ABSTRACT

Title of Thesis: CHEMICAL COMPOSITIONS OF SELECTED COLD-PRESSED SEED FLOURS AND THEIR FREE RADICAL SCAVENGING AND ANTI-PROLIFERATIVE CAPACITIES.

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The cold-pressed blackberry, broccoli, carrot, cucumber, and milk thistle seed flours were extracted with 100% ethanol and examined for their phytochemical compositions, total phenolic contents, $ABTS^{\bullet+}$ and relative $DPPH^{\bullet}$ scavenging capacities, and anti-proliferative activities in HCT116 and SW480 colon cancer cells. Eleven, eight, ten, and thirteen compounds were tentatively identified in the blackberry, broccoli, carrot, and milk thistle seed flour extracts, with ellagic acid, glucoraphanin, kaempferol-3-*O*-rutinoside, and silychristin isomers being the primary components in each, respectively. Milk thistle seed flour extract had the greatest total phenolic content. Blackberry seed flour extract possessed the strongest free radical scavenging capacities against both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. Milk thistle

seed flour extract was the only extract capable of significantly suppressing the growth of SW480 colon cancer cells, but not HCT116 cells.

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by

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Introduction

With the improvement of living standards, people begin to pay attention to the consumption of vegetables and fruits. Different kinds of vegetable and fruit products came into the market, such as juices, jellies and jams. During their processing, the seeds become a by-product, which would impose influence on the environment when dumped to soil or rivers directly (Rodríguez Couto, 2008). However, when utilized properly, the seeds can be high-value ingredients. Firstly, the oil extracted from the seed contains high amount of unsaturated fatty acids including α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6) (Parry & Yu, 2006). These fatty acids may play important roles in prevention of coronary heart disease and cancers (Connor, 2000; Tapiero, Nguyen Ba, Couvreur, & Tew, 2002). After oil extraction, what left over is the seed flour. The seed flour also contains many value-adding components, including essential fatty acids, different polyphenolics, vitamin E in the form of tocopherols and tocotrienols, and carotenoids. These chemical components confer antioxidant activities along with other beneficial properties, such as anti-inflammatory property to the seed flours.

In this study, selected fruit and vegetable seed flours including blackberry, broccoli, carrot, cucumber, and milk thistle were examined. These cold-pressed seed flours were tested for their chemical compositions, total phenolic content, antioxidant activities against $DPPH^{\bullet}$ and $ABTS^{\bullet}$, and anti-proliferative activities in two colon cancer cell lines. The results might lead to value-added utilization of the seed flours which is being simply discarded. Through the exploitation of seed flours, not only the pressure they exert on the environment will be relieved, but also

the fruit and vegetable processing industry, and the related agricultural market will benefit from it.

Chapter I: Literature Review

Fruit and Vegetable Seeds

There is no doubt that seeds play an essential role in the field of agriculture. They are not only the starting point of the growth of crops, but also the part of the edible part of the crop that people favor in many cases, such as corn, quinoa, chestnut, rice, and peanut. However, there are also circumstances in which other parts of the crops are consumed. For instance, we take the mesocarp of the pumpkin and both endocarp and mesocarp of the grape. In this scenario, the seeds usually become wastes since the other parts are directly consumed or made into products with longer storage time, such as jellies, juices, salad dressings, wines and jams. Though the seeds seem to be a small part of the fruit, billions of tons of seeds are throwed away due to the huge production of processed fruit and vegetable products. Seeds can be further divided into two parts: seed oil and seed flour which is the leftover after oil extraction. Nowadays, seed oils are common in the market and popular for their richness in unsaturated fatty acids and the health benefits these fatty acids bringing along. However, consumers are not very familiar with seed flours. In fact, the seed flour also contains many beneficial components, including phenolics, dietary fibers and so on. If this part of the seed is deeply studied, these precious chemical components can be properly applied and the surplus value of seeds may be explored and utilized, which would also relieve the pressure exerted on the environment when dumped directly as a waste.

Chemical Composition

Phenolic Compounds

Phenolic compounds are composed of an aromatic ring with one or more hydroxyl groups and can be either simple phenolic molecules or highly-polymerized structures. Natural phenolic compounds are usually conjugated with one or more sugar moieties. The sugar units can be linked to the hydroxyl group or the aromatic ring of the phenols (Bravo, 1998). Phenolic compounds are very common in seed flours of fruits and vegetables, which are presented by phenolic acids, flavonoids and tannins (King & Young, 1999). There's an increasing tendency in studying phenolic compounds because of their functions as antioxidants, antimutagens and prevention of cancers and cardiovascular diseases (Bravo, 1998). Phenolic compounds exhibit the property of antioxidant resulting from their capacities to scavenge free radicals, donate electrons or hydrogen atoms and chelate metal ions (Balasundram, Sundram, & Samman, 2006), which can be illustrated as the following reactions:

$$ROO\bullet + PPH \rightarrow ROOH + PP\bullet$$

$$RO\bullet + PPH \rightarrow ROH + PP\bullet$$

The phenoxy radical intermediates (PP•) are more stable, which means the new free radicals are harder to form. Besides, the phenoxy radical intermediate (PP•) can also act as a terminator in a chain reaction, which is shown as follow:

$$ROO\bullet + PP\bullet \rightarrow ROOPP$$

$$RO\bullet + PP\bullet \rightarrow ROPP$$

However, when the concentration of phenolic compounds is high, these antioxidants will act

as prooxidants as well (Bravo, 1998). The antioxidant property of phenolic compounds is related to the structure. Take phenolic acids as an example, the antioxidant activities are determined by the numbers of hydroxyl groups and their relative positions to the carboxyl groups. And the increase of hydroxylation degree will increase the antioxidant activity of phenolic acids (Rice-Evans, Miller, & Paganga, 1996).

A total of 20 different phenolic compounds were detected in the blackberry seed flour (Ayoub, de Camargo, & Shahidi, 2016; Fazio, Plastina, Meijerink, Witkamp, & Gabriele, 2013). Quercetin 3-*O*-glucoronide was reported to be the one with the highest amount (1439 μg/100 g), followed by epicatechin (463 μg/100 g), gallic hexoside (305 μg/100 g) and quercetin (304 μg/100 g) in the blackberry seed flour. The seed flour was provided by the Fruit Smart Company (Washington, USA) and extracted with methanol-acetone-water (7:7:6, v/v/v) using sonication (Ayoub et al., 2016). Apart from what's shown in Table 1.1, Fazio and others found cyanidin 3-glucoside, ellagic acid, galloyl-HHDP glucose, and galloyl-*bis*-HHDP glucose in the seed flour, which was from the blackberries grown wildly on the mountain slope in Contrada Pallone, Cosenza. This seed flour was extracted 100% methanol under room temperature (Fazio et al., 2013). Compared to the blackberry seed flour, blackberry seeds shared some common phenolics with the seed flour, including ellagic acid, catechin, epicatechin, and procyanidins (Siriwoharn & Wrolstad, 2004). Procyanidins were the major components in the seed with the total content ranging from 48.8 to 58.5 mg/100 g. The highest content was detected in a blackberry cultivar named Evergreen (harvested in 1999), which was

purchased from Conroy Packing Co. in Woodburn, OR, USA and extracted with acetone (Siriwoharn & Wrolstad, 2004).

Table 1.1. Phenolic profiles of blackberry seed and seed flour

	Seed flour (μg/100 g)	Seed (mg/100 g)	Reference
Ellagic acid		13.3 – 14.5	(Siriwoharn & Wrolstad, 2004)
Protocatechuic acid	182		(Ayoub et al., 2016)
p-Coumaric acid	64.7		(Ayoub et al., 2016)
Gallic acid	295		(Ayoub et al., 2016)
Caffeic acid	122		(Ayoub et al., 2016)
Gallic hexoside	305		(Ayoub et al., 2016)
Catechin	130	Detected	(Ayoub et al., 2016; Siriwoharn & Wrolstad, 2004)
Epicatechin	463	0.926 – 1.130	(Ayoub et al., 2016; Siriwoharn & Wrolstad, 2004)
Quercetin	304		(Ayoub et al., 2016)
Epigallocatechin	216		(Ayoub et al., 2016)
Myricetin	209		(Ayoub et al., 2016)
Quercetin 3-*O*-glucoronide	1439		(Ayoub et al., 2016)
Procyanidin dimer B1	78.5	Total procyanidins: 48.8-58.5	(Ayoub et al., 2016; Siriwoharn & Wrolstad, 2004)
Procyanidin dimer B2	162		
Procyanidin dimer B3	47.1		
Procyanidin dimer B4	64.0		
Peonidin-3-*O*-glucoside	244		(Ayoub et al., 2016)

The phenolic profile of broccoli seed flour was barely studied. It was only reported by Choe and others that seven chemical compounds were found in the broccoli seed flour, including glucoraphanin isomers, glucoerucin, sinapoylhexose, quercetin-3-glucoside, disinapoylgentiobiose, 1,2-disinapoylglucoside and 1,2,2'-trisinapoylgentiobiose (Choe et al., 2018). The seed flour was provided by the Botanic Oil Innovations Inc. (Spooner, WI, USA) and extracted with 50% acetone (v/v) using sonication. The chemical composition of the whole broccoli seed was studied as well. Twelve chemical compounds have been discovered in the broccoli seeds in total (Chuanphongpanich & Phanichphant, 2006; Pająk, Socha, Gałkowska, Rożnowski, & Fortuna, 2014). In the research of Pająk and others, the broccoli seed was a variety called "Ramoso Calabrese" obtained from Diet Food (Warsaw, Poland). 5 g of the ground seeds were extracted with 20 mL of methanol (99.8%) by shaking for 15 min. In this kind of seed, chlorogenic acid had the highest concentration (12.02 mg/100 g), followed by caffeic acid (3.25 mg/100 g), gallic acid (1.57 mg/100 g) and protocatechuic acid (1.33 mg/100 g). The concentrations of *p*-coumaric acid, ferulic acid, sinapic acid and quercetin in the whole broccoli seed were 0.84, 1.17, 0.56 and 0.38 mg/100 g, respectively (Pająk et al., 2014). Chuanphongpanich, and Phanichphant studied the seed of five broccoli cultivars: "Packman", "Pak Ging", "Rod Fai", "Green Queen" and "Top Green #067", which were all harvested between April and May, 2003. The ground seeds were extracted with 80% methanol (v/v) and 1% HCl (v/v). Six chemical compounds were detected in the seed extract: quercetin, catechin, epicatechin, epigallocatechin gallate, gallic acid and rutin (Chuanphongpanich & Phanichphant, 2006).

Eight phenolic compounds were found in the carrot seed flour extracted with 50% acetone using sonication. They were kaempferol-3-*O*-rutinoside isomer, diosmetin-7-rutinoside, apigenin-7-*O*-*β*-D-rutinoside, caffeoyldihexoside, lycibarbarphenylpropanoid C and luteolin. All those compounds mentioned above were detected in the carrot seed flour for the first time (Choe et al., 2018).

Table 1.2. Silymarin compositions of milk thistle seed flour and whole seed (mg/g)

	Seed flour	Seed	Reference
Silybin	10.78 – 12.32	10.05 – 13.20	(Omar, Hadad, & Badr, 2012; Saleh et al., 2017)
Silydianin	0.56 – 1.66	0.79 – 1.03	(Omar et al., 2012; Saleh et al., 2017)
Isosilybin	1.52 – 2.26	2.17 – 2.92	(Omar et al., 2012; Saleh et al., 2017)
Silychristin	10.5 – 11.42	4.59 – 6.21	(Omar et al., 2012; Saleh et al., 2017)
Taxifolin	6.26 – 10.20	17.5 – 31.5	(Omar et al., 2012; Saleh et al., 2017)

As shown in Table 1.2, the highest concentrations of silybin, silydianin, isosilybin, silychristin and taxifolin were 12.32. 1.66, 2.26, 11.42 and 10.20 mg/g in the seed flour, respectively. They were all found in the milk thistle seeds from a pharmaceutical company in Anshas, Egypt. The seeds were extracted with methanol by cold maceration after defatted with petroleum ether under 40 – 60 °C using Soxhlet extraction (Omar et al., 2012). Saleh and others examined the milk thistle seeds obtained from Cairo-Alexandria desert road in Egypt in April, 2013. The highest contents of silybin, silychristin, and taxifolin were 13.20, 6.21 and 31.5 mg/g in the whole milk thistle seed, respectively. The seeds were extracted with 80% methanol assisted

with microwave at 800 W for 15 min. The highest content of silydianin was yielded by extracting the seeds with 80% methanol under heat reflux for 30 min. And the highest content of isosilybin was 2.92 mg/g when the seeds were extracted with 80% methanol with microwave assistance at 400 W for 30 min. The seeds were obtained from Cairo-Alexandria desert road in Egypt (Saleh et al., 2017). The contents of silybin, silydianin, and isosilybin in the milk thistle seed flour and whole seed were close to each other. However, milk thistle seed flour seemed to have higher silychristin content, while the whole seed had higher taxifolin content. The differences of phenolic profiles between the seed flour and the whole seeds could be caused by many reasons, such as the places of production, extraction methods and varieties.

Table 1.3. Total Phenolic Contents (TPC) of selected seeds (mg GAE/g)

	Seed flour	Whole seed	Reference
Blackberry	5.13	14.6 – 54.4	(Ayoub et al., 2016; Bushman et al., 2004; Siriwoharn & Wrolstad, 2004)
Broccoli	N/A	7.3 – 15.2	(Aguilera et al., 2015; Pérez-Balibrea, Moreno, & García-Viguera, 2011)
Milk thistle	25.22	29	(Mhamdi, Abbassi, Smaoui, Abdelly, & Marzouk, 2016; Parry, Cheng, Moore, & Yu, 2008)

Total phenolic contents of blackberry, broccoli and milk thistle seeds were shown in Table 1.3. Ayoub and others used methanol-acetone-water (7:7:6, v/v/v) to extract phenolic compounds from defatted blackberry seed flour, the total phenolics contents were 5.13 mg GAE/g, which

consisted of 2.23 mg GAE/g soluble free phenolics and 2.90 mg GAE/g esterified phenolics (Ayoub et al., 2016). Bushman and others reported a very high TPC of 54.4 mg GAE/g in the blackberry whole seeds collected from Scenic Fruit Co. (Gresham, OR) extracted in methanol (Bushman et al., 2004). The highest TPC in the broccoli whole seed was found in a cultivar named Viola, which was obtained from Thompson & Morgan Ltd. Poplar Lane, Ipswich (England, United Kingdom) extracted with 70% methanol at 70 °C (Pérez-Balibrea et al., 2011). TPC of milk thistle seed flour and whole seed were 25.22 and 29 mg GAE/g, respectively (Mhamdi et al., 2016; Parry et al., 2008). The seed flour was collected from Botanic Oil Innovations Inc. (Spooner, WI) extracted with 50% acetone (v/v), while the whole seeds were from Amdoun region, North Western of Tunisia.

Tocopherols

Tocopherol is one of the two groups of vitamin E compounds. The other group is tocotrienol. Both tocopherols and tocotrienols have the similar activity to that of α-tocopherol. The difference between tocopherols and tocotrienols is that tocopherols are 2-methyl-2(4',8',12'-trimethyltridecyl)chroman-6-ols with saturated side chains, while tocotrienols have unsaturated side chains with three conjugated double bonds at positions 3', 7', and 11'. Tocopherols are the major compounds in foods having the activity of vitamin E. They are the derivatives of tocol with one or more methyl groups at different positions. The α, β, δ, and γ are four forms of tocopherols and tocotrienols classified by the number and position of the methyl groups. And different structures confer different vitamin E activities with α-tocopherol

exhibiting the highest of that (Damodaran, Parkin, & Fennema, 2008).

Both tocopherols and tocotrienols are nonpolar and mainly exist in the lipid phase of foods. When they are not esterified, they possess the antioxidant property, which is the ability to eliminate free radicals by donating an electron and H^+. It's well known that tocopherols are important components of all the biological membranes. And they make contributions to the stability of the membrane due to their antioxidant activity (Damodaran et al., 2008). In the vegetable and fruit seeds, tocopherols and tocotrienols act as antioxidants to protect the unsaturated oils as well.

Since tocopherols are fat-soluble vitamins, it will mostly in the seed oil. The existence of tocopherol can protect the unsaturated fatty acids from oxidation. While γ-tocopherol has a better free radical scavenging capacity, α-tocopherol owns a better singlet oxygen trapping capacity and a higher vitamin E bioactivity (Fazio et al., 2013). Researches about the tocopherol content in different seed oils have been conducted. After knowing the fat content of the seed flour, the kinds and even the contents of tocopherols in the seed flour can be inferred from the tocopherol content of seed oil.

Table 1.4. Tocopherol contents of the selected seed oils (mg/100 g oil)

	α-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherol	Reference
Blackberry	1.6 – 20.5	8.5 – 131.2	3.10 – 82.32	10.2 – 205.4	(Bushman et al., 2004; Hoed et al., 2009; Parry, 2006; Radočaj, Vujasinović, Dimić, & Basić, 2014; Xu, Zhang, Chen, & Tu, 2006)
Broccoli				0.013 – 0.022*	(Chuanphongpanich, Suttajit, Phanichphant, Buddhasukh, & Sirithunyalug, 2006)
Cucumber	0.4	7.5	91.3	108.6	(Matthaus, Vosmann, Pham, & Aitzetmüller, 2003)
Milk thistle	4.76 – 46.51	0 – 5.21	0 – 8.05	4.95 – 63.22	(Fathi-Achachlouei & Azadmard-Damirchi, 2009; Gruszka & Kruk, 2007; Meddeb, Rezig, Abderrabba, Lizard, & Mejri, 2017; Parry et al., 2006)

*Tocopherol contents of the whole seed (mg/100 g dry seeds).

Tocopherol contents of blackberry, broccoli, cucumber and milk thistle seed oils are shown in Table 1.4. Tocopherol contents of blackberry seed oil were reported in several literatures. And it appeared that the contents varied depended on the cultivar, oil extraction method, etc. The highest α-tocopherol concentration in the blackberry seed oil was 20.5 mg/100 g oil, which was from a cultivar called *Kiowa* extracted by petroleum ether (Xu et al., 2006). The highest concentration of γ-tocopherol in the blackberry seed oil was 131.2 mg/100 g oil. This concentration appeared in the cold-pressed seed oil grown in the Washington State, US (Hoed et al., 2009). Radočaj and others reported the highest concentrations of δ- and total tocopherol in the blackberry seed oil, which were 82.32 and 205.43 mg/100 g oil, respectively (Radočaj et al., 2014). This blackberry seed was a cultivated variety named *Čačanska beztrna* grown in the US.

Only the total tocopherol concentration in the broccoli seed was reported (Chuanphongpanich et al., 2006). Five different broccoli seed cultivars which were all grown in Thailand were ground by a pestle and mortar and then extracted by a reagent described as follows: 50 mL of ethanol, 10 mL of 40% potassium hydroxide, and 10 mL of 0.1% ascorbic acid solution to prevent the tocopherols from oxidation. Then the whole solution was extracted by n-hexane. The organic phase in the extract was removed by evaporation before entering a chromatographic system to measure the tocopherol content. It turned out that a cultivar named "Top Green #067" had a relatively high total tocopherol concentration, which was 0.022 mg/100 g dry weight.

The tocopherol content of the cucumber seed oil was obtained by using petroleum ether to extract the cucumber seed purchased from a typical Vietnamese market. The total tocopherol concentration turned out to be 108.6 mg/100 g oil with δ-tocopherol being a major part of it, which was 91.3 mg/100 g oil. However, δ-tocopherol possesses a relatively low bioactivity compared to the other tocopherols (Matthaus et al., 2003).

The tocopherol content of the milk thistle seed oil has been reported by many authors. The highest concentrations of α-, δ- and total tocopherol were all reported by Fathi-Achachlouei and others, which were 46.51, 8.05 and 63.22 mg/100 g oil, respectively. And they belonged to the same seed cultivar called "*Khoreslo*", which was grown in Ardabil, Iran. The seed was extracted by the mixture of hexane and isopropanol (3:2, v/v) to obtain the oil sample (Fathi-Achachlouei & Azadmard-Damirchi, 2009). Then the tocopherol content was analyzed by HPLC. The highest γ-tocopherol concentration in the milk thistle seed was 5.21 mg/100 g oil. This was actually the concentration of a γ- and β-tocopherol mixture because a C_{18} column was used in the experiment and it was unable to separate β-tocopherol from γ-tocopherol. However, β-tocopherol is a minor component in most oils, which means it has a trivial contribution to the concentration of γ-tocopherol. In this experiment, tocopherols in the milk thistle seed oil, which was purchased from a local market in Poland, was extracted with acetonitrile-methanol-water mixture (72:8:1, v/v) to determine the concentration (Gruszka & Kruk, 2007).

Oil Content

The oil contents of blackberry, broccoli, carrot, cucumber and milk thistle seeds are listed in Table 1.5. Most seeds have a big range of oil content depending on the cultivar, extraction methods and growing conditions.

