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Phytochemicals from the Roots of Northern Highbush Blueberry (*Vaccinium Corymbosum*)

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PHYTOCHEMICALS FROM THE ROOTS OF NORTHERN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM*)

BY

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ABSTRACT

Growing evidence from many *in vitro* studies suggest that plants produce secondary metabolites which may have potential physiological properties. The northern highbush blueberry (*Vaccinium corymbosum* L.) plant is commercially cultivated for its valuable dark-blue fruit, which has been extensively researched and has been shown to contain phenolic compounds recognized to have positive health benefits. Thus, an evaluation of other parts of the plant, that as of yet have not been investigated, could be worthwhile. There may be undiscovered bioactive compounds within the roots of this plant that may contribute to the improvement of human health. This rationale supports further research and investigation into the roots of the plant.

A preliminary study showed that the crude extract of the blueberry roots showed antioxidant activity. Using various chromatographic methods and spectroscopic techniques, the blueberry root compounds were isolated, fractionated and analyzed. Six compounds were identified by ^{1}H and ^{13}C NMR and mass spectrometry data. Three lignans, which were never previously reported in the blueberry plant, were identified as nudiposide, lyoniside, and ssioriside; one phenolic acid; sinapic acid glucoside, and two catechins; epigallocatechin (EGC) and dulcisflavan. The isolated compounds were evaluated for inhibitory effect on α-glucosidase and tyrosinase. Of the six compounds evaluated, ssioriside was a moderate inhibitor of α-glucosidase (IC_{50} = 650µM) and epigallocatechin showed weak tyrosinase enzyme inhibition activity (IC_{50} = 2001µM).

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I would like to express my sincere thanks to my advisor, Dr. Navindra Seeram, for providing me with the opportunity and encouragement to work on this project. His invaluable advice, guidance and support helped me throughout this program, which has granted me these years a rewarding and unforgettable experience. His patience and accessibility have helped me to grow as a scientist. While I have overcome many difficulties and setbacks during my research, Dr. Seeram has been helpful always; and, most importantly, he exhibited confidence in my ability to succeed. To have had the opportunity to work and learn from him has been an honor and an unforgettable experience. The University of Rhode Island College of Pharmacy would not be the same without him.

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PREFACE

This thesis has been written in manuscript format. It is broken into two sections. Part I is a literature review, covering previous work on *Vaccinium corymbosum*, the history behind natural product chemistry and the scientific methods behind extraction and isolation of secondary plant metabolites. Part II contains the research section, covering methods used to determine phenolic compounds using scientific instrumentation.

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PART I. INTRODUCTION

1. Review of Previous work on Northern Highbush Blueberry

The Northern highbush blueberry (*Vaccinium corymbosum* L.) is a deciduous plant in the family Ericaceae, which is often found in dense thickets (Rowland *et al*., 2012). The alternate names of this plant are southeastern highbush blueberry, Maryland highbush blueberry, black highbush blueberry, American blueberry, New Jersey blueberry, swamp blueberry, and whortleberry depending on the location of where it is grown (Rowland, Alkharouf, Darwish, Ogden, & Main, 2012).

Northern highbush blueberry is native to eastern North America, growing from Nova Scotia and Ontario south to Alabama and west to Wisconsin. The name "highbush" refers to the relatively tall stature of the plant, which can grow upwards of 6 to 12 feet tall (Rowland, Alkharouf, Darwish, Ogden, & Main, 2012). The stems are a reddish color in the winter and a yellow-green color from the spring to fall. The leaves are 1 to 3 inches long and elliptical in shape. Generally blooming around February to June, the small white flowers of the highbush plant are bell-shaped with 5 petals, and arranged in clusters of about 8 to 10 flowers per cluster. Fruiting occurs approximately two months after flowering. When mature, the round, small fruit contains many seeds and has a dark blue color which attracts the attention of numerous birds and mammals, providing them an important summer and early fall food. The consumption of the fruit by the animals helps aid in the dispersion of the undigested seeds elsewhere (Rowland, Alkharouf, Darwish, Ogden, & Main, 2012).

The highbush blueberries are commercially cultivated for their dark blue fruits and are known to be beneficial to human health (Torri, Lemos, Caliari, Kassuya,

Bastos, & Andrade, 2007). The use of blueberries for medicinal purposes has a long history. Early North American settlers consumed blueberries as a tea or syrup to help with cough and diarrhea (Kalt & Dufour, 1997). Today, blueberries are still being consumed, and are known to contain a wide range of phytochemicals that are beneficial to human health. The multitude of health benefits include; antioxidant, anti-cancer, anti-bacterial, anti-neurodegenerative and anti-viral (Alejandro, Eicholz, Rohn, Kroh, & Huyskens-Keil, 2008; Torri, Lemos, Caliari, Kassuya, Bastos, & Andrade, 2007; Wan, Yuan, Cirello, & Seeram, 2012). It is known that blueberries have one of the highest levels of antioxidant capacity among many fruits and vegetables (Zeng & Wang, 2003).

