenic acid (11.1-29.4 g/100 g oil) and had a low ratio of n-6/n-3 fatty acids, which is a good choice for improving human health (Wang, Niu, Zhao, Wang, Jiang, Liu, et al., 2017).

Passion fruit (*Passiflora edulis*) seed oil. *Passiflora edulis* is native to southern Brazil through Paraguay to Northern Argentina. In the United States, it is grown in Florida and California. There are three edible passion fruits and can be divided into three main types: purple ones (fruits of *Passiflora edulis* Sims), yellow ones (*Passiflora edulis f. flavicarpa* Deg.), and giant granadilla (*Passiflora quadrangularis* L.). The shape of passion fruits is round to oval. The inside of passion fruit is juicy and filled with numerous seeds. The major fatty acid of passion fruit seed is linoleic acid (72.4-73.0 g/100 g oil) and followed by oleic (16.0-16.5 g/100 g oil), palmitic (8.5-8.7 g/100 g oil), stearic (1.7 g/100 g oil), linolenic (0.3 g/100 g oil), palmitoleic (0.2 g/100 g oil) and myristic (0.03 g/100 g oil) acids (Liu, Yang, Li, Zhang, Ji, & Hong, 2008). The specific gravity, refractive index, acid value, peroxide value, saponification value, non-saponification matter, water and volatile matter, insoluble

impurities and iodine value were 0.9, 1.47, 1.96-2.48 (mg KOH/g oil), 1.28-2.35 (mmol/kg), 173.86-189.72 (KOH mg/g oil), 0.77-3.03 (%), 0.19-7.21 (%), 1.28-2.58 (%) and 118.67-130.54 (g I/100 g oil) for the oil, respectively (Liu, Yang, Li, Zhang, Ji, & Hong, 2008).

Guava (*Psidium guajava*) seed oil. Guavas are common fruits from tropical and subtropical regions. It is native to Mexico, Central America, and Northern South America. Guava fruits are 1.6 to 4.7 inches long and appear as round or oval. Guava fruits have a specific fragrance similar to lemon but less sharp. Guavas are rich in dietary fiber and vitamin C. Guava seed oil is used for culinary or cosmetics products. Guava seed oil contains tocopherols, phytosterol and phenolic compounds. The γ-tocopherol is the predominant tocopherol at a level of 92.9-93.3 mg/kg, and followed by α-tocopherol at a content of 45.6-46.0 mg/kg. In addition to tocopherols, guava seed oil contains β-sitosterol (437.6 mg/100g), stigmastanol (26.6 mg/100g) and cholesterol (1.2 mg/100g). Also, phenolic compounds are rich in guava seed oil including *p*-coumaric acid, salicylic acid and quercetin and their contents are 48.0-48.8, 35.2-37.4 and 13.5-14.3 mg/kg, respectively. The guava seed oil is particularly rich in linoleic acid (76.4-77.0 g/100 g oil), and followed by oleic acid (8.3 g/100 g oil), palmitic acid (7.6 g/100 g oil), stearic acid (3.9 g/100 g oil) and arachidic acid (0.3 g/100 g oil) (da Silva & Jorge, 2014).

Dragon fruit seed oil (*Hylocereus spp.*). Dragon fruit is originated from South America, and also known as pitaya or pitahaya. Dragon fruit is usually growing in tropical region including Southeast Asia (Taiwan, Malaysia and Vietnam) (Ariffin, Bakar, Tan, Rahman, Karim, & Loi, 2009). In the U.S., cultivation of dragon fruit is limited to several states. In 2013, California, Hawaii, and Florida were growing 100 to 200, 200, and more than 400 acres of dragon fruit, respectively. There are two kinds of dragon fruits, white-flesh dragon fruit and red-flesh dragon fruit, and they have some different characteristics such as seed and oil contents. The white-flesh dragon fruit has 7.1-8.7% of seed while red-flesh dragon fruit has 7.7-9.5%. Seed oil content of white-flesh dragon fruit is 32.9-35.3%, while red-flesh dragon fruit seed has 31.4-32.6% oil. However, both white and red dragon fruits contain many tiny black seeds. The seed of dragon fruit contains oil similar to grape or berry seed oil. Dragon fruit seed oil is rich in tocopherols (407-657 mg/kg), and α-tocopherol (292.94 mg/kg) is the predominant tocopherol which is about 72% of total tocopherol content and followed by γ-tocopherol (75.62 mg/kg) and δ-tocopherol (38.70 mg/kg). The dragon fruit seed oil contains linoleic acid (44.6-55.8 g/100 g oil) followed by oleic acid (18.5-23.7 g/100 g oil), palmitic acid (14.4-18.9 g/100 oil), stearic acid (6.9-8.7 g/100g oil) and α-linolenic acid (0.1-0.2 g/100g oil) (Liaotrakoon, Clercq, Hoed, Walle, Lewille, & Dewettinck, 2013).

Strawberry seed oil (*Frafaria F. × ananassa*). Strawberry is cultivated worldwide. Strawberries are consumed in many ways, either flesh or processed into juice, pie,

jam, ice cream and chocolates. The leading producing countries of strawberries are USA, Turkey and Spain. In the U.S., more than 30 million cwt (3,419 million pounds) were produced in 2017 (https://quickstats.nass.usda.gov/results/4B59B809-C4A0-3AF0-A491-545B27637B38). Strawberry seed oil contains numerous sterols including campesterol (5.1-5.7%), stigmasterol (2.2-2.4%), β-sitosterol (70.4-71.8%), Δ5-avenasterol (8.6-8.8%), Δ7-sitosterol (8.1-8.3%) and Δ7-avenasterol (4.2-4.4%). Also, strawberry seed oil is rich in tocopherols, and γ-tocopherol is the major tocopherol compound at a level of 246.6-274.0 mg/kg. The oil also contains δ-tocopherol at a concentration of 16.2-23.8 mg/kg (Hoed, Clercq, Echim, Andjelkovic, Leber, Dewettinck, et al., 2009). Strawberry seed oil contains high content of unsaturated fatty acids which is about 92.87% of the total seed oil (Pieszka, Migdał, Gąsior, Rudzińska, Bederska-Łojewska, Pieszka, et al., 2015). The oil contains high amount of linoleic acid (42.2 g/100 g oil), followed by linolenic (36.5 g/100 g oil), oleic (14.5 g/100 g oil), palmitic (4.3 g/100g oil) and stearic (1.7 g/100g oil) acids (Hoed, Clercq, Echim, Andjelkovic, Leber, Dewettinck, et al., 2009).

Sweet lemon seed oil (*Citrus limetta*). Sweet lemon is a species of *citrus*. It is originated from South Asia and Southeast Asia and grows in tropical and subtropical regions. The fruits are oval, and the color turns from green to yellow as they ripe. The flesh of sweet lemon is a good source of vitamin C and thus usually processed into juice. The seed of sweet lemon is a by-product from this juice-making process. It is important to note that sweet lemon seeds are not just a by-product. They are a good

source of nutrition and contain oil (29.2-30.3 %), protein (6.2-6.6 %), fiber (4.8-5.2 %) and ash (5.4-5.6 %). Sweet lemon seed oil's physical and chemical characteristics such as iodine value, refractive index, density, saponification value, unsaponifiable matter and acid value are evaluated, and values are as follows: 116.8-113.2 g of I/ 100g oil, 1.48 at 40°C, 0.89-0.99 mg/mL at 25°C, 178.2-183.6 mg of KOH/100g oil, 0.27-0.35 %, 2.12-2.24 mg KOH/g oi oil, respectively. For fatty acids, sweet lemon seed oils also contain linoleic acid (39.5-40.2 g/100 g oil), palmitic acid (26.0-27.0 g/100 g oil), oleic acid (23.0-24.2 g/100 g oil), linolenic acid (4.2-4.5 g/100g oil) and stearic acid (2.6-2.8 g/100g oil) (Anwar, Naseer, Bhanger, Ashraf, Talpur, & Aladedunye, 2008).

Lemon seed oils (*Citrus limon*). The lemon tree is a small, evergreen shrub native to Southeast Asia and now is cultivated worldwide. It is one of the most important species in the flowering plant family of Rutaceae, consisting of approximately 140 genera and 1300 species. In 2014, world production of lemons (data combined with limes) was 16.3 million tons and India, Mexico and China were the top three producer countries (http://www.fao.org/faostat/en/#data/QC/visualize). In the U.S., the annual market productions in 2016 and 2017 were 0.695 and 0.711 million tons, respectively (https://quickstats.nass.usda.gov/results/E73008CA-E4A0-3B5B-90D0-30DD0DC6558D). Lemon seed oil was found to have various beneficial for human health such as aiding with blood flow and reducing blood pressure. The lemon seed oil is rich in vitamins and minerals, which is ideal for treating irritated skin and dry

damaged hair. Moreover, lemon seed oil is a superb carrier oil in most cosmetic formulations. Lemon seed oil mainly consisted of linoleic acid (33.8-34.0 g/100 g oil). Other prominent fatty acids were oleic acid (30.3-30.9 g/100 g oil), palmitic acid (20.9 g/100 g oil), linolenic acid (8.3-8.6 g/100g oil) and stearic acid (4.3 g/100g oil) (Yilmaz & Güneşer, 2017). In addition, cold pressed lemon seed oils had a significantly higher content of α-tocopherol (155.0 mg/kg) than that of solvent extracted (110.20 mg/kg) (Yilmaz & Güneşer, 2017).

Grapefruit seed oil (*Citrus paradise*). The color of grapefruit is yellow-orange and diameter is in range of 3.9 to 5.9. The color of flesh is white, pink and red. Worldwide, China is the leading grapefruit producer, and followed by USA and Mexico. In 2012, China produced 3.8 million metric tons. According to USDA NASS, market production in the U.S. in 2017 was 0.393 million tons (https://quickstats.nass.usda.gov/results/09558434-796C-32EF-B946-5B7741E4AD55). Grapefruit is rich in vitamin C and fiber pectin. Also, pink and red hues flesh ones contain lycopene (Lee, 2000). A study showed grapefruit seeds contained linoleic (35.8-36.3 g/100 g oil) followed by palmitic (31.8-32.6 g/100 g oil), oleic (21.5-22.4 g/100 g oil), linolenic (4.2-4.5 g/100g oil) and stearic (3.5-3.8 g/100g oil) acids (Anwar, Naseer, Bhanger, Ashraf, Talpur, & Aladedunye, 2008).

Orange seed oil (*Citrus sinensis*). Orange trees were found to be the most cultivated trees in the world since 1987. In 2014, more than 70 million tons of oranges were

grown worldwide. Brazil is the leading country of orange producer and produced 16.9 million tons followed by China (7.8 million tons) and India (7.3 million tons) in 2014 (http://www.fao.org/faostat/en/#data/QC/visualize). The market production in the U.S. in 2017 was 1.85 million tons (https://quickstats.nass.usda.gov/results/208E7120-FC29-332F-BE3A-5447C9983728). Orange seed oil contains linoleic acid (35.7-36.9 g/100 g oil), palmitic acid (29.3-29.9 g/100 g oil), oleic acid (23.5-24.5 g/100 g oil), stearic acid (3.8-4.0 g/100g oil) and linolenic acid (3.3-3.5 g/100g oil). Also, orange seed oil contains α-tocopherol (220 mg/kg), γ-tocopherol (27.72 mg/kg) and δ-tocopherol (16.73 mg/kg). The orange seed oil's physical and chemical properties are: iodine value (99.85 g I/100 g oil), refractive index (1.4645, 40 °C), density (0.920 mg/mL), saponification value (189.50 mg KOH/g oil), unsaponifiable matter (0.50%), acid value (0.50 mg KOH/ g oil) (Anwar, Naseer, Bhanger, Ashraf, Talpur, & Aladedunye, 2008).

Mandarin orange seed oil (*Citrus reticulata*). Mandarin orange is also known as mandarin or mandarine. The leading producing country is China and in 2011, the production was 12.4 million tons and followed by Spain (2.1 million tons) and Brazil (1.0 million tons) (http://www.fao.org/faostat/en/#data/QC/visualize). The size of mandarin orange is smaller compare to orange *(Citrus sinensis)*. Mandarin orange seeds are rich in linoleic acid (39.3-39.8 g/100 g oil), and followed by palmitic acid (25.5-26.1 g/100 g oil), oleic acid (23.7-24.3 g/100 g oil), stearic acid (4.3-4.6 g/100g

oil) and linolenic acid (3.5-3.7 g/100g oil). Compared to other *citrus* species, mandarin orange seed oil contains relatively high concentration of α-tocopherol (557.82 mg/kg), γ-tocopherol (84.10 mg/kg) and δ-tocopherol (20.02 mg/kg). Physical and chemical properties including iodine value, refractive index, density, saponification value, unsaponifiable matter and acid value of mandarin orange seed oil are 104.80 g I/ 100 g oil, 1.4658 at 40 °C, 0.927 mg/mL, 186.00 mg KOH/g oil, and 1.30 mg KOH/g oil, respectively (Anwar, Naseer, Bhanger, Ashraf, Talpur, & Aladedunye, 2008).

Citron seed oil (*Citrus medica L.*). Citron is one of the four forefathers (the others are pomelo, mandarin and papeda) of the citrus tribe, from which all other citrus types were developed through natural hybrid speciation or artificial hybridization. Citron grows in a high temperature and humidity environment, and originated from Southeast Asia and now is spread all over the world. Citron seed oil contains numerous fatty acids including linoleic acid (35.2 g/100 g oil), oleic acid (31.3-31.5 g/100 g oil), palmitic acid (19.7-19.9 g/100g oil), linolenic acid (7.9 g/100g oil) and stearic acid (3.5-4.3 g/100 g oil) (da Silva & Jorge, 2016). Moreover, the α-tocopherol was predominant in citron seed oil, with a content of 57.53 mg/kg. And the total carotenoids in citron seed oils was 6.85 μg β-carotene/g oil. Total sterols content in citron seed oil is 1666.1 mg/kg, with campesterol and β-sitosterol at 331.6 and 1325.3 mg/kg, respectively (da Silva & Jorge, 2016).

Kumquat seed oil (*Citrus japonica*). Kumquats are a group of small fruit-bearing trees that are native to South Asia and Pacific, and have been cultivated as an important economically fruit in China, Japan, Philippines and South-East Asia for years. They were special in citrus species because kumquats are usually consumed as a whole fruit together with the peel, unlike most other citrus species. Studies have reported that the kumquat seed oil mainly consisted of linoleic (42.5 g/100 g oil), oleic (21.4 g/100 g oil), palmitic acid (20.7 g/100g oil), linolenic acid (8.5 g/100g oil) and stearic acids (5.9 g/100 g oil) (da Silva & Jorge, 2016). The total sterols in kumquat seed oil were 1437.4 mg/kg, including campesterol (152.4 mg/kg), stigmasterol (108.9 mg/kg) and β-sitosterol (1176.2 mg/kg). The total carotenoids were 21.64 μg β-carotene/g oil. In addition, content of phenolic compounds including p-coumaric acid, salicylic acid and quercetin in kumquant oil were 7.13, 30.70 and 6.53 mg/kg, respectively. (da Silva & Jorge, 2016).

Tomato seed oil (*Solanum Lycopersicum*). Tomato is an edible fruit of *Solanum lycopersicum*, commonly known as a tomato plant. Tomato originated in Central and South America and spread throughout the world. In 2014, world production of tomatoes was 170.8 million tons, with China accounting for 31% of the total, followed by India, the United States and Turkey as the major producers (http://www.fao.org/faostat/en/#data/QC/visualize). The major component of tomato pomace is the seeds, which are considered to be a good source of edible oils. Tomato seed oil contains high amount of linoleic acid (59.1 g/100 g oil), followed by oleic

acid (20.0 g/100 g oil), palmitic acid (13.4 g/100g oil), stearic acid (4.6 g/100 g oil) and linolenic acid (2.0 g/100g oil) (da Silva & Jorge, 2016). In tomato seed oil, the γ-tocopherol represents all of the total tocopherol, at a concentration of 330.80 mg/kg. In addition, the content of β-sitosterol and stigmastanol were 1697.9 mg/kg and 14149 mg/kg, respectively (da Silva & Jorge, 2016).

Table 1.4. Edible seed oils rich in oleic acid (18:1n-9)

Seed oil	Species
Mango	*Mangifera indica L.*
Cherry	*Prunus avium* L.
Date	*Phoenix dactylifera* L.
Fluted pumpkin	*Telfaria occidentalis*
Carob bean germ	*Ceratonia siliqua* L.
Pumpkin	*Curcubita* sp.
Naked seed squash	*Cucurbita pepo* L.
American ginseng	*Panax quinquefolium* L.
African mahogany	*Khaya senegalensi*s
Drumstick tree	*Moringa oleifera*
Nutmeg	*Myristica fragrans*
Rambutan	*Nephelium lappaceum L.*
Mangaba	*Hancornia speciosa var. pubescens*
Papaya	*Carica papaya L.*
Soursop	*Anona muricata L.*
Mangosteen	*Garcinia mangostana*

List of seed oils are adapted from Bailey's Industrial Oil and Fat Products' chapter, Oils from Herbs, Spices, and Fruit Seeds (Yu, Choe, Li, & Zhang, 2020).

Oleic acid is a ω-9 (n-9) monounsaturated fatty acid (MUFA). Growing evidence suggests that diets rich in oleic acid may serve as an alternative choice to a low-fat blood cholesterol reducing diet, may modulate immune function, and may delay the development of atherosclerosis (Hargrove, Etherton, Pearson, Harrison, & Kris-Etherton, 2001; Nicolosi, Wilson, Handelman, Foxall, Keaney, & Vita, 2002; Yaqoob, 2002). Oleic acid is the predominant fatty acid in olive, canola, peanut, and specially produced sunflower seed oils. Oleic acid is not an essential fatty acid; it is

synthesized *in vivo* through the desaturation of stearic acid (18:0). Oleic acid is also rich in a number of other edible oils including: mango, cherry, date, pumpkin, naked seed squash, fluted pumpkin, carob bean germ, American gingseng, *Khaya senegalensis*, *Moringa oleifera,* nutmeg, rambutan, mangaba, papaya, soursop and mangosteen seed oils.

Mango seed kernel oil (*Mangifera indica L.*). Mango is originated from southern Asia. It is cultivated in various tropical region (https://plants.usda.gov/plantguide/pdf/cs_main3.pdf). In 2013, world production of mango was 43 million tons. The leading mango producing countries are India (18 million tons), China (4.45 million tons), and Thailand (3.14 million tons) in 2013. In the US, total production of mango including mangosteen and guava was 816 tons in 2014 (http://www.fao.org/faostat/en/#data/QC/visualize). Mango has a large seed which accounts for 20% weight of the whole fruit. Mango seed kernel is reported to have 6.0% protein, 4-12% fat, 77% carbohydrate, 2.0% fiber, and 2.0% ash (Raihana, Marikkar, Amin, & Shuhaimi, 2015; Lakshminarayana, Rao, & Ramalingaswamy, 1983; Ali, Gafur, Rahman, & Ahmed, 1985; Rao,1994). Mango seed kernel oil is rich in oleic acid, ranging 34-59% (Lakshminarayana, Rao, & Ramalingaswamy, 1983; Rao,1994; Comes, Farines, Aumelas, & Soulier, 1992) of total fatty acids. Stearic acid is the other major fatty acid in mango seed kernel oil, and may account for up to 57% of total fat. In addition, palmitic and linoleic acids were detected in the oil along

with trace amount of α-linolenic acid (Lakshminarayana, Rao, & Ramalingaswamy, 1983; Rao,1994).

Cherry seed oil (*Prunus avium* L.). The cherry tree is a member of the Rosaceae family. Cherry seed contains about 18% oil on a dry weight basis (Comes, Farines, Aumelas, & Soulier, 1992). Significant level of oleic acid was detected in the cherry seed oils prepared by hexane extraction using a Soxhlet apparatus. Oleic acid consisted of 24-38% of the seed oils of three different varieties of cherry fruits, with minor amounts of other monounsatuarted fatty acids (Comes, Farines, Aumelas, & Soulier, 1992). Linoleic acid was the major fatty acid in the cherry seed oil, and ranged 40-49%, along with α-eleostearic (18:3n-5), palmitic, stearic, arachidonic and α-linolenic acids. α-Eleostearic acid ($\Delta^{9c,11t,13t}$), comprising 10-13% of cherry seed oil, is a conjugated isomer of α-linolenic acid ($\Delta^{9c,12c,15c}$). α-Eleostearic acid was not detected in other previously studied seed oils from prunoids including peach, apricot, and plum seed oils.

Date seed oil (*Phoenix dactylifera* L.). Dates are popular in most of Middle Eastern countries, and serve as an important food ingredient (Salem & Hegazi, 1971; Sawaya, Khatchadourian, Khalil, Safi, & Al-Shalhat, 1982). Oil contents and fatty acid profiles of date seeds may vary among individual varieties. Date seeds were shown to contain 20-24% total fat (Sawaya, Khalil, & Safi, 1984). Oleic acid was the primary fatty acid in the date seed oil, and had a concentration of 43.5-45% of total fatty

acids. This was followed by lauric (12:0), myristic (14:0), palmitic (16:0), linoleic (18:2n6), capric (10:0), and stearic (18:0) acids along with trace amounts of other fatty acids. Date seed oil may serve as an excellent dietary source of oleic acid with a minor amount of linoleic acid.

Fluted pumpkin seed oil (*Telfaria occidentalis*). The fluted pumpkin (*Telfaria occidentalis*) is a tropical gourd native to West Africa. It is taxonomically classified as a member of the Curcubitaceae family. The fruits are very large and weigh up to 13 kg but only the seeds are edible (Nwokolo & Sim, 1987). The seeds are very rich in both protein and fat, containing approximately 28 and 55% of each, respectively, for the whole oven dried fluted pumpkin seeds (Moussata & Akoh, 1998). The fatty acid profile of fluted pumpkin seeds demonstrated a high oleic acid content of 35.4%, and a total saturated fatty acid concentration over 34% (Moussata & Akoh, 1998). Significant level of linoleic acid (18:2n-6) was also detected in the seed oil.

Carob bean germ seed oil (*Ceratonia siliqua* L). The carob bean, a tree of the *Leguminosae* family, is widely cultivated in the south and east of the Mediterranean region. The pulp tastes sweet and is used in foods (Maza, Zamora, Alaiz, Hidalgo, Millán, & Vioque, 1989). The seed germ contains about 5% oil. Oleic acid contributed to 20-39% of the total fatty acids, but only a small portion of that was on the n-2 position of the triglyceride. Linoleic acid ranged 44-59%, while palmitic and stearic acids accounted for 8-14% and 3-10% of the total fatty acids, respectively,

along with minor amount of linolenic and myristic acids. In addition, β-sitosterol was the primary sterol compound and contributed to 74% of the total sterols. Other sterol compounds included stigmasterol (17% of total sterol), campesterol (6%) and cholesterol (4.4%).

Pumpkin seed oil (*Curcubita* sp.). Pumpkin, *Curcubita sp.*, is a member of the gourd family, Curcubitaceae, that also includes melons, cucumbers, squash, and gac. In 2016, the United States' production of pumpkins was approximately 16 million cwt (1800 million pounds) (https://quickstats.nass.usda.gov/results/861DB11B-55CF-328C-8E32-77CBA95AD1D7). In some mid-eastern African countries, dried pumpkin seeds have been used to treat tapeworm when eaten on an empty stomach (Younis, Ghirmay, & Al-Shihry, 2000). Also, for many years in Europe, pumpkin seeds have been used as a remedy for micturition. Pumpkin seed oil has also shown possible beneficial effects in retarding the progression of hypertension (Zuhair, El-Fattah, & El-Sayed, 2000), potential anti-inflammatory activity in arthritis (Fahim, Abd-El Fattah, Agha, & Gad, 1995), and may be effective in reducing the risk of bladder-stone disease (Suphakarn, Yarnnon, & Ngunboonsri, 1987).

The pumpkin seed oil contained 37.8% oleic acid and 43.1% linoleic acid, and was fairly high in unsaturated fats (81%). Among the four pumpkin seed oil samples analyzed by Spangenberg and Orgrinc (Spangenberg & Ogrinc, 2001), oleic acid content was consistent and ranged 30.2-33.9% of total fatty acids (Spangenberg & Ogrinc, 2001), along with 24.5-47.9% linoleic acid. In addition, the oil of unroasted

pumpkin seed kernel (*Cucurbita mixta*) contained 21.4% oleic acid and 58.9% linoleic acid (Kamel, Deman, & Blackman, 1982). A recent study of pumpkin seed oil detected 55.6% of linoleic acid and 20.4% of oleic acid in the total fatty acids, with a total unsaturated fatty acid concentration of 76.5% (Ghoreishi & Bataghva, 2011; El-Adawy & Taha, 2001). The iodine value for the pumpkin seed oil was 103 g I/100 g oil (Moussata & Akoh, 1998), and the refractive index was 1.4616 (at 40 ºC) (Lazos, 1986), 1.4706 (at 25 ºC) (El-Adawy & Taha, 2001; El-Adawy & Taha, 2001b), and 1.4615 (at 60 ºC) (Kamel, Deman, & Blackman, 1982). The ORAC value of roasted pumpkin seed oil was determined to be 1.1 Trolox equivalents/g oil, and was the lowest in comparison to six other fruit seed oils tested under the same experimental conditions that included blueberry, red raspberry, black raspberry, boysenberry, and cranberry seed oils (Parry & Yu, 2004b). Phytosterols were also detected in pumpkin seed oil. (El-Adawy & Taha, 2001; El-Adawy & Taha, 2001b).

The seed oils from African pumpkin (squash) (*Cucurbita pepo* L.) were evaluated for their fatty acid profiles and the presence of other phytochemicals (Younis, Ghirmay, & Al-Shihry, 2000). The seed oils contained 28-36% oleic acid. The primary fatty acid was linoleic acid, along with palmitic and stearic acids, with a total unsaturated fatty acid concentration of 77-83%. α-Tocopherol was determined at a concentration up to 3.0 mg/100 g. These data suggest that pumpkin seed oil may be a better choice for consumers who prefer high unsaturation, and/or both linoleic and oleic acids. Seed oils from species of *Cucurbita* with minimal pericarp, called ‘naked seed squash’ are discussed below.

Naked seed squash seed oil (*Cucurbita pepo* L.). Squash (*Curcubita foetidissima, C. pepo, and C. lagenaria*) has been consumed worldwide for thousands of years. The fruits are used as vegetables and desserts, and seeds are consumed as nuts or used to prepare edible oils (Lazos, 1992). Normally, each plant produces 1-9 fruits weighing 0.47-12.67 kg, and each fruit may have 16-393 seeds (Idouraine, Kohlhepp, Weber, Warid, & Martinez-Tellez, 1996). The weight of the individual naked seeds ranged from 46-223 mg. Seeds of the naked seed squash varieties are preferred for direct consumption and further processing. In 1996, Idouraine and others (Idouraine, Kohlhepp, Weber, Warid, & Martinez-Tellez, 1996) reported that the crude oil content in the 9 selected naked seed squash lines (*C. pepo* L.) ranged 34-44% on a dry weight basis. The seed oil of the selected naked seed squash was rich in oleic acid, which made up 47-60% of the total fatty acids. Other major fatty acids were linoleic, palmitic, and stearic acids, along with small amount of other long chain fatty acids. The total unsaturated fatty acids ranged 70-79% for the 9 tested seed oils (Idouraine, Kohlhepp, Weber, Warid, & Martinez-Tellez, 1996). These were in consistent with earlier observations that oleic acid was the major fatty acid and constituted 46-50% of the seed oils for all progeny lines (Bemis, Berry, Kennedy, Woods, Moran, & Deutschman, 1967; El-Gharbawi, 1967). In summary, the seed oil of *Curcubita pepo* may serve as an edible oil rich in unsaturated fatty acid and oleic acid.

In addition, other studies reported that linoleic acid as the predominant fatty acid in the seed oils of squash varieties (*Curcubita foetidissima*) with a range of 39-

77%, while the level of oleic acid was 10-32% (Lazos, 1986; Vasconcellos, Berry, Weber, Bemis, & Scheerens, 1980; Vasconcellos & Berry, 1982). Interestingly, a significant level of conjugated dienoic acids was detected in the seed oils of *Curcubita foetidissima*. It is widely accepted that conjugated linoleic acids may have a number of health benefits such as anticarcinogenesis, reducing body weight, anti-atherosclerotic activity, and antioxidant activity (Yu, Adams, & Gabel, 2002). Future research is required to further confirm the presence of the conjugated dienoic acids in the seed oils of *Curcubita foetidissima* and identify their chemical structures. In summary, seed oil of *Curcubita foetidissima* may serve as edible oil rich in unsaturated fatty acid and linoleic acid.

American ginseng seed oil (*Panax quinquefolium* L.). American ginseng, native to North America, is one of the most widely used medicinal herbs and a functional food ingredient. American ginseng seed oil prepared by hexane or methylene chloride extraction at ambient temperature was analyzed for fatty acid profile and phytosterol content (86). The seed oils contained about 86.8-87.5% oleic acid, 9.9-10.5% linoleic acid, and 2.6% total saturated fatty acids. Significant levels of phytosterols were observed in the American ginseng seed oils prepared with hexane and CH_2Cl_2 (Beveridge, Li, & Drover, 2002). Squalene was the primary phytosterol compound with a concentration of 502-514 mg/100 g oil, followed by β-sitosterol and stigmasterol at levels of 164-177 and 93-95 mg/100 g oil, respectively. Phytosterols are thought to benefit human health through lowering cholesterol and increasing

antioxidant activity (Ling & Jones, 1995; Kelly, 1999). American ginseng seed oil would be a desirable dietary source for oleic acid, squalene, and total phytosterols.

***Khaya senegalensis* seed oil**. *Khaya senegalensis* is a dry land mahogany that grows to about 30 m in height and 3 m in girth; it is widely distributed in the savanna region of Nigeria. The seed of *Khaya senegalensis* contains about 53% oil. The seed oil has been used to treat certain local ailments. Oleic acid was the predominant fatty acid in the seed oil and accounted for 65% of the total fatty acid, along with 21% palmitic and 10% stearic acids (Okieimen & Eromosele, 1999). The seed oil had a saponification value of 186 mg KOH/100 g oil, a density of 0.962, a refractive index of 1.4690 (20 °C), and an iodine value of 68.0 g I/100 g oil (Okieimen & Eromosele, 1999).

***Moringa oleifera* seed oil**. The family Moringaceae embraces only one genus, *Moringa*, which was, in the past, credited with only three species. Today, the number is usually shown as 10 or 12. The best known and most widely distributed species is *M. pterygosperma* Gaertn (Morton, 1991). *Moringa oleifera*, native to the western and sub-Himalayam tracts of India and other countries in Asia, Africa, the Middle East, Central America, and the Caribbean islands, is the most common and broadly distributed species of the Moringaceae family (Tsaknis, Spiliotis, Lalas, Gergis, & Dourtoglou, 1999). The leaves, flowers, fruits and roots of the tree are used as vegetables (Tsaknis, Spiliotis, Lalas, Gergis, & Dourtoglou, 1999). Each fruit

generally contains about 20 seeds, which have an average weight of 0.3 g with the kernel responsible for 70-75% of the weight. The seed oils of *Moringa oleifera* were extracted and analyzed for chemical compositions (Anwar & Bhanger, 2003; Vlahov, Chepkwony, & Ndalut, 2002). The seed oil was a rich source of monounsaturated fatty acid, especially 18:1 that contributed 74.0-78.6% of the total fatty acids (Anwar & Bhanger, 2003; Vlahov, Chepkwony, & Ndalut, 2002). A significant amount of phytosterols was detected in the seed oil (Anwar & Bhanger, 2003). The major sterols were β-sitosterol (46.65% of total sterol), stigmasterol (19%), campesterol (16%), and Δ^5-avenasterol (10.7%), along with a number of others.

The seeds of *M. oleifera* variety Mbololo yielded 26, 31, and 36% crude oil by cold-pressing, hexane extraction, and chloroform-methanol extraction (1:1, v/v), respectively (Tsaknis, Spiliotis, Lalas, Gergis, & Dourtoglou, 1999). The seed oil was rich in total monounsaturated fatty acids and contained 74-75% oleic acid. The total saturated fatty acids were 19-21% in the seed oil, with small amounts of linoleic and α-linolenic acids. The density of the seed oils prepared by solvent extraction and cold-pressing ranged from 0.8809-0.9182 g/mL at 24 °C which is similar to that of olive oil at 0.915 g/mL at the same temperature. The refractive index (n_D40°C) was 1.4549-1.4591, and the smoke point was 198-202 °C for the seed oils. The seed oil prepared by cold-pressing had a greater viscosity of 103 mPa•s, while the oil prepared by solvent extraction exhibited a viscosity of 57-66 mPa•s, which is more comparable with that of olive oil (74 mPa•s) (Tsaknis, Spiliotis, Lalas, Gergis, & Dourtoglou, 1999). In addition, the seed oil of *M. oleifera* variety Mbololo contained greater

concentrations of total sterols and tocopherols than olive oil. The primary sterol in the Mbololo seed oil was β-sitosterol along with significant levels of stigmasterol, campesterol, and Δ^5-avenasterol, as well as small amounts of other sterol compounds (Tsaknis, Spiliotis, Lalas, Gergis, & Dourtoglou, 1999). In summary, the seed oil of *M. oleifera* may serve as a dietary source of oleic acid, sterols, and tocopherols.

Nutmeg seed oils (*Myristica fragrans*). Nutmeg is a dark-leaved, evergreen tree belongs to the family of Annonaceae. As a widely-consumed spice and aromatic component, nutmeg is widely distributed from Asia, Africa, Central to South America, and Australia. The most abundant fatty acid in nutmeg seed oil was determined oleic acid (56.5-59.4 g/100 g oil). And the other two major components were linoleic acid and linolenic acid (13.6-13.8 and 1.8-1.9 g/100 g oil, respectively.). In addition, the content of phenolics in the nutmeg seed oil varied depending on the method of oil extraction. The total sterol content is much higher when using chloroform/methanol extraction (3104.85 mg/kg oil) than that using *n*-hexane extraction (2836.39 mg/kg oil) (Kozłowska, Gruczyńska, Ścibisz, & Rudzińska, 2016).

Rambutan seed oil (*Nephelium lappaceum L.*). Rambutan is originated from Southeast Asia. Rambutan is cultivated in China, India, Thailand, Taiwan, Malaysia and Australia (Manaf, Marikkar, Long, & Ghazali, 2013). The shape of rambutan fruit is ovoid or ellipsoid and color varies from pinkish to deep red. Each rambutan

has one seed inside the flesh, and it is around an inch long. The moisture, oil, protein, crude fiber, ash, and carbohydrate of rambutan seed are in range of 6.08-13.12, 33.64-42.36, 12.42-14.98, 7.46-7.74, 2.08-2.72, 28.27-29.13%, respectively. Rambutan seed oil may have an iodine value (50.176-50.424 g I/100g), saponification value (181.94-182.26 mg KOH/g oil), unsaponifiable matter (0.38-0.62%) and melting point (39.2 °C). Rambutan seed oil is rich in oleic acid at a level of 43.1 g/100 g oil, and followed by arachidic acid (31.5 g/100 g oil), stearic acid (7.9 g/100 g oil), gadoleic acid (5.9 g/100 g oil), palmitic acid, (4.6 g/100 g oil), linoleic acid (3.2 g/100 g oil), behenic acid (2.1 g/100 g oil), linolenic acid (0.7 g/100 g oil), palmitoleic acid (0.7 g/100 g oil), myristic acid (0.1 g/100 g oil) and erucic acid (0.1 g/100 g oil) (Manaf, Marikkar, Long, & Ghazali, 2013).