The blackberry seed has the oil content ranging from 4.81 to 18.17%. The lowest oil content was reported in a cultivar named Kiowa grown in Beijing, China. The seed was dried and ground. Then the seed oil was extracted with petroleum ether (Xu et al., 2006). The highest oil content of blackberry seed was reported by Fang and others. A hybrid cultivar named Young which was grown in Jiangsu, China, was dried, ground, and extracted with petroleum ether to obtain the oil content of 18.17% (Fang, Wu, Zhao, Lv, & Li, 2012).

The broccoli seed also showed a large range of oil content varying from 9.4 to 28.1%. The seed of a variety of broccoli grown in Mexico was extracted with chloroform/methanol (2:1, v/v). It yielded the lowest oil content, which was 9.36% (López-Cervantes et al., 2013). Another cultivar named Pirate possessed a much higher oil content of 28.1% when extracted with the same reagent (West, Tsui, Balch, Meyer, & Huth, 2002).

Topkafa (2016), and Özcan and Chalchat (2007) reported the oil content of carrot seed as 7.5 and 7.9%, respectively. Their seeds were both purchased from Konya, Turkey. The relatively low oil content was obtained through cold press procedure, while the relatively high one was

obtained with petroleum ether using Soxhlet extractor (Musa Özcan & Chalchat, 2007; Topkafa, 2016).

Table 1.5. Oil contents of the selected fruit and vegetable seeds

Seed	Oil content (g/100 g)	References
Blackberry	4.81 – 18.17	(Fang et al., 2012; Fazio et al., 2013; Xu et al., 2006)
Broccoli	9.4 – 28.1	(López-Cervantes et al., 2013; West et al., 2002)
Carrot	7.5 – 7.9	(Musa Özcan & Chalchat, 2007; Topkafa, 2016)
Cucumber	26.6 – 33.2	(Kaymak, 2012; Matthaus et al., 2003)
Milk thistle	16.1 – 32.7	(Afshar, Chaichi, Assareh, Hashemi, & Liaghat, 2014; Çelik & Gürü, 2015; Dabbour, Al-Ismail, Takruri, & Azzeh, 2014; Fathi-Achachlouei & Azadmard-Damirchi, 2009; Harrabi, Romdhane, Daassa, & Fellah, 2015; Keshavarz Afshar et al., 2015; Koláčková, Růžičková, Gregor, & Šišperová, 2015)

The oil content of the cucumber seed was 26.6 – 33.2%. The lowest oil content was yielded from a species called "Beith Alpha F1" extracted in chloroform/methanol (2:1, v/v) (Kaymak, 2012), while the highest content was from a species grown in Vietnam extracted in petroleum ether (Matthaus et al., 2003).

The oil content of milk thistle seed has been reported by many researchers and the results varied a lot. Among all the results, the milk thistle seed from Turkey, whose oil was extracted with

the supercritical CO_2 at 40 °C, 180 bar, 4 mL/min, had the highest oil content of 32.7% (Çelik & Gürü, 2015).

Fatty Acid Compositions

Fatty acids are not only the one of the major dietary energy materials, but also important components of human body cell membranes. They can be classified in several ways, one of which is by the saturation level. The fatty acids with no double bonds are called saturated fatty acids, while those with one or more than one saturated double bonds are named as monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), respectively. Different kinds of fatty acids have been found in the seed oils, but they mostly have the carbon chain lengths of 16, 18, and 20. Two essential fatty acids (EFAs) found in the seed oils are linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) (Parry, 2006). Linoleic acid is an omega-6 fatty acid and α-linolenic acid is an omega-3 fatty acid. Human bodies are not able to synthesize these EFAs using other components absorbed from daily diet. Therefore, they have to be taken from the diet.

The omega-3 fatty acids possess the ability to prevent many chronical diseases, such as coronary heart disease and type 2 diabetes (Simopoulos, 1999). They play important roles in vision development and cerebral cortex operation as well. Omega-3 fatty acids deficiency will cause vision impairment, abnormal electroretinograms and polydipsia. The first two diseases are caused by the abnormal biochemistry of the retina, which to be specific, is the decrease of

docosahexaenoic acid (22:6n-3) and the increase of n-6 fatty acids, particularly docosapentaenoic acid (22:5n-6) in the retinal phospholipids at the same time. The similar biochemical abnormalities can happen in the brain as well, which would result in the polydipsia (Connor, Neuringer, & Reisbick, 1992). Linoleic acid (18:2n-6), which is a dietary omega-6 fatty acid, has the potential to lower the plasma total cholesterol and the low-density lipoprotein (LDL) cholesterol, which are both coronary heart disease risk factors (Wijendran & Hayes, 2004). It was reported that not only the clearance rate but also the production rate of LDL was declined when saturated fatty acids were replaced by linoleic acid (Brousseau, Stucchi, Vespa, Schaefer, & Nicolosi, 1993).

Both linoleic acid and α-linolenic acid need to be metabolized *in vivo* to achieve some bioactivities. Linoleic acid will be converted into arachidonate acid (20:4n-6) by Δ6 and Δ5 desaturase and elongase. After that, arachidonate acid can either be converted by cyclooxygenases into prostaglandins and thromboxanes, which possess the ability to mediate inflammation, vascularization and tumor promotion, or be converted by lipoxygenases into hydroxyeicosatetraenoates (HETEs) then into leukotrienes which participate in immune response and inflammation (Belury, 2002). α-Linolenic acid will be converted into eicosapentaenoic acid (EPA) (20:5n-3) by Δ6 and Δ5 desaturase and elongase. EPA can be converted by cyclooxygenase and 5-lipoxygenase to 3-series prostanoids such as TXA_3, PGE_3 and PGI_3 and 5-series leukotrienes i.e. LTB_5, LTC_5 and LTE_5. These chemicals are able to reduce the proinflammatory activity *in vivo* (Mickleborough, Ionescu, & Rundell, 2004). It's

also possible that EPA is further converted by elongase, Δ6 desaturase and then through β-oxidation turned into docosahexaenoic acid (DHA) (22:6n-3) (Calder 2004). DHA exerts positive effects on many diseases including hypertension and thrombosis along with immunoregulatory activity and anticancer activity (Horrocks & Yeo, 1999; Hung et al., 1999; Jakobsen et al., 2008).

The ratio of n-6/n-3 fatty acids is also quite important apart from the intakes of these two fatty acids individually. Nowadays, the n-6/n-3 fatty acid ratio in the western diet reaches up to 16.7/1, which is exceedingly high compared to the recommended ratio range from 1/1 to 4/1 (Simopoulos, 2002, 2006). Researchers found that an extremely high n-6/n-3 fatty acid ratio could increase the risk of cardiovascular diseases, prostate cancer, inflammation and cause a stronger immunosuppressive effect. (Grimm et al., 1994; Kalogeropoulos et al., 2010; Riediger et al., 2008; Williams et al., 2011). Some of the seed oils have a relatively ideal n-6/n-3 fatty acid ratio, such as the cranberry seed oil and boysenberry seed oil, which can balance the n-6/n-3 fatty acid ratio of the present western diet if they are consumed reasonably (Parry, 2006).

Oleic acid (18:1n-9) is another major component in seed oils. Chan and others have reported that oleic acid was effective in decreasing the concentrations of total cholesterol, LDL cholesterol and apolipoprotein. And there was no significant difference between the hypocholesterolemic abilities of linoleic acid, linolenic acid and oleic acid when the same amount of saturated fatty acids was replaced by them (Chan, Bruce, & McDonald, 1991;

Gardner & Kraemer, 1995). Besides, it has been reported that oleic acid, as a MUFA, is able to lower the susceptibility of LDL oxidation when being a major part of the LDL compared to the LDL with high content of SUFA (Castro et al., 2000; Parry, 2006; Ramírez-Tortosa, Aguilera, Quiles, & Gil, 1998).

Table 1.6. Fatty acid compositions of the selected seed oils (g/100 g)

	Blackberry	Broccoli	Carrot	Cucumber	Milk thistle	References
C14:0	0.00 – 0.05	0.05 – 0.12	0.3	0.07	0.1	(Hoed et al., 2009; Matthaus et al., 2003; Parry et al., 2006; Radočaj et al., 2014; Topkafa, 2016; West et al., 2002)
C16:0	2.59 – 7.79	3.87 – 7.43	3.7 – 10.2	11.26 – 13.65	5.5 – 12.7	(Fang et al., 2012; Fazio et al., 2013; Harrabi et al., 2015; Kaymak, 2012; López-Cervantes et al., 2013; Matthaus et al., 2003; Meddeb et al., 2017; Topkafa, 2016; West et al., 2002; Yetim, Sagdic, & Ozturk, 2008)
C16:1	0.08 – 0.22	0.31 – 0.54	0.2 – 0.64	0.058 – 0.148	0.10 – 0.16	(Dabbour et al., 2014; Fazio et al., 2013; Harrabi et al., 2015; Kaymak, 2012; Musa Özcan & Chalchat, 2007; Radočaj et al., 2014; Topkafa, 2016; West et al., 2002)
C18:0	1.16 – 2.99	0.64 – 1.10	0.40 – 2.41	6.93 – 10.41	2.9 – 7.6	(Fang et al., 2012; Fazio et al., 2013; Kaymak, 2012; López-

						Cervantes et al., 2013; Malekzadeh, Mirmazloum, Mortazavi, Panahi, & Angorani, 2011; Matthaus et al., 2003; Meddeb et al., 2017; Parker, Adams, Zhou, Harris, & Yu, 2006; West et al., 2002; Yetim et al., 2008)
C18:1	9.41 – 26.48	10.06 – 15.44	60.07 – 82.08	10.98 – 18.62	15.50 – 36.67	(Fang et al., 2012; Fazio et al., 2013; Kaymak, 2012; López-Cervantes et al., 2013; Malekzadeh et al., 2011; Matthaus et al., 2003; Meddeb et al., 2017; Musa Özcan & Chalchat, 2007; Parker et al., 2006; West et al., 2002)
C18:2	48.76 – 69.28	11.31 – 18.15	11.8 – 13.3	54.32 – 69.88	39.7 – 60.8	(Fang et al., 2012; Fazio et al., 2013; Gao, Yang, & Birch, 2016; Kaymak, 2012; López-Cervantes et al., 2013; Malekzadeh et al., 2011; Musa Özcan & Chalchat, 2007; Parry et al., 2006; West et al., 2002)
C18:3	9.70 – 31.55	12.37 – 13.10	0.20 – 0.48	0.37	0.17 – 0.76	(Çelik & Gürü, 2015; Dabbour et al., 2014; Fang et al., 2012;

						Gao et al., 2016; López-Cervantes et al., 2013; Matthaus et al., 2003; West et al., 2002; Xu et al., 2006; Yetim et al., 2008)
C20:0	0.13 – 0.85	0.40 – 0.41	0.1 – 0.8	0.37 – 0.70	1.6 – 4.3	(Fang et al., 2012; Fathi-Achachlouei & Azadmard-Damirchi, 2009; Fazio et al., 2013; Harrabi et al., 2015; Kaymak, 2012; Matthaus et al., 2003; Musa Özcan & Chalchat, 2007; Topkafa, 2016; West et al., 2002)
C20:1	0.10 – 0.68	4.64 – 6.25	0.7	0.07	Trace – 0.95	(Çelik & Gürü, 2015; Fazio et al., 2013; López-Cervantes et al., 2013; Matthaus et al., 2003; Radočaj et al., 2014; Topkafa, 2016; West et al., 2002)
C20:4	-	0.14 – 40.87	0.2	-	-	(López-Cervantes et al., 2013; Topkafa, 2016; West et al., 2002)

C22:0	0.04	0.56 – 0.57	0.1	0.06	0.9 – 2.9	(Fathi-Achachlouei & Azadmard-Damirchi, 2009; Fazio et al., 2013; Matthaus et al., 2003; Meddeb et al., 2017; Topkafa, 2016; West et al., 2002)
C22:1	-	47.38 – 48.41	-	-	-	(West et al., 2002)
C24:0	-	0.34 – 0.35	0.2	0.16	0.31 – 0.92	(Harrabi et al., 2015; Matthaus et al., 2003; Meddeb et al., 2017; Topkafa, 2016; West et al., 2002)
SAFA	4.12 – 11.03	8.53	4.52 – 10.61	19.09 – 19.44	13.8 – 22.8	(Fang et al., 2012; Fathi-Achachlouei & Azadmard-Damirchi, 2009; Fazio et al., 2013; Kaymak, 2012; López-Cervantes et al., 2013; Parker et al., 2006; Parry et al., 2006; Yetim et al., 2008)
MUFA	9.49 – 26.80	20.08	81.5 – 82.1	11.03 – 13.98	16.2 – 29.7	(Fang et al., 2012; Fathi-Achachlouei & Azadmard-Damirchi, 2009; Fazio et al., 2013; Gao et al., 2016;

						Kaymak, 2012; López-Cervantes et al., 2013; Meddeb et al., 2017; Parker et al., 2006)
PUFA	62.17 – 83.27	71.39	12.5 – 13.8	-	49.9 – 61.1	(Fang et al., 2012; Fathi-Achachlouei & Azadmard-Damirchi, 2009; Fazio et al., 2013; Gao et al., 2016; López-Cervantes et al., 2013; Parry et al., 2006; Topkafa, 2016)
n-6/n-3	1.64 – 5.92	-	47.11	-	-	(Fang et al., 2012; Parker et al., 2006)

- Not detected.

SAFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. n-6/n-3: ratio of n-6 to n-3 fatty acids.

As is shown in Table 1.6, blackberry seed, as one of the berry seeds, has a high content of PUFA, which include the essential fatty acids (Hoed et al., 2009). From all the data reported by other researchers, the range of PUFA content was 62.17 – 83.27%. And it appeared that the two essential fatty acids, C18:2 (linoleic acid) and C18:3 (linolenic acid) were two major components of PUFA. The highest C18:2 content was found in a cultivar named "Hull", while the highest C18:3 content was found in "Young". These two cultivars were both grown in Jiangsu, China and extracted with diethyl ether by Soxhlet method (Fang et al., 2012). Following the contents of linoleic acid and linolenic acid, C18:1 had the third highest content in the blackberry seed, which was 9.41 – 26.48%. The highest one was obtained from the wild blackberry bushes in Cosenza, Italy (Fazio et al., 2013). Blackberry seed oil possessed a good ratio of n-6/n-3 ranging from 1.64 – 5.92, which almost completely fell into the recommended ratio range. And a relatively low ratio of n-6/n-3 can decrease the risk of the prostate cancer and coronary heart disease (Aronson et al., 2001; Connor, 2000). Although no experiment has been done on the fatty acid composition of blackberry seed flour, what kinds of fatty acids left in the seed flour can be reflected by those of the seed oil.

There was a great difference between two reports about the fatty acid composition of broccoli seed oil. West and others found the major component of broccoli seed oil was C22:1, especially C22:1ω-9 (erucic acid). The erucic acid content was 45.56 or 46.89% depending on the extraction methods (West et al., 2002). Erucic acid was suggested to possess cardiotoxic properties (Tvrzicka, Kremmyda, Stankova, & Zak, 2011). When rats were fed with oil

containing a large amount of erucic acid, their myocardial fatty acids increased dramatically (Beare-Rogers & Gordon, 1976). Humans who had adrenoleukodystrophy were treated with a erucic acid-rich diet and they turned out to have low platelet counts and bleeding diathesis (Stöckler et al., 1997). Therefore, it seems that broccoli seed oil is not a good choice as dietary oil based on the results of West and others. However, López-Cervantes and others found that C20:4 (arachidonic acid) was the predominant fatty acid in the broccoli seed oil, which took up to 40.87% (López-Cervantes et al., 2013), while the content of erucic acid was not reported. Arachidonic acid is rich in human immune cells typically. The intake of arachidonic acid can alter the fatty acid composition of immune cell membrane, whose change will affect T cell signaling pathways and antigen presentation capability (Calder, 2008). Besides, arachidonic acid is also a precursor to prostaglandins and leukotrienes, which will be released to respond the hormone stimulation or during the inflammatory response (Carrier III et al., 2011). The results of López-Cervantes and others showed that the content of arachidonic acid experienced a sharp drop during the germination (López-Cervantes et al., 2013). Hence, the great differences between the fatty acid compositions of these two seed oils might be caused by not only the cultivars and extraction methods, but also the status of the seeds.

It can be seen in Table 1.6 that carrot seed oil was largely composed of C18:1 (60.07 – 82.08%). The highest C18:1 content was reported by Parker and others, which belonged to the cold-pressed carrot seed oil provided by an American oil company (Parker et al., 2006). The lowest C18:1 content was from local herbal and vegetable suppliers in Konya, Turkey, extracted with

petroleum ether using a Soxhlet extractor, which, however, was still the major component of the carrot seed oil (Musa Özcan & Chalchat, 2007). Therefore, carrot seed oil is a good source of monounsaturated fatty acids.

The fatty acid profiles of cucumber seed oil and milk thistle seed oil were similar to each other (Table 1.7). Cucumber seed oil was rich in C18:2 (54.32 – 69.88%), followed by C18:1 (10.98 – 18.62%). For the milk thistle seed oil, C18:2 accounted for 39.7 – 60.8% and C18:1 took up 15.50 – 36.67% which was the second large portion. Blackberry seed oil also had the similar fatty acid composition, but cucumber and milk thistle seed oils were both short in C18:3 compared to that of blackberry. However, cucumber and milk thistle seed oils were still rich sources of the essential fatty acid.

Parry and others measured the oil content of milk thistle seed flour. After cold-press process, there was still 7.5 g oil/100 g left in the seed flour. The seed flour oil was composed of 37.6% linoleic acid, 27.4% palmitic acid, 17.7% oleic acid and other fatty acids. Besides, saturated fatty acids took up 54.8% while monounsaturated fatty acids and polyunsaturated fatty acids accounted for 40.6 and 4.6%, respectively. In 2006, Parry and others had analyzed the fatty acid composition of milk thistle seed oil with GC-FID. The result is shown in Table 1.7 (Parry et al., 2008; Parry & Yu, 2006).

Table 1.7. Fatty acid composition of milk thistle seed oil and flour lipid (g/100 g FA)

	seed oil	seed flour lipid
14:0	0.1	0.5
16:0	8.9	27.4
16:1	0.1	0.2
18:0	4.8	17.7
18:1	23.8	37.6
18:2	60.8	4.6
18:3	0.2	0
20:1	1.2	2.8
SFA	13.8	54.8
MUFA	25.2	40.6
PUFA	61.1	4.6

From Table 1.7, it is easy to tell that great changes of the fatty acid composition of oil in the milk thistle seed took place after the oil extraction. The percentage of polyunsaturated fatty acids decreased dramatically, which was mainly caused by the drop of linoleic acid (C18:2n-6) from 60.8 to 4.6%. The proportions of saturated fatty acids and monounsaturated fatty acids both increased with the original proportions of 13.8 and 25.2%, respectively. By the comparison of fatty acid composition of milk thistle seed before and after oil extraction, it appeared that although the kinds of fatty acids were almost the same, the proportion changed,

which might also apply to the fatty acid composition of other seed oils.

Health Beneficial Properties

Antioxidant

In the past, the term antioxidant referred to all substances that inhibited the oxidation reactions no matter what kind of mechanism is involved. More recently, antioxidants in the food sector usually apply to those compounds that interrupt the free-radical chain reaction involved in lipid oxidation and those that scavenge singlet oxygen (Damodaran et al., 2008). If a compound is to be defined as an antioxidant, it has to meet two criteria. First, when the compound is present at low concentrations compared to those of an oxidizable substrate, it should be able to significantly delay or prevent oxidation of that substrate. Second, the radical generated from the reaction ought to be relatively stable instead of being a chain-propagating radical (Croft, 1998; Halliwell, Aeschbach, Löliger, & Aruoma, 1995). As is known to all, oxidation reactions result in damages in many body tissues, which would further lead to different kinds of diseases, including cancers, Alzheimer's disease, liver disease, Parkinson's disease, cardiovascular disease and diabetes (Albano, 2008; Beal, 2003; Higashi, Noma, Yoshizumi, & Kihara, 2009; Moon & Shibamoto, 2009; Paz-Elizur et al., 2008; Rains & Jain, 2011; Smith, Rottkamp, Nunomura, Raina, & Perry, 2000; VIDELA et al., 2004; X. Wang et al., 2014). Therefore, the importance of antioxidants is quite obvious. Phenolics are the main components that possess antioxidant activities in food (Roginsky & Lissi, 2005). Apart from this, vitamin E and C are also common natural antioxidants which are proved to be capable of preventing the diseases

mentioned above (Moon & Shibamoto, 2009).