Generic and environmental conditions affect the distribution and composition of anthocyanins in blueberries worldwide. (Ayaz, Hayirlioglu-Ayaz, Gruz, Noyak, & M., 2005). Ten known anthocyanins were identified in the North American Highbush blueberry, which include; 3-monoarabinosides, 3-monogalacatosides and 3-monoglucosides of cyanidin, delphinidin, malvidin, peonidin and petunidin (Castrejon, Rohn, Kroh, & Huyskens-Keil, 2008; Meadows, Smith, Greenwood, Haight, Perez-Pons, Querol, et al., 1996). In Slovenia, Moze *et al*., fifteen anthocyanins were identified in planted Slovenian blueberries. This may be due to many factors such as climatic differences or the ripeness of the fruit when cultivated (Moze, Polak, Gasperlin, Koron, Vanzo, Ulrih, et al., 2011).

There are recent investigations of the non-edible plant parts due to numerous reports throughout the literature pertaining to the health benefits of the blueberry fruit. According to a study from Takeshita et al., other parts of the plant reveal potential

therapeutic values. The group confirmed that a crude extract from the leaves of the rabbit-eye blueberry (*V. ahsei*) inhibited Hepatitis C virus (HCV) RNA expression, *in vitro* (Takeshita, Yo-ichi, Akamatsu, Ohmori, U., Tsubouchi, et al., 2009). This data suggests that the compounds within the leaves extracts could be used to inhibit the viral replication of the HCV infection (Takeshita, et al., 2009). Another non-edible part of the blueberry plant that has been recently investigated is the flowers. Wan *et al*. showed that compounds isolated from the flowers were potent inhibitors of α-glucosidase enzymes. It was noted that the phenolic sub-classes were more potent than the positive control, acarbose (Wan *et al.*, 2012). The roots of the blueberry plant would make a great candidate to investigate the phytochemicals since there is numerous health benefits linked with this plant and there are no known phytochemical investigations of the roots

2. Natural Product Chemistry:

For thousands of years, natural products from plants, bacteria, sponges and fungi have been used to treat and cure illnesses (Butler, 2004). The most historically practiced medicinal chemistry is that of traditional Chinese medicine (TCM) and Ayurveda, the traditional Indian system of medicine. According to Butler, drugs derived from natural products have spiked global interest from pharmaceutical and biotech companies, which seek to produce drugs derived from natural sources. It is currently known that 15 natural product derived drugs are on the market and 15 are in Phase III clinical trials (Butler, 2004; Newman & Cragg, 2012; Newman, Cragg, & Snader, 1999). An example of a drug from natural product origin is, Taxol. Extracted

from the Pacific Yew tree, Taxol is a powerful anticarcinogenic clinical drug used in cancer chemotherapy (Cragg & Newman, 2005).

Natural Product Chemistry is a field of research that deals with the isolation and identification of these active compounds found in nature (Butler, 2004). There are two main techniques used to isolate pure compounds from natural sources. These techniques include: extraction of the crude extract and purification of the sample using chromatography. The most common methods used to identify the isolated pure, unknown compounds are: mass spectroscopy, nuclear magnetic resonance spectroscopy (NMR) and x-ray crystallography (Newman & Cragg, 2012).

Plant organisms contain an enormous variety of chemical compounds. These compounds are known as primary and secondary metabolites. Primary metabolites are required by the organism to survive. These essential biochemical compounds are: fats, proteins, carbohydrates and vitamins. Secondary metabolites are key components in aiding an organism's defense against herbivores and other interspecies. The health-promoting importance of these compounds has been recognized by researchers (Cragg & Newman, 2005). Therefore, there is an interest in the isolation and characterization of active phenolic compound due to their antioxidant, anti-inflammatory, anticarcinogenic and antimicrobial properties, to list a few (Eliane, Lemos, Caliari, Kassuya, Bastos, & Andrade, 2007; Wan, Yuan, Cirello, & Seeram, 2012). It is estimated that only 1/3 of the plants in the world have been investigated for their chemical constituents, leaving numerous possibilities for discovery of potential drug candidates (Butler, 2004; Cragg & Newman, 2005).