Mangaba seed oil (*Hancornia speciosa var. pubescens*). Mangaba tree belongs to the Apocynaceae family. It is native to Brazil and found spontaneously in various regions in South America. The tree reaches 5 to 10 m in height. The fruits are yellowish berries with red spots and stains and a meaty-viscous pulp with a sweet-acidic taste. The seeds are of the recalcitrant type and have a light brown color. Mangaba seeds correspond 12% of the fruit weight, which is a good south of essential fatty acid. It has been reported that mangaba seed oil was rich in oleic acid (63.9 g/100 g oil). And the other major fatty acids are palmitic acid (19.1 g/100 g oil), linoleic acid (7.3-7.5 g/100 g oil) and stearic acid (8.2 g/100 g oil). Total tocopherols in mangaba seeds oil were 36.77 mg/kg, mainly consist of α-tocopherol (36.77

mg/kg). Total carotenoids in the seeds oil were 15.91 μg β-carotene/g sample and total sterols in the seeds oil were 4565.7 mg/kg, including β-sitosterol (3471.1 mg/kg) and stigmastanol (1094.6 mg/kg) (da Silva & Jorge, 2016).

Papaya seed oil (*Carica papaya L.*). Papaya belongs to the family Caricaceae, and commonly distributes in tropical and subtropical regions of the world. In 2014, global production of papayas was 12.7 million tons, with India accounting for 44% of the world total production (http://edis.ifas.ufl.edu/fe913). The fruit is usually cylindrical, large (weighing 0.5-2.0 kg), and fleshy. The fresh fruit is yellow-orange color, soft, and juicy. The central cavity contains large quantities of seeds that comprise about 15% of the wet weight of the fruit. The seeds of papaya are edible and have some spicy flavor which makes it a substitute for black pepper. According to studies, the oil yield from the seeds was 29.16% and the oil was abundant in oleic acid (66.7-79.1 g/100 g oil), followed by palmitic acid (13.9-19.7 g/100 g oil), linoleic acid (0.4-6.1 g/100 g oil) and stearic acid (1.9-6.7 g/100 g oil) (da Silva & Jorge, 2016; Malacrida, Kimura, & Jorge, 2011). The saponification value, unsaponifiable matter and free fatty acid contents of freshly extracted papaya seed oil were 193.5 mg KOH/g oil, 1.52% and 0.91%, respectively (Malacrida, Kimura, & Jorge, 2011). In addition, Cassia and others reported that the α- and δ-tocopherol were the predominant tocopherols with 51.85 and 18.89 mg/kg, respectively (Malacrida, Kimura, & Jorge, 2011). However, Ana and others reported that α- and β-tocopherols were the

predominant tocopherols at levels of 27.20 and 3.97 mg/kg, respectively (da Silva, & Jorge, 2016).

Soursop seed oil (*Anona muricata L.*). Soursop is native to tropical regions of Central and South America, and it is wildly distributed across the equatorial belt of the planet, becoming a highly important economic crop in many countries. Soursop is a heart-shaped fruit with dark green color peel, creamy white edible pulp and black seeds inside (Leatemia & Isman, 2004). The seeds comprise 5% of fruit mass after commercial processing of soursop, and the total oil yield from the seeds were 40% of seeds weight (Kimbonguila, Nzikou, Matos, Loumouamou, Ndangui, Pamboutobi, et al., 2010). The analysis of soursop proximate composition showed on average (by mass), 20.5-40% oil (lipids), 8.5% protein, 34.1% total carbohydrates and 5.2% crude fiber, besides important minerals such as potassium, calcium, phosphorus, sodium and magnesium (Kimbonguila, Nzikou, Matos, Loumouamou, Ndangui, Pamboutobi, et al., 2010). Studies have reported that soursop seed oil mainly consisted of oleic acid (43.2-43.4 g/100 g oil), linoleic acid (29.7 g/100 g oil), palmitic acid (19.4 g/100g oil), stearic acid (4.8 g/100 g oil) and linolenic acid (0.8 g/100 g oil) (da Silva & Jorge, 2014, 2016). In addition, total tocopherols in soursop seeds oil were 29.57 mg/kg, mainly consist of α-tocopherol (20.67 mg/kg) and γ- tocopherol (8.90 mg/kg). Total carotenoids in the seeds oil were 4.39 μg β-carotene/g oil, and total sterols in the seeds oil were 1515.9 mg/kg, including campersterol (180.2 mg/kg), stigmasterol (472.2 mg/kg) and β-sitosterol (3471.1 mg/kg) (da Silva & Jorge, 2014).

Mangosteen seed oil (*Garcinia mangostana)*. Mangosteen is a tropical evergreen tree and mainly grows in Southeast Asia, Southwest India and other tropical regions. The fruit of mangosteen is sweet, tangy, and juicy. The almond-shaped seeds of mangosteen are by-products in food industry. The primary fatty acid of the seeds oil is palmitic acid (49.5 g/100 g oil) and followed by oleic acid (34.2 g/100 g oil), arachidic (8.8 g/100 g oil), stearic (1.3 g/100 g oil), linoleic (1.0 g/100 g oil), eicosadienoic (0.1 g/100 g oil) and gadoleic (0.1 g/100 g oil) acids. The seed oil is also rich in potassium (707 mg/kg), calcium (454 mg/kg), sodium (26.0 mg/kg), magnesium (865 mg/kg), zinc (19.0 mg/kg), iron (90.0 mg/kg) and manganese (18.0 mg/kg) (Ajayi, Oderinde, Ogunkoya, Egunyomi, & Taiwo, 2007).

Table 1.5. Edible seed oils rich in petroselinic acid (C18:1n-12)

Seed oil	Species
Dill	*Anethum graveolens*
Caraway	*Carum carvi*
Cumin	*Cuminum cyminum*
Coriander	*Coriandrum sativum L.*
Anise	*Pimpinella anisum*
Celery	*Apium graveolens*
Fennel	*Foeniculum vulgare*
Khella	*Ammi visnaga*

List of seed oils are adapted from Bailey's Industrial Oil and Fat Products' chapter, Oils from Herbs, Spices, and Fruit Seeds (Yu, Choe, Li, & Zhang, 2020).

Petroselinic acid (C18:1n-12), represents an important oleochemical raw material. Since there is a difference in structure with unsaturation occurs in the 6,7-

position, petroselinic acid is relatively rare among octadecenoic acids. Petroselinic acid offers a great opportunity to produce different chemical derivatives. For example, this acid has promising application in the chemical industry since they can be used as a precursor of both lauric acid (C12:0), which is a component of detergents and surfactants, and adipic acid, which is the monomeric component of nylon 66 (Murphy, Richards, Taylor, Capdevielle, Guillemot, Grison, et al., 1994; Murphy, 1996). Petroselinic acid were found relatively rich in seed oils from the Apiaceae family, such as dill, caraway, cumin, coriander, anise, celery and fennel (Nguyen, Aparicio, & Saleh, 2015).

Dill seed oil (*Anethum graveolens*). Dill is an annual herb in the Apiaceae family. It grows up to 40-60 cm tall with 4-5 mm long and 1 mm thick seeds. Dill is native to south-west Asia or south-east Europe and cultivated since ancient times. The leaves and seeds are commonly used as a medical herb or spice for flavoring food in Eurasia, South Africa and in many other countries. Recently, Thao Nguyen and others reported that oil yield from dill seeds was 20.5 g/100 g, in which petroselinic acid was the primary fatty acid with a content of 78.7-81.1 g/100 g oil. Linolenic acid was the second most abundant fatty acid with a content of 10.3-11.3 g/100 g oil. Furthermore, α-linolenic (0.7 g/100 g oil) and arachidic (0.5-0.6 g/100 g oil) acids were minor fatty acids in the dill seed oil (Nguyen, Aparicio, & Saleh, 2015).

Caraway seed oil (*Carum carvi*). Caraway is a biennial plant of the Apiaceae family which is original from Asia, Europe and North Africa. Caraway seeds are crescent-shaped with five pale ridges, and are about 2 mm long. Yield and composition of caraway seed oil various from production conditions, harvesting date, storage time and also depends on the cultivar and growth conditions (Galambosi & Peura, 1996). Recently, in Mariola and Thao's study, caraway seed oil is rich in petroselinic acid with a concentration range of 56.8-58.6 g/100 g oil. And the second major fatty acid was linoleic acid, ranging from 30.3 to 31.9 g/100 g oil (Kozłowska, Gruczyńska, Ścibisz, & Rudzińska, 2016; Nguyen, Aparicio, & Saleh, 2015). However, in an early report by Parker and others, caraway seed oil was rich in linoleic acid, which is about 55.82 g/100 g fatty acids (Parker, Adams, Zhou, Harris, & Yu, 2003).

Cumin seed oil (*Cuminum cyminum*). Cumin is believed to be native to Egypt and Syria, Turkestan and the East Mediterranean region (http://www.eolss.net/sample-chapters/c10/e1-05a-50-00.pdf). Cumin was introduced to the U.S. by Spanish and Portuguese colonists. Cumin seeds are used in various foods including curry, kebob, chilli, and sausage. In 2007, world's total cumin production was 300,000 tons. The leading producer of cumin is India. India produces more than 175,000 tons which is 70% of global cumin production (http://www.eolss.net/sample-chapters/c10/e1-05a-50-00.pdf). In India, cumin is often used as a traditional ingredient. Cumin seeds are rich in fat, protein, fiber and various minerals including iron and magnesium. Cumin seed oil has a strong aroma due to cumin aldehyde and cuminic alcohol. Cumin seed oil contains petroselinic acid (59.9-63.8 g/100 g oil) and linoleic (29.5-31.3 g/100 g

oil), palmitic (4.7-4.8 g/100 g oil), oleic (1.4 g/100 g oil), α-linolenic (0.6 g/100 g oil), palmitoleic (0.3 g/100 g oil) and arachidic (0.1 g/100 g oil) acids (Nguyen, Aparicio, & Saleh, 2015).

Coriander seed oil (*Coriandrum sativum L.*). Coriander is an annual herbaceous plant in the Apiaceae family that originated from the Mediterranean area. All parts of the plant are edible, but the dried seeds and fresh leaves are the parts most traditionally used as condiment or spice in eastern cooking, Indian food and Thai and Vietnamese cuisines. Coriander seeds may serve as a potential source of bioactive constituents that may have antimicrobial, antioxidant, anti-inflammatory and anti-cancer activities (Laribi, Kouki, Mhamdi, & Bettaieb, 2015). The principal fatty acids of coriander seed oil is petroselinic acid, ranging of 65.7-79.8 g/100 g oil, followed by linoleic acid (13.0-16.7 g/100 g oil) (Kozłowska, Gruczyńska, Ścibisz, & Rudzińska, 2016, Nguyen, Aparicio, & Saleh, 2015; Ramadan & Mörsel, 2002; Sriti, Talou, Wannes, Cerny, & Marzouk, 2009). However, in the report from Matthaus and others, oleic acid was the predominate fatty acid in coriander seed oil, which is about 78.3 g/100 g fatty acids (Matthaus, Al Juhaimi, Ozcan, El Babiker, & Ghafoor, 2016). Other representative fatty acids were oleic, palmitic and stearic acids with the contents of 5.5-7.8, 3.0-4.2 and 0.7-2.9 g/100 g oil, respectively (Sriti, Talou, Wannes, Cerny, & Marzouk, 2009). In addition, α-linolenic acid (0.1-0.2 g/100 g oil) and arachidic acid (0.1-0.2 g/100 g oil) were the minor fatty acids (Kozłowska,

Gruczyńska, Ścibisz, & Rudzińska, 2016; Ramadan & Mörsel, 2002; Sriti, Talou, Wannes, Cerny, & Marzouk, 2009).

Anise seed oil (*Pimpinella anisum*). Anise belongs to an aromatic plant family '*apiaceae'* family and originated from East Mediterranean to Southeast Asia (Surmaghi, 2010). Anise seeds have sweet aroma and due to its aroma, cuisines use anise seed for fish products, flavor dishes, ice cream, candies, gums and teas (Surmaghi, 2010; Özcan, Chalchat, Arslan, Ateş, & Ünver, 2006). In the ancient time, anise seeds were used as a medicine. Also, during the American civil war, anise seeds were used as a pain reliever. Anise seed oil is rich in petroselinic (64.4-68.1 g/100 g oil) acid, along with linoleic (24.5-24.7 g/100 g oil), palmitic (4.6-4.9 g/100 g oil), oleic (1.47 g/100 g oil), stearic (0.9-1.4 g/100 g oil), α-linolenic (0.7-0.8 g/100g oil) and palmitoleic (0.2-0.3 g/100 g oil) acids (Nguyen, Aparicio, & Saleh, 2015).

Celery seed oil (*Apium graveolens*). Celery is cultivated as a vegetable and often its stalks are eaten and used for cooking. Celery seeds are used as a spice. The origin of celery is thought to be the Mediterranean region. Today, celery grows in various continents such as North America, Europe, and Asia. In the U.S., total celery production in 2016 was 17 million cwt (1911 million pounds) and the primary celery producing state, California produced 1792 million pounds which is 94% of total U.S. production (https://quickstats.nass.usda.gov/results/6FC81E25-A976-3A71-B055-428208730684). Celery seed contains various flavoring ingredients and used in many

food products such as beverages, frozen dairy products, candy, baked goods, gelatins, puddings, meat products, soups, and snacks. Similar to other *apiaceae* family, celery seed oil is rich in petroselinic acid (64.0-67.6 g/100 g oil), along with linoleic (21.0-22.3 g/100 g oil), palmitic (7.5-9.5 g/100 g oil), stearic (2.0 g/100 g oil), α-linolenic (0.9-1.1 g/100 g oil), palmitoleic (0.3-0.4 g/100 g oil) and arachidic (0.3-0.4 g/100 g oil) acids (Nguyen, Aparicio, & Saleh, 2015).

Fennel seed oil (*Foeniculum vulgare*). Fennel is native to Mediterranean region (https://www.mdidea.com/products/new/new04203.html). The leading producers of fennel are India, Mexico and China. Fennel has a strong aroma and it is from compound 'anethole'. Anethole is also in anise seed. Because of this strong aroma and flavor, fennel is often used as culinary and medicinal purposes. Fennel seed is a good source of energy as well as nutrients. 100 grams of fennel seeds provide 345 kcal and contain 0.41 mg of thiamine (B_1), 0.35 mg of riboflavin (B_2), 6.1mg of niacin (B_3), 0.47 mg of vitamin B_6, 21 mg of vitamin C, 1196 mg of calcium, 385 mg of magnesium, 18.5 mg of iron, 6.5 mg of manganese, 1694 mg of potassium, 487 mg of phosphorous, 88 mg of sodium and 4 mg of zinc (https://ndb.nal.usda.gov/ndb/foods/show/268?fg=&man=&lfacet=&count=&max=35&sort=&qlookup=Spices%2C+fennel+seed&offset=&format=Full&new=&measureby=). Fennel seed oil is very rich in petroselinic (79.5-84.4 g/100 g oil) acid, followed by linoleic (10.7-11.3 g/100 g oil), palmitic (4.6-4.8 g/100 g oil), stearic (1.2-1.3

g/100 g oil), palmitoleic (0.4-0.5 g/100 g oil), α-linolenic (0.3 g/100 g oil) and arachidic (0.2 g/100 g oil) acids (Nguyen, Aparicio, & Saleh, 2015).

Khella seed oil (*Ammi visnaga*). Khella is also known as toothpickplant, toothpickweed and bisnaga. Khella grows wild in Mediterranean region especially in Egypt, Morocco and Islamic republic of Iran. In the ancient time, khella seed was used to treat kidney stone. Similar to other *apiaceae* family, khella seeds are small and in an oval shape. Khella seed oil contains petroselinic acid (72.8-77.1 g/100 g oil) as the major fatty acid, along with linoleic (16.5-16.8 g/100 g oil), α-linolenic (1.3-1.5 g/100 g oil), stearic (1.0-1.1 g/100 g oil), palmitoleic (0.2-0.3 g/100 g oil) and arachidic (0.2-0.3 g/100 g oil) acids (Nguyen, Aparicio, & Saleh, 2015).

Table 1.6. Other special seed oils of fruit, spice, and herb

Seed oil	Species
Gac	*Momordica cochinchinensis*
Pomegranate	*Punica granatum*

List of seed oils are adapted from Bailey's Industrial Oil and Fat Products' chapter, Oils from Herbs, Spices, and Fruit Seeds (Yu, Choe, Li, & Zhang, 2020).

Gac seed oil (Momordica cochinchinensis). Gac is another member of the gourd family (*Curcubitaceae*). The gac fruit is round and about 18-22 cm in diameter. It is native to Asia and is both consumed as food and used in traditional medicine. The seeds comprise about 16% of the total fresh weight of the fruit.

Ishida and others (Ishida, Turner, Chapman, & Mckeon, 2004) examined gac seed oil for its fatty acid composition. The oil contained an average of 63.1% stearic

acid (54.5-71.7 g/100 g oil). Palmitic acid (5.2-6.2 g/100 g oil) was the other saturated fatty acid found in the gac seed oil, and the total saturated fatty acids ranged from 60.5 to 79.2%. The most prevalent unsaturated fatty acid was linoleic acid (11.2-25.0 g/100 g oil) with an interesting variety of others including oleic acid (4.8-11.2 g/100 g oil), α-linolenic acid (0.4-0.6 g/100 g oil) and others (Ishida, Turner, Chapman, & Mckeon, 2004).

Pomegranate seed oil (*Punica granatum*)**.** Pomegranate, of the Punicaceae family, is a small tree grown in Iran, India and the United States, as well as in most Near and Far East countries. Pomegranate is used as a table fruit and is also processed to juice. Pomegranate preparations, including the juice of the fruit, the dried pericarp, the bark, and the roots, have been used in folk medicines to treat colic, colitis, dysentery, diarrhea, menorrhagia, oxyuriasis, parasis, headache, vermifugal, carminative, antispasmodic, taenicidal, and emmenagogue (Schubert, Lansky, & Neeman, 1999). Seeds are byproducts form the juice manufacture. Cold-pressed pomegranate seed oil was prepared and analyzed for fatty acid composition, inhibitory effects against cyclooxygenase and lipoxygenase, antioxidant properties, and total phenolic content (Schubert, Lansky, & Neeman, 1999). The major fatty acid was punicic acid (18:3n-5) (65.3 g/100 g oil), which comprised 65% of total fatty acids, along with linoleic (6.6 g/100 g oil), oleic (6.3 g/100 g oil), palmitic (4.8 g/100 g oil) and stearic (2.3 g/100 g oil) acids (Schubert, Lansky, & Neeman, 1999). The seed oil contained about 150 ppm total phenolics on an oil weight basis. The oil extract, at a concentration of 5

μg/mL total phenolics, exhibited 37% inhibition of the sheep cyclooxygenase activity under the experimental conditions (Schubert, Lansky, & Neeman, 1999). On a same weight concentration basis, the oil extract resulted in 75% inhibition of the soybean lipoxygenase activity, whereas butylated hydroxyl anisole (BHA) had a 92% inhibition under the same experimental conditions. The oil extract also showed strong antioxidant activity in the coupled oxidation system of carotene and linoleic acid, and the antioxidant capacity is comparable to that of BHA and green tea extract on the same weight concentration basis (Schubert, Lansky, & Neeman, 1999). These data suggest the potential application of pomegranate seed oil as anti-inflammatory agent and for general health promotion.

1.2.1. Nutraceutical properties

The oils of the selected fruit, spice and herb seeds may also contain significant levels of phytosterols, tocopherols, carotenoids, and natural antioxidants. The chemical composition of an edible seed oil determines the potential health benefit and applications for the oil. Individual edible seed oils may be preferred by special groups of consumers for preventing and treating a selected health problem or for general health promotion. Great opportunities are available in the research and development of specialty seed oils and the oil-based nutraceutical products from fruit, spice and herb seeds for improving human health. More research is required to screen and characterize the fatty acids and bioactive components in the fruit, spice, and herb seeds to develop novel edible seed oils for optimum human nutrition.

1.2.2. Impact to agriculture and oil industry

The seeds of many fruits, spices, and herbs are discarded every year at a cost or used as cheap animal feed. Therefore, developing specialty oils from these fruits, spices, and herbs can lead to the profit of both the agriculture and oil industry. At the same time, the utilization of these seeds can reduce environmental contamination. Recently, the grape industry made a huge profit using grape seeds as an ingredient for grape seed oils. Similarly, other seed oils may contain health-beneficial components and can be used as a potential food ingredient. However, currently, there are several hurdles for the oil industry to overcome. Such hurdles are consumer's acceptance, infrastructure, and research. Even though hurdles are slowing down the development of the specialty oils, specialty oil industry is growing each year and numerous oils will be displayed in the market in the future.

1.2.3. Antioxidants and their health benefits/impacts

Seed oils contain fat-soluble vitamins such as vitamin E and provitamin A and these vitamins work as an antioxidant. Vitamin E family includes eight different vitamers. These eight different vitamers are classified into 2 groups called tocopherols and tocotrienols. Each group has four forms, α, β, δ, and γ of either tocopherols or tocotrienols based on its methyl group's location on the phenol ring. All four tocopherols have vitamin E activity while only α-tocotrienol shows vitamin E activity. An important function of vitamin E is the maintenance of cell membranes. Vitamin E stops the chain reaction of lipid peroxyl radicals and prevents initial oxidation of polyunsaturated fatty acids (Gropper, Smith, & Carr, 2018)

Provitamin A such as α- and β-carotenes are often found in seed oils. The function of vitamin A includes visual system and eyesight, regulation of gene expression, immunity, red blood cell production and cancer prevention (Conaway, Henning, & Lerner, 2013) Other carotenoids such as lutein, cryptoxanthin, and zeaxanthin found in seed oils may possess antioxidant abilities.

1.2.4. Anti-inflammatory components and their health benefits/impacts

Alpha linolenic acid (18:3n-3), also known as omega-3 (ω-3) fatty acid, is rich in various seed oils and well-known for its anti-inflammatory effects. Alpha linolenic acid is synthesized from an omega-6 (ω-6) fatty acid, linoleic acid (18:2n-6) by desaturation, catalyzed by delta-15 desaturase. Animals including humans do not have the enzyme delta-15 desaturase. On the other hand, plants have delta-15 desaturase and possible to synthesize alpha linolenic acid. Even though animals do not synthesize alpha linolenic acid, they can metabolize it through desaturation and elongation (Calder, 2013). Alpha linolenic acid is converted to eicosapentaenoic acid (EPA) (20:5n-3) and further to DHA (22:6n-3).

It has been reported that consumption of omega-3 fatty acid may have effects in decreasing generation of arachidonic acid-derived eicosanoids, increasing generation of EPA-derived eicosanoids, increasing generation of EPA and DHA-derived resolvins, decreasing generation of inflammatory cytokines including tumor necrosis factor alpha, interleukin-1beta, interleukin-6, and interleukin-8, decreasing expression of adhesion molecules, decreasing leukocyte chemotaxis, decreasing generation of reactive oxygen species (Calder, 2006).

Throughout these anti-inflammatory effects, omega-3 fatty acids may have a benefit in chronic diseases such as rheumatoid arthritis, Crohn disease, ulcerative colitis, type 1 and 2 diabetes, multiple sclerosis, atherosclerosis, obesity, and cancer (Calder, 2006). Therefore, alpha linolenic acid rich oils such as black raspberry, red raspberry, boysenberry, marionberry, blackberry, blueberry, cranberry, kiwi, sea buckthorn, basil, hemp, and chia seed oils (Table 1.1) may possess health benefit previously described and can be utilized for agriculture, oil industry, and customers for the future.

1.2.5. Antiproliferative components and health benefits/impacts

Seed oils contain both ω-3 (n-3) and ω-6 (n-6) unsaturated fatty acids and oils rich in ω-3 (n-3) unsaturated fatty acids may have health benefits in preventing cancer by suppressing cancer cell's proliferation. It has been well-known that cyclooxygenase-2 (COX-2) is involved in carcinogenesis. The cyclooxygenase enzyme produces several prostanoids including prostaglandin D_2, E_2, F_2a, I_2, and thromboxane A_2 (Gu, Shan, Chen, & Chen, 2015). Among these, prostaglandin E_2 is highly expressed in many cancer cells such as colon, breast, head, neck, and lung (Gu, Shan, Chen, & Chen, 2015). Therefore, downregulate the prostaglandin E_2 levels by inhibiting or suppressing COX-2 plays an important role in cancer prevention. Omega-3 fatty acid works as an inhibitor of COX-2 by disrupting the signaling pathway.

Seed oils rich in alpha linolenic acid (18:3n-3) such as black raspberry, red raspberry, boysenberry, marionberry, blackberry, blueberry, cranberry, kiwi, sea

buckthorn, basil, hemp, and chia seed oils (Table 1.1) may have a potential health benefit in preventing cancer development through modulating COX-2 pathway.

In summary, there is an increasing demand for edible oils with special fatty acid profiles and other beneficial components for improving human health. A number of studies have been conducted to screen for and evaluate the chemical composition and potential nutraceutical applications of fruit, spice and herb seed oils. Among the discussed edible seed oils, some have unique fatty acid compositions, such as black raspberry and hemp seed oils rich in α-linolenic acid, date and naked seed squash seed oils rich in oleic acid, whereas blackcurrant seed oil is rich in γ-linolenic acid. The oils of the selected fruit, spice and herb seeds may also contain significant levels of phytosterols, tocopherols, carotenoids, and natural antioxidants. The chemical composition of an edible seed oil determines the potential health benefit and applications for the oil. Individual edible seed oils may be preferred by special groups of consumers for preventing and treating a selected health problem or for general health promotion. Great opportunities are available in the research and development of specialty seed oils and the oil based nutraceutical products from fruit, spice and herb seeds for improving human health. More research is required to screen and characterize the fatty acids and bioactive components in the fruit, spice, and herb seeds to develop novel edible seed oils for optimum human nutrition.

1.3. Fruit, vegetable, and spice seed flours

As discussed in the previous section, specialty fats and oils, a number of different types of seed oils were widely studied. This includes fatty acid compositions and health beneficial properties such as free radical scavenging, anti-inflammatory, and anti-proliferative capacities. On the other hand, studies focusing on seed flours are minimal. Even though fat-soluble components are extracted by oils, some literature suggests that seed flours from fruit, vegetable, and spice are rich in polyphenolic compounds and possess potential health beneficial properties. To lead the potential utilization of these seed flours in nutraceuticals and functional foods and add value to oil manufacturers and seed producers while reducing environmental contaminations, further studies on seed flours are warranted. In this section, health-beneficial components and properties of the fruit, vegetable, and spice seed flours from the previous studies will be discussed.

1.3.1. Total phenolic content (TPC) of fruit, vegetable, and spice seed flours

Phenolics found in foods are bioactive substances that are closely associated with the sensory and nutritional quality of foods. Many phenolic compounds in plants are good sources of natural antioxidants (Ho, Lee, & Huang, 1992). Also, phenolics have been reported to possess anti-inflammatory and anti-proliferative capacities (Yoon & Baek, 2005; Mileo & Miccadei, 2016).

In 2006, Parry and others evaluated total phenolic content (TPC) of seed flours from black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006).

The TPC values of the black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape seed flours ranged from 14.5 to 186.3 mg of gallic acid equivalents per gram of flour (mg GAE/g). The chardonnay grape seed flour had the highest TPC value among all tested seed flours, and pinot noir grape seed flour had the next highest value at 55.5 mg GAE/g. The red raspberry and black raspberry fruits had TPC values of 36.9 and 57.6 mg GAE/g, and the TPC values of the red raspberry and black raspberry seed flours were 25.1 and 41.2 mg GAE/g, respectively (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006).

Addition to fruit seed flours, Parry and others also evaluated total phenolic content of seed flours from pumpkin, parsley, mullein, cardamom, and milk thistle (Parry, Cheng, Moore, & Yu, 2008).

The TPCs of the pumpkin, parsley, mullein, cardamom, and milk thistle seed flours ranged from about 1.6 to 25.2 GAE mg/g. The milk thistle seed flour had the highest TPC value of 25.2 GAE mg/g followed by parsley (8.1 GAE mg/g), mullein (4.7 and 4.1 GAE mg/g), cardamom (1.9 GAE mg/g), and roasted pumpkin (1.6 GAE mg/g) seed flours (Parry, Cheng, Moore, & Yu, 2008).

1.3.2. Total anthocyanin content (TAC) of fruit, vegetable, and spice seed flours

Anthocyanins are pigments found in plants and contribute human health through antioxidant activity (Cheynier, 2005).

Previously, Parry and others evaluated total anthocyanin content (TAC) of black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). The TAC values of the

black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape seed flours ranged from 0 to 61.3 mg of cyanidin 3-glucoside equivalents per 100 g of flour (CGE mg/100 g). The highest TAC of 61.3 CGE mg/100 g was detected in the black raspberry seed flour, and no anthocyanin was detected in the red raspberry seed flour under the experiment condition. They found significant levels of TAC in the cranberry, blueberry, and chardonnay grape seed flours at a range of 6.9 to 7.4 CGE mg/100 g. Even though these data indicate that anthocyanins are not concentrated in fruit seeds, black raspberry seed may contain a significant level of anthocyanins (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006).

1.3.3. Free radical scavenging capacities of fruit, vegetable, and spice seed flours

Excessive free radicals are attacking human body and may cause chronic diseases such as obesity, diabetes, arthritis, and cancers (Lobo, Patil, Phatak, & Chandra, 2010). Therefore, scavenging excessive free radicals through dietary intervention can be health beneficial.

In 2006, Parry and others tested ORAC and DPPH free radical scavenging capacities of fruit seed flours from black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). They found that black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape seed flours exhibited significant oxygen radical absorbing capacity with ORAC values of 110.5−1076 TE μmol/g. The chardonnay grape seed flour had the highest ORAC (1076 TE μmol/g) among all tested fruit seed flours on a per weight basis. The ORAC value of the chardonnay

grape seed flour was more than 3 times higher than that of the pinot noir grape flour (312.8 TE μmol/g) and almost 10 times higher than that of the cranberry seed flour (110.5 TE μmol/g) (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). Red raspberry and black raspberry seed flours showed ORAC values of 275.5 and 296.2 TE μmol/g, respectively. The ORAC values of cranberry and blueberry seed flours were 110.5 and 152.9 TE μmol/g, respectively. These data suggest that black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape seed flours are excellent dietary sources for oxygen radical absorbing components on a per weight basis (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006).

For DPPH free radical scavenging capacity, the chardonnay seed flour contained the highest level of $DPPH^{\bullet}$ scavenging agents and had an $ED_{50\text{-}DPPH}$ value of 39 μg flour equivalents/mL. The $ED_{50\text{-}DPPH}$ value 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 (160 μg flour equivalents/mL) and 32 times lower than that for the cranberry seed flour (1260 μg flour equivalents/mL) (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006).

In 2008, Parry and others evaluated three free radical scavenging capacities including oxygen radical scavenging capacity (ORAC), hydroxyl radical scavenging capacity (HOSC), and relative DPPH radical scavenging capacity (RDSC) of pumpkin, parsley, mullein, cardamom, and milk thistle seed flours (Parry, Cheng, Moore, & Yu, 2008). The ORAC values of pumpkin, parsley, mullein, cardamom,

and milk thistle seed flours ranged from 35.3 to 1130.7 TE μmol/g (Parry, Cheng, Moore, & Yu, 2008). The milk thistle seed flour had the highest ORAC value of 1130.7 TE μmol/g followed by parsley (390.0 TE μmol/g), mullein (127.3 and 98.2 TE μmol/g), pumpkin (37.6 TE μmol/g), and cardamom (35.3 TE μmol/g) (Parry, Cheng, Moore, & Yu, 2008).

Similar to order of ORAC values, milk thistle seed flour showed the highest HOSC value of 893 TE μmol/g followed by parsley (311.5 TE μmol/g), mullein (75.3 and 74.3 TE μmol/g), cardamom (22.6 TE μmol/g), and pumpkin (22.2 TE μmol/g) (Parry, Cheng, Moore, & Yu, 2008).

For RDSC, milk thistle had the highest RDSC value of 61.1 TE μmol/g. However, the parsley seed flour which had the second highest ORAC and HOSC values, had lower RDSC value compared to mullein (24.0 and 21.2 TE μmol/g) and cardamom (19.5 TE μmol/g) seed flours. Among pumpkin, parsley, mullein, cardamom, and milk thistle seed flours, the pumpkin seed flour showed the lowest RDSC value of 2.2 TE μmol/g (Parry, Cheng, Moore, & Yu, 2008).

1.3.4. Chelating capacity of fruit, vegetable, and spice seed flours

Chelating capacity is an ability to form chelate complex with metal ions. Because metal ions may induce free radical oxidation by initiating oxidative chain reactions in food and biological system, forming chelate complex can block or suppress free radical oxidation.

In 2006, Parry and others evaluated chelating capacity of black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape seed flour

extracts (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). They found that black raspberry, red raspberry, blueberry, cranberry, pinot noir grape, and chardonnay grape seed flour extracts demonstrated significant chelating capacities against Fe^{2+}. The values ranged from 1.9 to 3.9 EDTA equivalents mg/g flour. The 50% acetone extract of red raspberry seed flour had the highest chelating capacity (3.9 EDTA equivalents mg/g flour) but was not significantly higher than that of the black raspberry seed flour (3.6 EDTA equivalents mg/g flour) (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006).

1.3.5. Anti-proliferative capacities of fruit, vegetable, and spice seed flours

In the previous study, fruit seed flours from chardonnay grape, black raspberry, and cranberry seed flours have been evaluated for their anti-proliferative capacities (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). To evaluate the anti-proliferative capacities, dimethyl sulfoxide (DMSO) solutions of chardonnay grape, black raspberry, and cranberry seed flours were treated to HT-29 colon cancer cell line. They found that chardonnay grape seed flour extract completely eliminated all living HT-29 cells at both 3 and 6 mg flour equivalents/mL media following 24 h of exposure, whereas the extracts of black raspberry and cranberry dose-dependently suppressed cell proliferation under the same experimental conditions. Interestingly, the order of anti-proliferative capacity against HT-29 cancer cells was same as that of TPC, suggesting that total phenolic contents may be an important indicator for their anti-proliferative activity (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006).