Hydroxyl Radical Scavenging Activity

According to the Fenton's reaction, hydroxyl radicals would be produced by hydrogen peroxide in the presence of ferrous ions. This reaction is of importance in terms of biology since hydroxyl radicals, which is highly reactive, may lead to the strand breakage of supercoiled deoxyribonucleic acid (DNA) (Shahidi, Alasalvar, & Liyana-Pathirana, 2007). Hydroxyl radical could lower the activity of brain glutamine synthetase (GS) under iron-mediated oxidative stress significantly as well. And the inactivation of GS happens in several neurodegenerative disorders such as Alzheimer's disease (Fernandes, Dringen, Lawen, & Robinson, 2011). Besides, hydroxyl radical induced cell damage in liver (Chen, Ye, Ji, & Liu, 2010). Apart from all those mentioned above, this radical could cause protein oxidation which results in the conformation modification of proteins. For instance, the eye lens proteins, crystallins, would cross link and gather together when oxidatively modified, leading to the lens opacification or cataracts (Guptasarma, Balasubramanian, Matsugo, & Saito, 1992). However, the phenolic compounds in these seed flour possess hydroxyl radical scavenging activity because of two possible ways. One is neutralizing hydrogen peroxide to water by donating hydrogen while the other one is chelating the ferrous ion. (de Camargo, Regitano-d'Arce, Gallo, & Shahidi, 2015; Wettasinghe & Shahidi, 2000).

Oxygen Radical Absorbing Capacity

Like hydroxyl radicals, peroxyl radicals are reactive oxygen species as well. They contribute to tissue cell damage, cardiovascular disease, cancer development and inflammation. The seed flour extracts with high phenolics content are promising in preventing oxidative-related diseases (Ayoub et al., 2016).

Reducing the Risk of Cancers

Anti-proliferation

The anti-proliferative activities of broccoli, carrot and cucumber seed flour extracts were tested by Choe and others using LNCaP prostate cancer cells. They found that all three seed flour extracts inhibited the proliferations of LNCaP prostate cancer cells after 48 h. Carrot seed flour extract showed the greatest anti-proliferative capacity among them, which was 46.2% at the concentration of 0.1% (v/v) for 96 h. Broccoli and cucumber seed flour extracts exhibited the anti-proliferative activities of 20.0 and 19.2% at the same concentration for 96 h, respectively (Choe et al., 2018).

The anti-proliferative effect of milk thistle seed flour extract was determined by using HT-29 human colon cancer cells. Compared to the control, milk thistle seed flour extract at 3 and 6 mg flour equivalents/mL media final concentrations inhibited the cell growth by 76.6 and 95.8%, respectively, which was in a dose-dependent way (Parry et al., 2008). Silymarins, as major components in the milk thistle seed flour extract, are a series of polyphenols who provide

anti-proliferative effects against different cancer cell lines, including prostate (Deep, Singh, Agarwal, Kroll, & Agarwal, 2006), colon (Yang, Lin, Chen, & Chiu, 2003), and lung (R. P. Singh et al., 2006) cancer cells.

Anti-inflammation

Fruit and vegetable seeds possess the anti-inflammatory properties, which is of great importance. Coussens and others wrote that inflammation was a critical contributor to tumor progression since the microenvironment of tumor was manipulated by inflammatory cells (Coussens & Werb, 2002). Fazio and others tested the in-vitro anti-inflammatory properties of the methanolic extracts of wild blackberry seed. They found that the blackberry seed extract decreased the release of nitric oxide, which is a late inflammatory marker formed during the inducible nitric oxide synthase (iNOS) activation, in a concentration-dependent way. When the concentration of blackberry seed extract reached 50 μg/mL, nearly 60% of the NO was inhibited. The effects of the extract on macrophage-inflammatory protein-3α/C-C motif ligand 20 (CCL20), which is a chemokine playing an important role in immune and inflammatory response (Varesio, Battaglia, Raggi, Ledda, & Bosco, 2010). CCL20 production was also decreased by blackberry seed extract in a concentration-dependent way. There was an over 90% inhibition when the concentration was 50 μg/mL (Fazio et al., 2013).

Choe and others reported that broccoli seed flour extract had a strong anti-inflammatory capacity which expressed in the way that 38.9% Interleukin-1β(IL-1β) mRNA expression was

inhibited compared to the lipopolysaccharide(LPS)-induced control. However, carrot and cucumber seed flour extracts only had the inhibition of 31.3 and 16.3%, respectively. IL-1β is a pro-inflammatory cytokine participating in regulating several physiological center nervous system (CNS) processes such as sleep and appetite regulation. It is also of great importance to the neutral immune responses to the damage and infection happened to tissues. Therefore, once the peripheral administration of IL-1β malfunctions, many symptoms concerning the operation of CNS such as fever, excess sleep will break out (Hansen, Taishi, Chen, & Krueger, 1998). Besides, broccoli seed flour extract showed a inhibition of 33.8% on the cyclooxygenase-2 (COX-2) mRNA expression while those of carrot and cucumber showed none (Choe et al., 2018). COX-2 can be induced by inflammation, which will lead to the release of prostanoids. Prostanoids are substances which sensitize peripheral nociceptor terminals, leading to the localized pain hypersensitivity. Then the neuronal excitability will be increased in the spinal cord as well, which contributes to the pain hypersensitivity in nearby uninjured tissue (Samad et al., 2001).

Vasudevan and others used three rat paw edema models induced by carrageenan, histamine and serotonin, respectively, to evaluate the acute anti-inflammatory ability of the ethanolic extract of defatted carrot seeds, which were purchased from a market in Haryana, India in January, 2005. The formaldehyde-induced arthritis rat model was used to measure the chronic anti-inflammatory ability. The study indicated that both 200 and 400 mg/kg body weight of the carrot seed ethanolic extract could effectively suppressed the paw edema induced by

carrageenan, histamine and serotonin. And 400 mg/kg body weight of the extract could inhibit the edema induced by formaldehyde successfully in a 10-day length (Vasudevan, Gunnam, & Parle, 2006). This formaldehyde-induced arthritis model was very suitable for the anti-inflammatory test because it closely resembles human arthritis (Greenwald, 1991).

Milk thistle seed possessed anti-inflammatory properties due to its capacity to scavenge free radical, which are those acting as pro-inflammatory agents. One of the important active substance in the milk thistle seed extract was silymarin. Silymarin was actually a mixture, mainly composed of three flavonolignans, which were silybin, silidianin, and silychristine (Dixit, Baboota, Kohli, Ahmad, & Ali, 2007). Silymarin and silibinin impeded the inflammatory process by preventing neutrophil migration and Kupffer cell, viz. stellate macrophages and Kupffer-Browicz cells inhibition. Besides, they inhibited the formation of prostaglandins and leukotrienes, which were both inflammatory mediators and prevented the release of histamine from basophils. Through the mechanisms mentioned above, milk thistle seed possesses anti-inflammation activity (Bhattacharya, 2011).

Diabetes

Several fruit and vegetable seeds have exhibited the anti-diabetic activity. Zhang and others used the diabetic mouse model induced by intravenous injection of alloxan to measure the anti-diabetic activity of the blackberry seed flour, which came from defatting the seeds purchased in Henan, China. The defatted seeds were extracted by petroleum ether, ethyl acetate and *n*-

butanol in turn. They found all three extracts could raise the hepatic glycogen content compared to the control, which means that the chemical components in the extracts could promote the synthesis of hepatic glycogen while hindering the degradation. These three extracts lowered the levels of triglyceride (TG) and total cholesterol (TCH) in serum of the diabetic mice. In this way, the severities of hyperlipidemia, one of the common diabetic complications, and lipid metabolism disorder were reduced. Besides, the petroleum ether extract and ethyl acetone extract raised the level of superoxide dismutase (SOD) and lowered the content of malonaldehyde (MDA) in serum, which would enhance the antioxidant capacity of the diabetic mice (Zhang, Yin, & Kang, 2014).

The findings of Ranjbar and others showed that 300 mg/kg body weight of carrot seed methanolic extract could decrease the serum glucose levels and increase the insulin serum levels significantly. The following histological study also indicated that the dose of 100 mg/kg of the extract improved the pancreas acini and islets significantly (Banafsheh Ranjbar, Pouraboli, Mehrabani, & Dabiri, 2010). In another study of Ranjbar and others, the results showed that in the streptozocin-induced diabetic rats, serum levels of triglycerides, total cholesterol, and LDL cholesterol all decreased and the serum level of high-density lipoprotein (HDL) cholesterol increased after treated with methanolic extract of carrot seed at 300 mg/ kg body weight In this case, the carrot seeds were purchased from a herbal store in Kerman, Iran (Banafshe Ranjbar, 2015).

The streptozotocin-induced diabetic rats were used to test the effect of cucumber seeds extract on the blood glucose level by Minaiyan and others. The cucumber seeds were obtained from a market from Isfahan, Iran and extracted with 75% ethanol (v/v) and butanol, respectively. The results indicated that both hydroalcoholic and butanolic extracts of cucumber seeds decreased the blood glucose levels of diabetic rats and improved the body weights compared to the controls after 9 days of treatments (0.2, 0.4, 0.8 g/kg body weight). However, the two extracts only showed effects during the sub-acute phase (9-day treatment), while there was no significant effect during the acute phase (first day treatment) (Minaiyan, Zolfaghari, & Kamal, 2011).

The milk thistle seed flour was fed to the alloxan-induced diabetic rabbits by Shakeel and others. Results showed that 500 mg oral administration of milk thistle seed flour for three times per day could recover the fasting blood glucose level and daily mean blood glucose to normal after 30 days treatment (Shakeel & Yar, 2014). The anti-diabetic activities were also tested on humans. Huseini and others reported that one group which received 200 mg milk thistle seed extract three times per day along with the conventional therapy for 4 months had significant decreases in fasting blood glucose level, glycosylated hemoglobin level, total cholesterol, LDL cholesterol and triglycerides in blood. On the contrary, the other group which received placebo instead of milk thistle seed extract had significant increases in fasting blood glucose level and glycosylated hemoglobin level and no change in other indicators (Huseini et al., 2006). Silibinin, which is a major bioactive chemical compound in milk thistle seeds, was

administrated to streptozotocin-induced diabetic mice every day by intramuscular injection for eight weeks. More than 25 mg/kg body weight/day of silibinin significantly lower the blood glucose compared to the diabetic control. 50 mg/kg body weight/day of silibinin attenuated the increase of glucosylated hemoglobin A1C, serum triglyceride, and serum cholesterol caused by diabetes. Silibinin of the same dosage increased serum HDL cholesterol level of the diabetic mice as well. Besides, the findings indicated that silibinin suppressed the levels of apoptosis and autophagy in pancreatic β-cells (Q. Wang et al., 2012).

Hepatoprotective Activities

Carrot seed possessed hepatoprotective activities. Thioacetamide was used to induce oxidative stress on rats and then increase the levels of some liver enzymes, including serum glutamic pyruvic transaminase, alkaline phosphatase and serum glutamic-oxaloacetic transaminase. Findings showed that the administration of methanolic carrot seed extract significantly suppressed the rises of these enzymes compared to the thioacetamide-induced control, which indicated the membrane stabilizing ability of carrot seed extract. In addition to that, the seed extract was also effective in increasing the levels of some antioxidant enzymes, including SOD, catalase and glutathione reductase (K. Singh, Singh, Chandy, & Manigauha, 2012). The findings of Rezaei-Moghadam and others showed the hepatoprotective activity of carrot seed extract as well. The ethanolic extract of carrot seeds was able to stimulate SOD, catalase and glutathione peroxidase and reduce malondialdehyde contents in the liver tissue at the same time (Rezaei-Moghadam et al., 2012).

Milk thistle seed extract was proved to possess hepatoprotective activities as well. El-Adawi and others found that the treatment of silymarin, the extract of milk thistle seeds, could alter down the serum activity of alanine aminotransferase, which was raised by fumonisin B1 before in rats (El-Adawi, El-Azhary, El-Shafeey, & Abdel-Mohsen, 2011). Shaker and others used the carbon tetrachloride-induced liver damage in rat model. The results indicated that both ethyl acetate extract and ethanol extract of milk thistle seed could significantly decrease in the serum glutamic-oxaloacetic transaminase, serum glutamic pyruvic transaminase, and alkaline phosphatase compared to carbon tetrachloride-induced control. And the stabilization of these enzymes was the proof of improvements of liver functions (Shaker, Mahmoud, & Mnaa, 2010). Amiridumari and others measured the hepatoprotective activity of milk thistle seeds on the broilers fed with aflatoxin B1. The levels of alanine amino-transaminase and aspartate amino-transferase were brought down by the three-week treatment of milk thistle seeds compared to the aflatoxin B1 control(Amiridumari, Sarir, Afzali, & FaniMakki, 2013). The elevation of these two liver enzymes in the control could be caused by the change in hepatic cell membrane permeability (Ozer, Ratner, Shaw, Bailey, & Schomaker, 2008). Therefore, the decrease of liver enzymes was the indication of liver function recover.

Other Health Beneficial Properties

McWalter and others fed the $nrf2^{+/+}$ mice with a diet consisting of 15% (w/w) crushed broccoli seeds, it turned out that the activities of NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferase (GST) were increased in the liver, stomach and small intestine

(McWalter et al., 2004). The increased levels of these two enzymes could possibly render protection against benzo[a]pyrene, which is a chemical carcinogen (Bonnesen, Eggleston, & Hayes, 2001; Fahey et al., 2002).

Mani and others fed the ethanolic extract of defatted carrot seeds to rats with aging-, diazepam-, or scopolamine-induced amnesia for seven continuous days. Their findings suggested that carrot seeds were promising in improving memory (Mani, Parle, Ramasamy, & Majeed, 2010). The ethanolic extract of defatted carrot seeds in relatively high doses (200 and 400 mg/kg body wt.) could also inhibit the brain cholinesterase activity in both young and aged rats. The cholinesterase would lyse center cholinergic neurotransmitters which is associated with Alzheimer's Disease (Vasudevan & Parle, 2006). Therefore, the decrease of cholinesterase activity might improve the cognitive functions.

Chapter II: Chemical Compositions of Cold-Pressed Blackberry, Broccoli, Carrot, Cucumber and Milk Thistle Seed Flours

Abstract

Flours of blackberry, broccoli, carrot, cucumber and milk thistle seeds were examined for their chemical compositions. All four samples were extracted with 100% ethanol using a Soxhlet extractor. A total of 11, 8, 10 and 13 compounds were detected in the blackberry, broccoli, carrot and milk seed flour extracts, respectively. Ellagic acid, disinapoylgentiobiose, kaempferol-3-*O*-rutinoside isomers and silychristin isomers are the primary component(s) in the blackberry, broccoli, carrot and milk thistle seed flour extracts, respectively. However, no chemical compounds at a detectable level was found in the cucumber seed flour extract. All those polyphenols, flavonoids and other bioactive compounds found in the seed flour extracts could endow the flours with antioxidant properties.

Introduction

Growing scientific evidence supports that phenolic compounds are one of the factors confers fruits and vegetables with many health benefits, including antioxidant activities, vision improvement abilities, cardiovascular diseases prevention, cancer prevention and more (Benvenuti, Pellati, Melegari, & Bertelli, 2004; Canter & Ernst, 2004; Chong, Macdonald, & Lovegrove, 2010; Scalbert & Williamson, 2000; Yi, Fischer, Krewer, & Akoh, 2005; Zheng & Wang, 2003). Although some of the fruit and vegetables have been well studied, such as

blackberry and milk thistle, less attention has been paid on their seeds, let alone the seed flour. And since many fruit and vegetables have high contents of phenolic compounds, it is reasonable to assume that their seeds also possess high phenolic contents. Therefore, identifying the chemical compounds in the seed flours can help people know more about the health beneficial properties of them and make the best of them by turning these seed flours from a leftover of seed oil extraction into food ingredient with high values.

The present study investigated the chemical compositions of blackberry (*Rubus fruticosus* L.), broccoli (*Brassica oleracea* L. var. italica), carrot (*Daucus carota*), cucumber (*Cucumis sativus*) and milk thistle (*Silibum marianum*) seed flours. The results may be used to elaborate the mechanisms of the health beneficial properties possessed by these seed flours and be helpful to discover new properties.

Materials and Methods

Materials One sample of each blackberry, broccoli, carrot, cucumber and milk thistle seed flour samples were provided by Botanic oil innovation Inc. (Spooner, WI, USA). These fruit and vegetable seed flours were the solid cakes from the cold-pressing process. Gallic acid and Folin & Ciocalteu's phenol reagent (FC) were purchased from Sigma-Aldrich (St. Louis, MO, 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). The other chemicals were the highest commercial grade and used without additional purification.

Seed Flour Extraction and Sample Preparation 1.5 grams of each ground seed flour sample was weighted accurately, extracted with 15 mL of 100% ethanol using Soxhlet extractor for 3 hours, and filtered through Whatman No.1 filter paper. The filtrate was transferred to a 25 mL volumetric flask, diluted with 100% ethanol to volume, and mixed. All experiments were performed in triplicate.

Chemical Compositions Identification Using 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 reported (Choe et al., 2018) before, using an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with an Agilent 1290 Infinity liquid chromatography linked with a DAD detector.

The scanning range of the UV-vis spectrum was 190 − 600 nm. The separations were carried out on a 4.6 mm i.d. × 250 mm, and 5 μm particle size Luna C18 column. HPLC-grade water containing 0.1% formic acid (v/v) was used as solvent A. Acetonitrile containing 0.1% formic acid (v/v) was used as solvent B. The gradient elution started with 5% of solvent B. Then solvent B was increased to 13% at 5 min through a linear gradient followed by being increased to 20% at 10 min. After that solvent B was increased to 27% at 25 min, followed by increasing to 33% at 40 min. Then solvent B was increased to 50% at 45 min and increased to 90% at 46 min. 90% of solvent B was kept until 51 min and then the 10 min post-run time was carried out for re-equilibration. The flow rate was 1.0 mL/min. The injection volume was 5 μL. And the oven temperature was set at 40 °C. The HRMS was set at a negative ionization mode together with optimized parameters shown below: capillary temperature at 325 °C, capillary voltage at −50 V, spray voltage at 4.5 kV, and the tube lens offset voltage at −120 V. The mass range was between m/z 100 and 2000 with the resolution of 30000. The data were then post-processed with QualBrowser part of Thermo Scientific Xcalibur 2.2 software. The concentrations of the chemicals identified in the milk thistle seed flour extract were determined. Chlorogenic acid and silibinin were used as standards. The standard curve was derived from the peak area of different concentrations of the standard.

Total Phenolic Contents The 100% ethanol seed flour extracts were analyzed for total phenolic contents following a previously described procedure using the Folin-Ciocalteu (FC) reagent with some adjustments (Stevanato, Fabris, & Momo, 2004). The final reaction mixture contained 50 μL seed flour extract, or standard, or solvent (blank), with 250 μL FC reagent, 750 μL 20% sodium carbonate, and 3.0 mL ultrapure water. After 2 hours of reaction at ambient temperature in the dark, the absorbance at 765 nm was measured to calculate the total phenolic contents of samples with gallic acid as the standard. Experiments were taken in triplicate.

Statistical Analysis Data were reported as mean ± standard deviation (SD) for each point. IBM SPSS Statistics (Version Rel. 22.0.0.0, IBM Inc., Armonk, NY) was used to identify the differences among means. A one-way analysis for variation (ANOVA) was applied for comparison. Statistical significance was declared at $P < 0.05$.

Results and Discussion

Chemical Composition of the Blackberry Seed Flour

Figure 2.1 showed the UHPLC chromatogram of the blackberry seed flour extract. The peak at 15.54 min in Figure 2.1 had a $[M-H]^-$ at m/z 300.9986 (Figure 2.2A), corresponding to the molecular formula of $C_{14}H_6O_8$ (1 ppm), which almost suggested the formula of ellagic acid.

The precursor ion yielded characteristic MS/MS fragments at m/z 257.0700 and 229.0322

(Figure 2B) which was the same to others' reports (J.-H. Lee, Johnson, & Talcott, 2005; Mullen, Yokota, Lean, & Crozier, 2003). Therefore, the peak at 15.54 min was believed to be ellagic acid (Figure 2.4A).

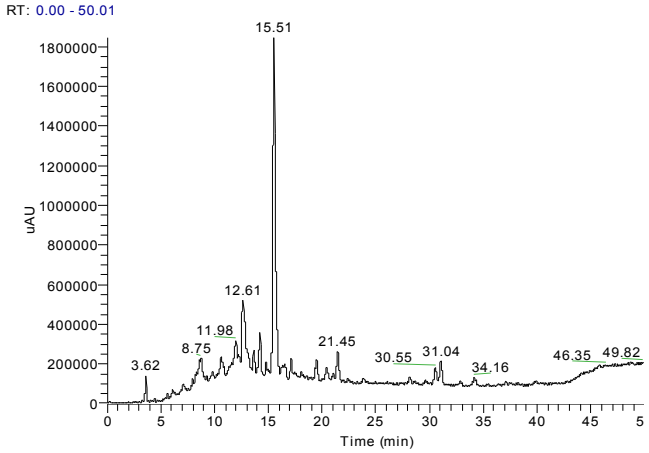
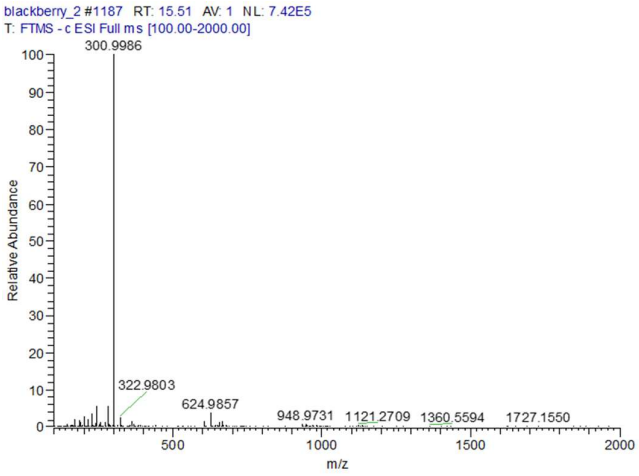


Figure 2.1. Typical UHPLC chromatogram of the blackberry seed flour extract detected at 348 nm

The peaks at 11.70 min, 12.06 min and 12.60 min in Figure 1 all had the same $[M-2H]^{2-}$ at m/z 934.0692 (Figure 2.3A), which gave the corresponding molecular formula of $C_{82}H_{54}O_{52}$ (2 ppm).