3. Extraction and Isolation:

Natural product extracts contain complex undetermined chemical entities that require proper characterization. These compounds are isolated from various parts of plants such as roots, barks, leaves, flowers, fruits and seeds (Newman, Cragg, & Snader, 1999). In this work, the crushed plant roots were partitioned using two different polarity solvents, butanol (more polar) and ethyl acetate (less polar). A polar solvent system is used to eliminate components of the plant such as waxes, fatty acids, sterols, chlorophylls and terpenes. The fractions are then further isolated through chromatography using different stationary phases. There are several types of resins that can be used to further purify a fraction. The choice of method depends on the stability of the analyte and the physical state of the sample. The most commonly used technique for separation of compounds involves developed HPLC methods. Common resins used to separate compounds are: Sephadex ^{TM} LH-20, C-18, XAD-16 and MCI.

Sephadex LH-20 is a cross-linked, dextran-based resin. It separates compounds based on molecular size and polarity. The bead cross-linked dextran contains a hydrophobic characteristic. Depending on the solvent used, the LH-20 resin will expand to different sizes, limiting what is eluted.

C-18 columns are packed with silica particles that are bonded to 18 carbon chain units in length, making this reverse phase column hydrophobic. When a sample is loaded onto the column, the polar compounds will elute first followed by the non-polar material which will elute later due to increased partitioning into the lipophilic stationary phase (like-dissolves-like rule). Generally, a gradient solvent system (organic: aqueous) with increasing organic content would be used to elute the

compounds. Common, organic solvents that are used to elute the compounds are methanol and acetonitrile.

XAD-16 resin is used to absorb hydrophobic molecules from polar solvents. Its characteristic pore size allows for the absorption of organic substances of relatively low to medium molecular weight. A gradient system of decreasing polarity is used.

MCI chromatography is a reverse-phase column that separates the compounds into structurally similar groups. A gradient system of decreasing polarity is used.

Once a compound is isolated and in its purest form it's structure can then be elucidated by NMR (Kwan & Huang, 2008). The mass of the pure compound can also be determined by using a Mass Spectrometer. With the help of these instruments, an analyst is frequently able to determine the chemical structure of the isolated compound (Stalikas, 2007).

3.1. Flavonoids:

A wide variety of flavonoids are introduced into the human diet through the consumption of fruits and vegetables (Lila, 2004). They make up a broad group of natural product compounds with useful biological properties. The common carbon-skeleton for these large groups consist of C6-C3-C6 structure characterization. Anthocyanins are common flavonoids responsible for the dark pigments found in fruits and vegetables (Takanori, 2012). They play an important role in plant life as a protecting agent against infection (bacterial, viral and fungal), UVB protection, pollination and plant and animal interaction (Su, 2012). They are also well known for efficiently scavenging and neutralizing free radicals which can cause damage to cells within the body (Lila, 2004). Flavonoids have been reported to have important

biological activity such as anti-spasmodic, hepatoprotective, anti-inflammatory, and antiviral activity in animal models (Van Acker, De Groot, Van Den Berg, Tromp, Kelder, Van Der Vijgh, et al., 1996).

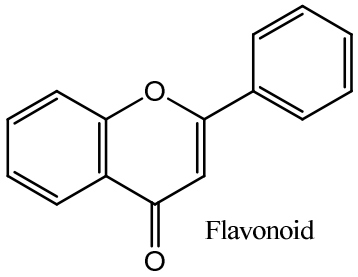

Figure 1: Basic skeletal structure of a Flavonoid

3.2. Hydroxycinnamic acids:

Hydroxycinnamic acids are non-flavonoid phenolics responsible for the integrity of the plant's cell wall structure as well as a defense mechanism against pathogens. These compounds are known to have UV protective and anticancer properties. (Faulds & Williamson, 1999).

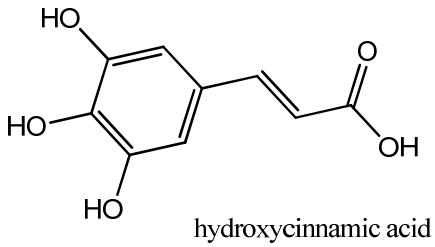

Figure 2: Basic skeletal structure of a Hydroxycinnamic acid

3.3. Lignans:

Lignans are a class of naturally occurring secondary metabolites within the plant and animal kingdoms. Lignans are produced through the oxidative dimerization of two linked phenylpropanoid units (C3-C6). They are frequently studied in the scientific community due to their ability to mimic the hormone estrogen. Compounds able to do this are known as phytoestrogens (Saleem, Kim, Shaiq, & Lee, 2005). Lignans are believed to be linked with breast cancer prevention. When there is an abundance of estrogen in the body, lignans may reduce the levels by displacing it from the cells. By decreasing estrogen levels, breast cancer cells are unable to grow and divide. Even though the biological activity of lignans is still unclear, they are known to have potent properties for antimicrobial, antifungal, antiviral, antioxidant, insecticidal and anti-feeding. Some speculate that lignans may participate in plant growth and defense mechanisms. Lignans have been isolated from various parts of the plant, including wooded parts, leaves, flowers, fruits and seeds (Smeds, Eklund, Sjohnolm, Willfor, Nishida, Deyama, et al., 2007).