Addition to fruit seed flours, Parry and others also evaluated anti-proliferative capacities of seed flours from pumpkin, parsley, mullein, cardamom, and milk thistle (Parry, Cheng, Moore, & Yu, 2008). The milk thistle seed-flour extract at both 3 and 6 mg flour equivalents/mL significantly inhibited HT-29 cell growth in a dose-dependent manner compared to the control at both 48 h and 96 h of treatment. The parsley seed flour extract showed inhibition only at 6 mg flour equivalents/mL after 48 h, while all other tested seed-flour extracts had no inhibitory effect against HT-29 cells under the experimental conditions (Parry, Cheng, Moore, & Yu, 2008).

In summary, previous studies found that seed flours from chardonnay grape, black raspberry, cranberry seed, pumpkin, parsley, mullein, cardamom, and milk thistle contained significant levels of natural antioxidants and showed potential health beneficial effects such as free radical scavenging, chelating and anti-proliferative capacities. Further research of seed flours may lead to value-added use of these by-products in improving human health while enhancing the profitability of seed production and seed oil industries.

1.4. Chemical compositions of broccoli, carrot, cucumber, milk thistle, and blackberry seed flours

As a continuous study of seed flours, the current study selected five seed flours including broccoli, carrot, cucumber, milk thistle, and blackberry seed flours. In this section, chemical compositions of broccoli, carrot, cucumber, milk thistle, and blackberry seed flours and their potential health beneficial effects including gut

microbiota modulation, free radical scavenging, anti-inflammatory, and anti-proliferative capacities have been reviewed.

1.4.1. Chemical composition of broccoli seed flour

Broccoli is a cruciferous vegetable, and cruciferous vegetables are an excellent dietary source of phytochemicals including glucosinolates, phenolics and other antioxidants such as vitamin C. Broccoli seed also contains health-beneficial phytochemicals such as glucosinolates. In 2004, McWalter and others detected glucosinolates including glucoiberin, sinigrin, glucoraphanin, progoitrin, gluconapin, gluconasturtiin and glucoalyssin in the 75% methanol extract of a broccoli seed flour (Mcwalter, Higgins, Mclellan, Henderson, Song, Thornalley, & Itoh, 2004). However, other than McWalter's glucosinolates identification, the chemical composition of broccoli seed flour has not been evaluated.

1.4.2. Chemical composition of carrot seed flour

The carrot is a root vegetable widely consumed in the human diet, either as fresh or processed in meals and beverages. The carrot has been ranked 10th among 39 fruits and vegetables for its multiple nutritional benefits (Dias, 2014). Carrot is a good source of anthocyanins, natural pigments widely occurring in plants, and which contribute to the nutritional value of vegetable and fruits, due to their molecular antioxidant properties and their involvement in anti-aging and anti-inflammatory processes (Blando, Calabriso, Berland, Maiorano, Gerardi, Carluccio, et al., 2018). In plants, anthocyanins are synthesized by the flavonoid biosynthetic pathway, which

leads also to the production of phenolic acids and other classes of polyphenols with healthy benefits (Blando, Calabriso, Berland, Maiorano, Gerardi, Carluccio, et al., 2018). In 2005, Kumarasamy and others reported luteolin as a primary component in the methanol extract of carrot seed flour along with luteolin 3'-*O*-beta-D-glucopyranoside and luteolin 4'-*O*-beta-D-glucopyranoside (Kumarasamy, Nahar, Byres, Delazar, & Sarker, 2005). In this study, the chemical composition of carrot seed flour was evaluated using 50% acetone as a solvent.

1.4.3. Chemical composition of cucumber seed flour

Cucumber contains a number of phytochemicals including cucurbitacins, apigenin, quercetin, and kaempferol (Mukherjee, Nema, Maity, & Sarkar, 2013). But, up to date, the chemical composition of cucumber seed has not been evaluated. Thus, this study evaluated the chemical composition of cucumber seed flour.

1.4.4. Chemical composition of milk thistle seed flour

Milk thistle seed contains silymarin, a group of polyphenolic flavonoid compounds (Barreto, Wallace, Carrier, & Clausen, 2003). In 2003, Barreto and others found silychristin, silybin A, silybin B, and taxifolin in the milk thistle seed flour extracted with water at 100 °C (Barreto, Wallace, Carrier, & Clausen, 2003). In 2005, Wallace and others reported silymarin content of an ethanolic extract of milk thistle seed flours. The primary component in the ethanol extract of the milk thistle seed flour was silybin B followed by silybin A, silychristin, and taxifolin (Wallace, Carrier, & Clausen, 2005). However, other chemical components of milk thistle seeds

have been seldom reported. Thus, the current study identified polyphenolic compounds along with the silymarin.

1.4.5. Chemical composition of blackberry seed flour

Previously, Hager and others reported that blackberry seed is rich in polyphenolic compounds (Hager, Howard, Liyanage, Lay, & Prior, 2008). From blackberry seed extract using acetone/water/acetic acid (70:29:0.5 v/v/v) as a solvent, they found phytochemicals including pedunculagin, castalagin, galloyl-hexahydroxydiphenoyl (HHDP) glucose, lambertianin C, sanguiin H-6, lambertianin D, and ellagic acid (Hager, Howard, Liyanage, Lay, & Prior, 2008). The current study used 50% acetone to extract the phytochemicals from blackberry seed flour for the comparison.

1.5. Gut microbiota and their impacts on human health

1.5.1. Interaction between gut microbiota and polyphenolic compounds

The human gut microbiota community is predominantly determined at birth because an infant's microbiota is delivered by the mother. Also, breast/bottle feeding, and epigenetics can alter the microbiota community of an infant. Therefore, an individual has different compositions and numbers of gut microbiota. Moreover, during the lifetime, gut microbial compositions may be changed by many factors. For example, using antibiotics or drugs can cause abnormal microbiota development. Also, disease, injury, surgery, and stress can affect the microbiota community (Conlon & Topping, 2016). Even though an individual has different gut microbiota

profile due to different environmental factors, a healthy diet can positively alter the gut microbiota profile. Also, the diet seems to have the biggest impact (Conlon & Topping, 2016; Mai, 2004). On the other hand, intestinal dysbiosis, a loss of beneficial microbial organisms and diversity, is associated with diseases such as inflammatory bowel disease, irritable bowel syndrome (IBS), celiac disease, asthma, metabolic syndrome, cardiovascular disease, and obesity (Carding, Verbeke, Vipond, Corfe, & Owen, 2015). Therefore, the role of foods and their components that possibly interact with gut microbiota has been noticeable. Usually, polyphenolic compounds in foods are known to interact with gut bacterial communities (Moco, Martin, & Rezzi, 2012).

1.5.2. Interaction between gut microbiota and oligosaccharides and polysaccharides

Oligosaccharides are polymer containing a small number of monosaccharides. Among oligosaccharides, non-digestible oligosaccharides can reach to the large intestine and interact with gut microbiota. Therefore, some oligosaccharides work as prebiotics. In 2018, Tingirikari reported that pectin-derived oligosaccharides significantly increased the population of beneficial bacteria and produced short chain fatty acids during fermentation (Tingirikari, 2018). Short chain fatty acids are recognized as the connector between gut microbiota and the immune system. For example, short chain fatty acid, butyrate, has functions such as homeostasis of the epithelium, antimicrobial activity, anti-inflammatory activity, and immune cell regulation (Tingirikari, 2018).

Polysaccharides are a major macromolecule found in plants. When plant polysaccharides are consumed, some polysaccharides are hydrolyzed in the stomach

by acid and enzymes, and sugar subunits are absorbed in the small intestine. However, some polysaccharides such as dietary fibers are resistant to hydrolysis. These indigestible polysaccharides are called dietary fiber. The common dietary fiber includes cellulose, hemicellulose, β-glucan, pectin, gum, and lignin (Zhang, Yang, Liang, Jiao, & Zhao, 2018). These indigestible polysaccharides can reach to the large intestine and fermented by gut microbiota and produce diverse metabolites.

Two major phyla, *Bacteroidetes* and *Firmicutes*, dominate the human gut. *Bacteroidetes* can degrade a wide range of polysaccharides and *Firmicutes* can metabolize some selected polysaccharides (Salyers, Vercellotti, West, & Wilkins, 1977). Often the ratio between *Bacteroidetes* and *Firmicutes* is determined by our daily diets and lifestyle. Thus, the proportion of these two phyla's inter-individual differences are huge. However, the proportion can be shifted through the diet and this suggests the health benefit of polysaccharides.

As mentioned above, polysaccharides can change the Ecology of the gut microbiota as well as the health status of the host. It has been reported that health beneficial effects of polysaccharides include prevention of large bowel cancer, inflammatory bowel disease, diabetes, metabolic syndrome, and ischemic brain, and immune enhancement (Zhang, Yang, Liang, Jiao, & Zhao, 2018).

To summarize, a combination of oligosaccharides and polysaccharides will help to provide a prebiotic effect throughout the intestine. Oligosaccharides and polysaccharides derived from agricultural waste such as seed flours can be efficiently

transformed into value-added dietary fibers, which could cater to the needs of the intestinal microbiota (Tingirikari, 2018).

1.5.3. Interaction between gut microbiota and food proteins and peptides

Protein is one of the most important components in food and provides essential amino acids. According to Cummings, approximately 12–18 g of protein per day reach the human colon and consist mainly of residual dietary protein and enzymes secreted in the small intestine (Cummings, 1997). The colon serves as a bioreactor for protein transformation and provides nitrogen and amino acids for bacterial growth and utilization. *Bacteroides* spp. and *Propionibacterium* spp. were identified as the predominant proteolytic bacteria in feces, and less abundant proteolytic bacteria belonged to the genera *Streptococcus*, *Clostridium*, *Bacillus*, and *Staphylococcus* (Macfarlane, Cummings, & Allison, 1986). When carbohydrate sources are insufficient, amino acids can be utilized by intestinal bacteria as an energy source (Macfarlane, Cummings, & Allison, 1986). Protein fermentation results in more diverse metabolites compared to carbohydrate fermentation. Bacterial deamination is the major pathway for amino acid fermentation in the human colon, leading to the production of short chain fatty acids, ammonia, and phenolic compounds (Scott, Gratz, Sheridan, Flint, & Duncan, 2013). Tryptophan is an essential amino acid that must be obtained from food such as red meat, fish, and eggs. Commensal bacteria expressing tryptophanase catabolize tryptophan into indole and certain other derivatives, some of which can activate the aryl hydrocarbon receptor and regulate the cytokine IL-22 production, which both play key roles in mucosal

immunity against pathogens. *Lactobacillus* spp. and *Clostridium sporogenes* are involved in this conversion (Zelante, Iannitti, Cunha, De Luca, Giovannini, Pieraccini, et al., 2013). Additionally, decarboxylation of amino acids and peptides are conducted by *Clostridium*, *Bifidobacterium*, and *Bacteroides* to produce a large number of amines, which are precursors for the formation of the carcinogenic nitrosamine (Smith & Macfarlane, 1996). In summary, the relationship between protein and gut microbiota and their impact on health seems to be complicated.

1.5.4. Interaction between gut microbiota and triglycerides and other oils components

The western diet contains high fat contents including triglycerides and cholesterol. The largest gut microbial composition studies in humans to date found an interesting observation. Microbial richness and diversity which is expressed by operational taxonomic units (OTUs) were inversely correlated with triglyceride levels (Allayee & Hazen, 2015). This suggests that high fat diet may reduce microbial richness and diversity.

Cholesterol composes about 30% of animal cell membranes and is required to build and maintain membranes and modulates membrane fluidity. However, high blood levels of cholesterol increase the risk of developing coronary heart disease, stroke, and peripheral vascular disease. Once cholesterol is consumed through the diet, cholesterol is metabolized by the liver into a variety of bile acids, which help the absorption of liposoluble nutrients because of their amphipathic properties. Approximately 95% of the bile acids are reabsorbed by the intestine and returned to the liver; this enterohepatic cycle between the gut and liver occurs approximately

eight times per day (Nicholson, Holmes, Kinross, Burcelin, Gibson, Jia, et al., 2012). Conjugated bile acids pass through the cecum and colon and are largely biotransformed by indigenous bacterial flora (*Bacteroides*, *Eubacterium*, and *Clostridium*), yielding secondary bile acids (Ridlon, Kang, & Hylemon, 2005). Bile acids as well as their secondary metabolites are characterized by strong antimicrobial activity and are major regulators for the gut microbial community. Only microbial populations able to tolerate the physiologic concentrations of bile acids can survive in the gut (Ridlon, Kang, & Hylemon, 2005). There is evidence that consuming animal-based foods causes an increase in bile-tolerant microorganisms (*Alistipes*, *Bilophila*, and *Bacteroides*), and the bacterial genes encoding microbial bile salt hydrolase, which are crucial for the production of secondary bile acids, also exhibit significantly higher expression when consumed as part of an animal-based diet (David, Maurice, Carmody, Gootenberg, Button, Wolfe, et al., 2013). Significant increases in bile acids, as well as their metabolic products in feces, may have contributed to the microbial disturbances and have implications for colonic cancer risk (Nagengast, Grubben, & Munster, 1995).

1.5.5. Interaction between gut microbiota and other food factors

Fermented foods are foods or beverages made with certain microorganisms that change or modify food components. There is an abundant diversity of fermented foods and the origin of these foods can date back to ~10,000 years ago. It was estimated that approximately one-third of the human daily diet was made up of fermented foods (Campbell-Platt, 1994). An increasing number of studies have

documented that fermented foods exhibit enhanced nutritional and functional properties because of the transformation of substrates and the formation of bioactive metabolic products by microorganisms. Most fermented foods also contain living microorganisms, some of which may contribute as probiotics to the promotion of human health. For example, fermented milk has been reported to have numerous benefits such as inhibiting GI infections, reducing serum cholesterol levels, and anti-mutation effects (Shiby & Mishra, 2013). Some of these benefits are carried out by the secreted bioactive substances from lactic acid bacteria such as lactic and acetic acids, SCFAs, and bacteriolytic enzymes (Belenguer, Duncan, Calder, Holtrop, Louis, Lobley, et al., 2006; Wouters, Ayad, Hugenholtz, & Smit, 2002). Other effects are related to the possible impacts of fermented milk on the host's autochthonous gut microbiota. The viable bacteria in fermented milk are approximately 10^6–10^9 CFU, and when passing through the human digestive tract, a relatively large proportion may survive (Derrien & van Hylckama Vlieg, 2015). Uyeno and others (Uyeno, Sekiguchi, & Kamagata, 2008) reported that consuming yogurt could alter the fecal microbial composition in healthy adults. Mcnulty and others (Mcnulty, Yatsunenko, Hsiao, Faith, Muegge, Goodman, et al., 2011) detected changes in the human fecal meta-transcriptome with fermented milk product consumption as well as altered expression of microbiome-encoded enzymes, especially those related to carbohydrate metabolism. Additionally, the trial conducted among adults with irritable bowel syndrome (IBS) also showed that the daily consumption of fermented milk may have short-term effects on certain strains (*Lactobacillus acidophilus* La-5

and *Bifidobacterium animalis* ssp. *lactis* BB-12) of patients' fecal microbiomes. In a subset of the patients, IBS symptoms were alleviated by the probiotic treatment (Matijašić, Obermajer, Lipoglavšek, Sernel, Locatelli, Kos, et al., 2016). Veiga and others (Veiga, Pons, Agrawal, Oozeer, Guyonnet, Brazeilles, et al., 2014) used a species-level metagenomics approach to identify that the improvement of IBS symptoms with the consumption of fermented milk production was related to a decreased level of a pathogen, *Bilophila wadsworthia*. An increase in butyrate and other health-promoting SCFAs produced by the human gut microbiota was also detected in the study (Veiga, Pons, Agrawal, Oozeer, Guyonnet, Brazeilles, et al., 2014). Additional recent research lends further support for the regulation of SCFAs through modulation of the gut microbiota by dietary fermented milk (Canani, Filippis, Nocerino, Laiola, Paparo, Calignano, et al., 2017). Furthermore, it was reported that the phenol production by gut microbiota could be inhibited by the intake of *Bifidobacterium* fermented milk and result in lower serum phenol levels, thus inhibiting the potential harm of phenols to the keratinization of skin and improving the skin conditions of adult women (Miyazaki, Masuoka, Kano, & Iizuka, 2014).

In addition to fermented milk and because of the benefits of the fermentation process, some new types of food material have been fermented. Fermented green tea extract was claimed to restore the changes in gut microbiota composition in diet-induced obese mice, including the *Firmicutes*:*Bacteroidetes* and *Bacteroides*:*Prevotella* ratios. The balance of these bacteria was closely related to the development of obesity and insulin resistance induced by a high-fat diet (Seo,

Jeong, Cho, Lee, Lee, Choi, et al., 2015). Also, a mixture of fermented blueberries altered the cecal microbiota of the N^G-nitro-L-arginine methyl ester in induced hypertensive rats and showed a protective effect against liver cell damage as well (Xu, Ahrén, Prykhodko, Olsson, Ahrné, & Molin, 2013). Fermented foods provide multiple elements, including probiotics, prebiotics, and novel bioactives, that may alter the gut microbiota and human health. However, the alteration of our intestinal microbiota by fermented foods is a transient change in most cases, therefore, continuous intake of fermented foods is necessary to maintain high levels of probiotics. Furthermore, the probiotic bacteria in fermented foods may be affected by diet and other gut-associated factors; thus, further studies are warranted to investigate the long-lasting effects of fermented food on resident gut microbiota and human health (Veiga, Pons, Agrawal, Oozeer, Guyonnet, Brazeilles, et al., 2014).

Food additives are substances added to food to maintain or improve the safety, freshness, taste, texture, and appearance of food. Some food additives such as salt, sugar, and sulfur dioxide have been in use for centuries for food preservation. With the advent of processed foods in the second half of the twentieth century, many food additives of both natural and artificial origin have been introduced. Food additives can be divided into several groups, including flavors, colorants, emulsifiers, sweeteners, and thickeners (Branen, Davidson, & Salminen, 2002). Given that food additives are more widely used, the impact of food additives on human health, particularly in gut microbiota, has gained attention in recent years. Emulsifiers and artificial sweeteners have been reported to promote the development of metabolic

syndrome by perturbing the host-gut microbiota relationship. For example, two emulsifiers, carboxymethylcellulose (CMC) and polysorbate-80 (P80), were reported to decrease the microbial diversity of the gut and significantly altered microbiota composition, including reduced levels of Bacteroidales and increased levels of *Ruminococcus gnavus* and Proteobacteria phyla (Chassaing, Koren, Goodrich, Poole, Srinivasan, Ley, et al., 2015). It has also been demonstrated that by using germ-free mice and fecal transplants, the microbiota changed by CMC were responsible for inducing inflammation and metabolic changes (Chassaing, Koren, Goodrich, Poole, Srinivasan, Ley, et al., 2015). An additional study from Chassaing and others (Chassaing, Wiele, Bodt, Marzorati, & Gewirtz, 2017) revealed that CMC and P80 altered microbiota with an increased level of fecal flagellin in the human intestinal microbial ecosystem. The increase of flagellin, a bacteria-derived protein, was sufficient to induce low-grade inflammation and metabolic syndrome (Chassaing, Wiele, Bodt, Marzorati, & Gewirtz, 2017).

Because of the strong correlation between overweight and obesity with sugar intake, there has been increased consumption of noncaloric sweetener instead of sugar. Suez and other (Suez, Korem, Zeevi, Zilberman-Schapira, Thaiss, Maza, et al., 2014) reported that consumption of noncaloric artificial sweeteners that induce the compositional and functional alteration of the gut microbiota might enhance the risk of glucose intolerance in both mice and humans. These adverse metabolic effects are eliminated by antibiotic treatment (Suez, Korem, Zeevi, Zilberman-Schapira, Thaiss, Maza, et al., 2014). Consistent with this finding, it was shown that eight weeks of

aspartame treatment in small quantities [5–7 mg/(kg·day) in drinking water] perturbed gut microbiota, resulting in elevated fasting glucose levels and impaired insulin tolerance in rats (Palmnäs, Cowan, Bomhof, Su, Reimer, Vogel, et al., 2014). Similar perturbation of gut microbiota in CD-1 mice was observed after a four-week treatment with the artificial sweetener acesulfame potassium, but the shifts in the gut bacterial composition and body weight were different in male and female mice (Bian, Chi, Gao, Tu, Ru, & Lu, 2017). Hence, accumulating data suggest that artificial food additives might contribute to metabolic disease through modulation of the microbiota. However, the related studies are limited, making it necessary to conduct additional studies to include the relationships between food additives, gut microbiota, and human health.

To summarize, a complex interactive relationship between food and the gut microbiota exists and is receiving intense scrutiny to provide information on how the interaction can produce either positive or negative effects on human health. The future holds exciting opportunities as well as challenges. In the current state of human health sciences, precision nutrition and individual medicine dominate our future direction on studies of human health. In this context, it will be critical to individualize gut microbiota and tailor individual foods that can modulate an individual's gut microbiota for health. The challenge will be to identify optimal combinations of the variables. This will open up the opportunity for food technologists to produce designer foods that help achieve optimal human health.

1.6. Research methods

1.6.1. 16S rRNA gene sequencing

Table 1.7. Sequence of real-time PCR primers

Bacteria	Direction	Sequence (5'-3')
Akkermansia	Forward	CAGCACGTGAAGGTGGGGAC
	Reverse	CCTTGCGGTTGGCTTCAGAT
Bacteroidetes	Forward	GGARCATGTGGTTTAATTCGATGAT
	Reverse	AGCTGACGACAACCATGCAG
Bifidobacteria	Forward	TCGCGTCYGGTGTGAAAG
	Reverse	CCACATCCAGCRTCCAC
Enterobacteriaceae	Forward	CATTGACGTTACCCGCAGAAGAAGC
	Reverse	CTCTACGAGACTCAAGCTTGC
Firmicutes	Forward	GGAGYATGTGGTTTAATTCGAAGCA
	Reverse	AGCTGACGACAACCATGCAC
Lactobacillus	Forward	GAGGCAGCAGTAGGGAATCTTC
	Reverse	GGCCAGTTACTACCTCTATCCTTCTTC

16S rRNA is the gene that encodes the RNA component of the smaller subunit of the bacterial ribosome. Amplifying 16S rRNA using polymerase chain reaction (PCR) allows identification of specific bacterial phylum, family, or, genus. For the sequencing, PCR machine such as ViiA 7 Real-Time PCR System is used.

16S rRNA gene sequencing is the most commonly used method in microbiota research for several reasons. First, it is a presence in almost all bacteria, often existing as a multigene family or operons. Second, the function of the 16S rRNA gene does not change over time. Third, the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001). After choosing the method, specific phyla and

genera were selected based on the literature review. Six phyla and genera will be tested in the present study. These six include *Bacteroidetes* and *Firmicutes* phyla and *Akkermansia, Bifidobacteria, Enterobacteriaceae,* and *Lactobacillus* genera. Based on the literature review, the functions of these phyla and genera are closely related to human health.

Mainly, the human gut microbiota is composed of *Bacteroidetes* and *Firmicutes*. In healthy adults, > 90% of gut microbiota community is dominated by these two phyla (Claesson, Jeffery, Conde, Power, O'Connor, Cusack, et al., 2012). Due to their abundance, *Bacteroidetes* and *Firmicutes* contribute to human health in various ways. For example, *Bacteroidetes* interact with the human immune system through activating T-cell mediated responses (Mazmanian, Round, & Kasper, 2008; Wen, Ley, Volchkov, Stranges, Avanesyan, Stonebraker, et al., 2008), whereas *Bacteroidetes* produce butyrate, an end product of colonic fermentation with possible anti-neoplastic properties (Kim & Milner, 2007). Also, *Bacteroidetes* may be involved in bile acid metabolism and transformation of toxic and mutagenic compounds (Smith, Rocha, & Paster, 2006).

The other major component of gut microbiota, *Firmicutes* phylum is involved in fatty acid metabolism (Turnbaugh, Ley, Mahowald, Magrini, Mardis, & Gordon, 2006). Also, *Firmicutes* may be closely related to aging. It has been reported that aging increases the total number of *Firmicutes* and therefore elderly people have a relative high portion of *Firmicutes* (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005).

Besides, a ratio between *Bacteroidetes* and *Firmicutes* has been reported to have a close relationship with aging and obesity (Nicholson, Holmes, Kinross, Burcelin, Gibson, Jia, et al., 2012). The ratio of *Bacteroidetes* and *Firmicutes* changes over the lifetime (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005). Compare to a healthy adult, an infant has a much higher portion of *Bacteroidetes*. On the other hand, elderly people have a relatively high portion of *Firmicutes*. There also has been evidence that the *Bacteroidetes*/*Firmicutes* ratio is closely related to obesity. According to Ley and colleagues, diet therapy for 52 weeks altered *Bacteroidetes* and *Firmicutes* ratio by increasing *Bacteroidetes* and decreasing *Firmicutes* (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005). The result from Ley's study suggested that the increased Bacteroidetes/*Firmicutes* ratio may correlate to weight loss.

Akkermansia is a mucin-degrading bacterium that resides in the mucus layer. *Akkermansia* has been considered as a contributor to the maintenance of gut health. Recently, Dao and others have shown that *Akkermansia* could reduce body fat mass, improve glucose homeostasis, decrease adipose tissue inflammation and increase gut integrity in mice (Dao, Everard, Aron-Wisnewsky, Sokolovska, Prifti, Verger, et al., 2015). Moreover, it has been reported that *Akkermansia* presence was inversely correlated with the consumption of polysaccharides and body weight gain in rodents and humans (Amar, Burcelin, Ruidavets, Cani, Fauvel, Alessi, et al., 2008).

Bifidobacteria and *Lactobacillus* genera are well-known probiotic bacteria. Maintaining a certain amount of these bacteria has several health beneficial effects

due to their functions. For example, *Bifidobacteria* and *Lactobacillus* can prevent or alleviate infectious diarrhea through their effects on the immune system and the reduction of colonization by pathogens (Picard, Fioramonti, Francois, Robinson, Neant, & Matuchansky, 2005). Also, there is some experimental evidence that certain *Bifidobacteria* may protect the host from the carcinogenic activity of intestinal flora (Rosenfeldt, Michaelsen, Jakobsen, Larsen, Møller, Tvede, et al., 2002). On the other side, *Bifidobacteria* and *Lactobacillus* are the major bacteria that produce lactic acid and overabundance of these genera can cause lactic acidosis.

Enterobacteriaceae is a gram-negative and emerging opportunistic pathogen and associated with rare but life-threatening cases of meningitis, necrotizing enterocolitis, and sepsis in premature and full-term infants (Drudy, Mullane, Quinn, Wall, & Fanning, 2006).

1.7. Free radical scavenging capacities

1.7.1. Relative DPPH scavenging capacity (RDSC)

A high-throughput relative 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging capacity (RDSC) assay is easy to perform and has acceptable accuracy (90−110% recovery), precision [3.9−7.0% pooled relative standard deviation (RSD)], and reproducibility (2.2 and 3.5% interday and intraday RSD). This assay has been successfully utilized for investigating antioxidant properties of wheat grain and bran, vegetables, conjugated linoleic acids, herbs, edible seed oils, and flours in several different solvent systems including ethanol, aqueous acetone, methanol, aqueous

alcohol, and benzene (Zhou, Lu, Niu, Liu, Zhang, Gao, et al., 2013; Valli, Gómez-Caravaca, Nunzio, Danesi, Caboni, & Bordoni, 2012; Whent, Lv, Luthria, Kenworthy, & Yu, 2011; Xie, Whent, Lutterodt, Niu, Slavin, Kratochvil, et al., 2011; Lu, Ross, Powers, Aston, & Rasco, 2011). However, it has been hard to compare the DPPH$^{\bullet}$ scavenging capacity data between different laboratories or from the same group at different times because most of the results using this radical system were reported in % DPPH$^{\bullet}$ remaining or quenched, which highly depends on the reaction time and the initial concentrations of DPPH$^{\bullet}$ and the antioxidant(s) in the assay mixture (Xie, Liu, Huang, Slavin, Zhao, Whent, et al., 2010). A high-throughput RDSC assay uses an area under the curve (AUC) for radical scavenging capacity estimation, expressed as Trolox equivalents (TEs) in μmol on a per-sample weight basis.

In 2006, Parry and others examined fruit seeds including black raspberry, red raspberry, blueberry, cranberry, and pinot noir and chardonnay grapes and detected an RDSC range of 39–1260 μmol TE/g (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). Zafra-Rojas and others evaluated blackberry residues consisted of peels, seeds, and pulp and found an RDSC value of 137 μmol TE/g (Zafra-Rojas, Cruz-Cansino, Quintero-Lira, Gómez-Aldapa, Alanís-García, Cervantes-Elizarrarás, et al., 2016). Our previous studies showed DPPH scavenging capacities of broccoli and carrot vegetables in a range of 0.3–0.5 and 0.7–0.9 μmol TE/g, respectively (Yu, Gao, Li, Wang, Luo, Wang, et al., 2018; Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017). Miller and others examined cucumber vegetable and detected a DPPH scavenging capacity

of 1.0 μmol TE/g (Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000). In 2008, Parry and others tested milk thistle seed flour. The milk thistle seed flour extract showed an RDSC value of 61 μmol TE/g (Parry, Cheng, Moore, & Yu, 2008).

1.7.2. Hydroxyl radical (HO•) scavenging capacity (HOSC)

Among physiologically relevant reactive oxygen species (ROS), the hydroxyl radical (•OH) is extremely reactive with almost every type of biomolecules and is possibly the most reactive chemical species known (Halliwell & Cross, 1994). The presence and pathological role of •OH *in vivo* have been demonstrated and include a direct attack of proteins and nucleic acids (Evans, Dizdaroglu, & Cooke, 2004; Lubec, 1996). Hydroxyl radicals may serve as an excellent target to investigate dietary antioxidants for their potential to directly react with and quench free radicals and protect important biomolecules from radical-mediated damage.

Previously, the HOSC value of blackberry or blackberry seed flour has not been reported as μmol TE/g. Thus, for the comparison, HOSC values of blueberry will be used. In 2017, Gao and others reported HOSC values of blueberry in a range of 566–1048 μmol TE/g (Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017). The previous study found HOSC values of fresh broccoli and carrot vegetables ranging in 390–509 μmoles TE/g (Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017) and 426–507 μmol TE/g (Yu, Gao, Li, Wang, Luo, Wang, et al., 2018), respectively. The milk thistle seed flour extract's HOSC value reported by Parry and others was 893 μmol TE/g (Parry, Cheng, Moore, & Yu, 2008).

1.7.3. Oxygen radical absorbing capacity (ORAC)

Peroxyl radical is an important physiological radical which may be involved in the propagating steps of lipid peroxidation chain reaction. The oxygen radical absorbing capacity (ORAC) assay was originally developed to measure the hydrophilic chain-breaking capacity of antioxidants against the peroxyl radical using β-cyclodextrin as a molecular probe (Cao, Alessio, & Cutler, 1993). Later, Huang and others adapted the ORAC method for high-throughput automated analysis using a microplate reader with a robotic liquid handling system (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). To measure the ORAC of lipophilic antioxidants and extracts, an alternative ORAC assay protocol was reported by Huang and others (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). The ORAC assay uses competitive kinetics to monitor the ability of antioxidants to compete with a molecular probe, fluorescein (FL), and scavenge peroxyl radical with FL yield a non-fluorescent product and can be monitored by measuring the loss of fluorescence of FL with a fluorometer. The ORAC values for a selected antioxidant are determined based on the areas under the reaction kinetic curves for the antioxidant sample, at least 4-5 concentrations of an antioxidant standard, and a blank. Trolox is a commonly used standard with results expressed as micromoles Trolox equivalents per unit of the sample.

In our previous studies, the fruit seeds such as black raspberry, red raspberry, blueberry, and cranberry had ORAC values in a range of 111–1076 μmol TE/g (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). ORAC values from fresh vegetables

including broccoli, carrot, and cucumber were in a range of 15–17, 1–4, 0.4–0.6 μmol TE/g, respectively (Yu, Gao, Li, Wang, Luo, Wang, et al., 2018; Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017; Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000). The milk thistle seed flour extract showed an ORAC value of 634 μmol TE/g (Parry, Cheng, Moore, & Yu, 2008).

1.7.4. $ABTS^{\bullet+}$ scavenging capacity

The ABTS cation radical scavenging capacity assay is a decolorization assay that measures the capacity of antioxidants to directly react with ABTS cation radicals generated by a chemical method. $ABTS^{\bullet+}$ is nitrogen centered radical with a characteristic of blue-green color which when reduced by antioxidants to its non-radical (ABTS) form becomes colorless. The method quantifies scavenging capacity by measuring the absorbance of the antioxidant-radical reaction mixture at 734 nm at a selected time point with a spectrophotometer. Results are generally expressed relative to a standard, commonly Trolox.

Previous studies tested ABTS scavenging capacities of carrot and carrot seed oil and reported values were 1 and 9 μmol TE/g, respectively (Yu, Zhou, & Parry, 2005). In our lab's previous study, broccoli had an ABTS value of 43 μmol TE/g and cucumber showed 7 μmol TE/g. There is no ABTS value reported for a milk thistle seed flour extract. Therefore, the value obtained from the current study will be compared to the widely consumed food, wheat. In 2002, Yu and others reported that three varieties of wheat (Arkon, Trego, and Platte) extracts using 100% ethanol had ABTS values of 1.31, 1.08 and 1.91 μmol TE/g, respectively (Yu, Haley, Perret, &

Harris, 2002). In 2006, Parry and Yu used two different solvents, 100% ethanol and 50% acetone for the black raspberry extraction and found $ABTS^{\bullet+}$ scavenging capacity values of 233 and 361 μmol TE/g, respectively (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). These values will be used to compare the ABTS value of blackberry seed flour extract.

1.8. Anti-inflammatory capacity

1.8.1. Pro-inflammatory markers

Inflammatory cells are known to produce pro-inflammatory cytokines at the site of inflammation. During the process, excessive pro-inflammatory cytokine production can cause oxidative stress and result in the development of chronic diseases. Therefore, inhibiting pro-inflammatory cytokines such as interleukin 1-beta has been an approach for reducing the risk of inflammation-induced chronic diseases.

Non-steroidal anti-inflammatory drugs (NSAIDs) have evolved from blocking both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) to selectively only blocking COX-2 to inhibit the inflammatory response and reduce the production of inflammatory prostaglandins and thromboxanes. However, by inhibiting COX-2 that blocks the production of prostacyclin (PGI2) there is unopposed thromboxane which will increase the clotting risk. Thus, inhibiting prostacyclin led to an increased risk of thrombotic cardiovascular and cerebrovascular events. Because of the significant side effect profiles of steroidal and NSAID medications, there is a greater interest in natural compounds, such as dietary supplement and herbal remedies, which have been

used for centuries to reduce pain and inflammation (Yu, Zhou, & Parry, 2005). Many of these natural compounds also work by similarly inhibiting the inflammatory pathways as NSAIDs. In addition to the COX pathway, many natural compounds act to inhibit nuclear factor-kB (NF-kB) inflammatory pathways.