A)



B)

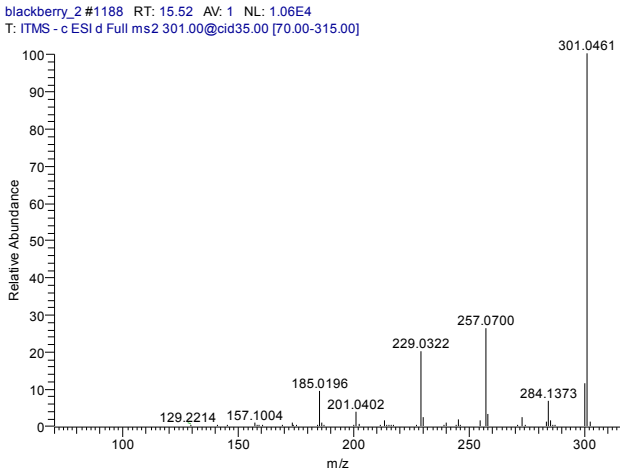
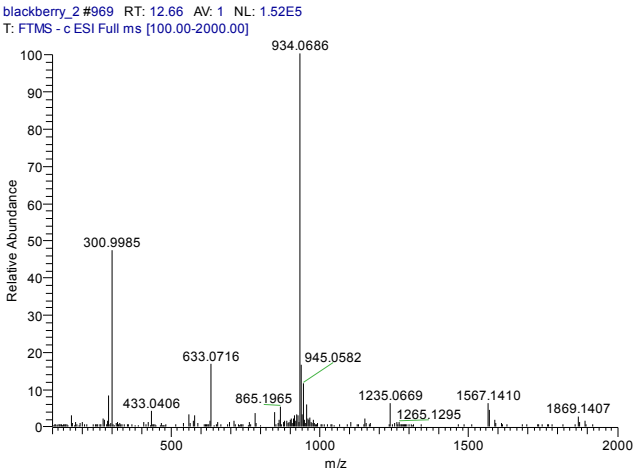


Figure 2.2. A) MS spectrum and B) MS/MS spectrum of ellagic acid

These double-charged ions were further fragmented into a series of single-charged products (Figure 2.3B). The products at m/z 1566.9896 were generated by the loss of a hexahydroxyphenoyl (HHDP) group. Then the products at m/z 1265.1393 were generated by further losing another HHDP group. Based on that, the loss of HHDP-galloyl-glucose yielded

products at m/z 633.1483. All these three fragments were characteristic fragments of sanguiin H6, matching others' reports (Aaby, Ekeberg, & Skrede, 2007; Buendia et al., 2009; Mullen et al., 2003). Therefore, this compound was tentatively identified as sanguiin H6 (Figure 2.4B).

A)



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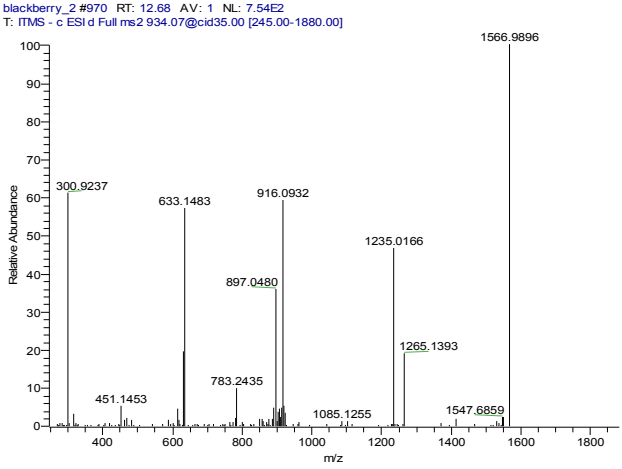


Figure 2.3. A) MS spectrum and B) MS/MS spectrum of sanguiin H-6

A) B)

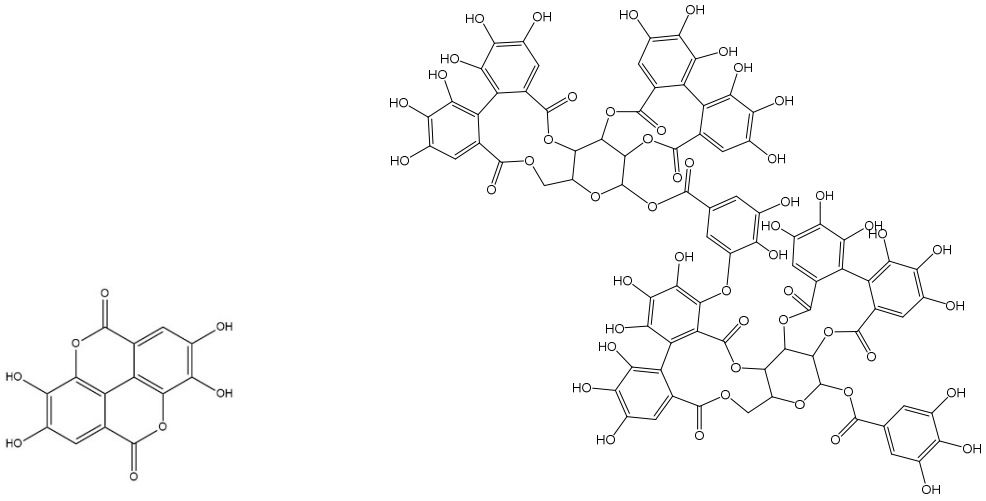


Figure 2.4. Structures of A) ellagic acid, and B) sanguiin H-6

There are eleven chemical compounds found in the blackberry seed flour in total, which include hexahydroxydiphenic acid hexoside, pedunculagin isomers, procyanidin B1, sanguiin H6 isomers, ellagic acid pentoside isomers and ellagic acid (Figure 2.1 and Table 2.1).

Hager and others used a mixed solution of acetone/water/acetic (70:29.5:0.5, v/v/v) to extract the blackberry seed. Then a high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) was used to detect the ellagitannins in the blackberry seed. A total of eleven ellagitannins have been found, including pedunculagin isomer, castalagin/vescalagin isomer, galloyl-HHDP glucose isomer, lambertianin C isomer, sanguiin H-6/lambertianin A, lambertianin D isomer and galloyl-bis-HHDP glucose isomer (Hager, Howard, Liyanage, Lay, & Prior, 2008). Apart from ellagitannins, ellagic acid was also detected from the blackberry seed. Compared to the current study, pedunculagin isomer, sanguiin H-6

and ellagic acid were found in common.

Table 2.1. Characterization of compounds present in the blackberry seed flour extract by Soxhlet extraction

Peak ID	t_R (min)	Theoretical $[M-H]^-$	Experimental $[M-H]^-$	Chemical Formula	Tentatively Identification
1	3.60	481.0618	481.0613	$C_{20}H_{18}O_{14}$	Hexahydroxydiphenic acid hexoside
2	7.11	783.0681	783.0656	$C_{34}H_{24}O_{22}$	Pedunculagin isomer
3	8.32	783.0681	783.0657	$C_{34}H_{24}O_{22}$	Pedunculagin isomer
4	8.65	783.0681	783.0658	$C_{34}H_{24}O_{22}$	Pedunculagin isomer
5	10.74	577.1346	577.1339	$C_{30}H_{26}O_{12}$	Procyanidin B1[3]
6	11.70	934.0713[a]	934.0679[b]	$C_{82}H_{54}O_{52}$	Sanguiin H6 isomer
7	12.06	934.0713[a]	934.0756[b]	$C_{82}H_{54}O_{52}$	Sanguiin H6 isomer
8	12.60	934.0713[a]	934.0692[b]	$C_{82}H_{54}O_{52}$	Sanguiin H6 isomer[2]
9	13.69	433.0407	433.0405	$C_{19}H_{14}O_{12}$	Ellagic acid pentoside isomer
10	14.23	433.0407	433.0407	$C_{19}H_{14}O_{12}$	Ellagic acid pentoside isomer
11	15.53	300.9984	300.9983	$C_{14}H_6O_8$	Ellagic acid[1]

[a] Theoretical m/z values of $[M-2H]^{2-}$. [b] Experimental m/z values of $[M-2H]^{2-}$. [1,2,3]Represented the three greatest peaks based on the typical UHPLC chromatogram peak area.

Chemical Composition of the Broccoli Seed Flour

The UHPLC chromatogram of the broccoli seed flour extract was shown in Figure 2.5. The peak at 21.38 min had a $[M-H]^-$ at m/z 753. 2244 (Figure 2.6), which was corresponding to the molecular formula of $C_{34}H_{42}O_{19}$ (0 ppm).

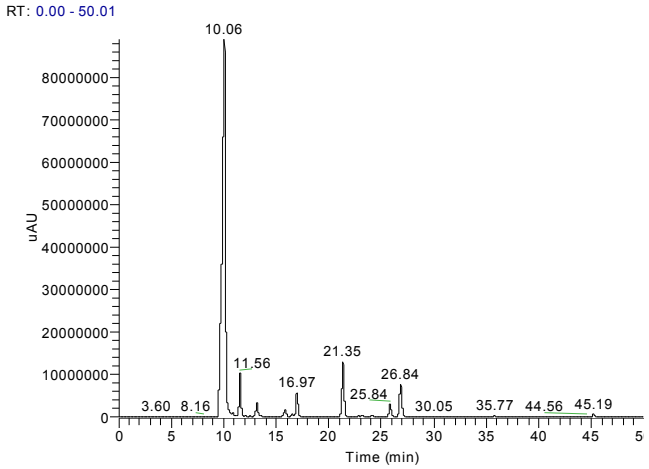


Figure 2.5. Typical UHPLC chromatogram of the broccoli seed flour extract detected at 348 nm

Fragmentation of this single-charged ion gave two single-charged diagnostic products at m/z 529.1564 and 223.0614. The first fragment was produced by the loss of a sinapinic acid from the single-charged parent ion, while the second one was generated by the losing a disaccharide gentiobiose from the precursor ion. Since these three characteristic ions were in accordance with the previous studies (Ferreres et al., 2007; Shao et al., 2014; Vallejo, Tomas-Barberan, & García-Viguera, 2003), the chemical at 21.38 min was provisionally identified as disinapoylgentiobiose (Figure 2.8A).

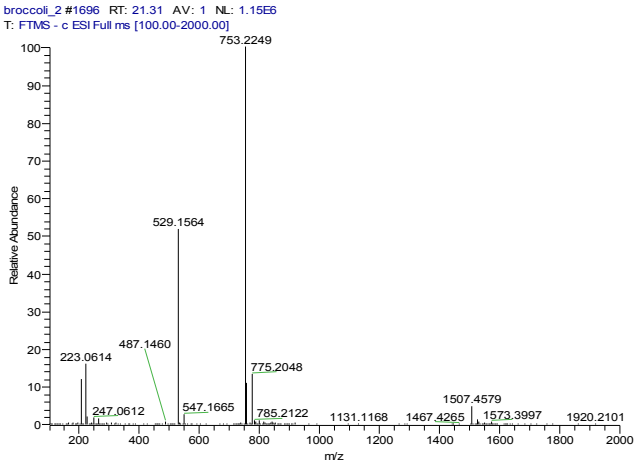


Figure 2.6. MS spectrum of disinapoylgentiobiose

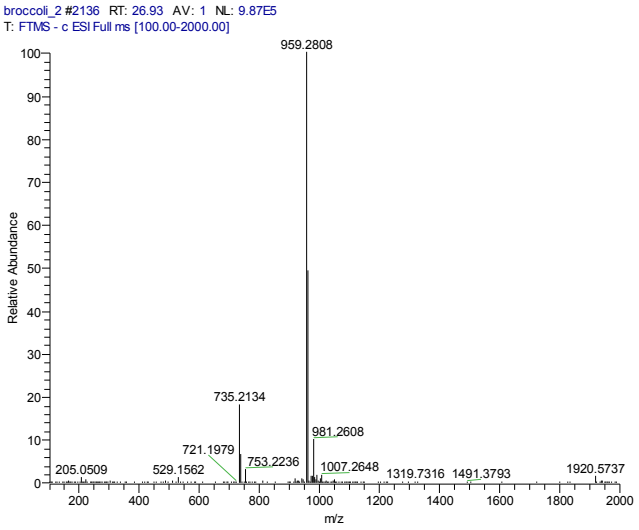


Figure 2.7. MS spectrum of 1,2,2'-trisinapoylgentiobioside

The peak at 26.86 min in Figure 2.5 had an $[M-H]^-$ at m/z 959.2808 (Figure 2.7), corresponding to the molecular formula of $C_{45}H_{52}O_{23}$ (1 ppm). From the mass spectrum in the negative ion mode, a daughter ion was seen at m/z 735.2134, which suggested the loss of a sinapinic acid

from the single-charged parent ion. The daughter ion in Figure 2.6 at m/z 529.1562 represented the loss of two sinapinic acids, while the other daughter ion at m/z 205.0509 was [sinapinic acid−H−18]$^-$. All these signature fragments were consistent with those of 1,2,2'-trisinapoylgentiobioside reported by previous researchers (Ferreres et al., 2009; Ferreres et al., 2006; Velasco et al., 2011). In addition to this, Choe and others have found 1,2,2'-trisinapoylgentiobioside in the broccoli seed flour (Choe et al., 2018). Therefore, the compounded detected was considered as 1,2,2'-trisinapoylgentiobioside (Figure 2.8B).

A) B)

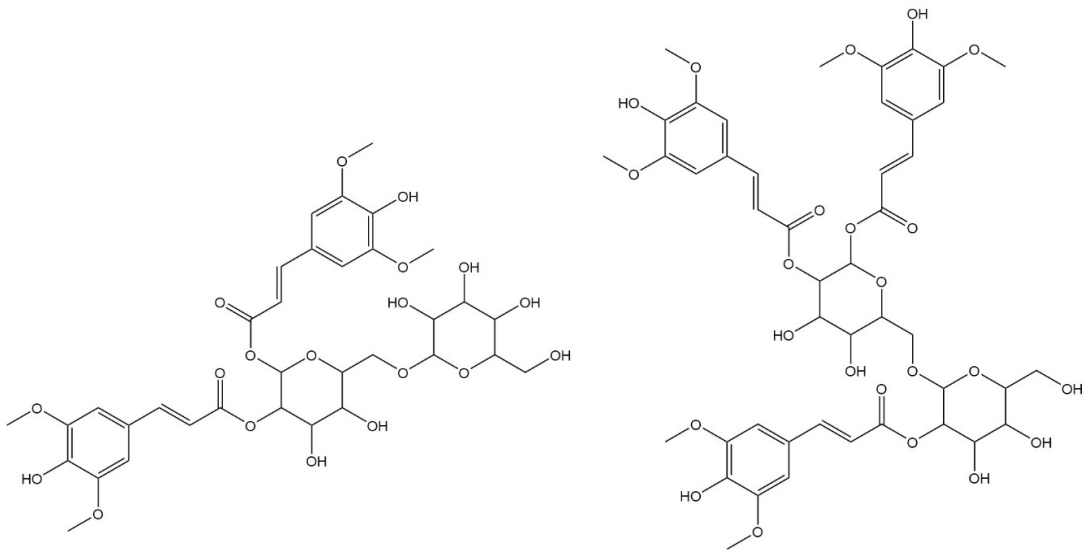


Figure 2.8. Structures of A) disinapoylgentiobiose and B) 1,2,2'-trisinapoylgentiobioside

Eight chemical compounds were provisionally identified in the broccoli seed flour, which included glucoraphanin isomers, glucoerucin, sinapoylhexose, disinapoylgentiobiose, 1,2-disinapoylglucoside and 1,2,2'-Trisinapoylgentiobiose (Figure 2.5 and Table 2.2).

Table 2.2. Characterization of compounds present in the broccoli seed flour extract by Soxhlet extraction

Peak ID	$t_{R\ (min)}$	Theoretical $[M-H]^-$	Experimental $[M-H]^-$	Chemical Formula	Tentatively Identification
1	3.62	436.0406	436.0407	$C_{12}H_{23}NO_{10}S_3$	Glucoraphanin isomer
2	3.99	436.0406	436.0408	$C_{12}H_{23}NO_{10}S_3$	Glucoraphanin isomer
3	4.22	436.0406	436.0400	$C_{12}H_{23}NO_{10}S_3$	Glucoraphanin isomer
4	9.87	420.0457	420.0457	$C_{12}H_{23}NO_9S_3$	Glucoerucin
5	11.61	385.1135	385.1136	$C_{17}H_{22}O_{10}$	Sinapoylhexose
6	21.38	753.2242	753.2244	$C_{34}H_{42}O_{19}$	Disinapoylgentiobiose[1]
7	25.89	591.1714	591.1708	$C_{28}H_{32}O_{14}$	1,2-disinapoylglucoside[*3]
8	26.86	959.2821	959.2809	$C_{45}\ H_{52}\ O_{23}$	1,2,2'-Trisinapoylgentiobioside[*2]

[1,2,3]Represented the three greatest peaks based on the typical UHPLC chromatogram peak area.

Compared to Choe and others' study, both analytical methods and the broccoli seed flour provider were the same as those in this study with the extraction methods being the only difference (Choe et al., 2018). Using sonication with 50% acetone (v/v) as extracting agent, Choe and others detected every compound identified in this study along with one more compound named quercetin-3-glucoside. And it was the first time that quercetin-3-glucoside was found in broccoli seed flour. The slight difference between these two studies' results could

be caused by the different extraction methods. McWalter and others extracted the defatted broccoli seed sample with 75% methanol (v/v) at 75 °C (McWalter et al., 2004). Then the extract was analyzed by liquid chromatography with triple quadrupole mass spectrometry detection. They have detected sinigrin, gluconapin, progoitrin, glucoiberin, glucoraphanin, glucoalyssin and gluconasturtiin in the defatted broccoli seed sample with glucoiberin being the primary component. The difference in the kinds of glucosinolate could be resulted from many factors, such as the differences in extraction methods, the cultivars of broccoli and the growing conditions.

Chemical Composition of the Carrot Seed Flour

The UHPLC chromatogram of the carrot seed flour extract was shown in Figure 2.9.

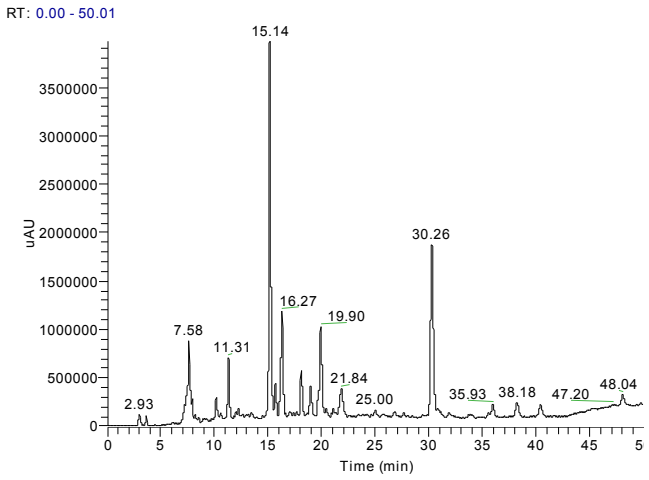
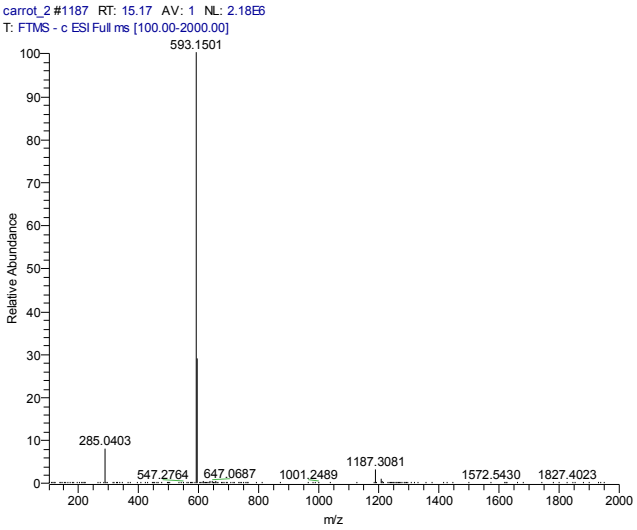


Figure 2.9. Typical UHPLC chromatogram of the carrot seed flour extract detected at 348 nm

The peaks at 11.38 min and 15.17 min in Figure 2.9 both had an $[M-H]^-$ at m/z 593.1505 (Figure 2.10A), which was in accordance with the molecular formula of $C_{27}H_{30}O_{15}$ (1 ppm).