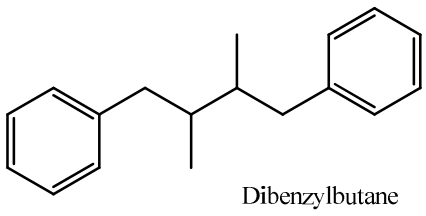

Figure 3: Basic skeletal structure of a Lignan

4. Family: Ericaceae

The Ericaceae family consists of 4000 species from 126 genera. This family is also commonly known as the heath family (Routray & Orsat, 2011). They are distributed worldwide, especially in the cooler areas of the northern hemisphere, but absent in continental Antarctica, central Australia and Greenland. They strive in acidic soils and the roots are usually closely associated with fungi for nutrients (Rowland, Alkharouf, Darwish, Ogden, & Main, 2012). Examples of some of the commercially well-known Ericaceae plants are; cranberries, blueberries, huckleberry and rhododendron. This family consists of a diverse group of plants. It contains plants that are shrubs, herbs, and trees and the petals are fused funnel shaped or widely bowl-shaped (Rowland, Alkharouf, Darwish, Ogden, & Main, 2012).

5. Genus: Vaccinium

Vaccinium is a morphologically diverse genus of shrubs or lianas. The common commercially grown *Vaccinium* species are; huckleberry, cranberry, whortleberry, lingonberries and blueberry (Routray & Orsat, 2011). This group of approximately 450 species is especially recognized for its wide range of therapeutic compounds (Su, 2012). Of these compounds, the production of flavonoids and anthocyanins are well documented from this genus (Routray & Orsat, 2011). The extracts and isolates from the leaves and fruits contain antioxidative, antinociceptive, anti-inflammatory, antitumor, antiviral, vasoprotective, and antiviral properties (Torri, Lemos, Caliari, Kassuya, Bastos, & Andrade, 2007). The phenolic compounds from the *Vaccinium* berry and leaves may have commercial value in the pharmaceutical and

cosmetic industry due to their potential health benefits. (Su, 2012; Torri, Lemos, Caliari, Kassuya, Bastos, & Andrade, 2007).

5.1. *Vaccinium corymbosum* (Northern Highbush Blueberry)

Vaccinium corymbosum, commonly known as the highbush blueberry is one of the most commercially cultivated fruit crops in North America (Routray & Orsat, 2011). It is known for its dark blue fruits that are beneficial to human health. Investigations of various species of *Vaccinium*, such as cranberry, bilberry, and huckleberry, have shown the fruits to contain a wide range of phytochemicals that are antioxidant, anti-cancer, anti-bacterial anti-viral and anti-aging (Adams, Phung, Yee, Seeram, Li, & Chen, 2010; Alejandro, Eicholz, Rohn, Kroh, & Huyskens-Keil, 2008; Wilson, Shukitt-Hale, Kalt, Ingram, Joseph, & Wolkow, 2006). It is known that blueberries have one of the highest levels of antioxidant capacity among most fruits and vegetables (Zeng & Wang, 2003).

Phytochemicals from the Roots of the Northern Highbush Blueberry (*Vaccinium corymbosum)*

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Abstract

Growing evidence from many *in vitro* studies suggest that secondary metabolites produced by plants may have potential physiological properties. The Northern Highbush blueberry (*Vaccinium corymbosum* L.) plant is commercially cultivated for its valuable dark-blue, sweet fruit. This small round fruit has been extensively researched and shown to contain phenolic compounds that are praised for having positive health benefits. Thus, it would be worthwhile to evaluate other parts of the plant that as of yet have not been investigated. There may be undiscovered bioactive compounds within the roots. Blueberry root compounds were extracted and their phytochemical constituents were isolated using chromatographic techniques and identified by spectroscopic techniques. Six phenolic compounds were identified by ^{1}H and ^{13}C NMR and mass spectrometry data. Three lignans, which were not previously reported in the blueberry plant, were identified as; lyoniside, nudiposide and ssioriside (**1-3**); one phenolic acid, sinapic acid glycoside and two catechins, dulcisflavan and epigallocatechin were identified (**4-6**). The isolated compounds were evaluated for inhibitory effect on of tyrosinase and α-glucosidase . Overall, of the six compounds elucidated, ssioriside (**3**) showed moderate inhibition of α-glucosidase enzyme and epigallocatechin (**6**) was a weak inhibitor of tyrosinase enzyme.