1.9. Anti-proliferative capacity

1.9.1. Prostate cancer and polyphenolic compounds

Prostate cancer is the most prevalent disease affecting males in many Western countries. Various dietary components, including polyphenols, have been shown to possess anti-cancer properties. To date, more than 8000 polyphenolic compounds have been identified in the human diet based on their chemical structures (Pandey & Rizvi, 2009). Generally considered as non-toxic, dietary polyphenols act as key modulators of signaling pathways and are therefore considered ideal chemo-preventive agents. Besides possessing various anti-tumor properties, dietary polyphenols also contribute to epigenetic changes associated with the fate of cancer cells and have emerged as potential drugs for therapeutic intervention.

1.10. Goal and specific objectives of the dissertation research

Fruit, vegetable, and spice seeds contain edible seed oils and flours. Edible seed oils play as a food ingredient and provide essential fatty acids. Fruit, vegetable, and spice seed flours are by-products from the manufacture of the seed oils. Even though many seed flours are wasted at a cost, previous studies suggest that these seed flours may have health-beneficial components and properties. Investigation of their

health-beneficial components and properties can lead to potential utilization of these seed flours in nutraceuticals and functional foods, and add value to oil manufacturers and the vegetable seed producers while reducing environmental contaminations. The specific objectives of this research are:

1. To identify chemical compositions of the selected cold-pressed seed flours.
2. To investigate the interaction between cold-pressed seed flours and gut microbiota.
3. To evaluate *in vitro* free radical scavenging capacities, anti-inflammatory properties,and anti-proliferative activities of the cold-pressed seed flours.

In the next three chapters, five seed flours from blackberry, broccoli, carrot, cucumber, and milk thistle were divided into categories of fruit, vegetable, and herb, and evaluated for their chemical compositions and health beneficial properties.

Chapter 2: Chemical compositions of cold-pressed broccoli, carrot and cucumber seed flours, and their in vitro gut microbiota modulatory, anti-inflammatory and free radical scavenging properties

Uyory Choe, Yanfang Li, Boyan Gao, Lu Yu, Thomas T. Y. Wang, Jianghao Sun, Pei Chen, Jie Liu, & Liangli Yu, Chemical Compositions of Cold-pressed Broccoli, Carrot and Cucumber Seed Flours, and Their in Vitro Gut Microbiota Modulatory, Anti-inflammatory and Free Radical Scavenging Properties.
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2.1. Abstract

Carrot, cucumber and broccoli seed flours were extracted with 50% acetone and evaluated for their phytochemical compositions, along with their potential gut microbiota modulating, and free radical scavenging and anti-inflammatory capacities. Nine and ten compounds were detected in the broccoli and carrot seed flour extracts, with kaempferol-3-*O*-rutinoside and glucoraphanin as the primary component, respectively. All three seed flour extracts enhanced total number of gut bacteria and altered the abundance of specific bacterial phylum or genus in vitro. The broccoli seed flour extract had the greatest RDSC, ORAC, and HOSC values of 85, 634 and 270 μmol trolox equivalent (TE)/g, respectively. Carrot seed flour extract showed the greatest $ABTS^{\bullet+}$ scavenging capacity of 250 μmol TE/g. Also, three seed flour extracts suppressed LPS induced IL-1β and COX-2 mRNA expressions in J774A.1 cells. The results might be used to promote the value-added utilization of these vegetable seed flours in improving human health.

2.2. Introduction

Vegetable seed flours including carrot, cucumber and broccoli seed flours are by-products from the manufacture of the seed oils. Investigation of their health beneficial components and properties can lead to potential utilization of these seed flours in nutraceuticals and functional foods, and add value to oil manufacturers and the vegetable seed producers, while reducing environmental contaminations. Previous studies have shown that pumpkin and parsley seed flours are rich in tocopherols, carotenoids, and free radical scavenging, anti-proliferative and anti-inflammatory components (Parry, Cheng, Moore, & Yu, 2008). Carrot and broccoli seed flours were reported to contain health promoting phytochemicals such as polyphenols and glucosinolates (Dias, 2014; West, Meyer, Balch, Rossi, Schultz, & Haas, 2004). Also, 95% methanol extracts of carrot seed flours showed *in vivo* antioxidant activity in toxin treated rat models (Singh, Singh, Chandy, & Manigauha, 2012) and 95% ethanol extracts of carrot seed flours showed anti-inflammatory activity in paw edema induced mouse models (Vasudevan, Gunnam, & Parle, 2006). Broccoli seeds (15% w/w) were able to induce antioxidant response element (ARE)-driven genes in experimental mice (Mcwalter, Higgins, Mclellan, Henderson, Song, Thornalley, et al., 2004). Taking together, these previous studies showed the potential health beneficial effects and components in the vegetable seed flours, suggesting a potential value-added utilization of these seed flours in nutraceuticals and functional foods, and warranting additional research to reveal the specific health components and properties of these seed flours.

Recently, with more and more interest of gut microbiota and human health, many foods including fruit and herb seeds and their components have been investigated for their potential interactions with gut microbiota. In 2017, Seo and others reported that 10% (w/w) chardonnay grape seed flour supplemented diet was capable in altering gut microbiota profile and lowering plasma cholesterol level and abdominal adipose tissue weight in a diet-induced obese mouse model (Seo, Kim, Jeong, Yokoyama, & Kim, 2017). This animal study has shown that gut microbiota may also play an important role in human health or disease development. Total number of gut microbiota genes exceeds human genes by a large number, and the number is critical for overall human health (Ley, Peterson, & Gordon, 2006). Besides the total number, gut microbiota composition is also very important for host health. To date, little is known whether vegetable seeds and their components such as seed flours may alter gut microbiota.

Dietary antioxidants capable of scavenging free radicals have long been recognized for their beneficial effects in reducing the risk of carcinogenesis, cardiovascular disease, inflammation, Alzheimer's and Parkinson's diseases, diabetes and other aging associated health problems (Uttara, Singh, Zamboni, & Mahajan, 2009). Dietary antioxidants have been identified in the seeds of several fruits and herbs including grape (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006), mango (Puravankara, Boghra, & Sharma, 2000), canola (Jun, Wiesenborn, & Kim, 2014), and flax seeds (Oomah, Kenaschuk, & Mazza, 1995). However, free radical

scavenging capacities of carrot, cucumber and broccoli seed flours have been seldom reported.

This study was conducted to evaluate the phytochemical compositions of carrot, cucumber and broccoli seed flours, and their radical scavenging and anti-inflammatory capacities, and effects on gut microbiota. This is the first time that cucumber seed flour was evaluated for its chemical composition and health beneficial effects, and was the first study evaluating the effects of carrot and broccoli seed four components on gut microbiota. The results from this study can serve as a scientific foundation for agricultural industry to add value to their production and to save their cost on by-products while reducing environmental hazes and enhancing human health.

2.3. Materials and methods

2.3.1. Materials

Carrot, cucumber and broccoli seed flour samples were gifted from the Botanic oil innovations (Spooner, WI, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein (FL), iron (III) chloride, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 30 percent ACS-grade hydrogen peroxide, DEPC-Treated water and nuclease-free water were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). 2,2′-Azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals

(Richmond, VA, USA). Ultrapure water was prepared by an ELGA Purelab ultra Genetic polishing system with < 5 ppb TOC and resistivity of 18.2 mΩ (Lowell, MA, USA). J774A.1 and LNCaP cells were purchased from American Type Culture Collection (Manassas, VA, USA). TRIzol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). TATA-binding protein (TBP), interleukin 1 beta (IL-1β) and cyclooxygenase 2 (COX-2) primers, SYBR®Green Real-Time PCR Master Mix and TaqMan Fast Universal PCR Master Mix were purchased from Applied Biosystems (Carlsbad, CA, USA). Affinity Script Multi Temperature cDNA Synthesis kit was purchased from Agilent Technologies (Savage, MD, USA). DMEM, RPMI1640, FBS, penicillin and streptomycin were purchased from GIBCO (Grand Island, NY, USA). LB broth was purchased from Quality Biological™ (Gaithersburg, MD, USA). QIAamp DNA Mini Kit was purchased from Qiagen (Gaithersburg, MD, USA). Precellys were purchased from Bertin Technologies (Rockville, MD, USA).

2.3.2. Seed flour extraction

Each seed flour (about 10 grams) was accurately weighed and extracted three consecutive times with 25 mL of 50% acetone (75 mL of 50% acetone in total) at ambient temperature with sonication. All experiments were performed in triplicate.

2.3.3. Ultra High-Performance liquid chromatography photo diode array high-resolution multi-stage mass spectrometry (UHPLC-PDA-ESI/HRMS^n)

The UHPLC-HRAM analysis was performed on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) with an Agilent 1290 Infinity liquid chromatography coupled with a DAD detector. The UV-vis spectrum scanning was from 190 to 600 nm. A luna C_{18} column, 4.6 mm × 250 mm and 5 μm particle size, was used. HPLC grade water with 0.1% formic acid (v/v) was used as solvent A, and acetonitrile with 0.1% formic acid (v/v) was used as solvent B. The elution was carried out with 5% of solvent B at the beginning, increasing via a linear gradient to 13% B at 5 min; increasing to 20% B at 10 min; increasing to 27% B at 25 min; increasing to 33% B at 40 min; increasing to 50% B at 45 min; increasing to 90% B at 46 min; keeping 90% until 51 min; and the post-run time for re-equilibration was 10 min. The injection volume was 5 μL, with a flow rate of 1 mL/min and an oven temperature of 40 °C. The HRMS was conducted in a negative ionization mode with the optimized parameters as follows: spray voltage at 4.5 kV, capillary temperature at 325 °C, capillary voltage at -50 V, and tube lens offset voltage at -120 V. The mass range was m/z 100-1000 with a resolution of 30,000. Data was post-processed using QualBrowser part of Thermo Scientific Xcalibur 2.2 software.

2.3.4. Bacterial growth and gut microbiota profile

Fecal samples were collected from regular chow diet fed C57BL/6J mice. Fecal bacteria were cultured in LB broth at 37 °C under an anaerobic condition one day prior to treatment. OD of the bacteria culture was measured at 600 nm and a

coefficient of OD_{600} of 1 = 8 $\times 10^8$ cells/mL was used to calculate the seeding concentration (Widdel, 2007). An initial concentration of 1×10^7 cells/mL was seeded in 96 well plates and 10 mL centrifuge tubes using M9 minimal broth with and without three (0.1% v/v) treatments, and cultured in an incubator shaker at 37 °C for 6 hours. OD_{600} values were measured at five different time points at 0, 2, 4, 5 and 6 hours of the treatment. At the end of the treatment, bacterial cells were collected by centrifuging at ambient temperature at 5000 rpm for 5 min, and the supernatant was aspirated. After cells were homogenized with Precellys, the bacterial DNA was extracted with QIAamp DNA MiniKit following the manufacturer's protocol. Real-Time PCR was performed with a reaction system of 10 µL SYBR®Green Real-Time PCR Master Mix, 0.25 µL 500 nM custom-made oligo primers, 4.5 µL water and 5 µL DNA. Primers specific for *Bacteroidetes*, *Firmicutes* phyla, and *Akkermansia*, *Bifidobacteria*, *Lactobacillus*, *Enterobacteriaceae* genus were used to determine the relative abundance of respective microorganisms (Parnell & Reimer, 2011; Wang, Bose, Kim, Hong, Kim, Kim, et al., 2014).

2.3.5. Relative DPPH radical scavenging capacity (RDSC)

The relative 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (RDSC) was evaluated according to a laboratory method (Cheng, Moore, & Yu, 2006). Sample extracts, Trolox standards, or blank solvent control was added to 0.1 mL of freshly prepared DPPH solution to initiate the reaction. The absorbance of the reaction mixture was measured at 515 nm every minute for 40 min of reaction in

dark. DPPH scavenging capacities were calculated using the areas under the curve and expressed as micromoles of Trolox equivalents (TE) per gram of dry flour.

2.3.6. Hydroxyl radical (HO•) scavenging capacity (HOSC)

The HOSC assay was performed following a previously reported laboratory procedure using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) (Moore, Yin, & Yu, 2006). Briefly, the reaction mixture contained 170 μL of 9.28×10^{-8} M FL, 30 μL of testing samples, solvent, or standards, 40 μL of freshly prepared 0.1990 M H_2O_2, and 60 μL $FeCl_3$. The fluorescence of the mixture was recorded every 2 min over 4 h at an ambient temperature. Excitation and emission wavelengths were 485 and 520 nm, respectively. HOSC was quantified using the area under the curve and expressed as micromoles of Trolox equivalent (TE)/g of the dry flour samples. Each seed flour sample was tested in triplicate.

2.3.7. Oxygen radical absorbing capacity (ORAC)

The oxygen radical absorbing capacity (ORAC) values were measured according to a previously reported laboratory protocol with minor modifications using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) (Moore, Hao, Zhou, Luther, Costa, & Yu, 2005). The final reaction mixture consisted of 225 μL of 8.16×10^{-8} M FL, 30 μL of sample or solvent blank or standard, and 25 μL of 0.36 M AAPH. The fluorescence of the mixture was recorded every 2 min over 2 h at 37 °C. Excitation and emission wavelengths were 485 and 520 nm, respectively. Trolox was

used as a standard, and the results were reported as μmol TE/g dry seed flour sample. Each flour sample was measured in triplicate.

2.3.8. ABTS$^{\bullet+}$ scavenging capacity

The scavenging ability against ABTS$^{\bullet+}$ was measured using a previously reported method (Moore, Luther, Cheng, & Yu, 2009). ABTS$^{\bullet+}$ working solution was prepared by reacting ABTS with manganese oxide and diluted to an absorbance of 0.700 ± 0.005 at 734 nm. The final reaction mixture consisted of 80 μL sample or solvent or standard, and 1 mL ABTS$^{\bullet+}$ working solution. After vortexing for 30 s the absorbance was read at 734 nm after 90 s of reaction. Trolox was used as a standard, and the results were expressed as micromoles of TE/g of flour. Each flour extract was measured in triplicate.

2.3.9. Anti-inflammatory capacity

6×10^5 cells/mL of J774A.1 mouse macrophage cells were sub-cultured using scrappers and cultured in DMEM with 10% FBS and 1% penicillin and streptomycin at 37 °C under 5% CO_2 in six well plates overnight to reach a 80% confluence. Cells were pre-incubated with each vegetable seed flour extract for 48 h. Medium was changed every 24 h. After 4 h of induction with10 ng/mL lipopolysaccharide (LPS), the culture media was discarded and the cells were collected for RNA isolation and Real-Time PCR analysis (Huang, Cheng, Shi, Xin, Wang, & Yu, 2011). cDNA synthesis kit was used to reverse transcribe cDNA. Real-Time PCR was performed on an ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR

Master Mix. IL-1β and COX-2 primers were used for inflammatory response and TBP was used for the control. The following amplification parameters were used for PCR: 50 °C for 2 min, 95 °C for 10 min, with 46 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min.

2.3.10. Anti-proliferative capacity

1×10^6 cells/mL of LNCaP prostate cancer cells were seeded in 24 well plates and cultured in RPMI1640 medium with 10% FBS and 1% antibiotic- antimycotic at 37 °C under 5% CO_2 with and without seed flour extracts (0.1% v/v) for 0, 24, 48, 72 and 96 hours. At each time point, Sulforhodamine B Cell Quantitation (SRB) (Skehan, Storeng, Scudiero, Monks, Mcmahon, Vistica, et al., 1990; Rubinstein, Shoemaker, Paull, Simon, Tosini, Skehan, et al., 1990) was used to count the cell numbers.

2.3.11. Statistical analysis

PRISM7 software was used for statistical analysis. Means ± standard deviation (SD) were used for each data point. For comparison, a one-way analysis of variation (ANOVA) ($P \leq 0.05$) followed by a post hoc test (Tukey test) was used, and $P < 0.05$ indicated a significant difference.

2.4. Results and discussion

To evaluate their beneficial effects and value-adding factors, the broccoli, carrot, and cucumber seed flours were extracted and examined for chemical compositions using UHPLC and UHPLC-HRMS, effects on gut microbiota by

measuring the total number of bacteria at five different time points (0, 2, 4, 5, 6 hours) and relative abundance of specific phylum or genus in gut microbiota using Real-Time PCR, free radical scavenging properties, and anti-inflammatory and anti-proliferative capacities. Radical scavenging properties were measured as relative diphenylpicrylhydrazyl (DPPH) radical scavenging capacity (RDSC), oxygen radical absorbance capacity (ORAC), relative hydroxyl radical scavenging capacity (HOSC) and $ABTS^{\bullet+}$ scavenging activity (ABTS).

2.4.1. Chemical compositions of broccoli, carrot and cucumber seed flours by UHPLC-PDA-ESI/HRMS^n

Table 2.1. Characterization of compounds present in broccoli seed flour.

Peak ID	$T_{R\ (min)}$	Theor. $[M-H]^-$	Exptl. $[M-H]^-$	Chemical Formula	Tentative Identification
1	3.79	436.0406	436.0402	$C_{12}H_{23}NO_{10}S_3$	Glucoraphanin isomer
2	3.85	436.0406	436.0402	$C_{12}H_{23}NO_{10}S_3$	Glucoraphanin isomer
3	4.38	436.0406	436.0400	$C_{12}H_{23}NO_{10}S_3$	Glucoraphanin isomer
4	8.83	463.0877	463.0882	$C_{21}H_{20}O_{12}$	Quercetin-3-glucoside[a]
5	11.09	420.0457	420.0453	$C_{12}H_{23}NO_9S_3$	Glucoerucin
6	12.36	385.1135	385.1141	$C_{17}H_{22}O_{10}$	Sinapoylhexose[a]
7	23.06	753.2242	753.2246	$C_{34}H_{42}O_{19}$	disinapoylgentiobiose[a]
8	27.71	591.1714	591.1709	$C_{28}H_{32}O_{14}$	1,2-disinapoylglucoside[a]
9	28.78	959.2821	959.2800	$C_{45}H_{52}O_{23}$	1,2,2'-Trisinapoylgentiobiose[a]

[a]The first time reported in broccoli seed flour. T_R stands for the retention time. Theor. $[M-H]^-$ and Exptl. $[M-H]^-$ were theoretical and experimental m/z of molecular ions, respectively.

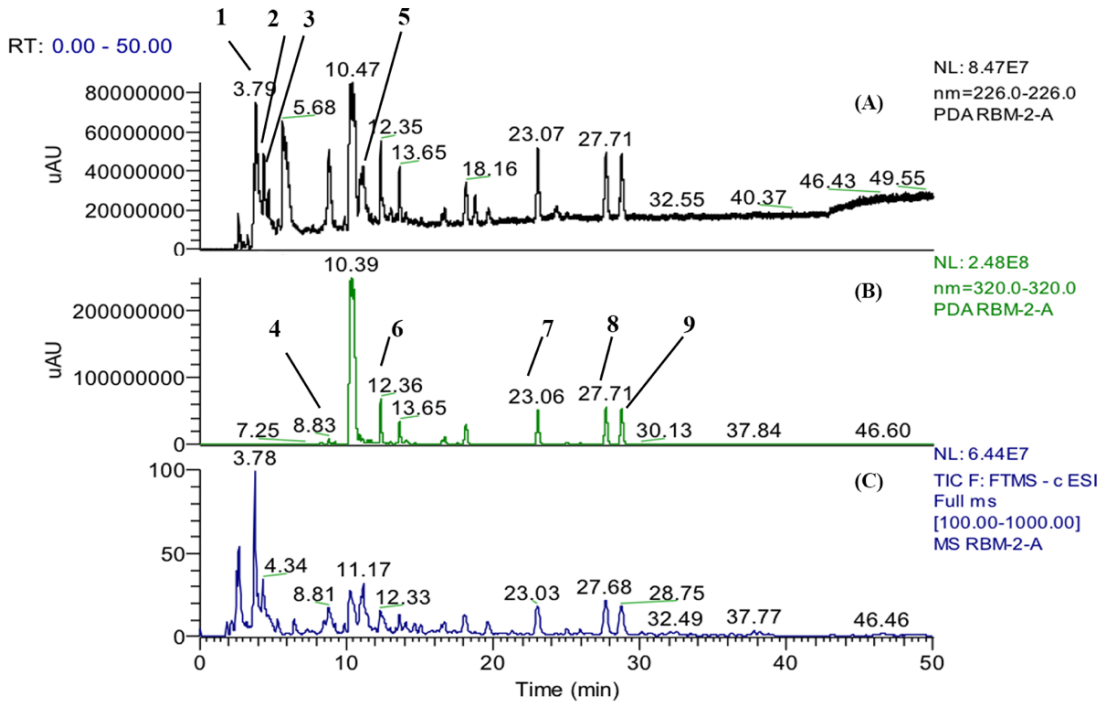

Figure 2.1. Typical UHPLC-UV chromatography of broccoli seed extract. A) 226 nm for optimal detection of glucosinolates, B) 320 nm for optimal detection of flavonols and flavonol glycosides and C) Typical UHPLC-Obitrap-MS chromatography of broccoli seed extract.

Total of nine chemical compounds including glucoraphanin isomers, quercetin-3-glucoside, glucoerucin, sinapoylhexose, disinapoylgentiobiose, 1,2-disinapoylglucoside and 1,2,2'-trisinapoylgentiobiose were tentatively identified in the broccoli seed flour, with glucoraphanin as the primary component (Table 2.1 and Figure 2.1). This observation was slightly different to McWalter and others' (2004) that glucoiberin was the primary component of the 75% methanol extract of a broccoli seed flour sample using liquid chromatography with mass spectrometry detection (Mcwalter, Higgins, Mclellan, Henderson, Song, Thornalley, et al., 2004). McWalter and others detected glucoiberin, sinigrin, glucoraphanin, progoitrin,

gluconapin, gluconasturtiin and glucoalyssin in the 75% methanol extract of a broccoli seed flour. The different glucosinolates composition may be due to the different extraction methods and solvents, as well as the possible different broccoli cultivars grown under different conditions. It needs to be pointed out that broccoli seed flour may contain significant level of erucic acid, which may have toxic effect (West, Tsui, Balch, Meyer, & Huth, 2002), although it was not detected under the experimental conditions. FDA regulates canola oil's erucic acid content of no more than 2 percent of the component fatty acids (https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1555). It has been reported that broccoli seeds contain high concentration of erucic acid compared to broccoli florets or sprouts (West, Tsui, Balch, Meyer, & Huth, 2002).

Table 2.2. Characterization of compounds present in carrot seed flour.

Peak ID	$t_{R\ (min)}$	Theor. $[M-H]^-$	Exptl. $[M-H]^-$	Chemical Formula	Tentatively Identification
1	8.40	503.1401	503.1410	$C_{21}H_{28}O_{14}$	Caffeoyldihexoside[a]
2	8.57	487.1452	487.1464	$C_{21}H_{28}O_{13}$	Cistanoside F[a]
3	8.73	517.1557	517.1560	$C_{22}H_{30}O_{14}$	Lycibarbarphenylpropanoid C[a]
4	12.25	593.1506	593.1505	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*-rutinoside isomer[a]
5	16.56	593.1506	593.1493	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*- rutinoside isomer[a]
6	17.84	447.0927	447.0940	$C_{21}H_{20}O_{11}$	Kaemprefol-3-*O*-glucoside isomer
7	19.91	577.1557	577.1568	$C_{27}H_{30}O_{14}$	Apigenin-7-*O*-β-D-rutinoside[a]
8	20.87	607.1663	607.1672	$C_{28}H_{32}O_{15}$	Diosmetin-7-rutinoside[a]
9	21.84	447.0927	447.0940	$C_{21}H_{20}O_{11}$	Kaempferol-3-*O*-glucoside isomer
10	32.61	285.0399	285.0402	$C_{15}H_{10}O_6$	Luteolin[a]

[a]The first time reported in carrot seed flour. T_R stands for the retention time. Theor. $[M-H]^-$ and Exptl. $[M-H]^-$ were theoretical and experimental m/z of molecular ions, respectively.

However, broccoli seed flours are by-product of broccoli seed oil production and most erucic acids might have been removed during the oil processing. It is important to consider the possible presence of erucic acid and its level in broccoli seed flours for food safety assurance (Table 2.1).

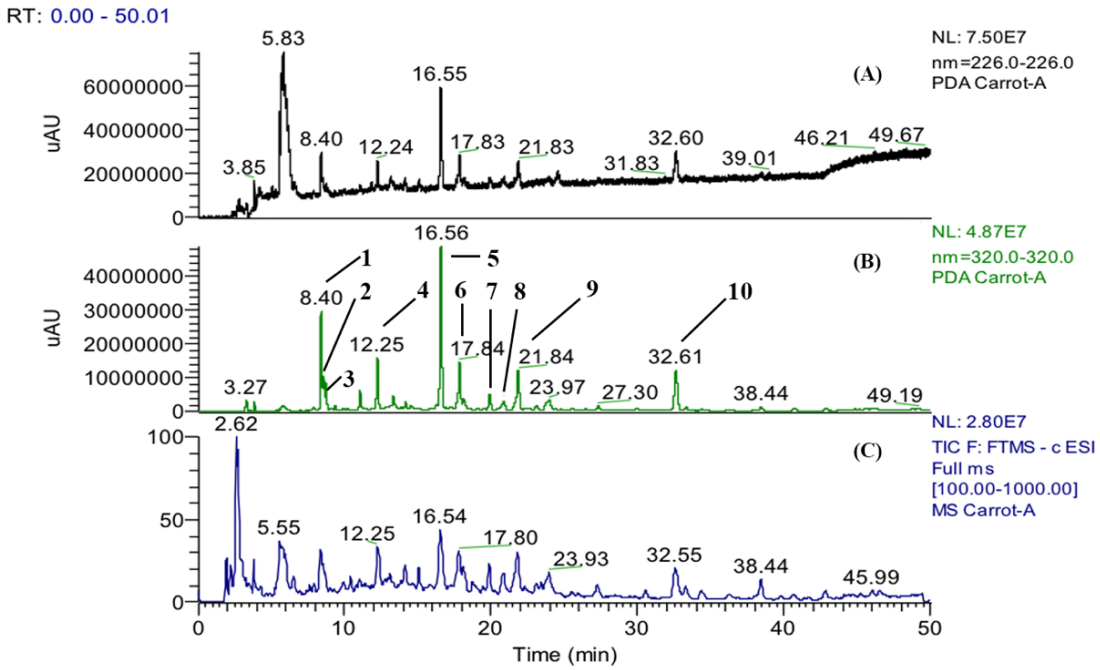

Figure 2.2. Typical UHPLC-UV chromatography of carrot seed extract.
A) 226 nm for optimal detection of glucosinolates, B) 320 nm for optimal detection of flavonols and flavonol glycosides, and C) Typical UHPLC-Obitrap-MS chromatography of carrot seed extract.

Ten compounds including kaempferol isomers, caffeoyldihexoside, lycibarbarphenylpropanoid C, cistanoside F, apigenin-7-*O*-β-D-rutinoside, diosmetin-7-rutinoside and luteolin were detected in the carrot seed flour extract, with kaempferol as the primary component and followed by luteolin and caffeoyldihexoside (Table 2.2 and Figure 2.2). Six compounds including caffeoyldihexoside, cistanoside F, lycibarbarphenylpropanoid C, kaempferol-3-*O*-

rutinoside isomers, apigenin-7-*O*-β-D-rutinoside and diosmetin-7-rutinoside were detected in the carrot seed flour extract for the first time. In 2005, Kumarasamy and others reported luteolin as a primary component in the methanol extract of carrot seed flour along with luteolin 3'-*O*-beta-D-glucopyranoside and luteolin 4'-*O*-beta-D-glucopyranoside (Kumarasamy, Nahar, Byres, Delazar, & Sarker, 2005). Even though the primary components were different, the results from the present study were in agreement to Kumarasamy and others that luteolin was one of the major compounds found in carrot seed flour extract.

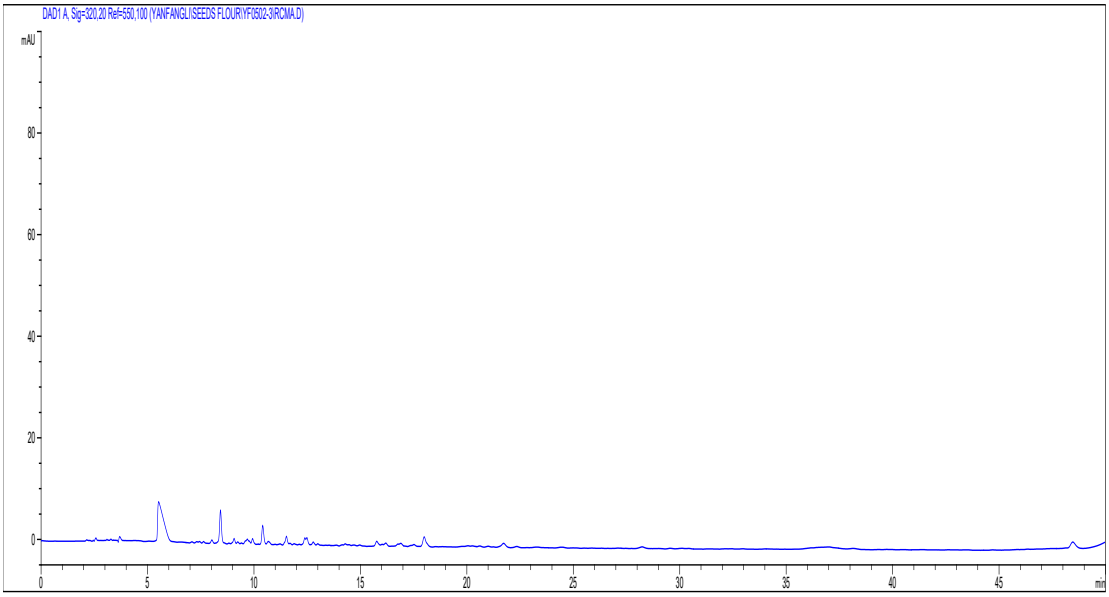

Figure 2.3. Typical UHPLC-UV chromatogram of cucumber seed extract.

Unfortunately, 50% cucumber seed flour extract did not have detectable level of chemical compounds under the experimental conditions (Figure 2.3).

2.4.2. Effects of seed flour extracts on gut microbiota

Growing evidence suggests that gut microbiota plays an important role in preventing the development of several chronic diseases including inflammatory bowel disease, obesity, type 2 diabetes mellitus, cardiovascular disease, and cancer (Singh, Chang, Yan, Lee, Ucmak, Wong, et al., 2017). Gut microbiota profile is critical to human health.

The human gut microbiota community is predominantly determined at birth because infant's microbiota is delivered by mother. Also, breast/bottle feeding and epigenetics can alter the microbiota community of an infant. Therefore, each individual has different compositions and numbers of gut microbiota. Moreover, during the life time, gut microbial compositions may be changd by many factors. For example, using antibiotics or drugs can cause abnormal microbiota development. Also, disease, injury, surgery and stress can affect microbiota community (Conlon & Topping, 2016). Even though each individual has different gut microbiota profile due to different environmental factors, healthy diet can alter gut microbiota profile in a positive way. In addition, diet seems to have the biggest impact (Conlon & Topping, 2016; Mai, 2004). There have been many studies over the last few decades showing the abundance enhancement and the composition alteration of the gut microbiota through diet treatments (Clavel, Fallani, Lepage, Levenez, Mathey, Rochet, et al., 2005; Bedani, Pauly-Silveira, Roselino, Valdez, & Rossi, 2010; Cavallini, Suzuki, Abdalla, Vendramini, Pauly-Silveira, Roselino, et al., 2011; Fernandez-Raudales, Hoeflinger, Bringe, Cox, Dowd, Miller, et al., 2012). As a result, probiotic foods such

as yogurt, pickles, miso soup and kimchi obtained consumer interests. However, there are some limitations in probiotic foods because health beneficial effects of probiotic foods are affected by temperature, pH and time. On the other hand, prebiotics are not affected by these factors. Prebiotics are defined as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon (Gibson & Roberfroid, 1995).

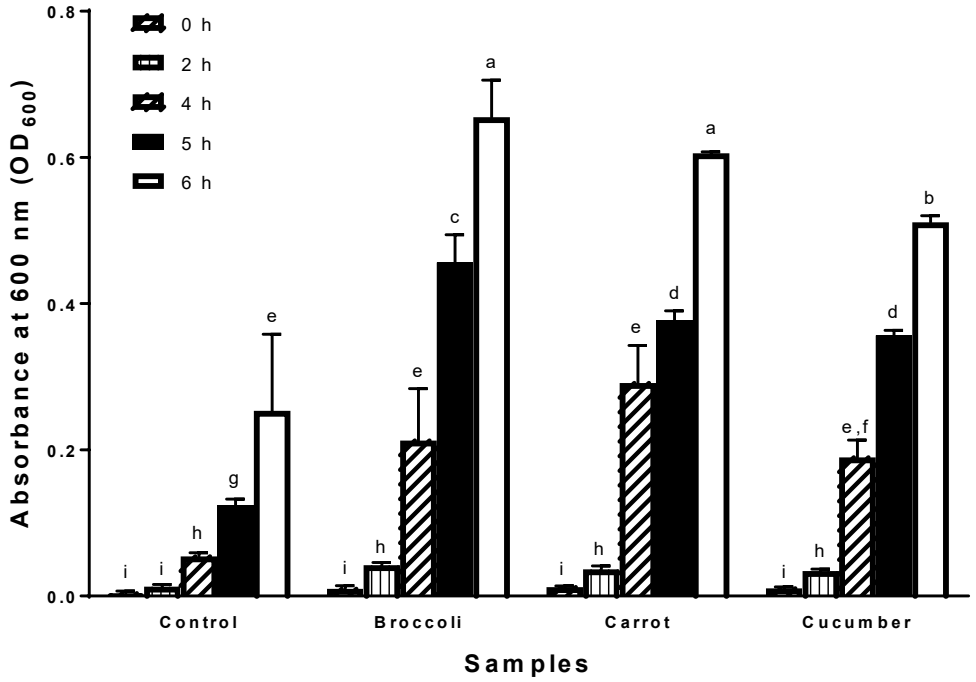

Figure 2.4. Bacterial growth curve.
1×10^7 cells/mL bacterial cells were seeded in 96 well plates and treated with and without seed flour extracts for 6 hours under an anaerobic condition. Total bacterial cell numbers were measured at five different time points (0, 2, 4, 5 and 6 hours) at 600 nm. Results are expressed as mean ± SD (n = 3). Bars with different letters indicate significant difference at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used). Broccoli stands for broccoli seed flour extract. Carrot stands for carrot seed flour extract. Cucumber stands for cucumber seed flour extract.

The present study showed that carrot, cucumber and broccoli seed flour extracts were able to time-dependently and significantly enhance gut bacterial growth under the experimental conditions (Figure 2.4). These enhancements are important because less gut microbiota has been reported in the patients with inflammatory bowel disorder (Qin, Li, Raes, Arumugam, Burgdorf, Manichanh, et al., 2010; Manichanh, 2006; Lepage, Häsler, Spehlmann, Rehman, Zvirbliene, Begun, et al., 2011), elderly patients with inflammation (Claesson, Jeffery, Conde, Power, O'Connor, Cusack, et al., 2012) or in obese individuals (Turnbaugh, Hamady, Yatsunenko, Cantarel, Duncan, Ley, et al., 2008). Taking together, consumption of carrot, cucumber and broccoli seed flours and components may enhance the total number of gut microbiota, and consequently reduce the risk of inflammation, inflammatory bowel disorder and obesity.