The MS/MS spectrum (Figure 2.10B) of the precursor deprotonated molecule displayed a diagnostic product ion at m/z 285.1403, confirming the presence of the aglycon skeleton, which was kaempferol. Another diagnostic fragment ion displayed in the MS/MS spectrum was at m/z 447.1625, which was generated by the loss of rhamnose moiety from the disaccharide rutinose. These signature fragments were in accordance with the MS spectrums of kaempferol-3-*O*-rutinoside reported by others (De Leo, De Abreu, Pawlowska, Cioni, & Braca, 2010; Falcão et al., 2013; Inbaraj, Lu, Kao, & Chen, 2010). Therefore, the peaks at 11.38 min and 15.17 min were provisionally considered as kaempferol-3-*O*-rutinoside (Figure 2.12A) or its isomers.

A)



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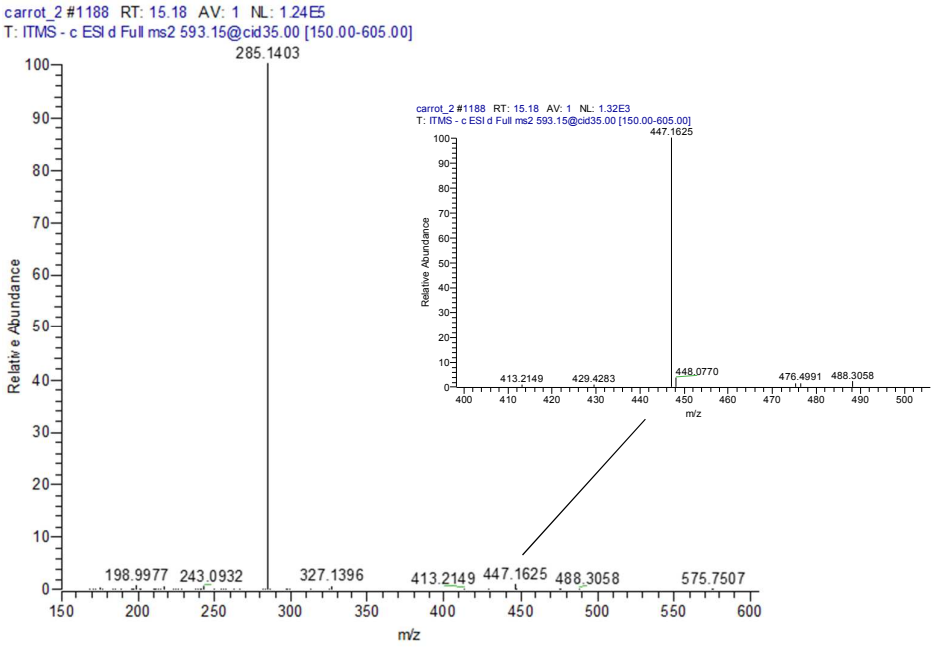
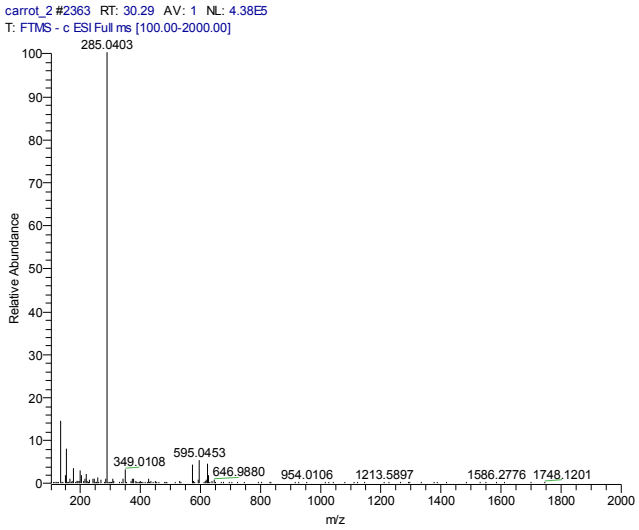


Figure 2.10. A) MS spectrum and B) MS/MS spectrum of kaempferol-3-*O*-rutinoside

A)



B)

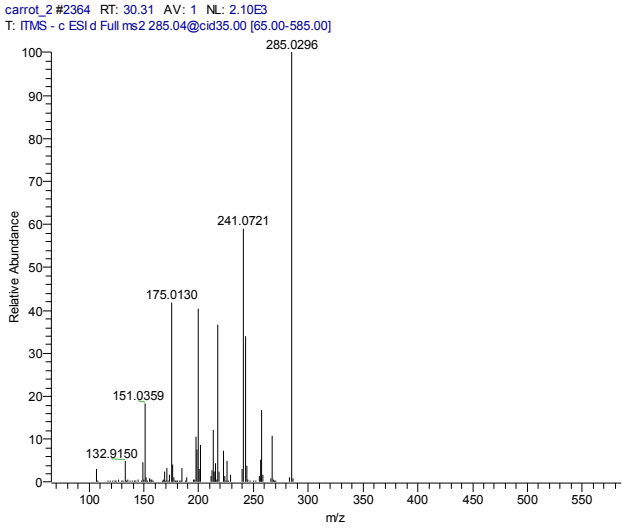


Figure 2.11. A) MS spectrum and B) MS/MS spectrum of luteolin

The chemical that had a retention time of 30.26 min had an $[M-H]^-$ at m/z 285.0402 (Figure 2.11A), which was in accordance with the molecular formula of $C_{15}H_{10}O_6$ (0 ppm). The MS/MS spectrum (Figure 2.11B) of this compound shared some common diagnostic ions with the

MS/MS breakdown spectrums of luteolin obtained by others at m/z 241, 175, 151 and 133 (De la Torre-Carbot et al., 2005; Ferreira, Quye, Hulme, & McNab, 2003). Hence, this compound was tentatively identified as luteolin (Figure 2.12B). And the fragment ion at m/z 151 could be the break-down of C ring in this flavone.

A) B)

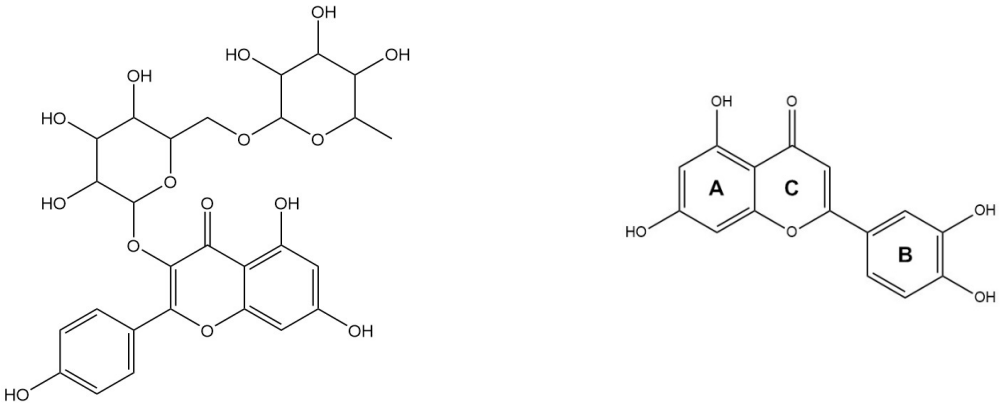


Figure 2.12. Structures of A) kaempferol-3-*O*-rutinoside, and B) luteolin

Ten chemical compounds were tentatively identified in the carrot seed flour, including caffeoyldihexoside, cistanoside F, lycibarbarphenylpropanoid C, kaempferol-3-*O*-rutinoside isomer, kaemprefol-3-*O*-glucoside isomers, apigenin-7-*O*-β-D-rutinoside, diosmetin-7-rutinoside, and luteolin (Figure 2.9 and Table 2.3). This result was in accordance with the finding of Choe and others (Choe et al., 2018).

Table 2.3. Characterization of compounds present in the carrot seed flour extract by Soxhlet extraction

Peak ID	$t_{R\ (min)}$	Theoretical $[M-H]^-$	Experimental $[M-H]^-$	Chemical Formula	Tentatively Identification
1	7.60	503.1401	503.1400	$C_{21}H_{28}O_{14}$	Caffeoyldihexoside
2	7.80	487.1452	487.1452	$C_{21}H_{28}O_{13}$	Cistanoside F
3	8.00	517.1557	517.1558	$C_{22}H_{30}O_{14}$	Lycibarbarphenylpropanoid C
4	11.38	593.1506	593.1505	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*-rutinoside isomer
5	15.17	593.1506	593.1501	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*-rutinoside isomer[1]
6	16.27	447.0927	447.0932	$C_{21}H_{20}O_{11}$	Kaempferol-3-*O*-glucoside isomer[3]
7	18.08	577.1557	577.1558	$C_{27}H_{30}O_{14}$	Apigenin-7-*O*-β-D-rutinoside
8	18.95	607.1663	607.1661	$C_{28}H_{32}O_{15}$	Diosmetin-7-rutinoside
9	19.90	447.0927	447.0940	$C_{21}H_{20}O_{11}$	Kaempferol-3-*O*-glucoside isomer
10	30.26	285.0399	285.0402	$C_{15}H_{10}O_6$	Luteolin[2]

[1,2,3]Represent the three greatest peaks based on the typical UHPLC chromatogram peak area.

Chemical Composition of the Cucumber Seed Flour

No chemical compounds in the detectable amount have been found in the 100% ethanol extraction of cucumber seed flour extract under the experiment conditions in this study (Figure 2.13). And the result was the same when the extracting agent was 50% acetone (v/v) (Choe et al., 2018). There were two reasons that might lead to the result: firstly, chemical compounds had relatively low concentrations in the cucumber seed flour, which made them harder to be detected; secondly, 100% ethanol was not a perfect solvent for extracting chemicals in the cucumber seed flour because 100% ethanol has weak polarity.

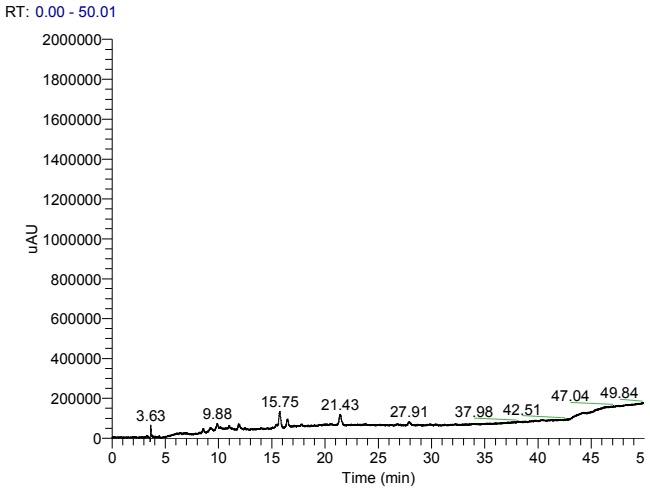


Figure 2.13. Typical UHPLC chromatogram of cucumber seed flour extract detected at 348 nm

Chemical Composition of the Milk Thistle Seed Flour

Figure 2.14 showed the typical UHPLC chromatogram of the milk thistle seed flour extract.

The peaks at 13.90 min and 15.32 min had the same $[M-H]^-$ at m/z 661.1764 in Figure 2.14, which was corresponding to the molecular formula of $C_{31}H_{34}O_{16}$ (1 ppm). The precursor ion yielded a product ion at m/z 499.1457 (Figure 2.15A), which indicated the loss of a caffeoyl group.

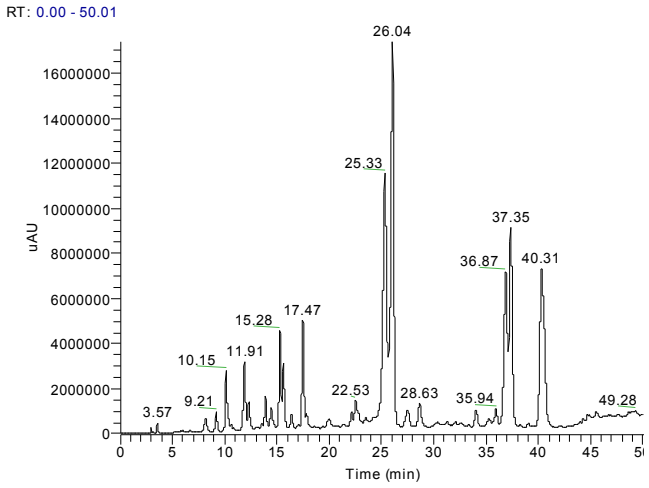
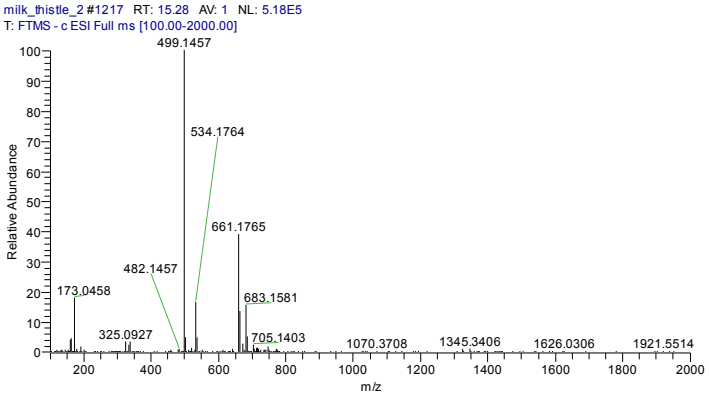


Figure 2.14. Typical UHPLC chromatogram of milk thistle seed flour extract detected at 348 nm

The ion at m/z 325.0728 could be generated by the further loss of a quinic acid from [M-H-caffeoyl]$^-$. Another product ion in the MS/MS spectrum (Figure 2.15B) at m/z 337.1077 could be resulted from the loss of a glucosyl group from [M-H-caffeoyl]$^-$. Therefore, the compounds at 13.90 min and 15.32 min were tentatively identified as 5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid (Figure 2.16).

A)



B)

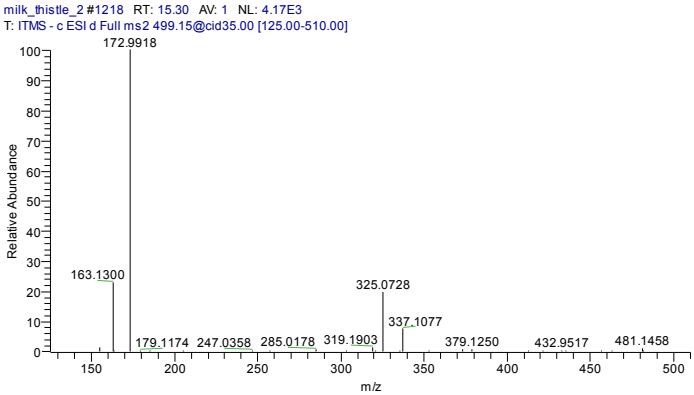


Figure 2.15. A) MS spectrum and B) MS/MS spectrum of 5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid

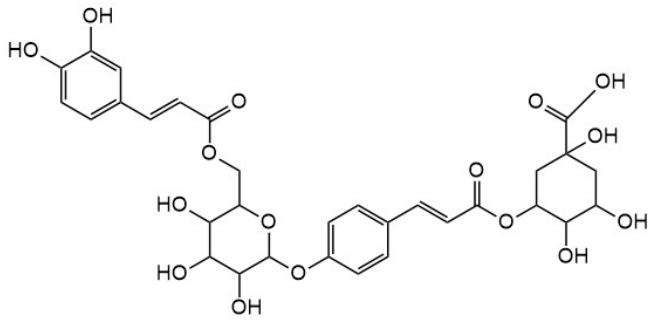
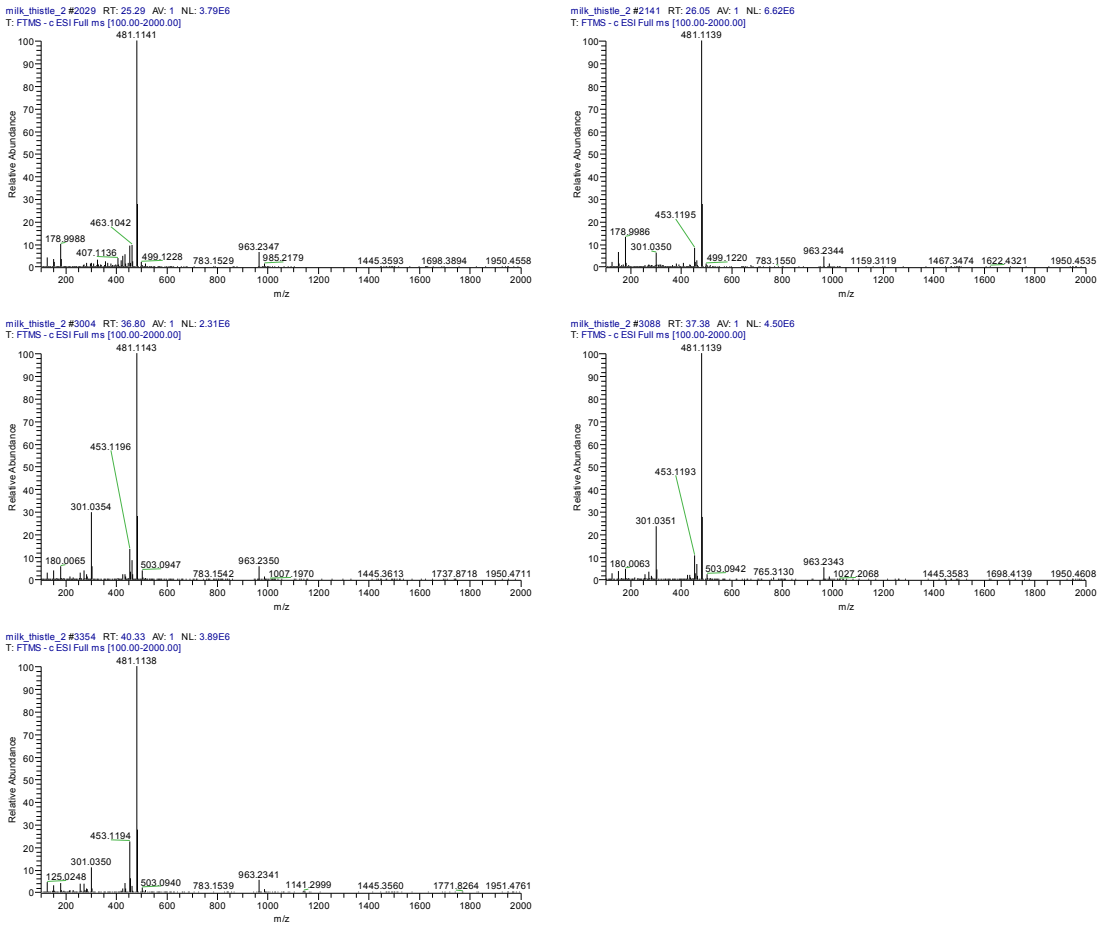


Figure 2.16. The structure of 5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid

The peaks at 25.33, 26.04, 36.78, 37.35 and 40.31 min had the $[M-H]^-$ ranging from m/z 481.1138 to 481. 1143 (Figure 2.17A), which were all in accordance to the same formula of $C_{25}H_{22}O_{10}$ (0 ppm).