Keywords: *Vaccinium corymbosum*; Highbush blueberry flowers; Phenolics; Antioxidant activity; α-glucosidase inhibition; Tyrosinase inhibition

1. Introduction:

Northern Highbush blueberries are commercially grown in northern states of North America (Wan, Yuan, Cirello, & Seeram, 2012). They are bred for consumption as fresh fruit along with being processed into jams, juice, syrups, teas and sweets. The highbush blueberry differs from that of the lowbush blueberry in the way it is grown. Highbush blueberries are grown on plantations while lowbush blueberries are wildly grown. They are also phenotypically different in height, leaf color and fruit size (Routray & Orsat, 2011). Both are noted for their high antioxidant capacities which have been linked to the anthocyanin pigment content of the fruit (Su, 2012). The major compounds found in blueberries are flavonoids and phenolic acids. It is also known that blueberries contain condensed tannins known as proanthocyanidins (Torri, Lemos, Caliari, Kassuya, Bastos, & Andrade, 2007). These complex compounds are known for their activity in inhibiting the beginning stages of chemically-induced carcinogenesis. A known cancer preventative compound from the fruit of the wild blueberry is quercetin, which is also found in wine (Bilyk & Sapers, 1986). Blueberries are one of the most abundant sources of phenolic compounds. The major glycosides found in Northern Highbush blueberry are derivatives of malvidin and delphinidin (Kader, Rovel, Girardin, & Metche, 1995). Stilbenes have been reported to be present in blueberries (Rimando, Kalt, Magee, Dewey, & Ballington, 2004). They are reported to exhibit significant effects against cell proliferation, inflammation, lowering cholesterol in animal models and to reverse the effects of aging in hamsters (Rimando & Cody, 2005; Rimando, Nagmani, Feller, & Wallace, 2005). Yi *et al*. reported that the phenolic compounds in rabbiteye blueberry (*V. ashei*) can inhibit

cancerous cell proliferation and induce cancer cell apoptosis (Yi, Fischer, Krewer, & Akoh, 2005).

Blueberries are not only known for their antioxidant ability; a preliminary study has shown that blueberry supplementation may improve memory in older adults (Krikorian, Eliassen, Nash, & Shidler, 2010; Rimando & Nagatomi, 2005). According to Krikorian *et al.*, adults with early stages of memory loss were analyzed for 12 weeks. During this time, they were given either blueberry juice or a placebo and were then tested for memory performance. Those given the blueberry juice tested had higher scores, suggesting that the juice may have neurocognitive benefits (Krikorian, Eliassen, Nash, & Shidler, 2010).

Our group recently isolated bioactive compounds from the flowers of the blueberry plant (Wan, Yuan, Cirello, & Seeram, 2012). The flowers were investigated and evaluated for their antioxidant and α-glucosidase inhibitory activities. Out of the 21 phenolics isolated the phenylpropanoid-substituted catechins and the flavonol glycosides showed superior antioxidant activity compared to the positive control, vitamin C and butylated hydroxytoluene. The phenolic sub-classes were more potent α-glucosidase inhibitors than the clinical drug, acarbose. This provided our group with stronger evidence that the non-consumed parts of food plants may be a source of bioactive compounds (Wan, Yuan, Cirello, & Seeram, 2012).

The objectives of this project were to isolate, elucidate, and biologically evaluate (for antioxidant activity and inhibition of tyrosinase and α-glucosidase enzymes) phytochemicals in the roots of the highbush blueberry plants. This is the first phytochemical and biological study of roots of the highbush blueberry species.