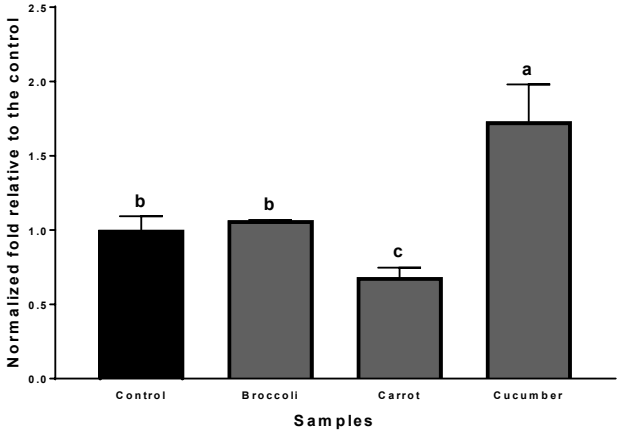

Figure 2.5. Relative abundance of *Bacteroidetes* phylum.
Broccoli stands for broccoli seed flour extract, carrot stands for carrot seed flour extract, and cucumber stands for cucumber seed flour extract. Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

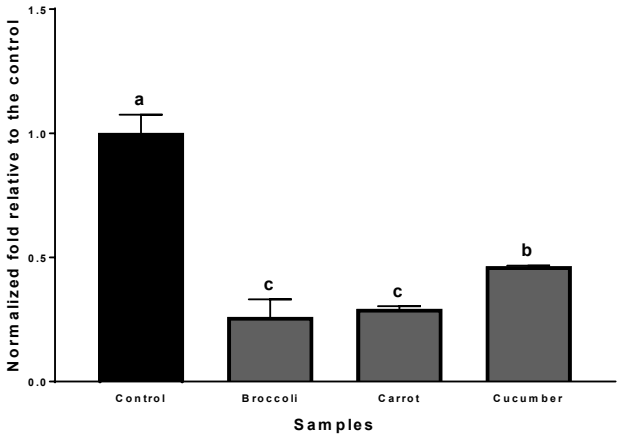

Figure 2.6. Relative abundance of *Firmicutes* phylum.
Broccoli stands for broccoli seed flour extract, carrot stands for carrot seed flour extract, and cucumber stands for cucumber seed flour extract. Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

The composition of gut microbiota is important as much as their total abundance. Mainly, human gut microbiota is composed of *Bacteroidetes* and *Firmicutes*. In healthy adults, > 90% of gut microbiota community is dominated by these two phyla (Louis, Hold, & Flint, 2014). Due to their abundance, *Bacteroidetes* and *Firmicutes* contribute to human health in various ways. For example, *Bacteroidetes* interact with human immune system through activating T-cell mediated responses (Mazmanian, Round, & Kasper, 2008; Wen, Ley, Volchkov, Stranges, Avanesyan, Stonebraker, et al., 2008), whereas *Bacteroidetes* produce butyrate, an end product of colonic fermentation with possible anti-neoplastic properties (Kim & Milner,2007). Also, *Bacteroidetes* may be involved in bile acid metabolism and transformation of toxic and mutagenic compounds (Smith, Rocha, & Paster, 2006). In this study, cucumber seed flour extract was most effective in increasing *Bacteroidetes*

phylum (Figure 2.5). The other major component of gut microbiota, *Firmicutes* phylum is involved in fatty acid metabolism (Turnbaugh, Ley, Mahowald, Magrini, Mardis, & Gordon, 2006). Also, *Firmicutes* may be closely related to aging. It has been reported that aging increases the total number of *Firmicutes* and therefore elderly people have relative high portion of *Firmicutes* (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005). The current study found that carrot, cucumber and broccoli seed flour extracts significantly inhibited the growth of *Firmicutes* phylum (Figure 2.6), suggesting possible effects of carrot, cucumber and broccoli seed flour extracts in fat metabolism and aging-related human health conditions.

In addition, a ratio between *Bacteroidetes* and *Firmicutes* has been reported to have a close relationship to aging and obesity (Nicholson, Holmes, Kinross, Burcelin, Gibson, Jia, et al., 2012). The ratio of *Bacteroidetes* and *Firmicutes* changes over the life time (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005). Compare to a healthy adult, an infant has much higher portion of *Bacteroidetes*. On the other hand, elderly people have relatively high portion of *Firmicutes*. There also has been an evidence that the *Bacteroidetes*/*Firmicutes* ratio is closely related to obesity. According to Ley and colleagues, diet therapy for 52 weeks totally altered *Bacteroidetes* and *Firmicutes* ratio by increasing *Bacteroidetes* and decreasing *Firmicutes* (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005). The result from Ley's study suggested that the increased Bacteroidetes/*Firmicutes* ratio may correlate to weight loss.

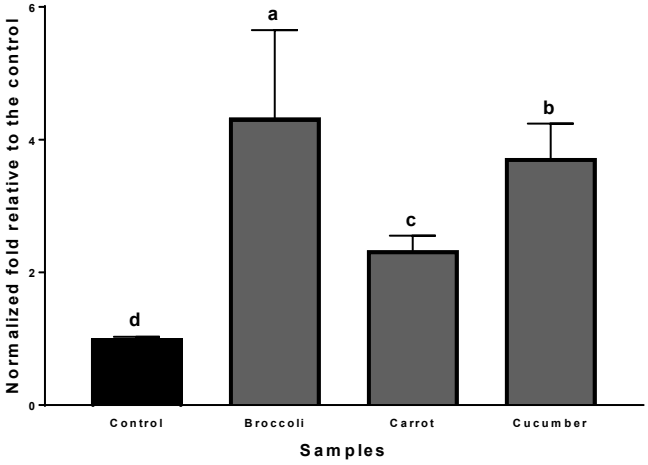

Figure 2.7. Bacteroidetes/Firmicutes ratio.
DNA from bacterial cells treated with and without seed flour extracts were extracted. and Real-Time PCR was performed using *Bacteroidetes* and *Firmicutes* primers. The ratio of *Bacteroidetes* and *Firmicutes* from seed flour samples were normalized to the control. Results are expressed as mean ± SD (n = 3). Bars with different letters indicate significant difference at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used). Broccoli stands for broccoli seed flour extract. Carrot stands for carrot seed flour extract. Cucumber stands for cucumber seed flour extract.

In this study, carrot, cucumber and broccoli seed flour extracts were able to significantly increase the *Bacteroidetes*/*Firmicutes* ratio (Figure 2.7). This was mainly due to a significant reduction of *Firmicutes* phylum (Figure 2.6). These results suggested that altering *Bacteroidetes*/*Firmicutes* ratio through dietary intakes of carrot, cucumber or broccoli seed flour or their components may have a potential to improve human health such as body weight reduction and healthy aging, and warrant additional investigations.

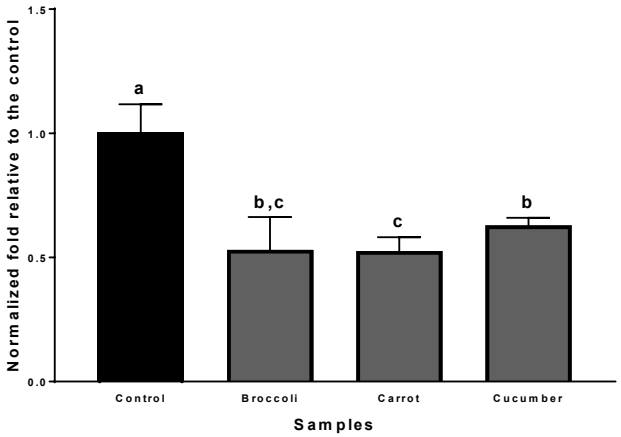

Figure 2.8. Relative abundance of *Akkermansia* genus.
Broccoli stands for broccoli seed flour extract, carrot stands for carrot seed flour extract, and cucumber stands for cucumber seed flour extract. Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

Effect of carrot, cucumber and broccoli flour extracts on *Akkermansia* was evaluated, and the results showed that reduction of *Akkermansia* was observed (Figure 2.8). *Akkermansia* is a mucin-degrading bacterium that resides in the mucus layer. *Akkermansia* has been considered as a contributor to the maintenance of gut health. Recently, Dao and others have shown that *Akkermansia* could reduce body fat mass, improve glucose homoeostasis, decrease adipose tissue inflammation and increase gut integrity in mice (Dao, Everard, Aron-Wisnewsky, Sokolovska, Prifti, Verger, et al., 2015). Moreover, it has been reported that *Akkermansia* presence was inversely correlated with consumption of polysaccharides and body weight gain in rodents and humans (Amar, Burcelin, Ruidavets, Cani, Fauvel, Alessi, et al., 2008). However, the precise physiological roles played by this bacterium during obesity and metabolic disorders are unknown (Everard, Belzer, Geurts, Ouwerkerk, Druart,

Bindels, et al., 2013). A recent study reported that polyphenols promoted growth of *Akkermansia* and attenuated high fat diet induced metabolic syndrome (Anonye, 2017). However, in the present study, reduction of *Akkermansia* was observed with all three extracts rich in polyphenolic compounds such as kaempferol and quercetin.

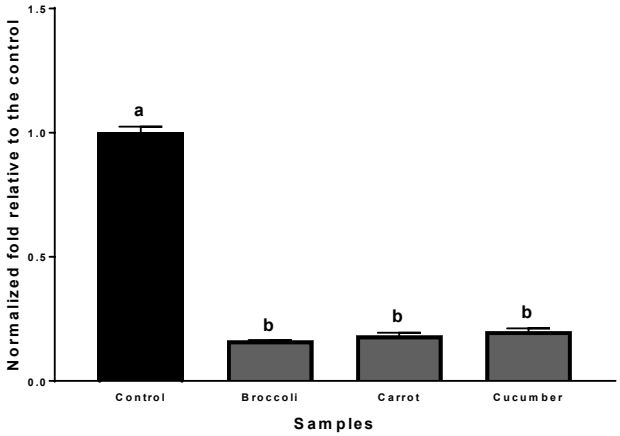

Figure 2.9. Relative abundance of *Bifidobacterium* genus.
Broccoli stands for broccoli seed flour extract, carrot stands for carrot seed flour extract, and cucumber stands for cucumber seed flour extract. Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

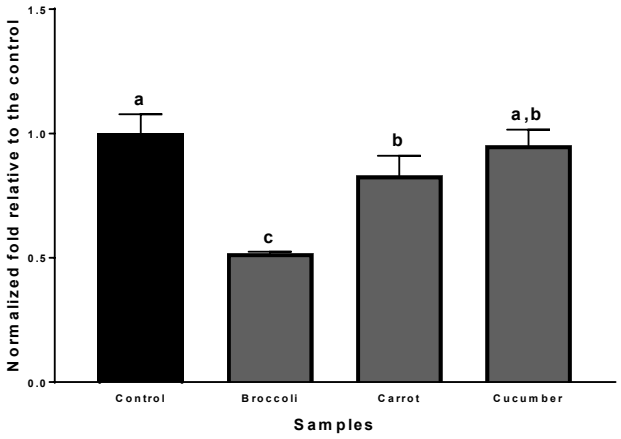

Figure 2.10. Relative abundance of *Lactobacillus* genus.
Broccoli stands for broccoli seed flour extract, carrot stands for carrot seed flour extract, and cucumber stands for cucumber seed flour extract. Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

As shown in Figure 2.9, a significant decrease of *Bifidobacteria* was detected for all the tested extracts. Carrot and broccoli seed flour extracts significantly reduced the *Lactobacillus* levels, whereas cucumber seed flour extract had no significant effect (Figure 2.10). *Bifidobacteria* and *Lactobacillus* genera are well-known probiotic bacteria. Maintaining certain amount of these bacteria has several health beneficial effects due to their functions. For example, *Bifidobacteria* and *Lactobacillus* are able to prevent or alleviate infectious diarrhea through their effects on the immune system and reduction of colonization by pathogens (Picard, Fioramonti, Francois, Robinson, Neant, & Matuchansky, 2005). Also, there is some experimental evidence that certain *Bifidobacteria* may actually protect the host from carcinogenic activity of intestinal flora (Rosenfeldt, Michaelsen, Jakobsen, Larsen, Møller, Tvede, et al., 2002). On the other side, *Bifidobacteria* and *Lactobacillus* are

the major bacteria that produce lactic acid and overabundance of these genera can cause a lactic acidosis. The results from current study suggest that carrot, cucumber and broccoli seed flours may be used for lactic acidosis treatment.

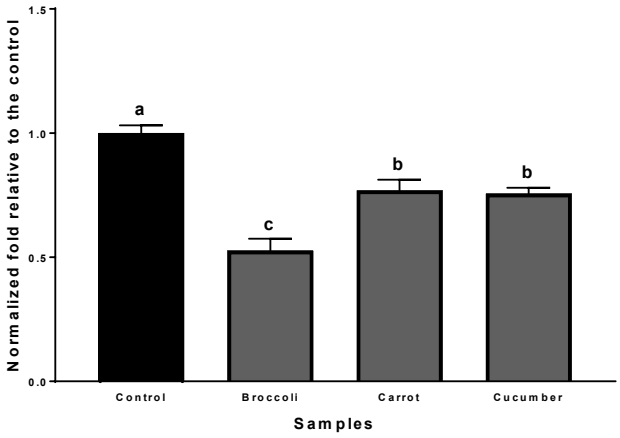

Figure 2.11. Relative abundance of *Enterobacteriaceae* genus.
Broccoli stands for broccoli seed flour extract, carrot stands for carrot seed flour extract, and cucumber stands for cucumber seed flour extract. Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

In addition, carrot, cucumber and broccoli seed flour extracts were examined for their effects on *Enterobacteriaceae* genus, known as "bad" bacteria (Huang, Krishnan, Pham, Yu, & Wang, 2016). A significant decrease of *Enterobacteriaceae* was observed for the treatments of all three seed flour extracts (Figure 2.11), suggesting their possible health beneficial effects. For instance, a recent study suggested that soy-based diet can decrease the number of *Enterobacteriaceae* genus, and that may reduce the risk of gastrointestinal disorders such as diarrhea and enteritis (Cheng, 2005).

Taking together, carrot, cucumber and broccoli seed flours may have potential health beneficial effects such as weight loss and anti-aging through their interactions with gut microbiota. These seed flours may also reduce the risk of inflammatory diseases and gastrointestinal disorders through altering gut microbiota abundance and composition.

2.4.3. Relative DPPH radical scavenging capacity (RDSC)

Table 2.3. Free radical scavenging capacities of cold-pressed vegetable seed flours[a]

	RDSC (μmol TE/g)	ORAC (μmol TE/g)	HOSC (μmol TE/g)	ABTS (μmol TE/g)
Broccoli	84.75 [a] ± 11.26	633.50 [a] ± 76.84	269.75 [a] ± 26.45	175.88 [b] ± 31.85
Carrot	16.00 [b] ± 0.61	143.91 [b] ± 6.36	112.35 [b] ± 7.86	250.00 [a] ± 7.35
Cucumber	2.64 [c] ± 0.15	28.63 [c] ± 2.60	51.50 [c] ± 5.33	6.81[c] ± 0.48

[a]Broccoli stands for broccoli seed flour extract. Carrot stands for carrot seed flour extract. Cucumber stands for cucumber seed flour extract. RDSC stands for relative DPPH scavenging capacity, ORAC stands for oxygen radical absorbing capacity, HOSC stands for hydroxyl radical scavenging capacity, and ABTS stands for ABTS radical scavenging capacity. Each column marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

The broccoli seed flour extract had the greatest DPPH radical scavenging capacity with a RDSC value of 85 μmol TE/g. The cucumber seed flour extract had the lowest DPPH radical scavenging capacity of 3 μmol TE/g (Table 2.3). These values are comparable to the RDSC values of fruit seeds and vegetables including

broccoli, carrot, and cucumber previously reported (Yu, Gao, Li, Wang, Luo, Wang, et al., 2018; Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017; Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000; Cao, Sofic, & Prior, 1996). Parry and others examined fruit seeds including black raspberry, red raspberry, blueberry, cranberry, and pinot noir and chardonnay grapes, and detected a RDSC range of 39–1260 μmol TE/g (Yu, Gao, Li, Wang, Luo, Wang, et al., 2018). Our previous studies (Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017; Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000) showed DPPH scavenging capacities of broccoli and carrot vegetables in a range of 0.3–0.5 and 0.7–0.9 μmol TE/g, respectively. Miller and others examined cucumber vegetable and detected a DPPH scavenging capacity of 1.0 μmol TE/g (Cao, Sofic, & Prior, 1996). Compared to fruit seeds, RDSC values of the three vegetable seed samples were lower. However, compared to seeds' counterpart vegetables, the three seed flour samples exhibited similar or greater RDSC values on a per weight basis. This is the first report of DPPH scavenging capacities for the three de-fatted seed flours.

2.4.4. Hydroxyl radical (HO•) scavenging capacity (HOSC)

The hydroxyl radical is one of the reactive oxygen species (ROS) generated under physiological conditions. By scavenging hydroxyl radical, oxidative damage at cellular level can be suppressed and therefore possibly reduce the risk of chronic diseases. The hydroxyl radical scavenging capacities of the three seed flour extracts were in a range of 52–270 μmol TE/g (Table 2.3). Among the three samples, broccoli seed flour extract had the greatest HOSC value of 270 μmol TE/g followed by carrot seed flour extract (112 μmol TE/g) and cucumber seed flour extract (52 μmol TE/g),

respectively. These values are comparable to HOSC values of fresh broccoli and carrot vegetables ranging in 390-509 μmoles TE/g (Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017) and 426–507 μmol TE/g (Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000), respectively. Compared to fresh broccoli and carrot extracts, broccoli and carrot seed flour extracts had lower HOSC values on a per dry weight basis. This is the first report of hydroxyl radical scavenging capacities for the three de-fatted seed flours.

2.4.5. Oxygen radical absorbing capacity (ORAC)

All tested seed samples exhibited oxygen radical absorbing capacities, with ORAC values in a range of 29–634 μmol TE/g (Table 2.3). The broccoli seed flour extract had the highest ORAC value of 634 μmol TE/g, whereas cucumber seed flour extract had the lowest ORAC value of 29 μmol TE/g. This range is comparable to the ORAC values of fruit seeds and fresh vegetables (Yu, Gao, Li, Wang, Luo, Wang, et al., 2018; Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017; Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000; Tiveron, Melo, Bergamaschi, Vieira, Regitano-D'Arce, & Alencar, 2012). The fruit seed extracts had ORAC values in a range of 111–1076 μmol TE/g (Yu, Gao, Li, Wang, Luo, Wang, et al., 2018). ORAC values from fresh vegetables including broccoli, carrot and cucumber were in a range of 15–17, 1–4, 0.4–0.6 μmol TE/g, respectively. Compared to counterpart vegetables, carrot, cucumber and broccoli seed flour extracts had a similar or greater ORAC values on a per dry weight basis. This is the first report of oxygen radical absorbing capacities for the three de-fatted seed flours.

2.4.6. $ABTS^{\bullet+}$ scavenging capacity

Three seed flour extracts had $ABTS^{\bullet+}$ scavenging capacity values of 7–250 µmol TE/g (Table 2.3). The carrot seed flour extract had the greatest ABTS value of 250 µmol TE/g and the cucumber seed flour extract had the lowest ABTS value of 7 µmol TE/g. Interestingly, the carrot seed flour extract showed a greater $ABTS^{\bullet+}$ scavenging capacity compared to its counterpart vegetable, carrot (1 µmol TE/g) and carrot seed oil (9 µmol TE/g) (Yu, Zhou, & Parry, 2005). Similarly, broccoli seed flour extract showed a greater $ABTS^{\bullet+}$ scavenging capacity of 176 µmol TE/g compare to its counterpart vegetable, broccoli (43 µmol TE/g) (Yu, Zhou, & Parry, 2005). However, ABTS value from cucumber seed flour extract (7 µmol TE/g) was lower than its counterpart vegetable, cucumber (10 µmol TE/g) (Yu, Zhou, & Parry, 2005). This is the first report of $ABTS^{\bullet+}$ scavenging capacities for the three de-fatted seed flours.

2.4.7. Anti-inflammatory and anti-proliferative capacities

Anti-inflammatory capacity is an important factor since various chronic diseases including cancer is an expanded concept of inflammation (Coussens & Werb, 2002).

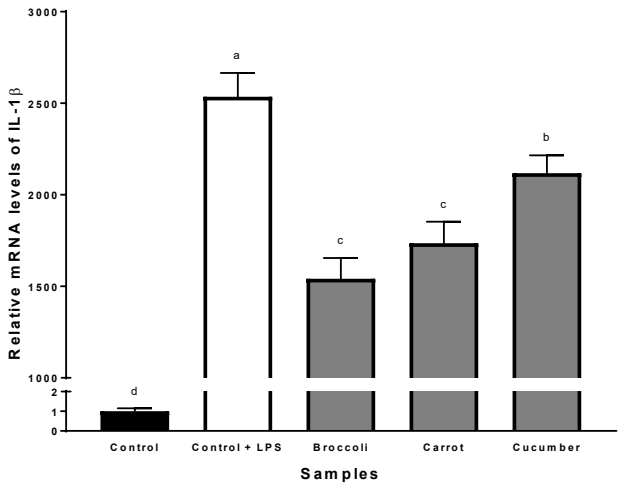

Figure 2.12. Anti-inflammatory capacity of seed flour extracts in J774A.1 mouse macrophage cells; Interleukin 1 beta (IL-1β).
LPS stands for lipopolysaccharide, broccoli stands for broccoli seed flour extract + LPS, carrot stands for carrot seed flour extract + LPS, and cucumber stands for cucumber seed flour extract + LPS. Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

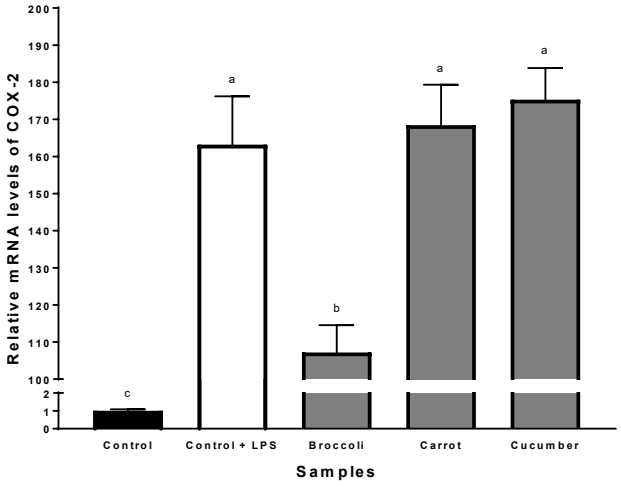

Figure 2.13. Anti-inflammatory capacity of seed flour extracts in J774A.1 mouse macrophage cells; Cyclooxygenase-2 (COX-2).
LPS stands for lipopolysaccharide, broccoli stands for broccoli seed flour extract + LPS, carrot stands for carrot seed flour extract + LPS, and cucumber stands for cucumber seed flour extract + LPS. Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

In this study, all three tested seed flour extracts exhibited potential anti-inflammatory capacities. The broccoli seed flour extract showed the greatest inhibition of 38.9% on IL-1β mRNA expression with compared to the LPS induced control. Also, carrot and cucumber seed flour extracts showed inhibition rates of 31.3 and 16.3%, respectively (Figure 2.12). Only broccoli seed flour extract showed a significant inhibition on COX-2 mRNA expression with an inhibition rate of 33.8% (Figure 2.13).

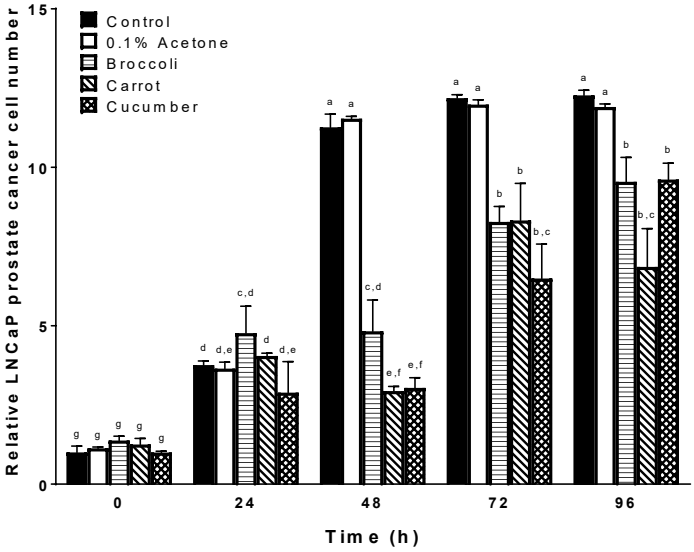

Figure 2.14. Anti-proliferative capacity of the seed flour extracts in LNCaP prostate cancer cells.
0.1% acetone stands for 0.1% v/v acetone added to the media, broccoli stands for broccoli seed flour extract, carrot stands for carrot seed flour extract, and cucumber stands for cucumber seed flour extract. Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

Also, anti-proliferative capacities were tested for the three seed flour extracts. After 48 h of treatment, all the extracts inhibited LNCaP prostate cancer cell

proliferations (Figure 2.8). After 96 h of treatment, carrot seed flour extract exhibited the greatest anti-proliferative capacity of 46.2% followed by broccoli seed flour extract (20.0%) and cucumber seed flour extract (19.2%) on a per extract weight concentration basis (Figure 2.8).

In summary, for the first time, the present study showed that carrot, cucumber and broccoli seed flour components could alter gut microbiota abundance and profile, scavenge free radicals, and suppress cancer cell proliferation and inflammation, suggesting a potential utilization of these seed flours in nutraceuticals and functional foods. This potential utilization may add value to the seed oil processing industry as the flours are by-products from oil production, while reducing environmental hazardous waste disposal. Their phenolic compositions could only explain partial of the beneficial effects, warranting additional studies to further investigating chemical compositions including polysaccharide and oligosaccharide compositions and small molecular compounds, as well as their roles in human health.

Chapter 3: Chemical composition of cold-pressed milk thistle seed flour extract, and its potential health beneficial properties

Choe et al., *Food Funct.* **2019**, *10* (5), 2461–2470.

3.1. Abstract

The cold-pressed milk thistle seed flour was extracted with 50% acetone and evaluated for its phytochemical composition, and gut microbiota modulating, free radical scavenging, anti-inflammatory and anti-proliferative capacities. UHPLC-MS analysis detected fifteen compounds in the milk thistle seed flour extract with silychristin as the primary component and followed by silybin B and isosilybins A & B. Milk thistle seed flour extract enhanced the total bacteria number and altered the abundance of specific bacterial phylum or genus under the experimental conditions. The extract had RDSC, ORAC, HOSC, and $ABTS^{\bullet+}$ scavenging capacities of 49, 634, 10420 and 116 μmol Trolox equivalent (TE)/g flour, respectively. In addition, milk thistle seed flour extract suppressed LPS induced IL-1β mRNA expressions in the cultured J774A.1 mouse macrophages and the proliferation of LNCaP prostate cancer cells. The results suggest milk thistle seed flour's potential health benefits in functional foods.

3.2. Introduction

Milk thistle seed flour is a byproduct from the manufacture of the seed oil. Investigation of milk thistle seed flour's health-beneficial components and properties can lead to potential utilization of milk thistle seed flour in nutraceuticals and functional foods, and add value to oil manufacturers and the milk thistle seed producers while reducing environmental contaminations.

Milk thistle seeds have been used for centuries since the time of ancient Greece. According to Dioscorides, a Greek herbalist, a tea of milk thistle seeds could cure the bite of a poisonous snake (Flora, Hahn, Rosen, & Benner, 1998). Later in 2013, the seeds were reported to protect toxins from attaching to the liver cells (Siegel & Stebbing, 2013). In addition to the hepatoprotective effect, milk thistle seeds can neutralize free radicals. In recent years, ethanolic extract of milk thistle seeds has shown free radical scavenging capacity related health beneficial effects such as reduced risk of DNA oxidation, protein damage and lipid peroxidation (Serçe, Toptancı, Tanrıkut, Altaş, Kızıl, Kızıl, et al., 2016). Moreover, milk thistle seed extracts have been recognized as potential candidates for reducing the risk of several cancers (Davis-Searles, Nakanishi, Kim, Graf, Oberlies, Wani, et al., 2005; Fan, Ma, Liu, Zheng, & Huang, 2014). Scientists believe that silymarin, a group of polyphenolic flavonoid compounds found in milk thistle seed extract, may be associated with these health beneficial effects. However, other chemical components of milk thistle seeds have been seldom reported. Also, milk thistle seeds and flours have not been investigated for their potential gut microbiota modulation activities,

though phenolics have been shown to interact with gut microbiota and impact host health conditions (Surai, 2015).

In this study, milk thistle seed flour extract is investigated for its chemical composition, and potential influence on gut bacteria *in vitro* for the first time. In addition, free radical scavenging, anti-inflammatory and anti-proliferative capacities of milk thistle seed extract were evaluated. The results from this study can add value to milk thistle seed flours and extend their utilization for enhancing human health.

3.3 Materials and methods

3.3.1. Materials

Milk thistle seed flour was gifted from the Botanic oil innovations (Spooner, WI, USA). HPLC grade water, acetone, acetonitrile, formic acid, silybin (HPLC grade), chlorogenic acid (HPLC grade), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein (FL), iron (III) chloride, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 30 % ACS-grade hydrogen peroxide, DEPC-Treated water, and nuclease-free water were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). 2,2′-Azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA, USA). J774A.1 and LNCaP cells were purchased from American Type Culture Collection (Manassas, VA, USA). TRIzol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). TATA-binding protein (TBP)

and interleukin 1 beta (IL-1β) primers, SYBR®Green Real-Time PCR Master Mix and TaqMan Fast Universal PCR Master Mix were purchased from Applied Biosystems (Carlsbad, CA, USA). Affinity Script Multi-Temperature cDNA Synthesis kit was purchased from Agilent Technologies (Savage, MD, USA). DMEM, RPMI1640, FBS, penicillin and streptomycin were purchased from GIBCO (Grand Island, NY, USA). LB broth was purchased from Quality Biological™ (Gaithersburg, MD, USA). QIAamp DNA Mini Kit was purchased from Qiagen (Gaithersburg, MD, USA). Precellys were purchased from Bertin Technologies (Rockville, MD, USA).

3.3.2. Sample preparation

10 g of milk thistle seed flour was extracted three times with 25 mL of 50% acetone at ambient temperature with sonication (Li, Sun, Shi, Yu, Ridge, Mazzola, et al., 2017; Choe, Li, Gao, Yu, Wang, Sun, et al., 2018). The concentration of the extract was 0.4 g flour equivalents/mL. The extracts were kept at 4 °C and used for chemical composition determination and bioactivity evaluations. All experiments were performed in triplicate.

3.3.3. Ultra High-Performance liquid chromatography photo diode array high-resolution multi-stage mass spectrometry (UHPLC-PDA-ESI/HRMS^n)

The UHPLC-HRMS analysis was performed as previously reported (Li, Sun, Shi, Yu, Ridge, Mazzola, et al., 2017; Liu, Li, Yang, Wan, Chang, Wang, et al., 2017). Briefly, the UHPLC-HRMS system consisted of an LTQ Orbitrap XL mass

spectrometer (Thermo Scientific, Waltham, MA, USA) with an Agilent 1290 Infinity liquid chromatography coupled with a DAD detector. The UV-vis spectrum scanning was from 190 to 600 nm. A Luna C_{18} column, 4.6 mm × 250 mm and 5 μm particle size, was used. HPLC grade water with 0.1% formic acid (v/v) was used as solvent A, and acetonitrile with 0.1% formic acid (v/v) was used as solvent B. The elution was carried out at 5% of solvent B at the beginning, increasing via a linear gradient to 13% B at 5 min; increasing to 20% at 10 min; increasing to 27% at 25 min; increasing to 33% at 40 min; increasing to 50% at 45 min; increasing to 90% at 46 min; keeping 90% until 51 min; and the post-run time for re-equilibration was 10 min. The injection volume was 5 μL, with a flow rate of 1 mL/min and an oven temperature of 40 °C. The HRMS was conducted in a negative ionization mode with the optimized parameters as follows: spray voltage at 4.5 kV, the capillary temperature at 325 °C, the capillary voltage at -50 V, and tube lens offset voltage at -120 V. The mass range was m/z 100-1000 with a resolution of 30,000. Data were post-processed using QualBrowser part of Thermo Scientific Xcalibur 2.2 software.

3.3.4. Bacterial growth and gut microbiota profile

Fecal samples were collected from a regular diet fed C57BL/6J mouse. Fecal bacteria were cultured in LB broth one day prior to treatment at 37 °C under an anaerobic condition according to a laboratory protocol (Li, Sun, Shi, Yu, Ridge, Mazzola, et al., 2017; Whent, Slavin, Kenworthy, & Yu, 2009). Bacteria culture and seeding concentration were measured at 600 nm using a coefficient of OD_{600} of 1 = 8 $\times 10^8$ cells/mL. Bacterial cells (1×10^7 cells/mL) were seeded in 96 well plates and 15

mL centrifuge tubes using M9 minimal broth with and without milk thistle seed flour extract treatments and cultured in an incubator shaker at 37 °C for 6 hours. The final flour extract concentration was 0.4 g flour equivalents/mL in the culture medium. OD_{600} values were measured at five different time points at 0, 2, 4, 5 and 6 hours of the treatment. At the end of the treatment, bacterial cells were collected by centrifuging 15 mL centrifuge tubes at ambient temperature at 5000 rpm for 5 min, and the supernatant was aspirated. After cells were homogenized with Precellys, the bacterial DNA was extracted with a QIAamp DNA mini kit following the manufacturer's protocol. Real-Time PCR was performed with a reaction system of 10 µL SYBR®Green Real-Time PCR Master Mix, 0.25 µL 500 nM custom-made oligo primers, 4.5 µL water and 5 µL DNA. Primers specific for *Bacteroidetes*, *Firmicutes* phyla, and *Akkermansia*, *Bifidobacteria*, *Lactobacillus*, *Enterobacteriaceae* genera were used to determine the relative abundance of the respective microorganisms.

3.3.5. Relative DPPH radical scavenging capacity (RDSC)

A laboratory assay of a relative 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (DPPH) was performed using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). To initiate the reaction, 0.1 mL of the milk thistle seed flour extract, Trolox standard, or blank solvent control was added to 0.1 mL of freshly prepared DPPH solution. The final flour extract concentration was 0.2 g flour equivalents/mL in the reaction mixture. The absorbance was measured at 515 nm every minute for 40 min of reaction at ambient temperature in dark (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018; Yu, Gao, Li, Wang, Luo, Wang, et al., 2018). DPPH

scavenging capacities were calculated using the areas under the curve and expressed as micromoles of Trolox equivalents (TE) per gram of dry flour.