A)



B)

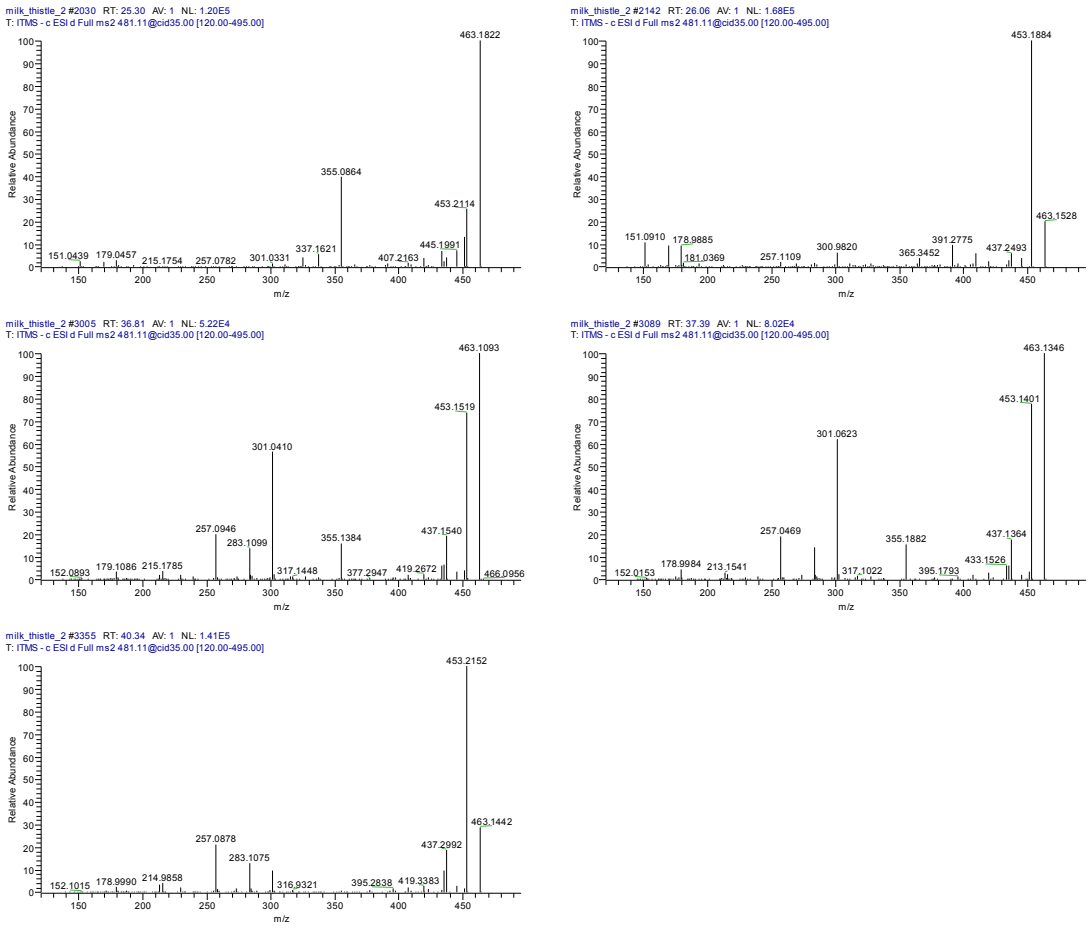
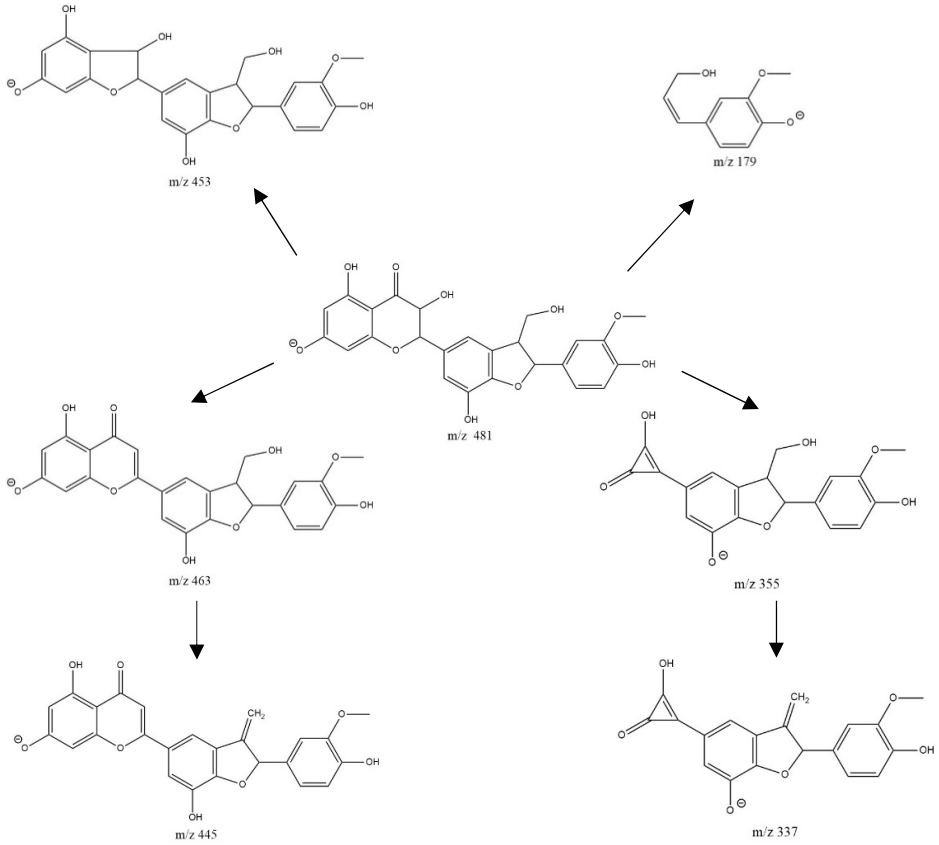


Figure 2.17. A) MS spectrum and B) MS/MS spectrum of silychristin isomers

The shared product ions observed in these five peaks were at m/z 463 $[M-H-H_2O]^-$ and 453 $[M-H-CO]^-$. Some other major product ions were observed at m/z 437, 355, 301, 257 and 179 in the MS/MS spectrum (Figure 2.17B). All these diagnostic fragment ions were reported by other researchers when HPLC-MS was used to detect and differentiate silymarins in the milk thistle extract and the possible fragmentation pathways were shown in Figure 2.18 (J. I. Lee, Hsu, Wu, & Barrett, 2006; Shibano, Lin, Itokawa, & Lee, 2007). Hence, these five peaks were

tentatively identified as silymarins, including silychrisin, silybin, isosilybin or silydianin (Figure 2.19), which were a group of isomers.



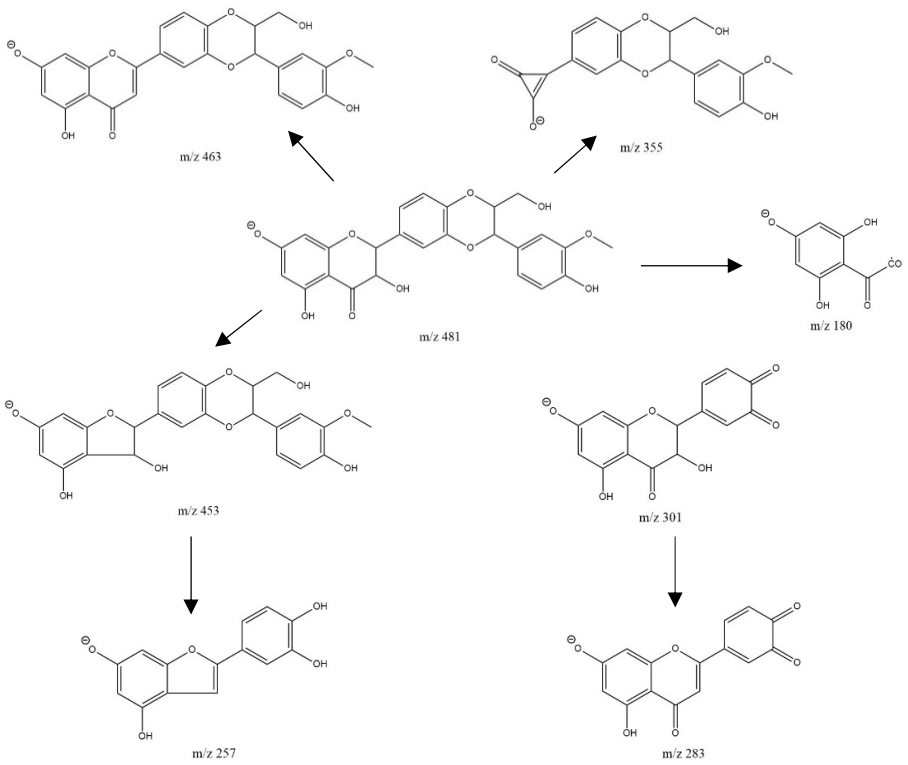


Figure 2.18. Possible fragmentation pathways of the silychristin isomers

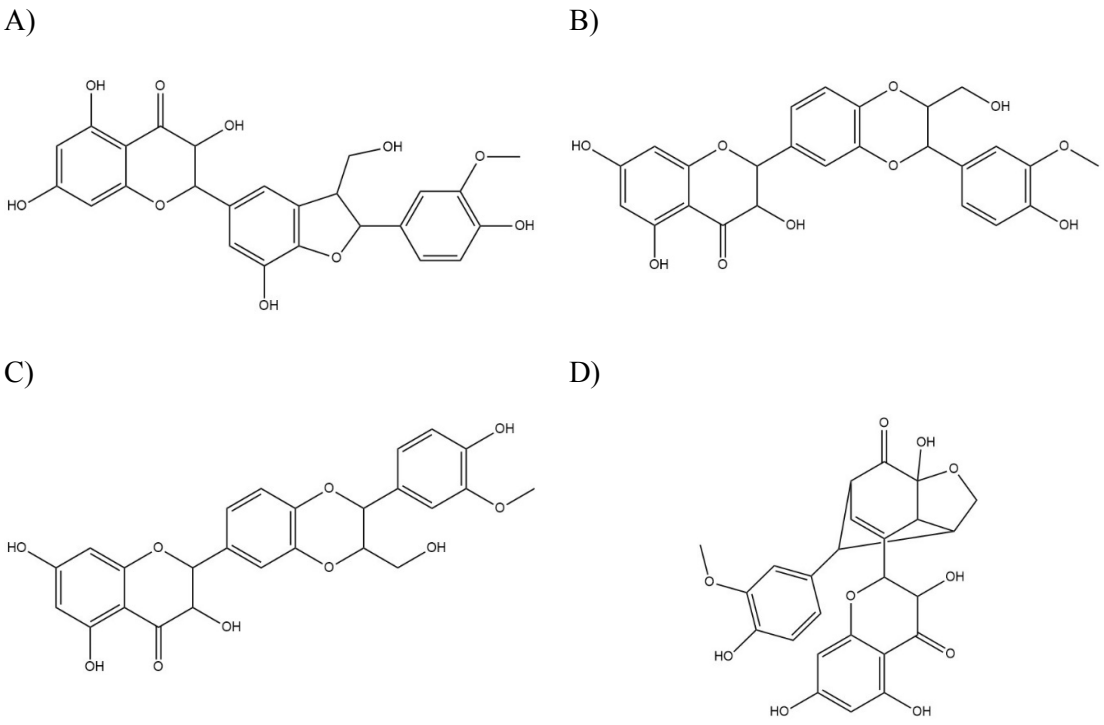


Figure 2.19. The structures of A) silychrisin, B) silybin, C) isosilybin, D) silydianin

A total of thirteen chemical compounds were tentatively identified in the milk thistle seed flour, including chlorogenic acid isomers, 5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomers, methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid, taxifolin, silychristin isomers, silybin A, silybin B, and isosilybin (Figure 2.14 and Table 2.5).

Table 2.4. Characterization of compounds present in the milk thistle seed flour extract by Soxhlet extraction

Peak ID	$t_{R\ (min)}$	Theoretical $[M-H]^-$	Experimental $[M-H]^-$	Chemical Formula	Tentatively Identification	Concentration* (μg/g)
1	8.24	353.0873	353.0875	$C_{16}H_{18}O_9$	Chlorogenic acid isomer	22.69 ± 0.66
2	10.15	353.0873	353.0876	$C_{16}H_{18}O_9$	Chlorogenic acid isomer	110.82 ± 9.67
3	13.90	661.1769	661.1760	$C_{31}H_{34}O_{16}$	5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomer	52.78 ± 7.16 CE
4	15.32	661.1769	661.1764	$C_{31}H_{34}O_{16}$	5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid isomer	137.97 ± 2.87 CE
5	15.62	691.1874	691.1886	$C_{32}H_{36}O_{17}$	Methyl 5-(6-Caffeoyl-glucopyranosyl)-caffeoylquinic acid	87.94 ± 3.81 CE
6	17.47	303.0505	303.0511	$C_{15}H_{12}O_7$	Taxifolin	
7	22.83	481.1135	481.1141	$C_{25}H_{22}O_{10}$	Silychristin isomer	tr
8	24.74	481.1135	481.1142	$C_{25}H_{22}O_{10}$	Silychristin isomer	2854.16 ± 60.56 SE
9	25.33	481.1135	481.1141	$C_{25}H_{22}O_{10}$	Silychristin isomer[2]	4253.89 ± 68.69 SE

10	26.04	481.1135	481.1139	$C_{25}H_{22}O_{10}$	Silychristin isomer[1]	4845.24 ± 54.55 SE
11	36.87	481.1135	481.1142	$C_{25}H_{22}O_{10}$	Silybin A	4942.16 ± 263.01 SE
12	37.35	481.1135	481.1141	$C_{25}H_{22}O_{10}$	Silybin B	5916.55 ± 126.53 SE
13	40.31	481.1135	481.1137	$C_{25}H_{22}O_{10}$	Isosilybin[3]	5103.81 ± 63.07 SE

[1,2,3]Represented the three greatest peaks based on the typical UHPLC chromatogram peak area. * CE stands for a chlorogenic acid equivalent. SE stands for a silibinin equivalent. tr stands for trace.

In addition, this study measured the concentrations of the major chemical compounds in the milk thistle seed flour (Table 2.5). The concentrations of the silychristin isomers in the seed flour were 2854 – 4845 μg silibinin equivalents per g. Silybin A, silybin B, and isosilybin were at the levels of 4942, 5917, and 5104 μg silibinin equivalents per g, respectively. Wallace and others extracted the defatted milk thistle seed flour purchased from Frontier Herbs (Norway, IA, USA) with boiling ethanol (79 □) for 10 h to achieve maximum yields. They detected silymarin, taxifolin, silychristin, silydianin, silybinin A and silybinin B in the extraction with HPLC. Among all the chemical compounds that have been identified, silybinin B, silybinin A, silychristin and taxifolin were the four major ones with the concentrations of 6.86, 4.04, 3.89 and 0.62 mg/g defatted seed, respectively (Wallace, Carrier, & Clausen, 2005). Barreto and others extracted the defatted milk thistle seed with 100 °C water for 210 min, the yields of taxifolin, silychristin, silybin A, and silybin B were 1.2, 5.0, 1.8, and 3.3 mg/g seed, respectively. The seeds were obtained from Frontier Herbs (Norway, IA, USA) (Barreto, Wallace, Carrier, & Clausen, 2003). Mudge and others purchased milk thistle seeds from two places, which were Midmore Organic Farm (Morinville, AB, Canada) and Horizon Herbs (Williams, OR, USA). Both sources of seeds were defatted first and then extracted with 100% methanol using sonication extraction. The levels of silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B were 2.6 – 3.7, 10.4 – 10.7, 2.0 – 2.9, 3.0 – 4.5, 2.1 – 2.5, and 1.4 – 1.6 mg/g, respectively (Mudge, Paley, Schieber, & Brown, 2015). In 2019, Choe and others reported the silychristin concentration in the milk thistle seed flour extracted with 50% acetone (v/v) under room temperature with sonification. The concentration was 2580 – 2610

μg silibinin equivalents per g which was lower than the value reported in this study. The differences of the kinds of the chemical components and the concentrations could be caused by the growing environment, cultivars, extraction conditions and many other factors.

Apart from silymarins, this study also found other polyphenols including chlorogenic acid and its derivatives, 5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid and methyl 5-(6-Caffeoyl-glucopyranosyl)-caffeoylquinic acid (Table 2.5). These two chemical compounds were firstly reported in the milk thistle seed flour by (Choe et al., 2019). Choe and others used sonication with 50% acetone (v/v) to extract the seed flour and found that the greatest concentration of 5-*p*-(6-caffeoyl-glucopyranosyl)-coumaroylquinic acid was 729 μg chlorogenic acid equivalents per g and the concentration of methyl 5-(6-Caffeoyl-glucopyranosyl)-caffeoylquinic acid was 519 μg chlorogenic acid equivalents per g, which were both higher than the concentrations obtained in this study. The concentrations of the chlorogenic acid isomers were 23 and 111 μg/g, which were lower than those (102 and 330 μg/g) in the study of Choe and others (Choe et al., 2019).

Total Phenolic Content (TPC) of the Seed Flours

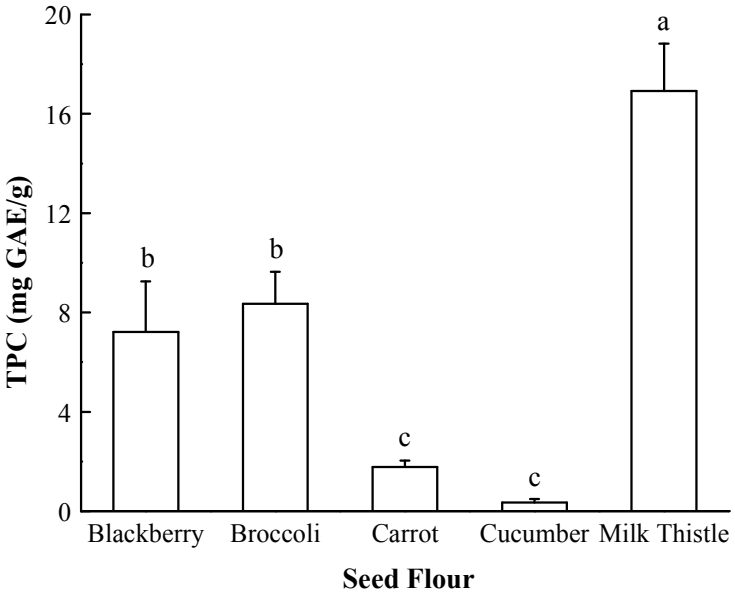


Figure 2.20. Total phenolic content (TPC) of seed flour samples. Values marked by different letters are significantly different ($P < 0.05$)

Among the five seed flour samples, milk thistle seed flour had the highest total phenolic content of 16.9 mg GAE/g, followed by Broccoli seed flour (8.4 mg GAE/g) and blackberry seed flour (7.2 mg GAE/g) (Figure 2.20). Parry and others reported a higher TPC of milk thistle seed flour, which was 25.2 mg GAE/g. In their studies, the milk thistle seed flour was first extracted by hexane in a Soxhlet apparatus to remove the oil. Then the processed seed flour was extracted by 50% acetone to examine TPC (Parry et al., 2008). The difference could be resulted from the different growing conditions and the extraction solutions. Ayoub and others measured the phenolic compounds in the blackberry seed flour extracted by methanol–acetone–water (7:7:6, v/v/v). The total phenolic content was 5.1 mg GAE/g including free and esterified phenolics

(Ayoub et al., 2016). This result was a little lower that in the current study. The TPC of fresh blackberry was in a range of 3.6 − 5.5 mg GAE/g depending on the species, cultivation, harvesting time and so on (Heinonen, Meyer, & Frankel, 1998; Sellappan, Akoh, & Krewer, 2002). However, the blackberry seed flour has a higher phenolic content than the fresh blackberry fruit in general.

Chapter III: Antioxidant Capacities of Cold-Pressed Blackberry, Broccoli, Carrot, Cucumber and Milk Thistle Seed Flours

Abstract

Antioxidant properties of blackberry, broccoli, carrot, cucumber and milk thistle seed flours, which were byproducts of their seed oil productions, were investigated. The free radical scavenging capacities against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ of ethanolic sample extracts differ from each other significantly. The blackberry seed flour had the strongest $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging capacities which were 41.5 μmol TE/g and 92.8 μmol TE/g, respectively, while the milk thistle seed flour had the greatest total phenol content which was 16.9 mg GAE/g. Antioxidant properties of these seed flours can promote the use of them as potential functional food ingredients.

Introduction

Epidemiological studies showed that the consumption of fruits and vegetables could relieve the oxidative stress in the human body and prevent diseases including atherosclerosis, cancers, stroke, arthritis, heart attack, retinal damage, hepatitis, liver injury, and so on, which are all associated with reactive oxygen species (Kaur & Kapoor, 2001; J. Lee, Koo, & Min, 2004). Therefore, it is considered health-beneficial to consume more antioxidants in diet. There are some synthetic antioxidants which have high manufacturing costs but lower antioxidant activities and even show toxicity (Soong & Barlow, 2004). Hence, it would be better if natural

food ingredient with efficient antioxidants can be utilized at a relatively low cost. Natural ingredients rich in antioxidants such as oil seeds, herbs, fruits and vegetables have already been well studied and applied, while the fruit and vegetable seeds don't get much attention because they don't have that wide range of applications compared to oil seeds. However, since the seeds of fruits and vegetables are leftovers of their processed products, they usually have great outputs and low costs. Therefore, it is necessary and profitable to fully uncover the antioxidant activities of seeds. In the present study, the seed flours of blackberry, broccoli, carrot, cucumber and milk thistle were investigated for their free radical scavenging capacities and total phenolic contents.

Materials and Methods

Materials

Blackberry, broccoli, carrot, cucumber and milk thistle seed flour samples were provided by Botanic Oil Innovation Inc. (Spooner, WI, USA). These fruit seed flours were the solid cakes from the cold-pressing process. (±)-6-Hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox), fluorescein (FL), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Corning Inc. (Corning, NY, USA). Fetal bovine serum was purchased from Hyclone

Laboratories Inc. (Logan, UT, 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).

Seed Flour Extraction and Sample Preparation

The methods were the same as described in the corresponding part in Chapter II.

$ABTS^{\bullet+}$ Scavenging Capacity

The radical scavenging capacities of seed flour extracts were evaluated against $ABTS^{\bullet+}$ generated by a chemical method according to a published protocol (Moore, Cheng, Su, & Yu, 2006). $ABTS^{\bullet+}$ was prepared by oxidizing a 5 mM solution of ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, with manganese dioxide at ambient temperature for 30 min. The final reaction mixture contained 1.0 mL of $ABTS^{\bullet+}$ solution with an absorbance of 0.7 at 734 nm and 80 μL of 100% ethanol for the control or 80 μL of the working sample or standard solution. The absorbance after 90 s of reaction time was measured at 734 nm, and the Trolox equivalent was calculated using a standard curve prepared with Trolox. Experiments were conducted in triplicate.