2. Materials and Methods

2.1. General Experimental Procedures

Optical rotations were measured on an Auto Pol III automatic polarimeter (Rudolph Research, Flanders, NJ, USA). Compounds **1-3** were analyzed on a Bruker 400 MHz NMR whereas compound **6** was analyzed on a Bruker 300 MHz NMR and compounds **4** & **5** were analyzed on a Varian 500 MHz NMR instrument with TMS as an internal standard. The chemical shifts (δ) are expressed in parts per million and coupling constants (J), in hertz. Deuterated methanol (methanol-d_4) was used as a solvent for all NMR experiments. All mass spectral data was obtained on a Sciex Q-Star Elite (Applied Biosystems MDS) equipped with a Turbo-Ionspray source. The UV spectra were measured on a SHIMADZU UV-2550 UV-visible spectrophotometer. High performance liquid chromatography (HPLC) were performed on a Hitachi Elite LaChrom system consisting of an L2130 pump, an L-2200 autosampler and an L-2455 diode array detector using a Waters Sunfire C_{18} column (250 x 10 mm 5 µM), were operated by EZChrom Elite software. All solvents were ACS or HPLC grade and were purchased from Wilkem Scientific (Pawtucket, RI). α-Glucosidase (yeast, EC 3.2.1.20) powder, mushroom tyrosinase, 4-nitrophenyl-α-D-glucopyranoside, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid (vitamin C) and butylated hydroxytoluene (BHT), were all purchased from Sigma-Aldrich (St. Louis MO). Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Extraction of Blueberry Roots

The whole root material of the highbush blueberry species (*Vaccinium corymbosum* var. 'Jersey') were collected locally from Morgan farms (North Kingstown, RI, USA) in December 2009. The roots were dried in an oven for 48 hours and then grounded using a Draper® Garden Shredder. Voucher specimens (16JPM51-VCBR12809R) are deposited in the Heber-Youngken Garden at the College of Pharmacy, University of Rhode Island (Kingstown, RI, USA). The ground roots (400 g, fresh weight) were submerged in methanol (2000 mL) and mixed for 72 hours to extract all phenolic compounds. The roots were then filtered and the solvent was dried to obtain a methanol extract (80g).

The crude extract was re-dissolved in water (1000 mL). The extract was partitioned using two polar solvents. The solvents used were butanol vs. water and ethyl acetate vs. water as shown in Figure 1. All the masses of extracts and fractions are presented in more detail in the Results and Discussion section.