3.3.6. Hydroxyl radical ($HO^{\bullet}$) scavenging capacity (HOSC)

The HOSC assay was performed using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) with a fluorescent probe according to a previously reported laboratory protocol (Moore, Yin, & Yu, 2006). Trolox was used as a standard. Briefly, the reaction mixture contained 170 μL of 9.28×10^{-8} M FL, 30 μL of the milk thistle seed flour extract, solvent, or standards, 40 μL of freshly prepared 0.1990 M H_2O_2, and 60 μL $FeCl_3$. The final flour extract concentration was 40 mg flour equivalents/mL in the reaction mixture. The fluorescence of the mixture was recorded at ambient temperature every 2 min over 4 h. The wavelengths of 485 and 520 nm were used for excitation and emission, respectively. HOSC was quantified using the area under the curve and expressed relative to Trolox as micromoles of Trolox equivalent (TE)/g of the dry flour samples.

3.3.7. Oxygen radical absorbing capacity (ORAC)

A previously reported laboratory protocol was used to conduct an ORAC assay (Slavin, Lu, Kaplan, & Yu, 2013; Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017). Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) with a fluorescent probe was used to measure fluorescence. Trolox was used as a standard. The final reaction mixture consisted of 225 μL of 8.16×10^{-8} M FL, 30 μL of the milk thistle seed flour extract or solvent blank or standard, and 25 μL of 0.36 M AAPH. The final

flour extract concentration was 43 mg flour equivalents/mL in the reaction mixture. The fluorescence of the mixture was recorded every 2 min over 2 h at 37 °C. Excitation and emission wavelengths were 485 and 520 nm, respectively. The results were expressed as μmol TE/g dry seed flour sample.

3.3.8. ABTS$^{\bullet+}$ scavenging capacity

Radical scavenging capacities of milk thistle seed flour extract were evaluated against the ABTS$^{\bullet+}$ generated according to a laboratory protocol (Yu, Gao, Li, Wang, Luo, Wang, et al., 2018; Moore, Cheng, Su, & Yu, 2006). ABTS$^{\bullet+}$ working solution was prepared by reacting ABTS with manganese oxide and diluted to an absorbance of 0.700 ± 0.005 at 734 nm. Trolox was used as a standard. The final reaction mixture consisted of 80 μL of the milk thistle seed flour extract or solvent or standard, and 1 mL ABTS$^{\bullet+}$ working solution. The final flour extract concentration was 30 mg flour equivalents/mL in the reaction mixture. After vortexing for 30 s, the absorbance was read at 734 nm after 90 s of reaction. Results were expressed as micromoles of TE/g of flour.

3.3.9. Anti-inflammatory capacity

The evaluation was carried out according to a laboratory protocol (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018; Huang, Fletcher, Niu, Wang, & Yu, 2012). J774A.1 mouse macrophage cells (6×10^5 cells/mL) were cultured in DMEM with 10% FBS and 1% penicillin and streptomycin at 37 °C under 5% CO_2 in six-well plates overnight and reached an 80% confluence. Cells were pre-incubated with milk

thistle seed flour extract at a final concentration of 0.4 mg flour equivalents/mL for 48 h. The medium was changed every 24 h. After 4 h of induction with10 ng/mL lipopolysaccharide (LPS), the culture medium was discarded, and the cells were collected for RNA isolation. cDNA synthesis kit was used to reverse transcribe cDNA and Real-Time PCR was performed on an ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR Master Mix. IL-1β primer was used for inflammatory response and TBP was used for the control. The following amplification parameters were used for PCR: 50 °C for 2 min, 95 °C for 10 min, with 46 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018).

3.3.10. Anti-proliferative capacity

Milk thistle seed flour extract was evaluated for their anti-proliferative capacity using prostate cancer LNCaP cells (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018; Huang, Cheng, Shi, Xin, Wang, & Yu, 2011). LNCaP cells (1×10^4 cells/mL) were cultured at 37 °C under 5% carbon dioxide in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. The milk thistle seed flour extract was mixed with DMSO in the ratio of 1:10. The final flour extract concentration was 0.4 mg flour equivalents/mL in the culture mixture. The final concentration of 1% DMSO was used as a vehicle for the treatment. An ATP-Lite 1 step kit (Perkin–Elmer Life and Analytical Sciences, Shelton, CT) was used to determine cell proliferation (Lv, Huang, Yu, Whent, Niu, Shi, et al., 2012). The emitted luminescence was determined using a Victor^3 plate reader (Perkin–Elmer,

Turku, Finland) immediately prior to treatment and at 0, 24, 48, 72 and 96 h after initial treatment. Treatment media were replaced every 24 h. All experiments were performed in triplicate.

3.3.11. Statistics

PRISM8 software was used for statistical analysis. Means ± standard deviation (SD) were used for each data point. For comparison, a one-way analysis of variation (ANOVA) ($P \leq 0.05$) followed by a post hoc test (Tukey test) was used.

3.4. Results and discussion

3.4.1. Chemical composition of the milk thistle seed flour extract

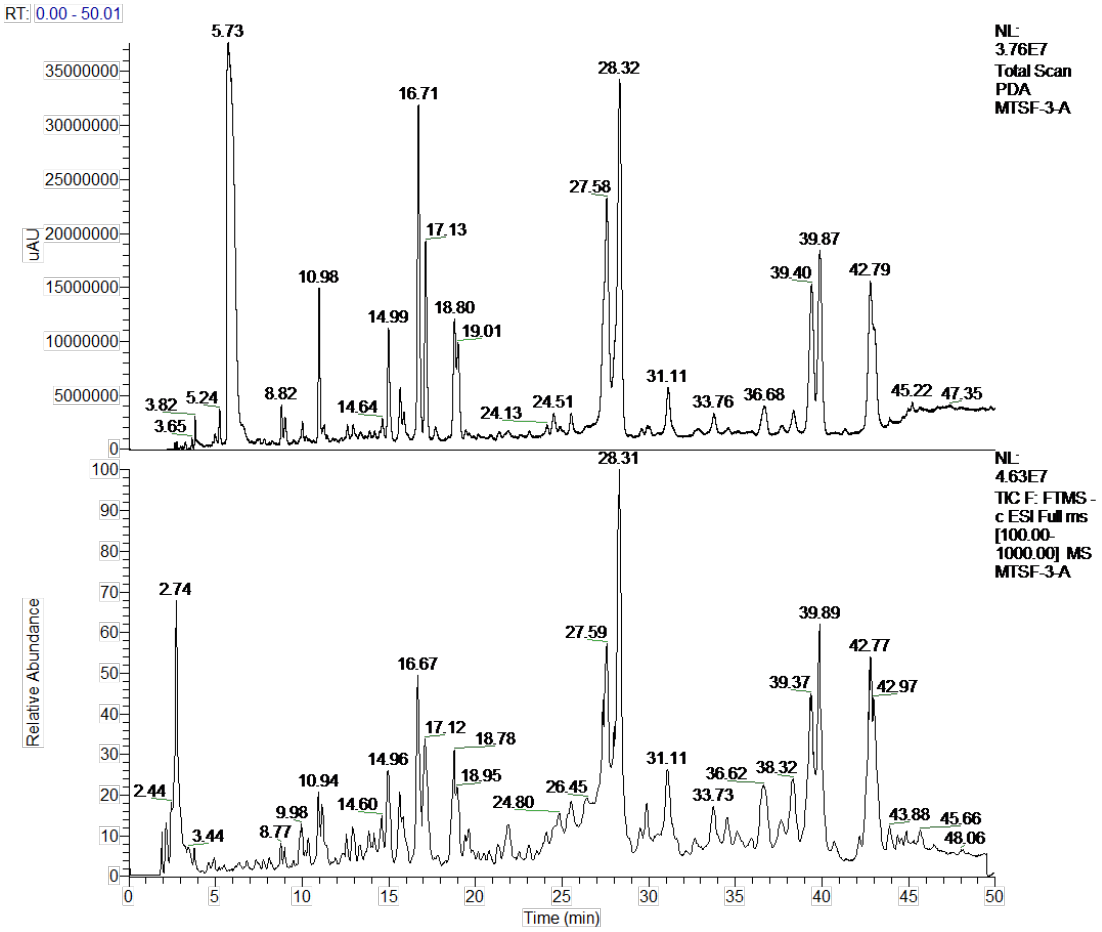

Figure 3.1. Typical UHPLC chromatogram and UHPLC-Obitrap-MS chromatogram of milk thistle seed extract.

In the milk thistle seed flour extract, fifteen compounds including chlorogenic acid and its derivatives, naringin, taxifolin and silybin isomers were detected with silychristin as the primary component (Table 3.1 and Figure 3.1). Among fifteen compounds, 5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomers (peak

IDs 3 and 4) and methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid (peak ID 5) were identified for the first time in milk thistle seeds.

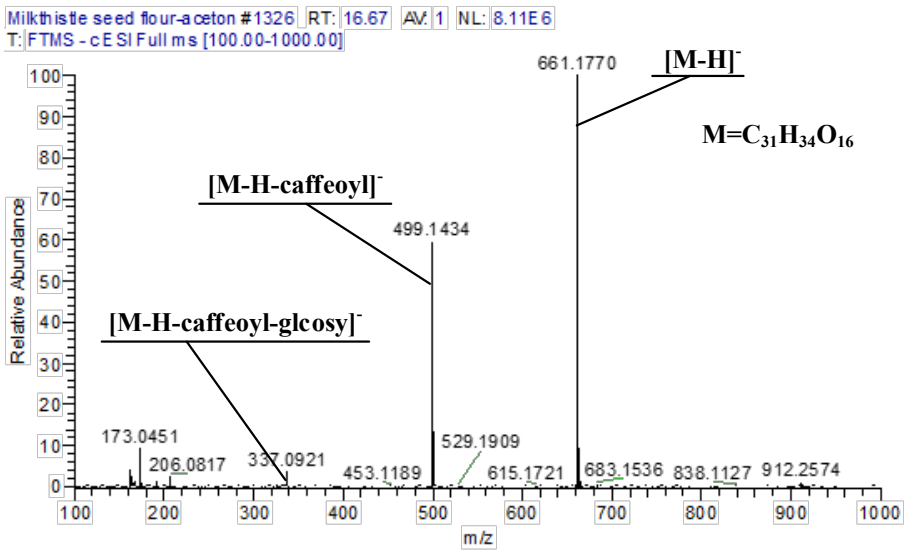

Figure 3.2. Identification of compounds 3 and 4, 5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid, MS spectrum.
The high resolution ESI-MS of 5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid showed the 661.1770 of $[M-H]^-$ corresponding to the formula of $C_{31}H_{34}O_{16}$ (0 ppm).

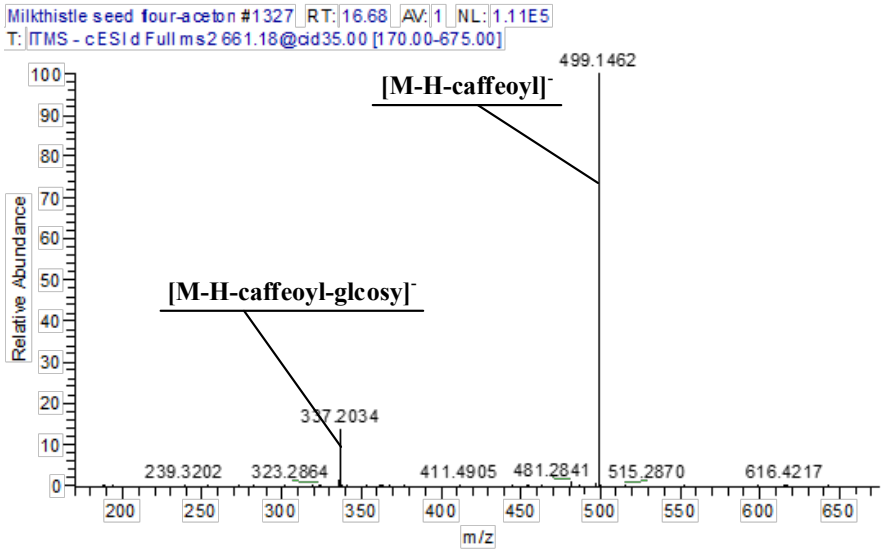

Figure 3.3. Identification of compounds 3 and 4, 5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid, MS^2 spectrum in a negative mode.
The detailed analysis of the fragmental ions of 5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid showed peaks of 499.1462 $[M\text{-}H\text{-caffeoyl}]^-$ ($C_{22}H_{27}O_{13}$) and 337.0721 $[M\text{-}H\text{-caffeoyl-glucosyl}]^-$ ($C_{16}H_{17}O_8$) in a negative mode.

The 5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomers had the same $[M\text{-}H]^-$ of 661.1770, which is corresponding to the formula of $C_{31}H_{34}O_{16}$ (0 ppm). The detailed analysis of the fragmental ions showed peaks of 499.1434 $[M\text{-}H\text{-caffeoyl}]^-$ ($C_{22}H_{27}O_{13}$) and 337.0721 $[M\text{-}H\text{-caffeoyl-glucosyl}]^-$ ($C_{16}H_{17}O_8$) in a negative mode (Figures 3.2 and 3.3), indicating that the skeleton of this compound is 5-p-coumarolyquinic acid, which has been reported by Pereira and others (Pereira, Barros, Carvalho, Santos-Buelga, & Ferreira, 2015).

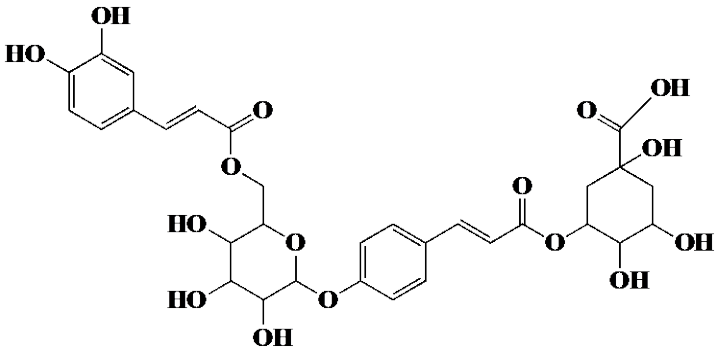

Figure 3.4. Structure of compound 3 or 4, 5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid.

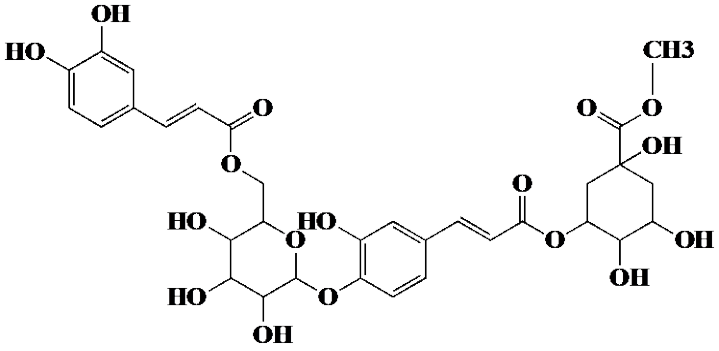

Figure 3.5. Structure of compound 5, methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid.

However, Pereira and others reported the molecular ion of 661 with fragments of 449 and 337 as 5-p-coumarolyquinic acid dihexoside, which, according to the high-resolution ESI-MS, is not correct. Thus, the 5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomers were tentatively identified (Figure 3.4).

Table 3.1. Characterization of compounds present in milk thistle seed flour.

Peak ID	T_R (min)	Theor. $[M-H]^-$	Exptl. $[M-H]^-$	Chemical Formula	Tentative Identification	Concentrationb (μg/g)
1	9.02	353.0873	353.0872	$C_{16}H_{18}O_9$	Chlorogenic acid isomer	101.54 ± 0.76
2	10.98	353.0873	353.0872	$C_{16}H_{18}O_9$	Chlorogenic acid isomer	330.49 ± 1.45
3	14.99	661.1769	661.1782	$C_{31}H_{34}O_{16}$	5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomer[a]	351.43 ± 0.69 CE
4	16.71	661.1769	661.1772	$C_{31}H_{34}O_{16}$	5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomer[a]	729.32 ± 1.02 CE
5	17.13	691.1874	691.1886	$C_{32}H_{36}O_{17}$	Methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid[a]	519.03 ± 3.18 CE
6	18.80	579.1714	579.1705	$C_{27}H_{32}O_{14}$	Naringin	tr
7	19.01	303.0505	303.0498	$C_{15}H_{12}O_7$	Taxifolin	tr
8	27.39	481.1135	481.1130	$C_{25}H_{22}O_{10}$	Silychristin isomer	2609.86 ± 6.16 SE
9	27.58	481.1135	481.1128	$C_{25}H_{22}O_{10}$	Silychristin isomer	2609.86 ± 6.16 SE
10	28.32	481.1135	481.1124	$C_{25}H_{22}O_{10}$	Silychristin isomer	2579.96 ± 5.8 SE
11	33.76	479.0978	479.0986	$C_{25}H_{20}O_{10}$	2,3-Dehydrosilymarin isomer	tr
12	39.40	481.1135	481.1127	$C_{25}H_{22}O_{10}$	Silybin A	907.99 ± 10.33 SE
13	39.87	481.1135	481.1125	$C_{25}H_{22}O_{10}$	Silybin B	1581.96 ± 4.38 SE
14	42.79	481.1135	481.1123	$C_{25}H_{22}O_{10}$	Isosilybin A	1512.01 ± 38.07 SE
15	43.00	481.1135	481.1123	$C_{25}H_{22}O_{10}$	Isosilybin B	1512.01 ± 38.07 SE

[a]The first time reported in milk thistle seeds. T_R stands for the retention time. Theor. $[M-H]^-$ and Exptl. $[M-H]^-$ were theoretical and experimental m/z of molecular ions, respectively. CE and SE stand for a chlorogenic acid and a silybin equivalent, respectively. The tr stands for trace.

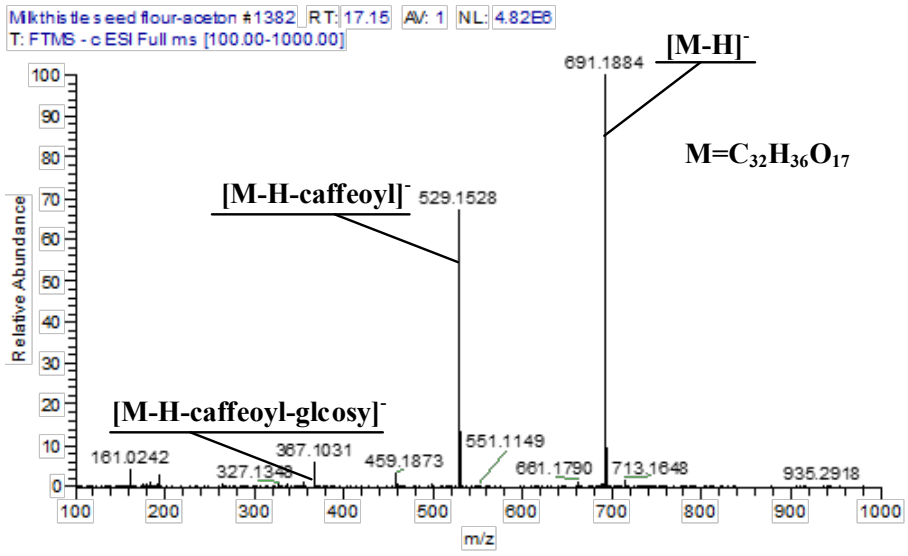

Figure 3.6. Identification of compound 5, Methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid, MS spectrum.
The high resolution ESI-MS of Methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid showed the 691.1884 of 27 $[M-H]^-$ corresponding to the formula of $C_{32}H_{36}O_{17}$ (0 ppm).

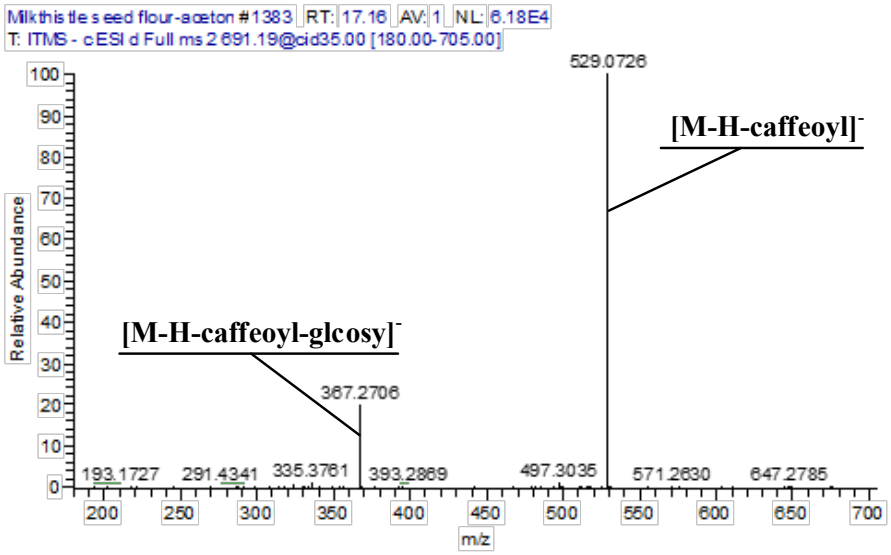

Figure 3.7. Identification of compound 5, Methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid, MS^2 spectrum in a negative mode.
The detailed analysis of the fragmental ions of Methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid showed peaks of 529.1528 $[M\text{-}H\text{-caffeoyl}]^-$ ($C_{23}H_{29}O_{14}$) and 367.1031 $[M\text{-}H\text{-caffeoyl-glucosyl}]^-$ ($C_{17}H_{19}O_9$) in a negative mode.

Similarly, the high-resolution ESI-MS of methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid showed the m/z 691.1884 of $[M\text{-}H]^-$ corresponding to the formula of $C_{32}H_{36}O_{17}$ (0 ppm). The detailed analysis of the fragmental ions of methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid showed peaks of 529.1528 $[M\text{-}H\text{-caffeoyl}]^-$ ($C_{23}H_{29}O_{14}$) and 367.1031 $[M\text{-}H\text{-caffeoyl-glucosyl}]^-$ ($C_{17}H_{19}O_9$) in a negative mode (Figures 3.6 and 3.7), and these fragmental ions were similar to that of methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid reported by Wu and others in the eggplant genus *Solanum* (Solanaceae) (Wu, Meyer, Whitaker, Litt, & Kennelly, 2013). Thus, this compound was tentatively determined

as methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid (Figure 3.5), which is reported in milk thistle seed for the first time.

In addition, the current study measured the concentrations of the selected compounds found in milk thistle seeds (Table 3.1). The concentration of the primary compound, silychristin isomer was 2580–2610 µg silybin equivalent/g. The other major compounds were silybin B, isosilybin A & B, and silybin A, at levels of 1582, 1512 and 908 µg silybin equivalent/g, respectively (Table 3.1). In 2003, Barreto and others found silychristin, silybin A, silybin B and taxifolin in the milk thistle seed flour extracted with water at 100 °C. The concentration of silychristin, silybin B, silybin A and taxifolin found in the water extract of milk thistle seed flour were 5.0, 3.3, 1.8 and 1.2 mg/g of seed, respectively (Barreto, Wallace, Carrier, & Clausen, 2003). In 2005, Wallace and others reported silymarin content of an ethanolic extract of milk thistle seed flours. The primary component in the ethanol extract of the milk thistle seed flour was silybin B at a level of 6.86 mg/g of seed and followed by silybin A (4.04 mg/g of seed), silychristin (3.89 mg/g of seed) and taxifolin (0.62 mg/g of seed) (Wallace, Carrier, & Clausen, 2005). The results from the current study were in agreement to Barreto and others that silychristin was the major compound found in milk thistle seed extract. The different silymarin compositions in these studies may be explained by the plant cultivar, and different plant growing and extraction conditions.

Interestingly, the current study found significant concentrations of non-silymarin polyphenols such as chlorogenic acid and its derivatives (Table 3.1). Among chlorogenic acid and its derivatives, 5-p-(6-caffeoyl-glucopyranosyl)-

coumaroylquinic acid (Peak ID 4) had the greatest concentration of 729 µg chlorogenic acid equivalent/g followed by methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid at 519 µg chlorogenic acid equivalent/g, 5-p-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomer at 351 µg chlorogenic acid equivalent/g and chlorogenic acid isomers at 102 and 330 µg/g, respectively (Table 3.1). Previously, Lucini and others found chlorogenic acid in milk thistle seeds. They examined 15 cultivars and reported the highest concentration of 395 µg/g for chlorogenic acid (Lucini, Kane, Pellizzoni, Ferrari, Trevisi, Ruzickova, et al., 2016). This concentration is comparable to the chlorogenic acid concentration of 330 µg/g in this study (Table 3.1).

3.4.2. Potential effects of the milk thistle seed flour extract on gut microbiota

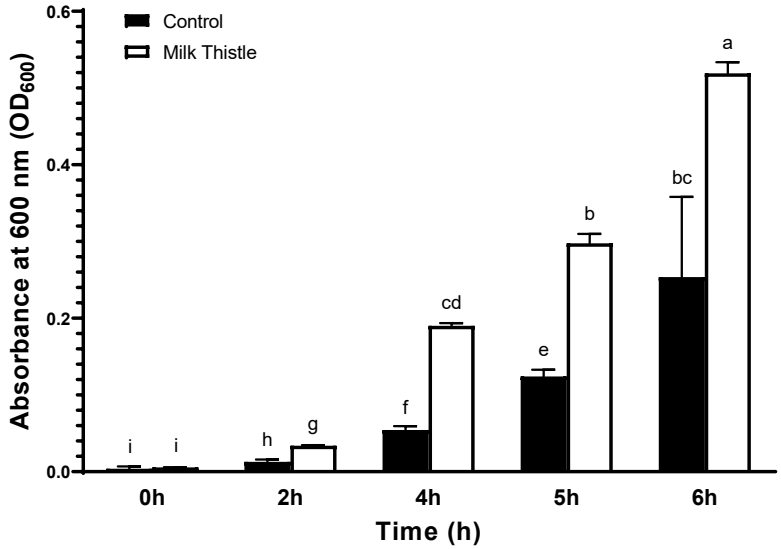

Figure 3.8. Effects of milk thistle seed flour extract on gut bacterial growth. Milk thistle stands for milk thistle seed flour extract. Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

In this study, milk thistle seed flour extract was able to enhance gut bacterial growth in a time-dependent manner (Figure 3.8). This is the first time that milk thistle seed flour extract was shown to influence gut bacterial communities *in vitro*. The presence of chlorogenic acid, chlorogenic acid derivatives and silymarin in the milk thistle seed flour extract (Table 3.1) might partially explain its gut bacterial enhancing effect as gut bacteria has been reported capable of utilizing these compounds (Tomas-Barberan, García-Villalba, Quartieri, Raimondi, Amaretti, Leonardi, et al., 2013; Shen & Ji, 2016).

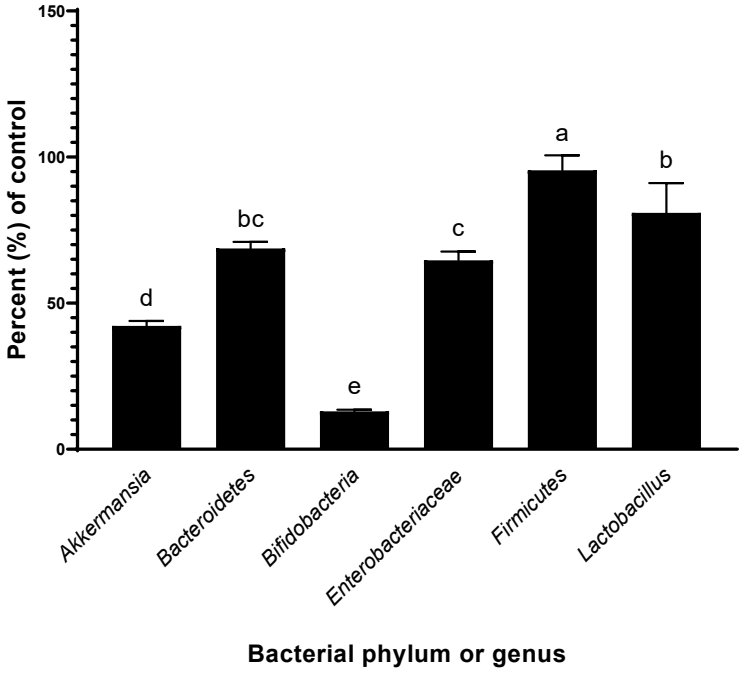

Figure 3.9. Effects of milk thistle seed flour extract on the relative abundance of specific phylum or genus.
Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

The milk thistle seed flour extract also was able to modulate specific bacterial phylum under the experimental conditions. The relative abundance of *Bacteroidetes* phylum was reduced by the milk thistle seed flour extract (Figure 3.9). On the other hand, milk thistle flour extract had no effect on *Firmicutes* phylum (Figure 3.9). Usually, the ratio between *Bacteroidetes* and *Firmicutes* phyla is known to may be correlated to obesity risk. Previous studies suggest that a decrease in *Bacteroidetes* and an increase in *Firmicutes* are observed in obese people (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005; Ley, Turnbaugh, Klein, & Gordon, 2006; Turnbaugh, Ley, Mahowald, Magrini, Mardis, & Gordon, 2006). The result from the current study suggests that milk thistle seed flour may not be used for body weight control.

In addition to *Bacteroidetes* and *Firmicutes* phyla, the effects of milk thistle seed flour extract on four genera including *Akkermansia*, *Bifidobacteria*, *Lactobacillus*, and *Enterobacteriaceae* have been evaluated. Milk thistle flour extract resulted in more than 50% reduction of *Akkermansia* compared to the control (Figure 4). *Akkermansia* abundance has been negatively associated with a metabolic disorder in many clinical and preclinical studies (Derrien, Belzer, & Vos, 2017). It has also been reported that *Akkermansia* protected mice from diet-induced obesity when administered alive. A decrease in *Akkermansia* was associated with an increased risk of obesity and type 2 diabetes (Yassour, Vatanen, Siljander, Hämäläinen, Härkönen, Ryhänen, et al., 2016). On the other hand, a very high level of *Akkermansia* has been associated with multiple sclerosis (Jangi, Gandhi, Cox, Li, Glehn, Yan, et al., 2016).

The result from the current study suggests that milk thistle seed flour components may be used for people with multiple sclerosis or at higher risk of the disorder accompanied by a very high *Akkermansia* level.

Both *Bifidobacteria* and *Lactobacillus* genera are probiotics. Probiotics are health beneficial bacteria and their benefits may include reducing gastrointestinal discomfort, improving immune health, relieving constipation, and avoiding the common cold (Rijkers, Vos, Brummer, Morelli, Corthier, & Marteau, 2011). In the current study, the milk thistle seed flour extract reduced the relative abundance of *Bifidobacteria* and had no effect on *the Lactobacillus* genus (Figure 3.9). It has been reported that extracts rich in polyphenolics commonly showed growth inhibition of probiotic bacterial strains. For example, Tabasco and others tested grape seed extracts on the growth of several lactic acid bacteria and *bifidobacteria* and found growth inhibition by the extracts (Tabasco, Sánchez-Patán, Monagas, Bartolomé, Moreno-Arribas, Peláez, et al., 2011). In addition, the previous study from our lab found that carrot, cucumber, and broccoli seed flour extracts reduced relative abundance of *Bifidobacteria* and *Lactobacillus* genera (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018), depending on the polyphenol composition (Tabasco, Sánchez-Patán, Monagas, Bartolomé, Moreno-Arribas, Peláez, et al., 2011). Since milk thistle seed flour extract contains different phenolics (Table 3.1), additional research is needed to further investigate whether and how each of the phenolic compounds may contribute to the overall changes of *Bifidobacteria* and *Lactobacillus* contents. In addition, it needs to be pointed out that the milk thistle seed

flour extract contained no fibers, which is a major food component known to enhance probiotic bacteria such as *Bifidobacteria* and *Lactobacillus*. Therefore, to evaluate the effects of the milk thistle seed flour requires additional *in vivo* studies using the whole flour.

Enterobacteriaceae are a gram-negative and emerging opportunistic pathogen and associated with rare but life-threatening cases of meningitis, necrotizing enterocolitis, and sepsis in premature and full-term infants (Drudy, Mullane, Quinn, Wall, & Fanning, 2006). In this study, milk thistle seed flour extract significantly reduced the relative abundance of *Enterobacteriaceae* genus (Figure 3.9). Milk thistle has been used from ancient times as herbal medicine due to its anti-microbial activity. The anti-microbial activity is believed from silymarin (Hao, 2015; Mojgan & Roya, 2016; Saa, 2017).

In 2007, the human microbiome project was launched to characterize the influence of the microbiota on the human body and to correlate changes in these microbial populations with human health. Another project called the Irish ELDERMET Project (2007–2013) focused on the characterization of the fecal microbiota associated with aging and aimed to correlate the composition, diversity and metabolic potential of the fecal microbial metagenome with health, diet, and lifestyle (Duda-Chodak, Tarko, Satora, & Sroka, 2015). Today, many studies focusing on the interaction between diet and gut microbiota suggest that dietary interventions can alter gut microbial communities in a positive way in humans (Singh, Chang, Yan, Lee, Ucmak, Wong, et al., 2017; David, Maurice, Carmody,

Gootenberg, Button, Wolfe, et al., 2013; Graf, Cagno, Fåk, Flint, Nyman, Saarela, et al., 2015; Maslowski & Mackay, 2010). On the other hand, intestinal dysbiosis, a loss of beneficial microbial organisms and diversity, is associated with diseases such as inflammatory bowel disease, irritable bowel syndrome (IBS), celiac disease, asthma, metabolic syndrome, cardiovascular disease, and obesity (Carding, Verbeke, Vipond, Corfe, & Owen, 2015). Therefore, the role of foods and their components that possibly interact with gut microbiota has been noticeable. Usually, polyphenolic compounds in foods are known to interact with gut bacterial communities (Moco, Martin, & Rezzi, 2012). Taking together, milk thistle seed flour rich in dietary phenolics was able to interact with gut bacterial communities *in vitro* and warrants future *in vivo* study to confirm health beneficial potentials of milk thistle seed flour through modulating gut microbiota.

3.4.3. Free radical scavenging capacities

In a human body, excessive free radicals often generate oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA) (Willcox, Ash, & Catignani, 2004). Therefore, scavenging free radicals through dietary intervention is important for reducing the risk of oxidative stress-induced human chronic diseases.

Relative DPPH radical scavenging capacity (RDSC) assay has been accepted for its capacity to directly compare water soluble and oil soluble

antioxidants with desirable accuracy, precision, and reproducibility (Cheng, Moore, & Yu, 2006).

Table 3.2. Free radical scavenging capacities of cold-pressed milk thistle seed flour[a]

	RDSC	ORAC	HOSC	ABTS
	(μmol TE/g)			
Milk thistle	48.61 ± 6.47	633.57 ± 267.17	10420.28 ± 607.58	116.16 ± 16.81

[a]Milk thistle stands for milk thistle seed flour extract. RDSC, ORAC, HOSC, and ABTS stand for relative DPPH scavenging capacity, oxygen radical absorbing capacity, hydroxyl radical scavenging capacity and ABTS radical scavenging capacity. TE stands for Trolox equivalent.