Relative $DPPH^{\bullet}$ Scavenging Capacity

The relative $DPPH^{\bullet}$ scavenging capacity values of the seed flour extracts were obtained using the high throughput assay described by Cheng and others (Cheng, Moore, & Yu, 2006). This

assay was carried out using a Victor^3 multilabel plate reader with 96-well plates. The reaction mixture consisted of 100 μL 0.2 mmol/L DPPH and 100 μL of the standards, control or samples at different concentrations. Absorbance was read at 515 nm and read once every minute for 1.5 hours. The standard curve was derived from the area under the curve from different concentrations of Trolox. Experiments were conducted in triplicate.

Anti-proliferative Activities

The selected five seed flour extracts were evaluated for their anti-proliferative activities using colorectal cancer (CRC) cells (HCT116, SW480). CRC cells (5×10^3 cells per mL) were cultured at 37 °C under 5% carbon dioxide in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. After 18 h, the cells were treated with media containing the seed flour extract whose final concentration was 30 μg flour equivalent per mL in the culture mixture for 24 and 48 h. After that, the supernatant was removed and the mixture of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and serum-free media (1:5, v/v) was added. After 3 h, DMSO was added and the absorbance at 540 nm was measured.

Statistical Analysis

Data were reported as mean ± standard (SD) deviation for each point. IBM SPSS Statistics (Version Rel. 22.0.0.0, IBM Inc., Armonk, NY) was used to identify the differences among

means. A one-way analysis for variation (ANOVA) was applied for comparison. Statistical significance was declared at $P < 0.05$.

Results and Discussion

DPPH• Scavenging Capacities

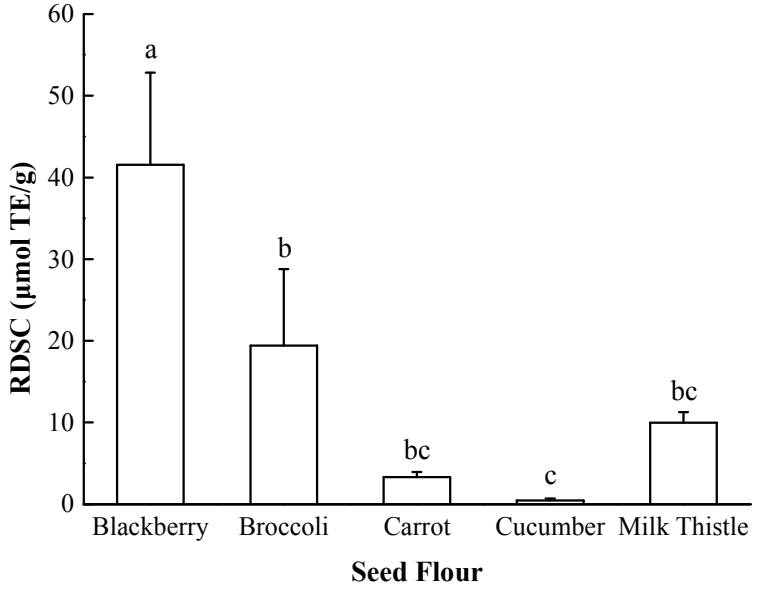


Figure 3.1. Relative DPPH• scavenging capacities (RDSC) of the seed flour samples.

Values marked by different letters are significantly different ($P < 0.05$).

All five seed flour samples exhibited relative DPPH• scavenging capacities, with the RDSC value in a range of 0.5 – 41.5 μmol TE/g (Figure 3.1). Blackberry seed flour extract possessed the highest RDSC value of 41.5 μmol TE/g among all followed by broccoli and milk thistle seed flour extracts with the values of 19.4 and 10.0 μmol TE/g, respectively. Choe and others

reported that the RDSC of broccoli, carrot and cucumber seed flour extracts were 84.8, 16.0 and 2.64 μmol TE/g, respectively (Choe et al., 2018). Parry and others found that milk thistle flour extract had the RDSC of 61.1 μmol TE/g (Parry et al., 2008). The values got by, Choe et al. (2018) and Parry, et al. (2008) were higher than those obtained in this study. This could be caused by the difference in solvents used in the extraction. The solvent used in Choe and Parry's study was 50% acetone (v/v), while in this study the solvent was 100% ethanol. The type and concentration of solvent, which have different polarities, will affect the yield of phenolic compounds (de Camargo et al., 2014). It has been found that there's a significant correlation between total phenolics content and antioxidant assays, such as relative $DPPH^{\bullet}$ scavenging capacity and $ABTS^{\bullet+}$ radical scavenging capacity (da Silva et al., 2016; de Camargo et al., 2015). Therefore, the change of solvent for the extraction will alter the result of RDSC assay. Furthermore, Ayoub's findings showed that the yield of extracted phenolics was higher, especially for the esterified phenolics when acetone was used as a solvent compared to methanol (Ayoub et al., 2016). This might lead to the lower outcoming in RDSC assay.

$ABTS^{\bullet+}$ Scavenging Capacities

The $ABTS^{\bullet+}$ radical scavenging capacities of these five seed flour samples were from 1.2 to 92.8 μmol TE/g (Figure 3.2) with the blackberry seed flour extract being the highest. The second highest one was broccoli seed flour extract with the value of 54.4 μmol TE/g, followed by carrot seed flour extract (39.2 μmol TE/g) and milk thistle seed flour extract (32.8 μmol TE/g). No research about the $ABTS^{\bullet+}$ radical scavenging capacities of blackberry and milk

thistle seed flours has been published yet. But the $ABTS^{\bullet+}$ radical scavenging capacities of broccoli, carrot and cucumber seed flour samples extracted by 50% acetone (v/v) have been measured, which were 175.9, 250.0 and 6.8 μmol TE/g, respectively (Choe et al., 2018). The reason that the values were lower in this study might be the same as the one mentioned in the above RDSC part.

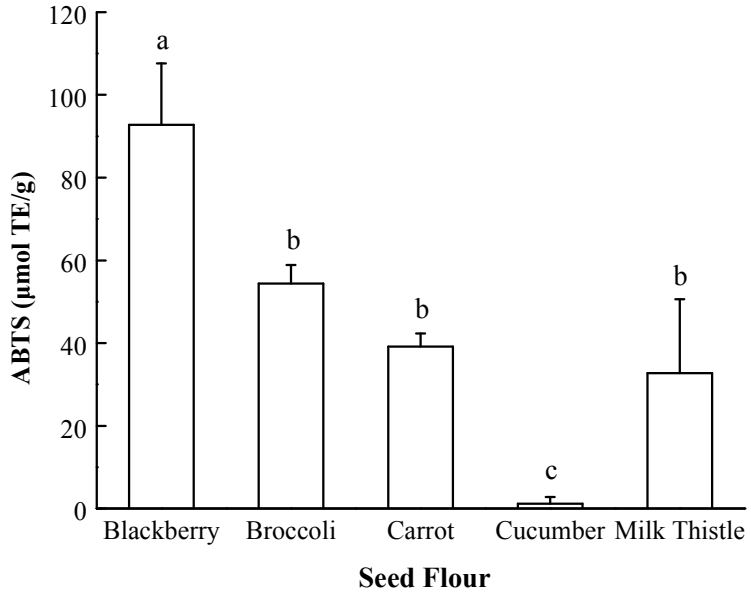


Figure 3.2. $ABTS^{\bullet+}$ scavenging capacities of seed flour samples. Values marked by different letters are significantly different ($P < 0.05$).

Merlot grape seed flour (2008) from AprèsVin (Prosser, Washington, U.S.A.) was defatted with n-hexane (70 °C) first and extracted with 70% ethanol (v/v) using a shaking water bath (24 °C) for 12 h. The radical scavenging capacity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ of this ethanolic extract was 269.26 and 1873.3 μmol TE/g dry weight, respectively (Ross, Hoye, & Fernandez-Plotka,

2011). The other four cultivars (muscadine, chardonnay, concord, and ruby red) of defatted grape seed flours from Botanic Oil Innovations Inc. (Spooner, WI, USA) were extracted with 100% ethanol using Soxhlet method for 3 h. The RDSC of these four seed flour extracts had the range of 11.8 – 15.0 mmol TE/g (Lutterodt, Slavin, Whent, Turner, & Yu, 2011). Black raspberry seed flour provided by Badger Oil Co. (Spooner, WI, USA) was extracted with 50% acetone (v/v) for 15 h at ambient temperature or with 100% ethanol using Soxhlet extractor for 2.5 h. $ABTS^{\bullet+}$ scavenging capacities of the acetonic and ethanolic extracts were 232.8 and 361.0 μmol TE/g, respectively (Parry & Yu, 2006). Compared to blackberry seed flour, grape and black raspberry seed flours appeared to have stronger antioxidant activities against both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, especially the grape seed flours.

The cold-pressed seed flours of pumpkin and parsley were obtained from Botanic Oil Innovations Inc. (Spooner, WI, USA) and extracted with 50% acetone (v/v) at ambient temperature. The RDSC of pumpkin and parsley seed flour extracts were 2.2 and 18.1 μmol TE/g, respectively (Parry et al., 2008) . Parsley seed flour extract had similar RDSC value to that of broccoli seed flour, while pumpkin, carrot, and cucumber all had relatively low RDSC value.

The cold-pressed mullein and cardamom seed flours from Botanic Oil Innovations Inc. were extracted with 50% acetone (v/v) at ambient temperature. The acetonic extracts of mullein and cardamom seed flours had the RDSC of 21.2 – 24.0 and 19.5 μmol TE/g (Parry et al., 2008).

The DPPH$^{\bullet}$ scavenging capacities of these two seed flours were stronger than that of milk thistle seed flour. However, the difference could be caused by different extracting solvents. It appeared that fruit seed flours had much stronger radical scavenging capacities compared to vegetables, herbs, and spices.

Anti-proliferative Activities

The cell toxicity of blackberry, broccoli, carrot, cucumber, and milk thistle seed flours were tested on the 3T3-L1 preadipocytes. Only the milk thistle seed flour showed cell toxicity at the concentration of 60 μg seed flour equivalent per mL (Figure 3.3).

As shown in Figure 3.4 and Figure 3.5, blackberry, broccoli, carrot, and cucumber seed flours had no anti-proliferative activities against human colon cancer cells HCT116 and SW480 at the testing concentration in 24 and 48 h. 60 μg seed flour equivalent per mL of the milk thistle seed flour inhibited the cell growth of SW480 by 17% and the that of HCT116 by 21% in 48 h. Therefore, the milk thistle seed flour potentially possessed the anti-proliferative capacity against colon cancer cells SW480 and HCT116.

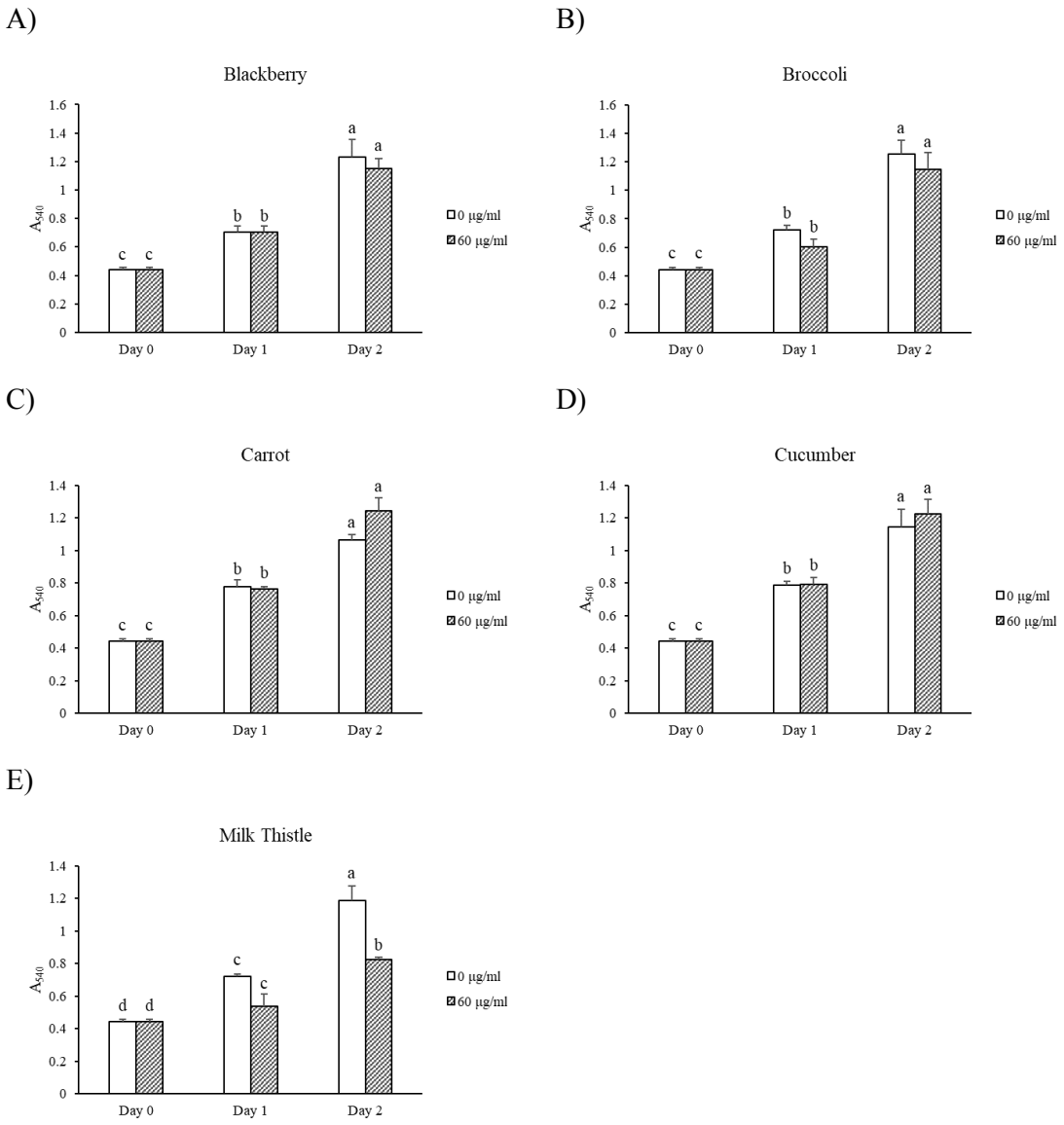


Figure 3.3. Cell toxicity of selected seed flour extracts. Different letters within the same day represent significant difference ($P < 0.05$).

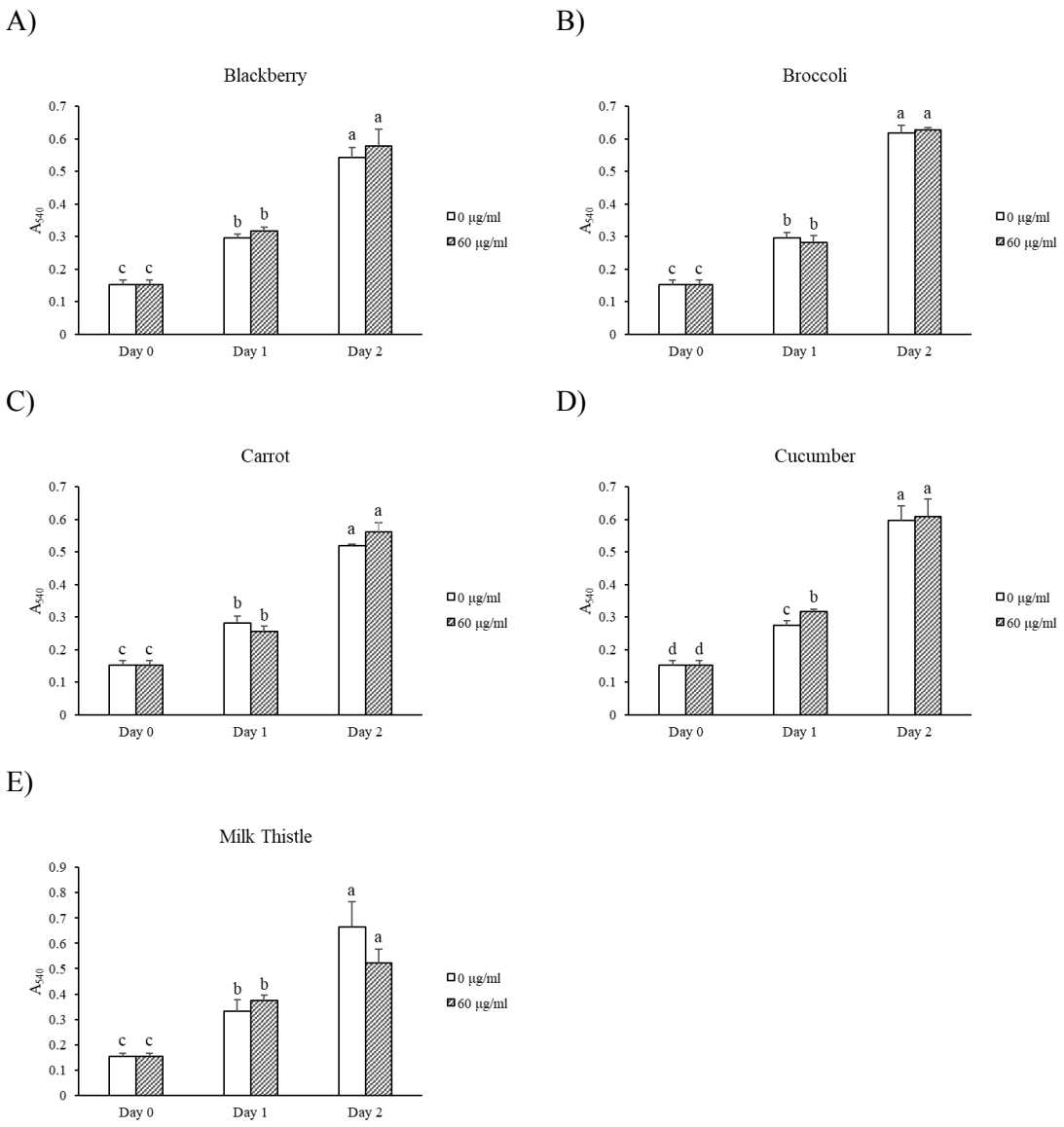


Figure 3.4. Anti-proliferation of HCT116 colon cancer cells treated with seed flour extracts. Different letters within the same day represent significant difference ($P < 0.05$).

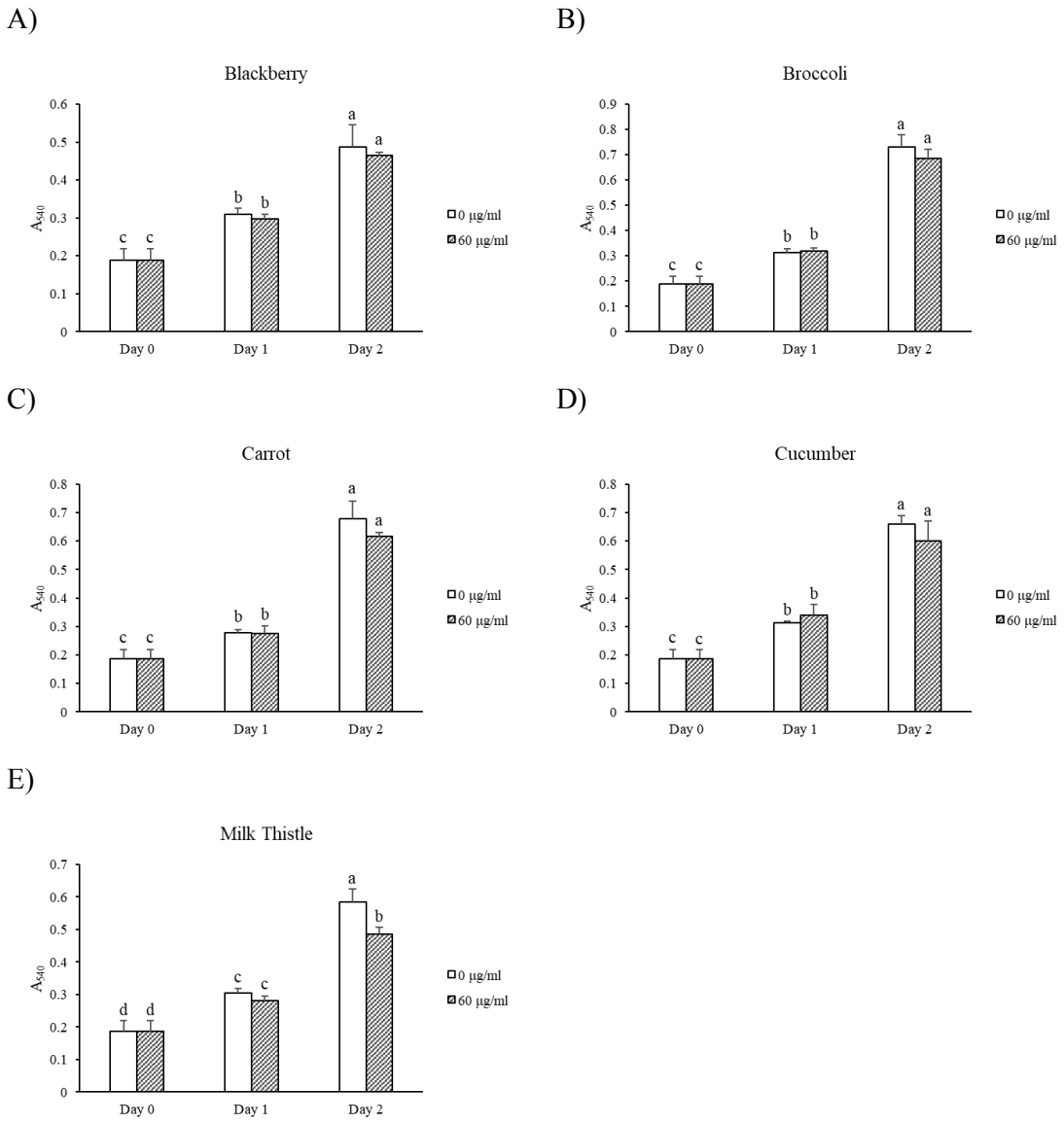


Figure 3.5. Anti-proliferation of SW480 colon cancer cells treated with seed flour extracts. Different letters within the same day represent significant difference ($P < 0.05$).