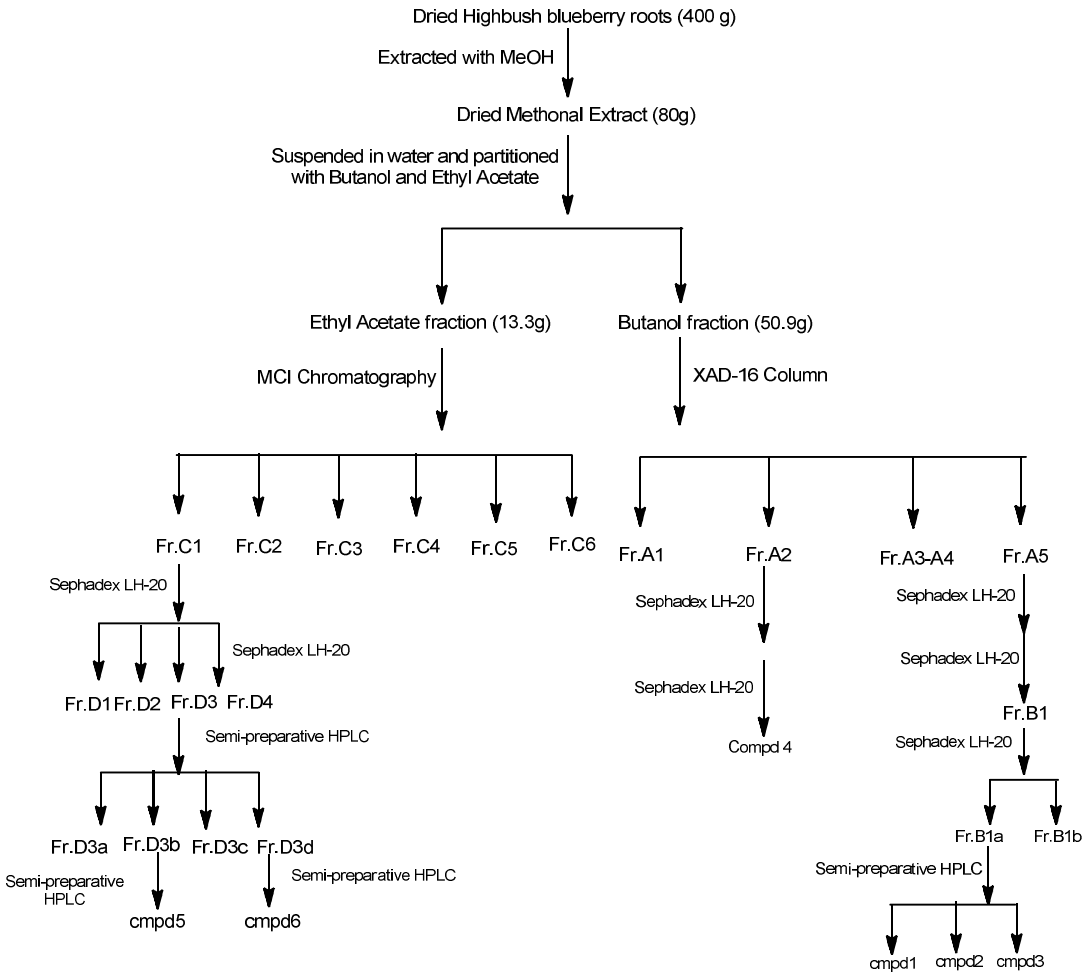

Figure 4. Extraction and isolation scheme of compounds 1–6 from Northern Highbush blueberry roots.

2.3 Isolation of Compounds

Figure 4 shows a detailed flow chart of the isolation of compounds from the highbush blueberry roots. Briefly, the butanol extract (50.9 g) was loaded onto an XAD-16 resin column (45 x 3 cm), and eluted with a gradient solvent system of methanol in aqueous water (0.1% trifluoroacetic acid, TFA) to obtain five major fractions A-1 through A-5 which were combined based on analytical HPLC analyses. Fraction A-5 (0.79g) was

further purified over an LH-20 column using a methanol/water gradient system to obtain five fractions, B1 through B5. Fraction B1 (400 mg) was then absorbed onto a smaller LH-20 column using an isocratic solvent system of methanol to obtain two sub-fractions. Similar fractions were combined by analyzing their chromatogram on an HPLC. Of those fractions, one fraction (15.45 mg) containing three peaks was individually purified by semi-preparative HPLC using a Waters Sunfire Prep C_{18} column (10 x 250 mm, i.d., 5 µm; flow 2mL/min.) with a gradient elution system of methanol water. The program used was: 0–40 minutes 30% methanol; 40–45 minutes 60% methanol; 45–50 minutes 30% methanol. The peaks were collected over several runs according to the retention time (RT) and absorbance wavelength. The compounds isolated, according to NMR and Mass spectra data were: lyoniside (**1**) (2.6 mg; dark yellow powder), nudiposide (**2**) (2.6 mg; dark yellow powder), and ssioriside (**3**) (1.9 mg; dark yellow powder).

Fraction A2 (0.43 g) was further purified and loaded onto a small LH-20 column. Through repeated analytical HPLC, similar fractions were combined. Of the similar compounds, one fraction was loaded onto a semi-preparative HPLC C_{18} column at 50 µL of sample per injection. According to NMR is sinapic acid glucoside (**4**) (2.5 mg; yellow powder). The ethyl acetate fraction (13.3 g) was loaded onto an MCI resin column (45 x 3 cm), and eluted with a gradient solvent system of methanol and water (0.1% trifluoroacetic acid, TFA) to obtain six major fractions C-1 through C-6. Fraction C-1 (4.89g) was further purified over an LH-20 column (45x3cm) using a methanol/water gradient system to obtain four sub-fractions D1 through D4. Fraction D3 (1.1g) was then absorbed onto a smaller LH-20 column using an isocratic solvent

system of methanol to obtain sub-fractions. Through repeated analytical HPLC analysis, similar fractions were combined. Of those fractions, D3b (310.7mg) which contained four peaks, was further purified using preparative HPLC and a Waters Sunfire Prep C_{18} column (10 x 250 mm, i.d., 5 µm; flow 2mL/min.) with an isocratic system of 25% methanol in water for 35 minutes. The peaks were collected over several runs according to the RT and absorbance wavelength. The compound isolated according to NMR and Mass spectral data was dulcisflavan (**5**) (3.2 mg; light brown powder).

The similarly profiled fractions from Fraction D-3 were combined to give D3d. This fraction was loaded onto a semi-preparative HPLC C_{18} column at 50 µL of sample per injection with an isocratic system of 80% aqueous and 20% organic for 55 minutes. The compound isolated was epigallocatechin (EGC) (**6**) (3.6 mg; light brown powder).

2.4 Identification of Compounds

All of the isolated compounds **1-6** (chemical structures shown in Figure 2) were identified by analyzing the ^{1}H and/or ^{13}C NMR along with the Mass spectral data. These findings were then compared with previously published literature reports. The ^{1}H and ^{13}C NMR data for all the compounds are shown in Tables 1 (compounds **1**-**2**), Table 2 (compounds 3-4) and Table 3(compounds **5**-**6**).

Lyoniside (**1**): colorless needles; $[M+Na]^{+}$ 575.1151 $C_{27}H_{36}O_{12}$; $[\alpha]_D$+43^{o}. The ^{1}H and ^{13}C NMR data were consistent with previous literature (Szakiel & Henry, 2007).