The milk thistle seed flour extract showed an RDSC value of 49 μmol TE/g (Table 3.2). This RDSC value is comparable to that of 61 μmol TE/g for milk thistle flour seed extract reported by Parry and others in 2008 using the same extraction solvent (Parry, Cheng, Moore, & Yu, 2008). Also, this value is comparable to RDSC values of vegetable seed flours such as carrot (16 μmol TE/g) and broccoli (85 μmol TE/g) (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018).

The hydroxyl radical is one of the reactive oxygen species found in biological systems. The hydroxyl radical can attack DNA, membrane lipids, and proteins to induce the development of many health problems such as cancer, atherosclerosis, and autoimmune disorders (Lobo, Patil, Phatak, & Chandra, 2010). The milk thistle seed flour extract had an HOSC value of 10420 μmol TE/g (Table 3.2). The HOSC value for milk thistle seed flour extract is much greater than that of 893 μmol TE/g in the seeds reported by Parry and others (Parry, Cheng, Moore, & Yu, 2008).

The ORAC assay measures antioxidants' scavenging capacity against the peroxyl radical. Similar to hydroxyl radical, peroxyl radical is a physiologically relevant free radical (Parry, Cheng, Moore, & Yu, 2008). The milk thistle seed flour extract showed an ORAC value of 634 μmol TE/g. This value is lower than that of 1131 μmol TE/g for milk thistle seed flour extract reported by Parry and others (Parry, Cheng, Moore, & Yu, 2008).

The milk thistle seed flour extract had an ABTS value of 116 μmol TE/g (Table 3.2). This is the first report of the ABTS value for milk thistle seed flour extract. In 2002, Yu and others reported that three varieties of wheat (Arkon, Trego, and Platte) extracts using 100% ethanol had ABTS values of 1.31, 1.08 and 1.91 μmol TE/g, respectively (Yu, Haley, Perret, & Harris, 2002). Compared to the wheat extracts, milk thistle seed flour extract showed a significantly greater ABTS radical scavenging capacity.

3.4.4. Anti-inflammatory capacity

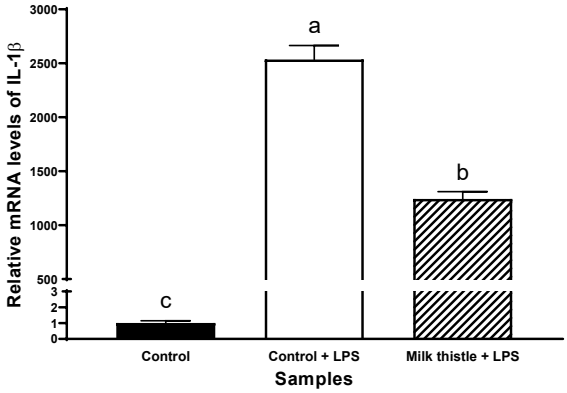

Figure 3.10. Effects of milk thistle seed flour extract on the interleukin 1 beta (IL-1β) levels in J774A.1 mouse macrophage cells.
LPS stands for lipopolysaccharide and milk thistle stands for milk thistle seed flour extract. Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

In this study, milk thistle seed flour extract at an initial concentration of 0.4 mg flour equivalents/mL suppressed mRNA expression of pro-inflammatory cytokine IL-1β in the LPS stimulated J774A.1 mouse macrophages (Figure 3.10). The concentration of milk thistle seed flour extract was chosen as no cell number reduction was observed for this concentration treatment in a preliminary cell cytotoxicity evaluation (data not shown). Milk thistle seed is known for its anti-inflammatory capacity (Ashkavand, Malekinejad, Amniattalab, Rezaei-Golmisheh, & Vishwanath, 2012). This effect is considered mainly from polyphenolic compounds such as chlorogenic acid and silymarin. Inflammatory cells are known to produce pro-inflammatory cytokines at the site of inflammation. During the process, excessive pro-inflammatory cytokines production can cause oxidative stress and result in the

development of chronic diseases. Therefore, inhibiting pro-inflammatory cytokines has been an approach for reducing the risk of inflammation-induced chronic diseases. Several studies have reported the anti-inflammatory capacity of milk thistle seeds in osteoarthritis (Siegel & Stebbing, 2013; Ashkavand, Malekinejad, Amniattalab, Rezaei-Golmisheh, & Vishwanath, 2012; Hussain, Jassim, Numan, Al-Khalifa, & Abdullah, 2009). Osteoarthritis is a type of joint disease with a degradative condition of the articular cartilage. During the development of osteoarthritis, immune cells including T cells, B cells, and macrophages infiltrate the joint tissues and release cytokines and chemokines, and damage articular cartilage (Haseeb & Haqqi, 2013). In 2012, Ashkavand and others have shown that silymarin has anti-inflammatory effects on osteoarthritis in rats. Silymarin was also found to reduce IL-1β level in the blood serum (Ashkavand, Malekinejad, Amniattalab, Rezaei-Golmisheh, & Vishwanath, 2012).

In addition, silymarin has shown anti-inflammatory effect against lipopolysaccharide (LPS) induced sepsis in mice model (Kang, Jeon, Park, Yang, & Kim, 2004). Kang and others found that silymarin improved the survival rate of LPS treated mice from 6 to 38 %. They also investigated mechanism responsible for anti-inflammatory effect in sepsis and found that silymarin had an inhibitory effect on IL-1β and prostaglandin E2 production in mouse peritoneal and RAW 264.7 macrophages in a dose-dependent manner (Kang, Jeon, Park, Yang, & Kim, 2004). Moreover, silymarin suppressed LPS stimulated IL-1β and COX-2 mRNA expressions in a dose-dependent manner in RAW 264.7 macrophage.

Taking together, milk thistle seed flour components may reduce the risk of IL-1β mediated inflammation and related chronic diseases such as osteoarthritis and sepsis.

3.4.5. Anti-proliferative capacity

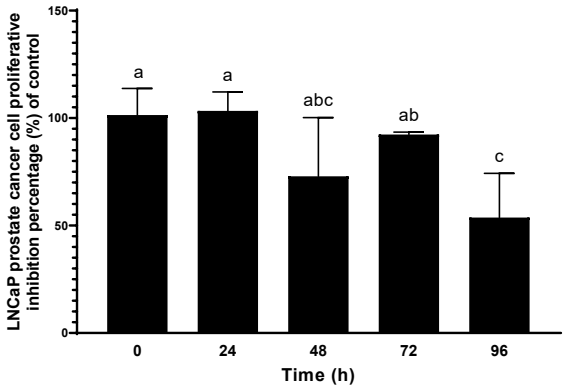

Figure 3.11. Anti-proliferative capacity of milk thistle seed flour extract in LNCaP prostate cancer cells.
Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

The milk thistle seed flour extract at an initial concentration of 0.4 mg flour equivalents/mL significantly inhibited LNCaP cell growth after 96 h treatment (Figure 3.11). This observation was consistent with findings in previous studies that silymarin compounds in milk thistle seeds had anti-proliferative activities in LNCaP, PC3, and DU145 human prostate carcinoma cell lines (Davis-Searles, Nakanishi, Kim, Graf, Oberlies, Wani, et al., 2005; Ramasamy & Agarwal, 2008). Silymarin was reported to induce G1 and/or G2-M arrests in the LNCaP, PC3 and DU145 human prostate carcinoma cells (Ramasamy & Agarwal, 2008). In the last decade,

throughout *in vitro* and *in vivo* studies, understanding of how compounds from milk thistle seeds affected cell cycle and signaling to arrest cancer cell growth has been improved. For examples, Davis-Searles and others compared anti-proliferative effects of four silymarin compounds in prostate carcinoma cell lines ((Davis-Searles, Nakanishi, Kim, Graf, Oberlies, Wani, et al., 2005). They found that isosilybin B had the most significant anti-proliferative capacity in the PC3 human prostate carcinoma cells followed by isosilybin A, silybin A and silybin B. Also, isosilybin B showed the most significant anti-proliferative capacity in the LNCaP human prostate carcinoma cells (Davis-Searles, Nakanishi, Kim, Graf, Oberlies, Wani, et al., 2005). In 2008, Deep and others compared the isosilibinin (a 50:50 mixture of isosilybin A and isosilybin B) with silymarin and silibinin (a 50:50 mixture of silybin A and silybin B) using a mouse xenograft (DU145 human prostate carcinoma cell) model (Deep, Raina, Singh, Oberlies, Kroll, & Agarwal, 2008).

In addition, silymarin has shown anti-proliferative activities in HepG2 (human hepatoblastoma-derived cell line) and U937 (human histiocytic lymphoma) cells (Manna, Mukhopadhyay, Van, & Aggarwal, 1999; Saliou, Rihn, Cillard, Okamoto, & Packer, 1998). Previously, it has been reported that in both HepG2 and U937 cell lines, NF-κB/Rel was involved in the proliferation. Later, Kang and others investigated how silymarin affected the DNA binding activity of NF-κB/Rel. They found that silymarin significantly suppressed LPS induced NF-κB/Rel DNA binding in a concentration dependent manner (Kang, Jeon, Park, Yang, & Kim, 2004).

In summary, the results showed that isosilibinin, silymarin and silibinin were able to significantly suppress cancer cell proliferation under the experimental conditions, suggesting the potential use of milk thistle seed flour in reducing the risk of carcinogenesis.

3.5. Conclusions

The current study found several possible health beneficial polyphenolic compounds in the milk thistle seed flour extract. For the first time, the present study provided evidence that milk thistle seed flour components could interact with gut microbiota, and warrant further research to investigate how the interaction may contribute to human health. In addition, the present study evaluated milk thistle seed flour extract for its free radical scavenging, anti-inflammatory, and anti-proliferative capacities. The results might be used to promote the value-added utilization of milk thistle seed flour in improving human health.

Chapter 4: Chemical composition of cold-pressed blackberry seed flour extract and its potential health beneficial properties

Choe et al., *Food Sci. Nutr.* **2020**, *8*, 1215–1225.

4.1. Abstract

The blackberry seed flour was cold-extracted using 50% acetone and examined for its phytochemical composition and health beneficial properties including *in vitro* gut microbiota modulatory, free radical scavenging, anti-inflammatory, and anti-proliferative capacities. Among identified thirteen components of blackberry seed flour extract through UHPLC-MS analysis, sanguiin H6 was the primary component and followed by ellagic acid and pedunculagin. For health beneficial properties, the blackberry seed flour extract increased the total number of gut bacteria and shifted the abundance of specific bacterial phylum, family or genus. The extract had RDSC, ORAC, HOSC, and $ABTS^{\bullet+}$ scavenging capacities of 362, 304, 2531, and 267 μmol Trolox equivalents (TE)/g, respectively. In addition, the blackberry seed flour extract showed capacities for anti-inflammation and anti-proliferation by suppressing LPS induced IL-1β mRNA expressions in the cultured J774A.1 mouse macrophages and the proliferation of LNCaP prostate cancer cells. The results suggest potential health benefits and further utilization of blackberry seed flour as functional foods.

4.2. Introduction

Blackberries contain many large seeds that are not preferred by consumers. Food industry typically removes seeds when processing blackberries (Bushman, Phillips, Isbell, Ou, Crane, & Knapp, 2004). Blackberry seeds are used to produce the seed oil, and the blackberry seed flour is a by-product from oil processing. Studying the health-promoting components and properties of the blackberry seed flour can lead to its potential utilization in nutraceuticals and functional foods and create additional value to blackberry producers and related industries while reducing environmental contaminations.

Previously, studies have demonstrated that blackberry seed flour is rich in polyphenolic compounds such as sanguiin H6 and ellagic acid (Hager, Howard, Liyanage, Lay, & Prior, 2008), and has potential free radical scavenging capacity (Bushman, Phillips, Isbell, Ou, Crane, & Knapp, 2004). In 2004, Siriwoharn and Wrolstad showed that the 70% acetone (v/v) extracts of both Marion (*Rubus* sp. hyb) and Evergreen (*Rubus laciniatus*) blackberry seeds had significantly greater free radical scavenging capacities compared to whole blackberry or blackberry without seeds (Siriwoharn & Wrolstad, 2004). Also, a few studies have shown anti-inflammatory capacities of polyphenolic compounds found in blackberry seed flours such as ellagitannins and ellagic acid. It has been reported that these compounds inhibited pro-inflammatory biomarkers such as IL-1, IL-6, and TNF-α (Landete, 2011). In addition, polyphenolic compounds may play an important role in human health by interacting with gut microbiota (Ozdal, Sela, Xiao, Boyacioglu, Chen, &

Capanoglu, 2016). For example, ellagitannins and ellagic acid found in walnut, strawberry, raspberry, and blackberry are metabolized by gut microbiota (Ozdal, Sela, Xiao, Boyacioglu, Chen, & Capanoglu, 2016). Interestingly, a recent study isolated specific bacterial species that is involved in ellagitannins and ellagic acid metabolism (Selma, Beltrán, Luna, Romo-Vaquero, García-Villalba, Mira, et al., 2017). However, up to date, the influence of blackberry seed flour components on gut microbiota profile has not been evaluated.

In the current study, the chemical composition of blackberry seed flour has been examined. Its potential health-promoting effects including gut microbiota profile modulatory, free radical scavenging, anti-inflammatory and anti-proliferative capacities have also been evaluated.

4.3. Materials and methods

4.3.1. Materials and chemicals

Blackberry seed flour was gifted from the Botanic Innovations (Spooner, WI, USA). 2,2′-Azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA, USA). Precellys lysing kits were purchased from Bertin Technologies (Rockville, MD, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 10 ml Pyrex screw-cap tubes, 37% Hydrochloric acid (HCl), 10 M sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), and ellagic acid were purchased from Sigma Aldrich (Saint-Louis, MO, USA). Lysogeny broth (LB) was purchased from Quality Biological™ (Gaithersburg,

MD, USA). Iron (III) chloride, fluorescein (FL), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), DEPC-Treated water and nuclease-free water, and 30% ACS grade hydrogen peroxide were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). cDNA Synthesis kit was purchased from Agilent Technologies (Savage, MD, USA). J774A.1 and LNCaP cells were purchased from American Type Culture Collection (Manassas, VA, USA). DMEM, RPMI1640, FBS, penicillin and streptomycin were purchased from GIBCO (Grand Island, NY, USA). QIAamp DNA Mini Kit was purchased from Qiagen (Gaithersburg, MD, USA). TRIzol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). TaqMan Fast Universal PCR Master Mix, TATA-binding protein (TBP) and interleukin-1beta (IL-1β) primers, and SYBR®Green Real-Time PCR Master Mix were purchased from Applied Biosystems (Carlsbad, CA, USA).

4.3.2. Preparation of blackberry seed flour extract

Blackberry seed flour (10 g) was cold-extracted using 25 ml of 50% acetone at room temperature. Sonication was applied for 1 min and extraction was performed three times (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018). The extract's concentration was 0.4 g flour equivalents/mL. All experiments were performed in triplicate.

4.3.3. Ultra High-Performance liquid chromatography photo diode array high-resolution multi-stage mass spectrometry (UHPLC-PDA-ESI/HRMS^n)

UHPLC-HRMS analysis was carried out as previously reported (Choe, Li, Gao, Yu, Wang, Sun, et al., 2019) with an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) and Agilent 1290 infinity liquid chromatography system equipped with a DAD detector. The UV-vis spectrum scanning range was 190 to 600 nm. Luna C18 column with 4.6 mm × 250 mm and 5 μm particle size was used. A formic acid (0.1% v/v) added HPLC grade water was used as solvent A, and a formic acid (0.1% v/v) added acetonitrile was used as solvent B. The elution was 5% of solvent B at the beginning and increased linearly to 13% B at 5 min; increased to 20% B at 10 min; increased to 27% B at 25 min; increased to 33% B at 40 min; increased to 50% B at 45 min; increased to 90% B at 46 min; keeping 90% B until 51 min; the re-equilibration post-run time was 10 min. The oven temperature was 40 °C and an injection volume was 5 μL with a flow rate of 1 ml/min. The HRMS was conducted in a negative ionization mode with the optimized parameters as follows: the spray voltage at 4.5 kV, the capillary temperature at 325 °C, the capillary voltage at −50 V, and the tube lens offset voltage at −120 V. The mass range was m/z 100–1000 with a resolution of 30 000. Data were post-processed using the QualBrowser part of the Thermo Scientific Xcalibur 2.2 software. For quantitative analysis, 3.34 ml of blackberry seed flour extract was added to 1.66 ml of 37% HCl in 10 ml Pyrex tube, vortexed for 1 min and kept at 90 °C for 24 h. The resulted hydrolyte solution was cooled down to ambient temperature,

and the pH was adjusted to 2.5 using 5 and 10 M NaOH (García-Villalba, Espín, Aaby, Alasalvar, Heinonen, Jacobs, et al., 2015). HPLC grade ellagic acid was used to generate the standard curve and the standard curve was used to quantify the compounds including pedunculagin, sanguiin H6, and ellagic acid derivatives.

4.3.4. Bacterial incubation and microbiota analysis

Gut bacteria were extracted from a chow diet fed C57BL/6J mouse's fecal and cultured in LB following a previously described laboratory protocol (Choe, Li, Gao, Yu, Wang, Sun, et al., 2019). Bacterial culture and seeding concentration were calculated using wavelength of 600 nm and an OD_{600} value of $1 = 8 \times 10^8$ cells/mL. Using 15 ml tubes and 96 well plates, 1×10^7 cells/mL of bacterial cells were cultured with and without the extract in M9 minimal broth and incubated in a shaker incubator at 37 °C for 6 h. The concentration of flour extract used in the culture medium was 0.4 g flour equivalents/mL. OD_{600} values were measured at 0, 2, 4, 5 and 6 h. After 6 h, bacterial cells were centrifuged at 5000 rpm for 5 min for collection. Precellys lysing and QIAamp DNA mini kits were used to extract the bacterial DNA. Real-time PCR was performed as reported by a laboratory protocol (Choe, Li, Gao, Yu, Wang, Sun, et al., 2019). To determine the relative abundance, *Bacteroidetes* and *Firmicutes* phyla, *Enterobacteriaceae family, Akkermansia*, *Bifidobacterium*, and *Lactobacillus* genera primers were used.

4.3.5. Relative DPPH radical scavenging capacity (RDSC)

The DPPH radical scavenging capacity was evaluated according to the laboratory procedure (Cheng, Moore, & Yu, 2006), using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). DPPH radical solution was prepared in 50% acetone. Trolox was used as the standard. The final reaction mixture contained 0.1 ml of the blackberry seed flour extract, Trolox standard or 50% acetone (the control), and 0.1 ml 0.2 mM DPPH solution. The absorbance was read at 515 nm every minute for 40 min. The relative radical scavenging capacity (RDSC) was calculated from the area under the curve and reported in micromoles of Trolox equivalents/g of dry flour (μmol TE/g).

4.3.6. Hydroxyl radical (HO•) scavenging capacity (HOSC)

A fluorescent probe equipped Victor^3 plate reader (PerkinElmer, Turku, Finland) was used to perform the HOSC assay previously described (Moore, Yin, & Yu, 2006). For a standard, Trolox was used. Fluorescein (170 μL of 9.28×10^{-8} M), 30 μL of the solvent, standards, or sample, 60 μL of $FeCl_3$, and 40 μL of 0.1990 M of H_2O_2 were used as an assay mixture. The concentration of the extract was 40 mg flour equivalents/mL in the reaction mixture. The fluorescence was recorded every 2 min over 4 h. For excitation and emission, the wavelengths of 485 and 520 nm were used. The HOSC was quantified using the area under the curve and expressed relative to Trolox as μmol TE/g of the dry flour samples.

4.3.7. Oxygen radical absorbing capacity (ORAC)

The oxygen radical absorbing capacity (ORAC) value was determined following a previously reported laboratory procedure (Cheng, Su, Moore, Zhou, Luther, Yin, et al., 2006), with fluorescein (FL) as the fluorescent probe. Trolox standards were prepared in 50% acetone and other reagents were prepared in 75 mM pH 7.4 phosphate buffer. In the initial reaction, 225 μL of 8.16×10^{-8} M FL was combined with 30 μL of sample, standard, or solvent in a 96-well plate. The plate was heated at 37 °C for 20 min in a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). Twenty-five microliter of 0.36 M AAPH was added to each well and the fluorescence of the mixture was recorded every 2 min over 2 h at 37 °C. The blackberry seed flour extract's initial concentration was 43 mg flour equivalents/mL in the reaction mixture. For excitation, 485 nm wavelength was used and for emission, 535 nm was used. The ORAC value was reported as μmol TE/g of the dry flour samples.

4.3.8. $ABTS^{\bullet+}$ scavenging capacity

The ABTS radical scavenging capacity of the blackberry seed flour extract was assessed based on a previously reported laboratory protocol (Moore, Cheng, Su, & Yu, 2006). ABTS with manganese oxide were used for $ABTS^{\bullet+}$ working solution preparation. The prepared $ABTS^{\bullet+}$ working solution's absorbance was measured at 734 nm and adjusted to 0.700 ± 0.005. For a standard, Trolox was used. The reaction was carried with 1 ml $ABTS^{\bullet+}$ working solution, and 80 μL of the blackberry seed flour extract or standard

or solvent. The initial concentration of blackberry seed flour extract was 30 mg flour equivalents/mL in the reaction mixture. The reaction mixture was vortexed for 30 s and absorbance value was recorded at 734 nm after 90 s of the reaction. The ABTS value was reported as μmol TE/g of the dry flour samples.

4.3.9. Anti-inflammatory capacity

Anti-inflammatory capacity was examined based on the laboratory procedure (Whent, Slavin, Kenworthy, & Yu, 2009). The density of 6×10^5 cells/mL J774A.1 mouse macrophages were cultured to reach 80% confluency in DMEM with 10% FBS and 1% penicillin and streptomycin at 37 °C under 5% CO_2 in six well plates. Then, macrophages were incubated with the blackberry seed flour extract concentration of 0.4 mg flour equivalents/mL for 48 h. In every 24 h, the medium was changed. For inflammatory response, 10 ng/m lipopolysaccharide (LPS) was delivered to the medium and incubated for 4 h. After 4h, RNA was isolated from the macrophages and cDNA was synthesized using the cDNA synthesis kit. Real-time PCR was performed using ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR Master Mix. For primers, TBP was used as a control and an IL-1β as an inflammatory response. The PCR amplification parameters are as follow: 46 amplification cycles, 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min (Whent, Slavin, Kenworthy, & Yu, 2009).

4.3.10. Anti-proliferative capacity

The anti-proliferative capacity was evaluated using the earlier laboratory procedure (Whent, Slavin, Kenworthy, & Yu, 2009). The density of 1×10^4 cells/mL

LNCaP prostate cancer cells were cultured with blackberry seed flour extract (0.4 mg flour equivalents/mL). In response to the blackberry seed flour extract delivery, the extract was dissolved in DMSO (1:10 v/v). In a final mixture, the vehicle, 1% DMSO, was delivered for the treatment. For cell proliferation calculation, An ATP-Lite 1 step kit (Perkin–Elmer Life and Analytical Sciences, Shelton, CT) was used. The Victor^3 plate reader (Perkin–Elmer, Turku, Finland) was used to determine the emitted luminescence. Media were changed daily, and data were collected at five different time points including 0, 24, 48, 72 and 96 h. All experiments were performed in triplicate.

4.3.11. Statistics

For statistical analysis, PRISM8 software from GraphPad was used. Each data point was obtained through Means ± standard deviation (SD). For the value comparison, multiple t-tests or a one-way analysis of variance (ANOVA) ($P \leq 0.05$) followed by a post hoc test (Tukey test) was used.

4.4. Results and discussion

4.4.1. Chemical composition of the blackberry seed flour extract

Table 4.1. Characterization of compounds present in blackberry seed flour.

Peak ID	$T_{R\ (min)}$	Theor. $[M-H]^-$	Exptl. $[M-H]^-$	Chemical Formula	Tentative Identification	Concentration (µg/g)
1	3.79	481.0618	481.0621	$C_{20}H_{18}O_{14}$	Hexahydroxydiphenic acid (HHDP) hexoside	tr
2	7.64	783.0681	783.0687	$C_{34}H_{24}O_{22}$	Pedunculagin isomer	186.65 ± 3.28 EAE
3	8.54	633.0728	633.0730	$C_{27}H_{22}O_{18}$	Galloyl-HHDP-hexoside	tr
4	9.20	783.0681	783.0687	$C_{34}H_{24}O_{22}$	Pedunculagin isomer	tr
5	9.23	783.0681	783.0694	$C_{34}H_{24}O_{22}$	Pedunculagin isomer	tr
6	11.55	577.1346	577.1349	$C_{30}H_{26}O_{12}$	Procyanidin B1	tr
7	12.06	1868.1425	934.0761[a]	$C_{82}H_{54}O_{52}$	Sanguiin H6 isomer	457.03 ± 40.64 EAE
8	12.60	1868.1425	934.0692[a]	$C_{82}H_{54}O_{52}$	Sanguiin H6 isomer	565.91 ± 48.91 EAE
9	13.26	1868.1425	934.0757[a]	$C_{82}H_{54}O_{52}$	Sanguiin H6 isomer	675.10 ± 47.32 EAE
10	13.84	447.0927	447.0939	$C_{21}H_{20}O_{11}$	Kaempferol-3-glucoside	tr
11	14.44	433.0407	433.0418	$C_{19}H_{14}O_{12}$	Ellagic acid pentoside isomer	373.57 ± 14.03 EAE
12	15.07	433.0407	433.0420	$C_{19}H_{14}O_{12}$	Ellagic acid pentoside isomer	261.61 ± 9.83 EAE
13	16.51	300.9984	300.9983	$C_{14}H_6O_8$	Ellagic acid	653.81 ± 66.84

T_R stands for the retention time. Theor. $[M-H]^-$ and Exptl. $[M-H]^-$ were theoretical and experimental m/z of molecular ions, respectively. EAE stands for ellagic acid equivalent. The tr stands for trace. [a]Experimental m/z values of $[M-2H]^{-2}$.

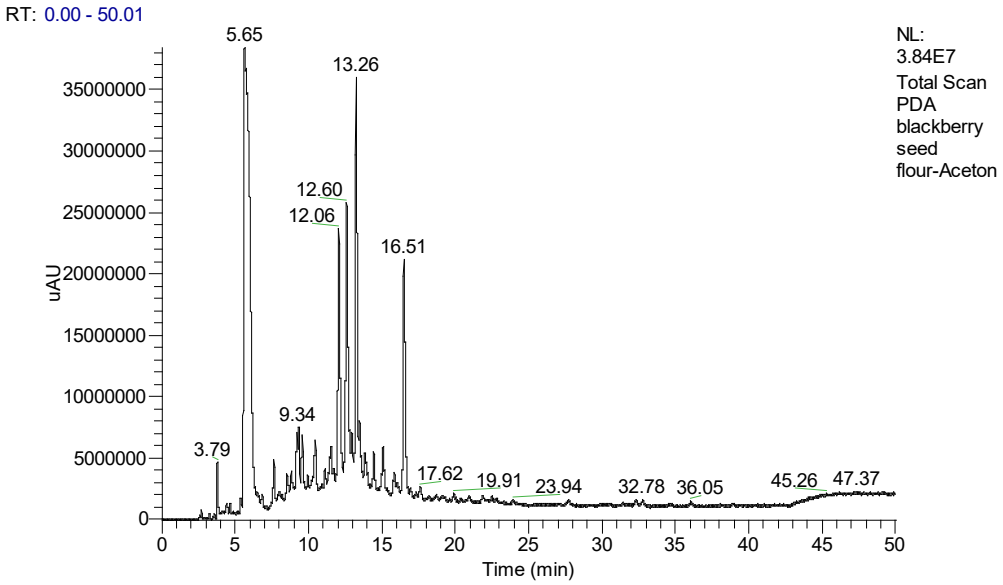

Figure 4.1. Typical UHPLC chromatogram showing peaks of major compounds identified in the blackberry seed flour extract and their corresponding retention times.

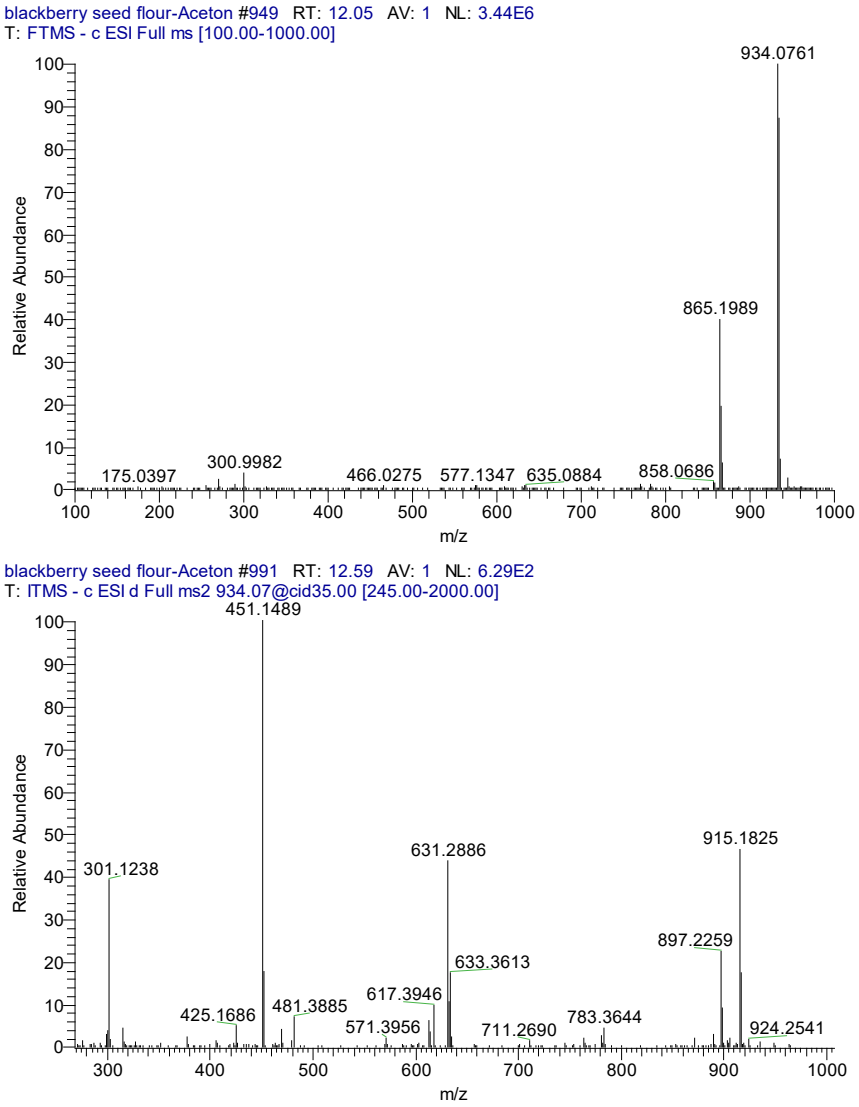

Figure 4.2. MS and MS^2 spectra of sanguiin H6 and peaks of fragmental ions in a negative mode.

The high resolution ESI-MS of sanguiin H-6 showed $[M-H]^{-2}$ of 934.0761, which corresponds to the formula $C_{82}H_{54}O_{52}$ (Table 4.1). A detailed analysis of the fragmental ions had peaks of *m/z* 783.3644 due to the loss of digalloyl-gallagyl-hexoside, *m/z* 633.3613 due to the loss of di-HHDP-glucose-galloyl-ellagic acid, and *m/z* 301.1238 due to the loss of galloyl-glucose residue from galloyl-HHDP-hexoside in a negative mode.

In the blackberry seed flour extract, thirteen compounds including hexahydroxydiphenic acid (HHDP) hexoside, pedunculagin isomers, galloyl-HHDP-hexoside, procyanidin B1, sanguiin H6 isomers, kaempferol-3-glucoside, ellagic acid, and ellagic acid derivatives were identified with sanguiin H6 as the primary compound (Table 4.1 and Figure 4.1). In addition to the chemical composition, this study quantified the selected compounds in the blackberry seed flour extract (Table 4.1). The primary compound, sanguiin H6's concentration was 457–675 μg ellagic acid equivalents/g (μg EAE/g). The second compound was ellagic acid and concentration was 654 μg/g. The following compounds were ellagic acid derivatives and pedunculagin, at levels of 262–374 and 187 μg EAE/g, respectively (Table 4.1).

The most abundant component, sanguiin H6, found in blackberry seed flour extract had a $[M-H]^-$ of 1868.1425, which corresponds to the formula $C_{82}H_{54}O_{52}$ (Table 1). A detailed analysis of the fragmental ions had peaks of *m/z* 783.3644 due to the loss of digalloyl-gallagyl-hexoside, *m/z* 633.3613 due to the loss of di-HHDP-glucose-galloyl-ellagic acid, and *m/z* 301.1238 due to the loss of galloyl-glucose residue from galloyl-HHDP-hexoside in a negative mode (Figure 4.2). This fragmentation matched with sanguiin H6 reported from a previous study (Mena, Calani, Dallasta, Galaverna, García-Viguera, Bruni, et al., 2012). Therefore, this compound was tentatively identified as sanguiin H6.

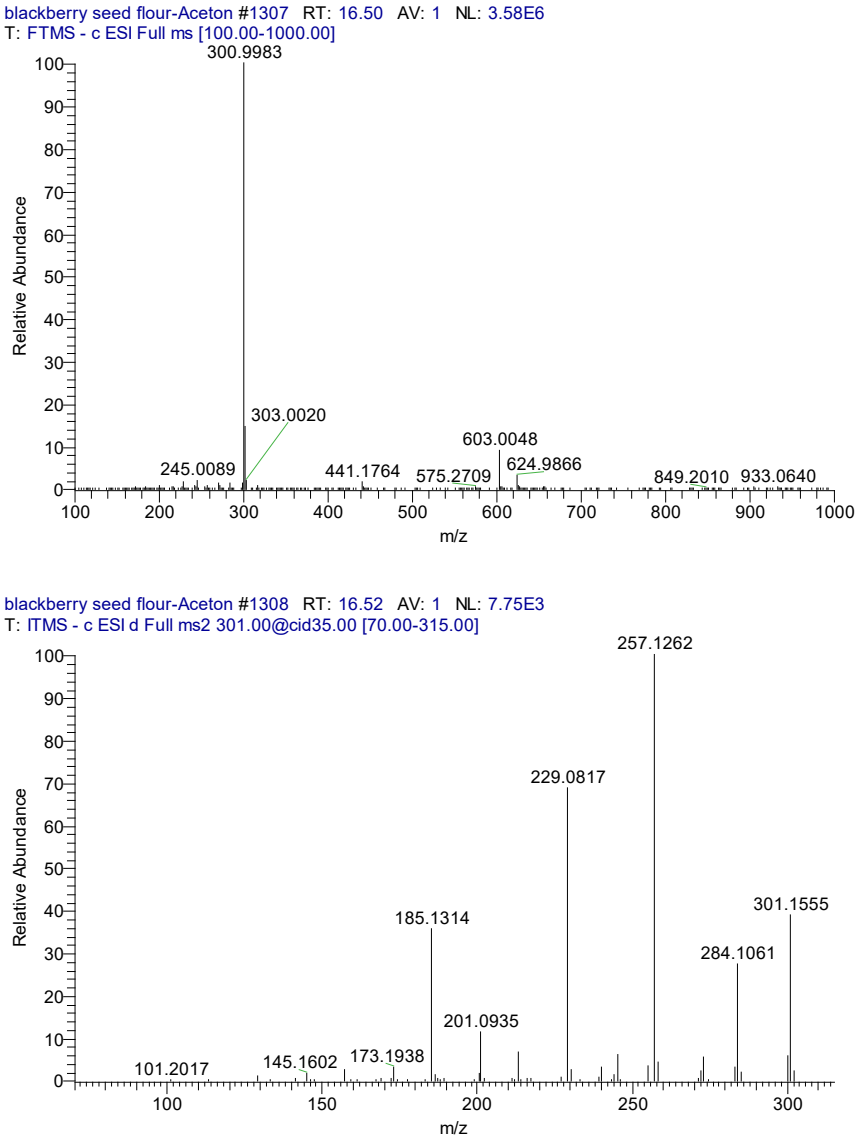

Figure 4.3. MS and MS^2 spectra of ellagic acid and peaks of fragmental ions in a negative mode.