Eo and others reported that silymarin (silybin, silydianin, silychristin, tamoxifen, and quercetin) could inhibit the proliferation of HCT116 by 11, 22 and 48% at 24 h and 16, 36 and 54% at 48 h with the concentrations of 50, 100 and 200 µg/mL, respectively. The cell growth of SW480

was reduced by 13, 28 and 47% at 24 h and 24, 39 and 59% at 48 h with the concentrations of 50, 100 and 200 μg/mL, respectively. The suppression was both dose- and time- dependent (Eo et al., 2015). The difference between the results of Eo and others and those in the current study could be caused by different concentrations of bioactive components. In the study of Eo and others, the final concentrations in the culture mix were higher and the samples had a higher purity of silymarin.

Appendix

MS spectrums of chemicals found in selected seed flour extracts

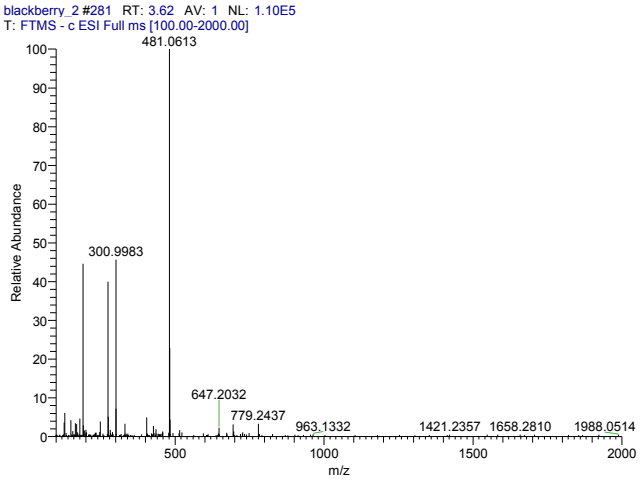


Figure S1. MS spectrum of hexahydroxydiphenic acid hexoside found in the blackberry seed flour extract

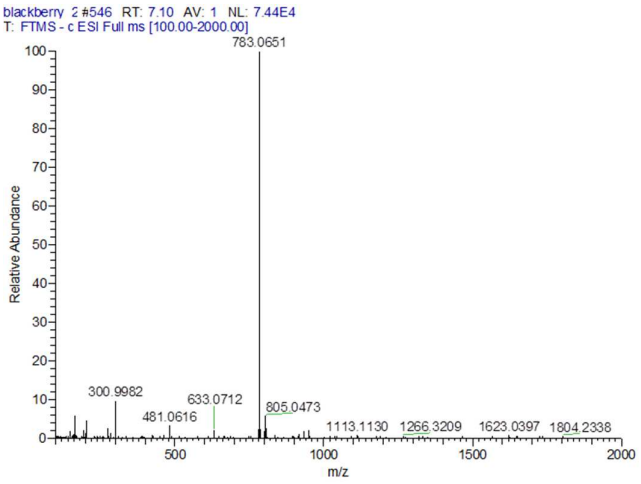


Figure S2. MS spectrum of pedunculagin found in the blackberry seed flour extract

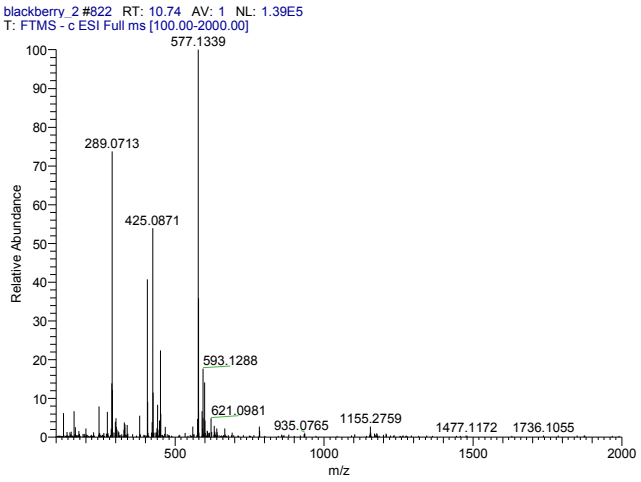


Figure S3. MS spectrum of procyanidin B1 found in the blackberry seed flour extract

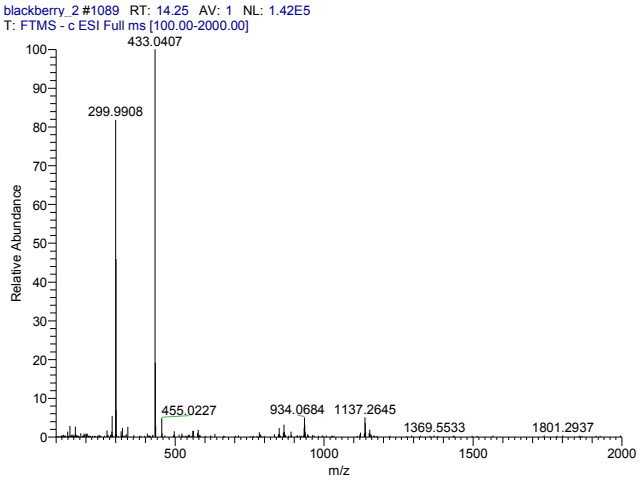


Figure S4. MS spectrum of ellagic acid pentoside found in the blackberry seed flour extract

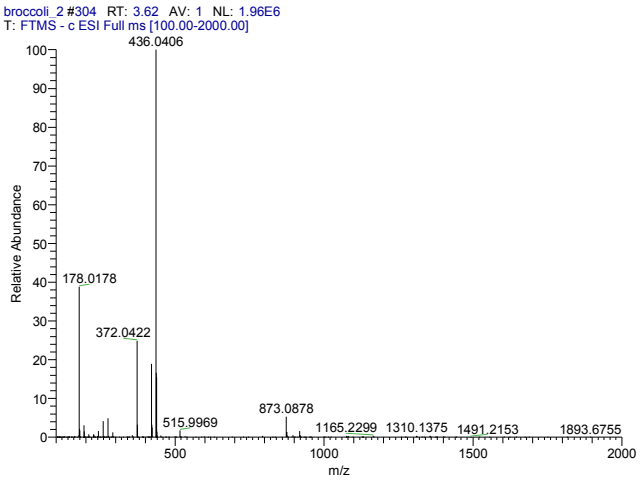


Figure S5. MS spectrum of glucoraphanin found in the broccoli seed flour extract

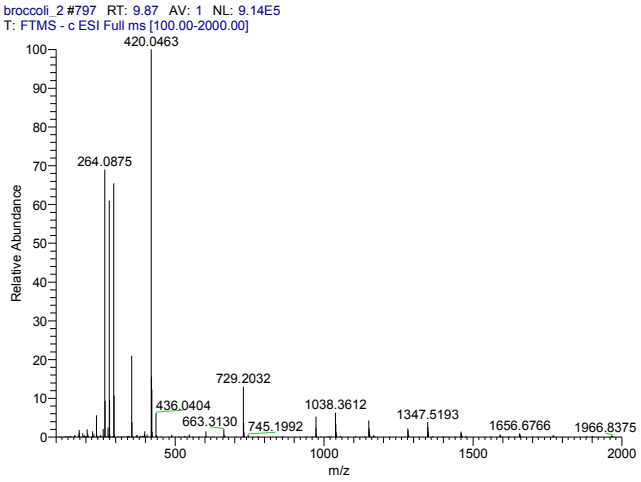


Figure S6. MS spectrum of glucoerucin found in the broccoli seed flour extract

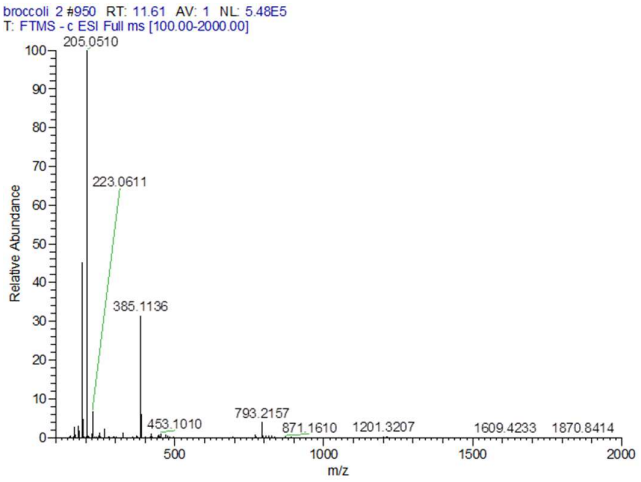


Figure S7. MS spectrum of sinapoylhexose found in the broccoli seed flour extract

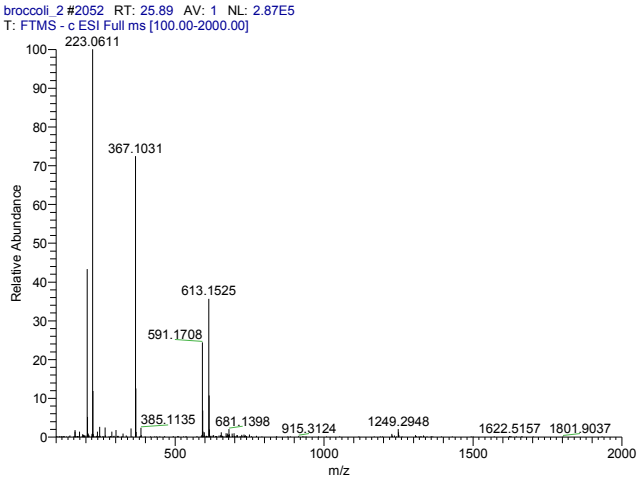


Figure S8. MS spectrum of 1,2-disinapoylglucoside found in the broccoli seed flour extract

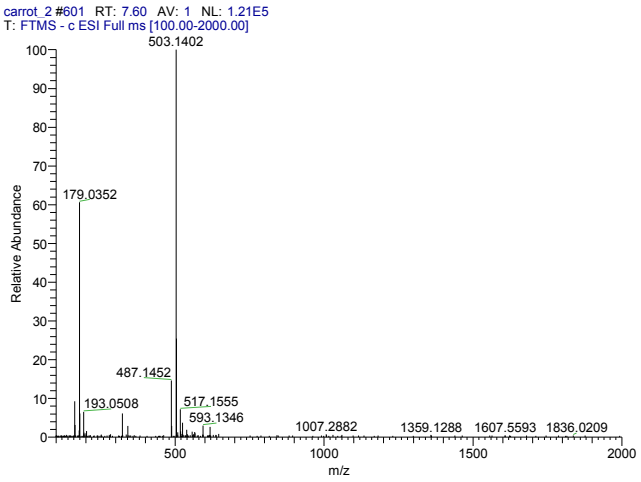


Figure S9. MS spectrum of caffeoyldihexoside found in the carrot seed flour extract

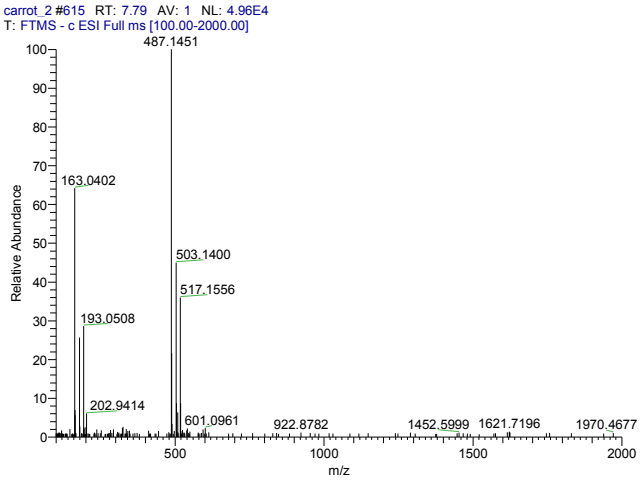


Figure S10. MS spectrum of cistanoside F found in the carrot seed flour extract

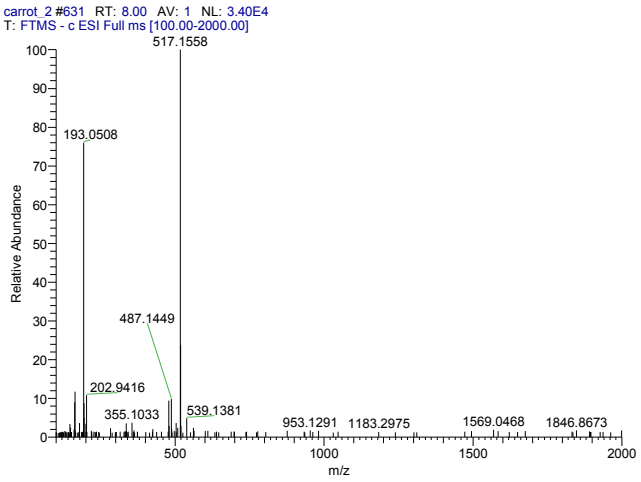


Figure S11. MS spectrum of lycibarbarphenylpropanoid C found in the carrot seed flour extract

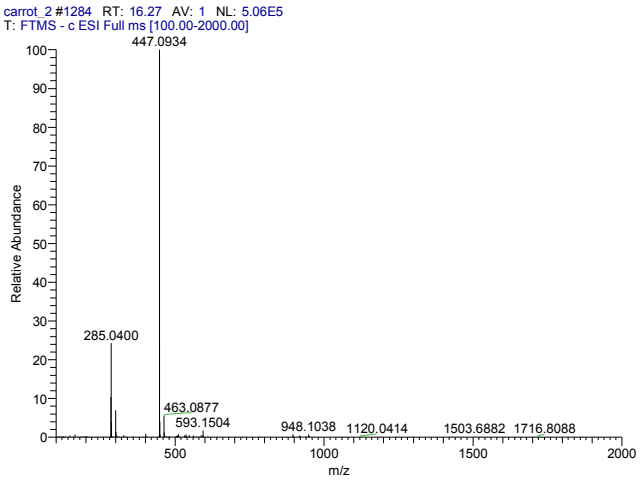


Figure S12. MS spectrum of kaempferol-3-*O*-glucoside found in the carrot seed flour extract

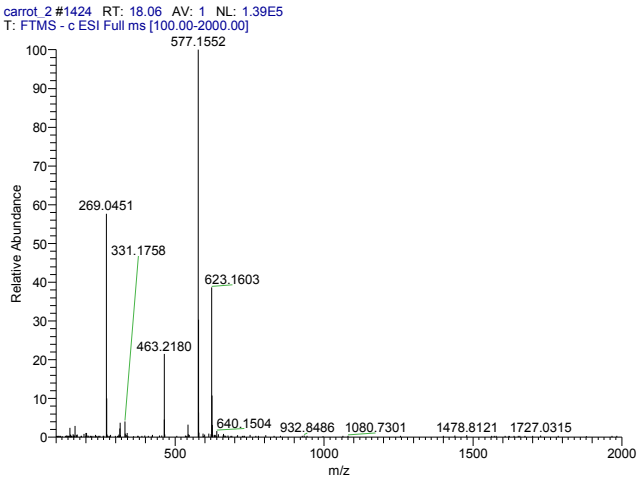


Figure S13. MS spectrum of apigenin-7-*O*-β-D-rutinoside found in the carrot seed flour extract

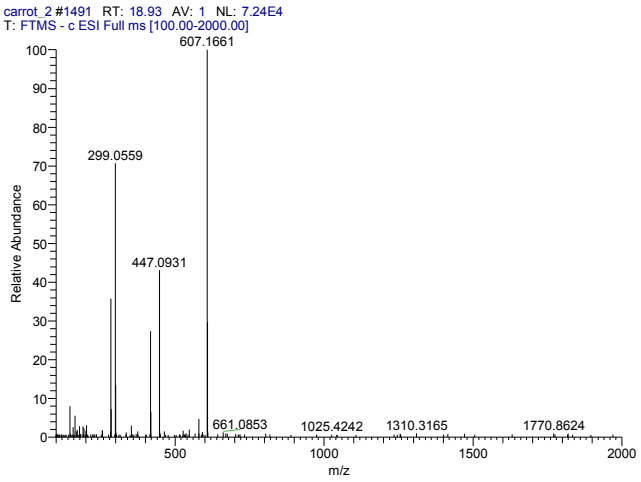


Figure S14. MS spectrum of diosmetin-7-rutinoside found in the carrot seed flour extract

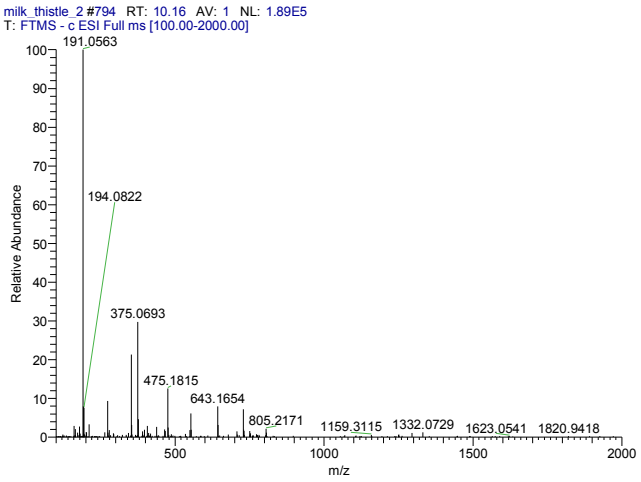


Figure S15. MS spectrum of chlorogenic acid found in the milk thistle seed flour extract

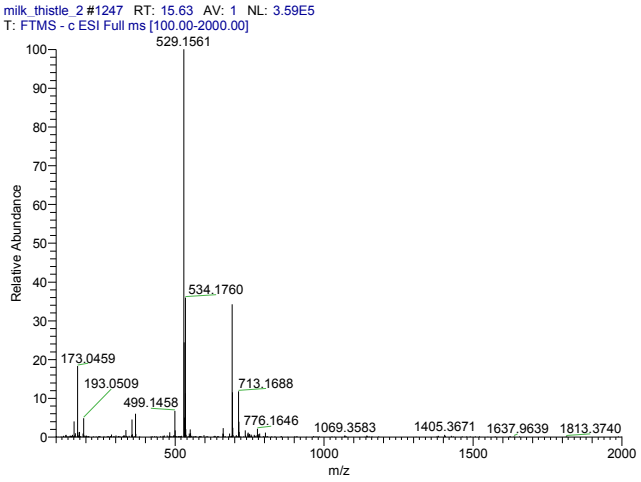


Figure S16. MS spectrum of methyl 5-(6-caffeoyl-glucopyranosyl)-caffeoylquinic acid found in the milk thistle seed flour extract

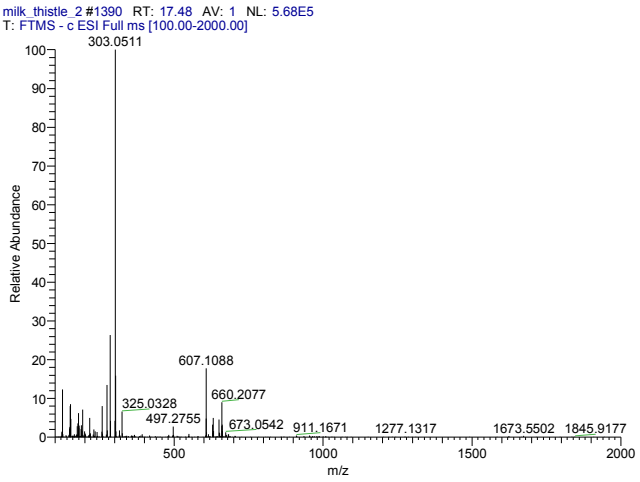


Figure S17. MS spectrum of taxifolin found in the milk thistle seed flour extract

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