The high resolution ESI-MS of ellagic showed $[M-H]^-$ of 300.9983, which corresponds to the formula $C_{14}H_6O_8$ (Table 4.1). A detailed analysis of the fragmental ions had peaks of *m/z* 284.1061, 257.1262, 229.0817, and 185.1314 in a negative mode.

Correspondingly, the high resolution ESI-MS of ellagic acid had an *m/z* of 300.9984 of $[M-H]^-$ corresponding to the formula of $C_{14}H_6O_8$ (Table 4.1). A detailed analysis of the fragmental ions of ellagic acid had peaks of 284.1061, 257.1262,

229.0817, and 185.1314 in a negative mode (Figure 4.3) (Mena, Calani, Dallasta, Galaverna, García-Viguera, Bruni, et al., 2012). Based on ellagic acid standard's retention time and reported fragmental ions, this compound was tentatively determined as ellagic acid.

Two major compounds found in blackberry seed flour, pedunculagin and sanguiin H6, are ellagitannins. The ellagitannins are water soluble and high molecular weight phenolic compounds. When exposed to acids or bases, ester bonds are hydrolyzed and the hexahydroxydiphenic acid (HHDP) spontaneously rearranges into the water insoluble ellagic acid. Therefore, to quantify the ellagitannins found in blackberry seed flour extract, ellagic acid was used as the standard. In the current study, the total ellagic acid concentration after hydrolysis was about 3 mg/g of the dry seed flour (Table 4.1). Previously, it has been reported that ellagic acid concentration in blackberry after hydrolysis was 1.5 mg/g dry weight (Daniel, Krupnick, Heur, Blinzler, Nims, & Stoner, 1989). Interestingly, compared to the ellagic acid concentration found in the whole blackberry fruit, blackberry seed had a significantly greater ellagic acid concentration.

4.4.2. Potential effects of the blackberry seed flour extract on gut microbiota

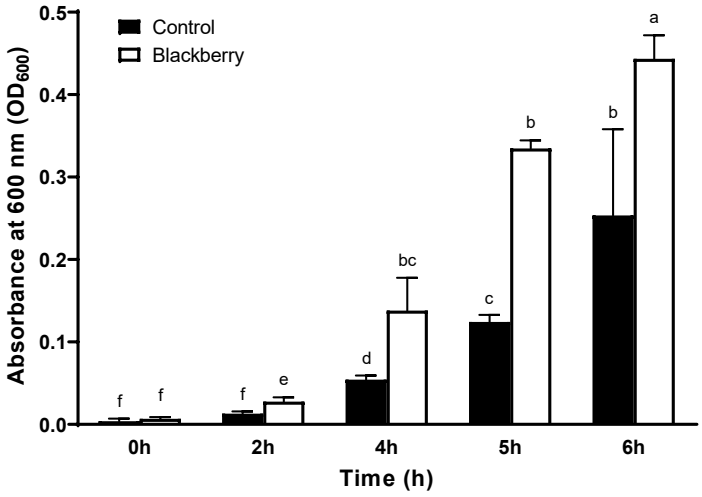

Figure 4.4. Effects of blackberry seed flour extract on gut bacterial growth. Blackberry stands for blackberry seed flour extract. Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

In this study, the blackberry seed flour extract was able to enhance gut bacterial growth in a time dependent manner (Figure 4.4). Previously, our group reported that extracts rich in polyphenols were able to enhance the gut bacterial populations (Choe, Li, Gao, Yu, Wang, Sun, et al., 2019). Similarly, Bialonska and others reported that the addition of pomegranate by-product and pomegranate polyphenols significantly enhanced the growth of total bacteria (Bialonska, Ramnani, Kasimsetty, Muntha, Gibson, & Ferreira, 2010). Also, it has been reported that several other polyphenols including punicalagin, punicalin, ellagic acid, gallic acid (Bialonska, Kasimsetty, Schrader, & Ferreira, 2009), catechin, epicatechin (Tzounis, Vulevic, Kuhnle, George, Leonczak, Gibson, et al., 2007) and resveratrol (Larrosa, Yañéz-Gascón, Selma, González-Sarrías,

Toti, Cerón, et al., 2009) could interact with gut microbiota. In this study, the presence of pedunculagin, sanguiin H6, and ellagic acid in the blackberry seed flour extract (Table 4.1) might have close relation to the total bacterial growth since gut bacteria have been reported to utilizing these compounds (Bialonska, Kasimsetty, Schrader, & Ferreira, 2009).

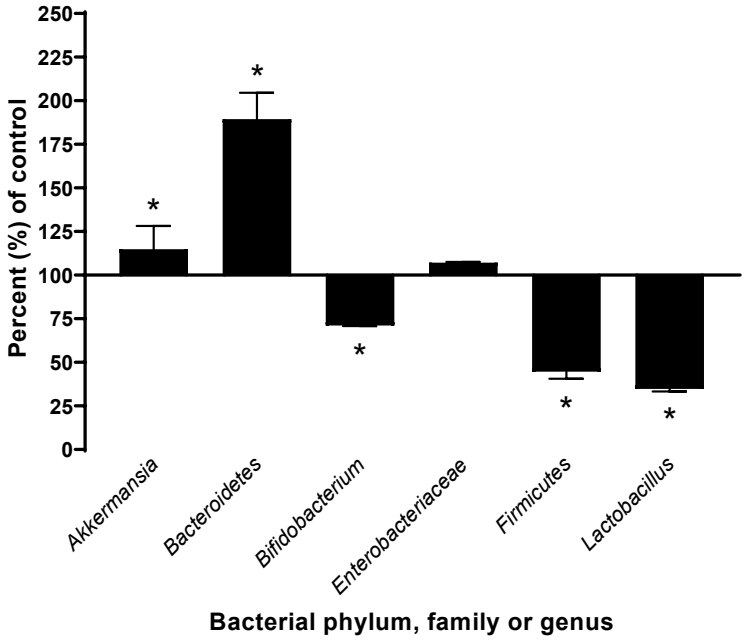

Figure 4.5. Effects of the blackberry seed flour extract on the relative abundance of a specific bacterial phylum, family, or genus.
All values were normalized to the control. Each column represents the mean ± SD (n = 3). Columns marked with an asterisk are significantly different from the control at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

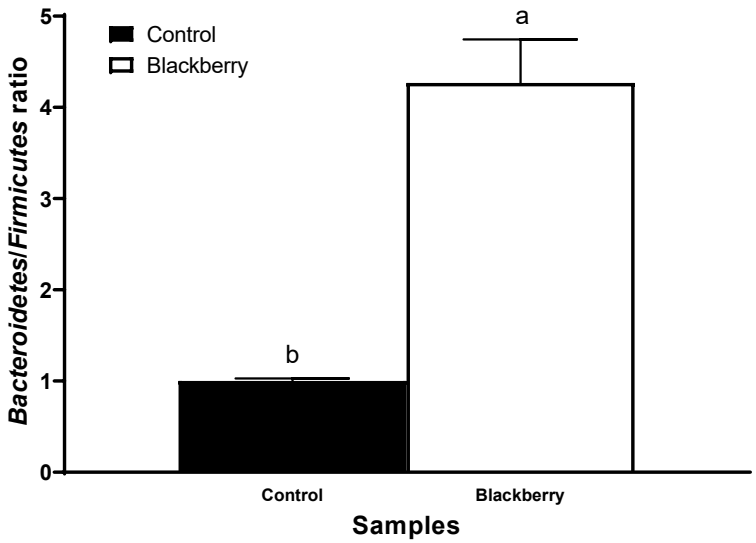

Figure 4.6. Effects of the blackberry seed flour extract on *Bacteroidetes*/*Firmicutes* ratio.
Blackberry stands for blackberry seed flour extract. All values were normalized to the control. Each column represents the mean ± SD (n = 3). Columns marked with different letters are significantly different from each other at $P \leq .05$ (multiple t-tests were used).

The blackberry seed flour extract was able to increase the abundance of *Bacteroidetes* phylum by two-fold compared to the control (Figure 4.5). In contrary, *Firmicutes* phylum was reduced by half compared to the control (Figure 4.5). The *Bacteroidetes* and *Firmicutes* phyla are predominant phyla that consist of more than 90% of gut microbiota community in human. In addition, these two phyla are closely associated with human health. For example, it has been reported that *Bacteroidetes* phylum is known to carry very large number of genes that encode carbohydrate active enzymes (Flint, Scott, Duncan, Louis, & Forano, 2012). Also, *Bacteroidetes* phylum is involved in T-cell activation, bile acid metabolism, and transformation of toxic and mutagenic compounds (Mazmanian, Round, & Kasper, 2008). The *Firmicutes* is associated with metabolism of

fatty acid and aging (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005). Ley and others found an increase in number of *Firmicutes* during aging progress (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005). This increase in *Firmicutes* phylum alters the ratio between *Bacteroidetes* and *Firmicutes* phyla. In 2018, Spychala and others found that the ratio of *Firmicutes* to *Bacteroidetes* (F:B) increased about nine-fold in aged mice compared to the young mice (Spychala, Venna, Jandzinski, Doran, Durgan, Ganesh, et al., 2018). It is also recognized that the ratio of *Bacteroidetes* to *Firmicutes* phyla is associated with obesity. Ley and others found a significant increase of the *Firmicutes* and decrease of the *Bacteroidetes* levels in obese (ob/ob) mice compared to wild-type mice (Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005). In the current study, the ratio of *Bacteroidetes* to *Firmicutes* increased over four-fold in blackberry seed flour extract added culture compared to the control (Figure 4.6). This result is consistent with the observation by Roopchand and others that grape polyphenols decreased the proportion of *Firmicutes* to *Bacteroidetes* in mice model (Roopchand, Carmody, Kuhn, Moskal, Rojas-Silva, Turnbaugh, et al., 2015). The result from this study proposes that blackberry seed flour may be used for body weight control and for health promotion in the aging population.

The blackberry seed flour extract also altered three bacterial genera and one family including *Akkermansia*, *Bifidobacterium*, and *Lactobacillus*, and *Enterobacteriaceae*, respectively (Figure 4.5). Among three genera, the blackberry seed flour extract increased *Akkermansia* genus (Figure 4.5). *Akkermansia* genus is a mucin degrading bacterium that resides in the human intestinal tract and known as a contributor to the maintenance of gut

health. Recent studies suggest that *Akkermansia* may reduce the risk of obesity, diabetes, and inflammation (Everard, Belzer, Geurts, Ouwerkerk, Druart, Bindels, et al., 2013). Previously, it has been reported that grape polyphenols promote the growth of *Akkermansia* (Roopchand, Carmody, Kuhn, Moskal, Rojas-Silva, Turnbaugh, et al., 2015). Together, the results suggested the possible contribution of phenolics in the blackberry seed flour extract in increasing *Akkermansia* genus (Figure 4.5). However, our previous studies found that polyphenols found in broccoli, carrot, cucumber, and milk thistle seeds decreased the abundance of *Akkermansia* genus (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018, 2019), suggesting the specificity of the individual polyphenolic compound in interacting with a selected bacterial species.

The blackberry seed flour extract lowered the abundance of *Bifidobacterium* and *Lactobacillus* genera compared to the control (Figure 4.5). *Bifidobacteria* and *Lactobacilli* are probiotic bacteria. *Bifidobacteria* are believed to benefit on host health. Due to their health-beneficial properties, *Bifidobacteria* have been incorporated into many functional foods as active ingredients (O'Callaghan & Sinderen, 2016). *Lactobacilli* are well-known probiotic bacteria. Previous studies suggest that together with *Bifidobacteria*, *Lactobacilli* can lower the concentration of carcinogenetic enzymes in colon flora through normalizing intestinal permeability and microflora balance as well as the production of antimutagenic organic acids and enhancing the host immune system (Kumar, Kumar, Nagpal, Mohania, Behare, Verma, et al., 2010). The result from the current study is consistent with our previous studies that extracts rich in polyphenols decreased the abundance of both

Bifidobacterium and *Lactobacillus* genera (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018, 2019). Also, the result was supported by the observation of the previous study using grape seed extracts by Tabasco and others (Tabasco, Sánchez-Patán, Monagas, Bartolomé, Moreno-Arribas, Peláez, et al., 2011). Tabasco and others detected a growth inhibition effect of several lactic acid bacteria and *Bifidobacteria* using the grape seed extracts (Tabasco, Sánchez-Patán, Monagas, Bartolomé, Moreno-Arribas, Peláez, et al., 2011).

The blackberry seed flour extract did not change the abundance of *Enterobacteriaceae* family compared to the control (Figure 4.5). The *Enterobacteriaceae* are a large family of gram-negative bacteria and some species naturally inhabit in human. Even though *Enterobacteriaceae* are non-spore-forming bacteria, there are number of foodborne pathogens including *Salmonella*, *Yersinia enterocolitica*, pathogenic *Escherichia coli* (including *E. coli* O157:H7), *Shigella* spp. and *Cronobacter* spp. (Baylis, 2006). Thus, the increase of *Enterobacteriaceae* family in gut microbiota may cause health problems.

Recently, the gut microbiota has been spotlighted due to their health beneficial properties. Besides their health beneficial properties, the impact of gut microbiota on food metabolism has obtained significant attention. Ellagitannins found in fruits, legumes and edible seeds are hydrolyzed to ellagic acid during digestion. Then, part of ellagic acid is metabolized by gut microbiota to produce urolithins (Landete, 2011). In 2017, Selma and others were able to isolate the bacterial species that is involved in ellagitannins and ellagic acid metabolism (Selma, Beltrán, Luna, Romo-

Vaquero, García-Villalba, Mira, et al., 2017). Interestingly, it has been reported that urolithins, metabolites of ellagitannins and ellagic acid, showed anti-inflammatory, anticarcinogenic, and free radical scavenging capacities (González-Sarrías, Espín, Tomás-Barberán, & García-Conesa, 2009). These results suggest the benefits of consuming ellagitannins and ellagic acid rich foods including blackberry seed flours. To summarize, the current study showed that blackberry seed flour extract was able to change the gut microbiota profile and may have potential health benefits.

4.4.3. Relative DPPH radical scavenging capacity (RDSC)

Table 4.2. Free Radical Scavenging Capacities of Cold-pressed Blackberry Seed Flour.

	RDSC	ORAC	HOSC	ABTS
	(μmol TE/g)			
Blackberry	362.09 ± 22.07	304.01 ± 15.65	2531.44 ± 375.09	266.82 ± 16.52

Abbreviations: Blackberry, blackberry seed flour extract; RDSC, Relative DPPH scavenging capacity; ORAC, Oxygen radical absorbing capacity; HOSC, Hydroxyl radical scavenging capacity; ABTS, ABTS radical scavenging capacity; TE, Trolox equivalent.

The blackberry seed flour extract demonstrated DPPH radical scavenging capacity with an RDSC value of 362 μmol TE/g (Table 4.2). This value is about three times greater than 137 μmol TE/g reported by Zafra-Rojas and others for the blackberry residues consisted of peels, seeds, and pulp (Zafra-Rojas, Cruz-Cansino, Quintero-Lira, Gómez-Aldapa, Alanís-García, Cervantes-Elizarrarás, et al., 2016). Also, the RDSC value obtained from the current study is compatible with the RDSC

values of berry seeds that Parry and others previously reported (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). Parry and others tested black raspberry, red raspberry, blueberry and cranberry seeds using 50% acetone as a solvent and found their RDSC values of 200, 510, 670, and 1260 μmol TE/g, respectively (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). Blackberry seed flour had a DPPH scavenging capacity comparable to other berry seeds.

4.4.4. Oxygen radical absorbing capacity (ORAC)

The blackberry seed flour extract showed an oxygen radical absorbing capacity of 304 μmol TE/g (Table 4.2). Previously, it has been reported that extracts of fruit seeds such as black raspberry, red raspberry, blueberry, and cranberry had ORAC values of 296, 276, 153 and 111 μmol TE/g, respectively (Parry, Su, Moore, Cheng, Luther, Rao, et al., 2006). Compared to other berry seed flour extracts, blackberry seed flour extract showed high oxygen radical absorbing capacity.

4.4.5. Hydroxyl radical (HO•) scavenging capacity (HOSC)

The blackberry seed flour extract showed HOSC value of 2531 μmol TE/g (Table 2). This is the first HOSC report of blackberry seed flour. In 2017, Gao and others reported HOSC values of blueberry in a range of 566–1048 μmol TE/g (Gao, Yu, Liu, Wang, Luo, Yu, et al., 2017). Compared to the whole blueberry fruit extract, blackberry seed flour extract showed a HOSC value 2.5 to 4.5-fold greater. Previously, our group tested HOSC of vegetable seed flours such as broccoli, carrot and cucumber using 50% acetone as a solvent (Choe, Li, Gao, Yu, Wang, Sun, et al.,

2018). The broccoli, carrot, and cucumber seed flour extracts had HOSC values of 270, 112, and 52 μmol TE/g, respectively (Choe, Li, Gao, Yu, Wang, Sun, et al., 2018). Compared to vegetable seed flours, the blackberry seed flour had a significantly greater HOSC value.

4.4.6. $ABTS^{\bullet+}$ scavenging capacity

The blackberry seed flour extract showed an $ABTS^{\bullet+}$ scavenging capacity value of 267 μmol TE/g (Table 4.2). This value is compatible with the black raspberry seed extract previously reported (Parry & Yu, 2006). In that study, Parry and Yu used two different solvents, 100% ethanol and 50% acetone for the extraction and found $ABTS^{\bullet+}$ scavenging capacity values of 233 and 361 μmol TE/g, respectively (Parry & Yu, 2006).

Free radicals are generated inside the human body internally and externally. Internal sources may include mitochondria, xanthine oxidase, peroxisomes, inflammation, phagocytosis and exercise. External sources include smoking, environmental pollutants, radiation, certain drugs, industrial solvents and ozone. Either from internal or external, excessive free radicals in the human body can cause oxidative stress, a deleterious process in which can vigorously change the cell membranes, lipids, lipoproteins, and DNA. Thus, scavenging free radicals using dietary intervention is regarded as a potential health benefit. In this study, blackberry seed flour extract showed promising free radical scavenging capacities. This result suggests that blackberry seed flour may be used for diminishing the risk of oxidative stress-caused human chronic diseases.

4.4.7. Anti-inflammatory capacity

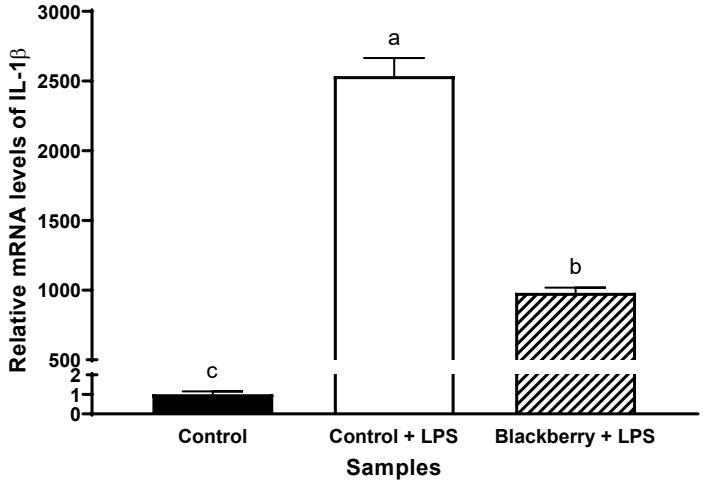

Figure 4.7. An anti-inflammatory capacity of the blackberry seed flour extract in J774A.1 mouse macrophage cells: interleukin 1 beta (IL-1β).
LPS stands for lipopolysaccharide and blackberry stands for blackberry seed flour extract. All values were normalized to the control. Each column represents the mean ± SD (n = 3). Columns marked with different letters indicate a significant difference at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

In the current study, blackberry seed flour extract showed significant inhibition of pro-inflammatory marker gene, interleukin 1 beta (IL-1β), induced by lipopolysaccharides (LPS) compared to the LPS stimulated J774A.1 mouse macrophages (Figure 4.7). Growing evidence suggests that chronic health conditions such as diabetes, cardiovascular disease, cancer, rheumatoid arthritis, and inflammatory bowel disease may all be linked to inflammation (Clark, Kroger, & Tisch, 2017). Therefore, inflammation has been considered a target for reducing the risk of chronic diseases. During the process of inflammation, IL-1β plays an important role. IL-1β is a potent pro-inflammatory cytokine and a key mediator of the inflammatory response. Previous studies reported that blocking or inhibiting IL-1β

can significantly lower the risk of chronic diseases such as rheumatoid arthritis, type 3 diabetes, and cancers (Dinarello, Simon, Jos, & van der Meer, 2012). Also, ellagic acid, a compound identified in blackberry seed flour extract (Table 4.1), has been reported for its anti-inflammatory capacity (Chen, Chen, & Zhou, 2018; El-Shitany, El-Bastawissy, & El-desoky, 2014). The mechanism of action for ellagic acid's anti-inflammatory capacity is still not clear. But, it is believed that ellagic acid may modulate the production of cyclooxygenase-2 (COX-2) mRNA mainly through the inhibition of reactive oxygen species (ROS) production which in turn inhibited nuclear kappa light-chain-enhancer of activated B cells (NF- κB) activation (El-Shitany, El-Bastawissy, & El-desoky, 2014). The other possible mechanism of action is by blocking the COX-2 receptor. El-Shitany and others tested molecular docking between ellagic acid and COX-2 active site and found high affinity. The binding affinity of ellagic acid and COX-2 was even greater than that of the selected anti-inflammatory drugs including diclofenac and meloxicam (El-Shitany, El-Bastawissy, & El-desoky, 2014).

In addition, pedunculagin, a compound found in blackberry seed flour, may possess anti-inflammatory activity. Previously, Ishii and others extracted *Melastoma dodecandrum* Lour using 80% acetone and tested the anti-inflammatory activity of the hydrolyzable tannins found in *Melastoma dodecandrum* Lour (Ishii, Saito, Horie, Shibano, Kitanaka, & Amano, 1999). *Melastoma dodecandrum* Lour is a flower found in Asia including India, and Australia that contains hydrolyzable tannins such as casuarinin, casuarictin, pedunculagin, and nobotannin. Ishii and others found that

these tannins were able to inhibit nitric oxide (NO) production in the lipopolysaccharide (LPS) stimulated RAW264.7 macrophage (Ishii, Saito, Horie, Shibano, Kitanaka, & Amano, 1999). Also, the protein amounts of inducible nitric oxide synthase (iNOS) was remarkably decreased when LPS and interferon-gamma (IFN-γ) treated RAW264.7 macrophage cells were treated with hydrolyzable tannins (Ishii, Saito, Horie, Shibano, Kitanaka, & Amano, 1999).

Taking together, components found in blackberry seed flour may abate the risk of IL-1β mediated inflammation and related chronic diseases.

4.4.8. Anti-proliferative capacity

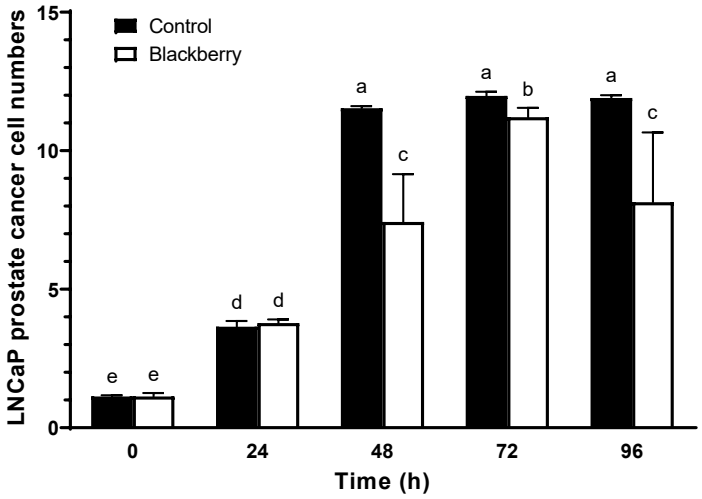

Figure 4.8. Anti-proliferative capacity of the blackberry seed flour extract in LNCaP prostate cancer cells.
A final concentration of 0.4 mg flour equivalent/mL blackberry seed flour extract was treated in LNCaP prostate cancer cells. LNCaP prostate cancer cell numbers were measured every 24 h. Blackberry stands for blackberry seed flour extract. Each column represents the mean ± SD (n = 3). Columns marked with different letters indicate significant difference at $P \leq .05$ (one-way ANOVA and Tukey for post-hoc were used).

The blackberry seed flour extract at an initial treatment concentration of 0.4 mg flour equivalents/mL suppressed the growth of LNCaP prostate cancer cells after 48 h (Figure 4.8). Previously, it has been reported that ellagic acid treatment induced a significant increase in DNA damage in LNCaP and DU145 prostate cancer cell lines in a dose-dependent manner (Vanella, Barbagallo, Acquaviva, Giacomo, Cardile, Abraham, et al., 2013). The mechanism behind this anti-proliferative capacity includes several signaling pathways. In cancer cells, aberrant accumulation of intracellular β-catenin is a well-recognized characteristic. Vanella and others found a reduction in β-catenin levels when LNCaP prostate cancer cells were treated with ellagic acid (Vanella, Barbagallo, Acquaviva, Giacomo, Cardile, Abraham, et al., 2013). Also, they found that ellagic acid was able to reduce the mammalian target of rapamycin (mTOR) activation. Previously, the capacity of phospho-protein kinase B (p-Akt) to phosphorylate/activate mTOR has been reported in several cancer cell lines (Pratheeshkumar, Budhraja, Son, Wang, Zhang, Ding, et al., 2012; Diersch, Wenzel, Szameitat, Eser, Paul, Seidler, et al., 2013). Their finding suggests that ellagic acid might have an anti-proliferative effect by reducing PI3K/Akt downstream signaling through inhibition of mTOR phosphorylation (Vanella, Giacomo, Acquaviva, Barbagallo, Cardile, Kim, et al., 2013). The second possible mechanism might involve the NAD-dependent deacetylase sirtuin-1 (SIRT1) protein. SIRT1 functions as an oncogenic protein and plays a role in tumorigenesis (Chen, Jeng, Yuan, Hsu, & Chen. 2012). This protein is overexpressed in human prostate cancer cells including DU145, LNCaP, 22Rv1, and PC3. In the LNCaP cell, the treatment of 25 and 50 μM

of ellagic acid significantly reduced the SIRT1 protein level (Vanella, Giacomo, Acquaviva, Barbagallo, Cardile, Kim, et al., 2013).

In addition, fruits containing high amounts of ellagitannins seemed to be more promising for treating prostate cancer compared to other fruits, vegetables, and culinary herbs (Seeram, Aronson, Zhang, Henning, Moro, Lee, et al., 2007). An ellagitannin rich fruit, pomegranate, has been extensively tested *in vivo* for prostate cancer inhibitory properties (Seeram, Aronson, Zhang, Henning, Moro, Lee, et al., 2007). Interestingly, higher concentrations of ellagitannins were recorded in prostate and colon tissues as compared to the others in animal models (Seeram, Aronson, Zhang, Henning, Moro, Lee, et al., 2007). Also, Malik and others reported dose-dependent anti-proliferative and pro-apoptotic effects of pomegranate fruit extracts (10–100 µg/mL) against aggressive PC3 human prostate cancer cells (Malik, Afaq, Sarfaraz, Adhami, Syed, & Mukhtar, 2005). As a proposed mechanism of action, compounds found in pomegranate fruit extracts may induce pro-apoptotic mediators including Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) and downregulate B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL). As a result of this signaling pathway, reduced expressions of cyclin-dependent kinase 2, 4, 6, and cyclins D1, D2, and E may be observed (Malik, Afaq, Sarfaraz, Adhami, Syed, & Mukhtar, 2005).

Ellagitannins and ellagic acid also had anti-proliferative capacities in liver, skin, esophageal and oral, cervical, lung, breast, and colon cancer cell lines (Ismail,

Calcabrini, Diaz, Fimognari, Turrini, Catanzaro, et al., 2016). These results suggest the potential use of blackberry seed flour in reducing the risk of carcinogenesis.

4.5. Conclusions

The current study identified the chemical composition of blackberry seed flour and evaluated its potential health beneficial properties including gut microbiota modulatory, free radical scavenging, anti-inflammatory, and anti-proliferative capacities. The presence of ellagitannins and ellagic acid in blackberry seed flour and their potential health beneficial properties suggest the potential value-added utilization of blackberry seed flour in enhancing human health.

Summary

The current study identified chemical compositions and evaluated health beneficial properties of blackberry, broccoli, carrot, cucumber, and milk thistle seed flours. The result showed that these seed flours are rich in phytochemicals such as polyphenolic compounds and showed promising health beneficial properties including free radical scavenging, suppressing inflammatory responses, cancer cell growth, and interaction with gut microbiota. Therefore, blackberry, broccoli, carrot, cucumber, and milk thistle seed flours may be used in nutraceuticals and functional foods.

The design of the current study was *in vitro*. Due to the limitation of the *in vitro* environment, health beneficial effects of these seed flours *in vivo* are still unknown. Therefore, to have a better idea and understanding, an animal study using a seed flour supplemented diet is necessary and animal study will provide a better understanding of seed flours' health beneficial effects.

Future perspective

For the future, there are many extension studies of broccoli, carrot, cucumber, milk thistle, and blackberry seed flours can be done and possible studies are as follow:

First, the activity and molecular mechanism of free radical scavenging, anti-inflammatory, and anti-proliferative capacities of each compound found in broccoli, carrot, cucumber, milk thistle, and blackberry seed flour extracts may be investigated. This can help in developing nutraceuticals and functional foods.

Second, the synergistic effects of compounds found in broccoli, carrot, cucumber, milk thistle, and blackberry seed flour extracts may be investigated to optimize the health beneficial effects of these seed flours.

Third, the anti-microbial activity of milk thistle seed flour extract and its components may be investigated for possible anti-microbial reagent development and .gut microbiota alteration.

Lastly, beneficial and safety issues of broccoli, carrot, cucumber, milk thistle, and blackberry seed flour may be investigated for their usage.

Appendices

Liangli (Lucy) Yu, Uyory Choe, Yanfang Li, & Yaqiong Zhang, Oils from Fruit, Spice, and Herb Seeds.
In *Bailey's Industrial Oil and Fat Products* (7th ed.). **2020**, John Wiley & Sons, Ltd.

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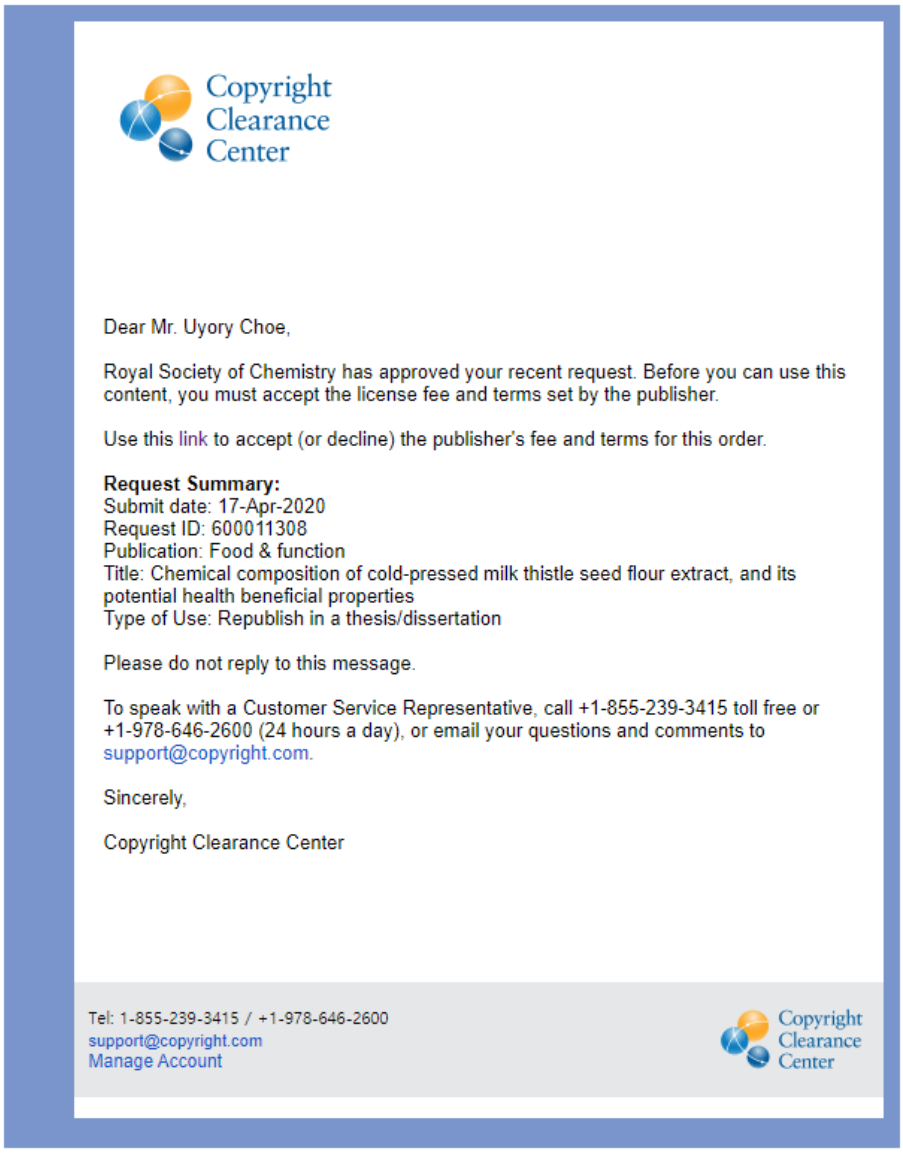

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