Nudiposide (**2**): dark yellow needles; $[M+ Na]^+$ 575.1151 $C_{27}H_{36}O_{12}$; $[\alpha]_D$ -21.0°. The ^1H and ^{13}C NMR data were consistent with previous literature (Dada & Corbani, 1989). See Table 1.

Ssioriside (**3**): dark yellow amorphous powder: $[M+ Na]^+$ 577.1291 $C_{27}H_{38}O_{12}$. The ^1H and ^{13}C NMR data were consistent with previous literature (Kiyoshi, Yutaka, & Hiroko, 2005).

Sinapic acid glucoside (**4**): light yellow color, $C_{17}H_{22}O_{10}$. The NMR data for this compound are being reported here for the first time.

Dulcisflavan (**5**): light brown color, $C_{15}H_{14}O_8$. The ^1H NMR data was consistent with previous literature (Deachathai, Mahabusarakam, Phongpaichit, & Taylor, 2005).

Epigallocatechin (EGC) (**6**): light brown color, $[M+ Na]^+$ 329.0633 $C_{15}H_{14}O_7$. The ^1H NMR data was consistent with previous literature (Yang, Chang, Chen, & Wang, 2003).

2.5 Analytical HPLC

All analyses were conducted with a Luna C18 column (250 x 4.6 mm i.d., 5 µm; Phenomenex) with a flow rate at 0.8 mL/min and injection volume of 20 µL. A gradient solvent system consisting of solvent A (0.1% aqueous trifluroacetic acid) and solvent B (MeOH) was controlled throughout all analytical runs.

2.6 Antioxidant Assay

The antioxidant potential of the blueberry crude extract were determined on the basis of their ability to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical as previously reported (Li & Seeram, 2010). The positive controls that were

used for this study ascorbic acid (vitamin C) and butylated hydroxytoluene (BHT). The assay was conducted on a 96-well format using serial dilutions of 50 µL aliquots of test compounds, vitamin C and BHT. After this, 100µL DPPH (80mg/mL) was added to each well. Absorbance was determined after 30 min of reaction in the dark at 515 nm with a micro-plate reader (SpectraMax M2, Molecular Devices Corp., operated by Softmax Pro v. 4.6 software, Sunnyvale, CA, USA). The scavenging capacity (SC) was calculated as $\%SC = [(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance of the reagent blank and A_1 is the absorbance of the test samples. All tests were performed in triplicate. IC_{50} values denote the concentration of sample required to scavenge 50% DPPH free radicals.

2.7 α-glucosidase Inhibitory Assay

The assay was conducted as previously reported. Briefly, a mixture of 50 µL of different concentrations of the compounds and 100 µL of 0.1 M phosphate buffer (pH 6.9) containing yeast α-glucosidase solution (1.0 U/mL) was incubated in 96 well plates at 25^{o}C for 10 min. After pre-incubation, 50 µL of 5 mM p-nitrophenyl- α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25^{o}C for 5 min. Absorbance was recorded at 405 nm before and after incubation with a micro-plate reader (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA, USA) and compared to that of the control which had 50 µL acarbose instead of the test compounds. The α-glucosidase inhibitory activity was expressed as % inhibition and was calculated as follows:

$$\%\,\text{inhibition} = \left(\frac{\Delta\text{Abs}_{\text{control}} - \Delta\text{Abs}_{\text{sample}}}{\Delta\text{Abs}_{\text{control}}} \right) \times 100$$

2.8 Tyrosinase Inhibitory Assay

The enzymatic assay was conducted as previously reported. Briefly, L-DOPA was used as the substrate in this experiment. The activity was measured by spectrophotometrically. A mixture of 1 mL of L-DOPA and 1.8 mL of 0.1 mL of PBS (pH= 6.8) was incubated in 96 well plates at 25^{o}C for 10 min. After pre-incubation, 0.1 mL of each compound and 0.1 mL of the tyrosinase solution were added to separate wells. The positive control was kojic acid. The samples were then compared to the control which is measured to have 100% inhibition. The reaction mixtures were incubated at 25^{o}C for 5 min. Absorbance was recorded at 490 nm before and after incubation with a micro-plate reader (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA, USA). The tyrosinase inhibitory activity was expressed as % inhibition and was calculated as follows:

$$\%\,\text{inhibition} = \left(\frac{\Delta\text{Abs}_{\text{control}} - \Delta\text{Abs}_{\text{sample}}}{\Delta\text{Abs}_{\text{control}}} \right) \times 100$$

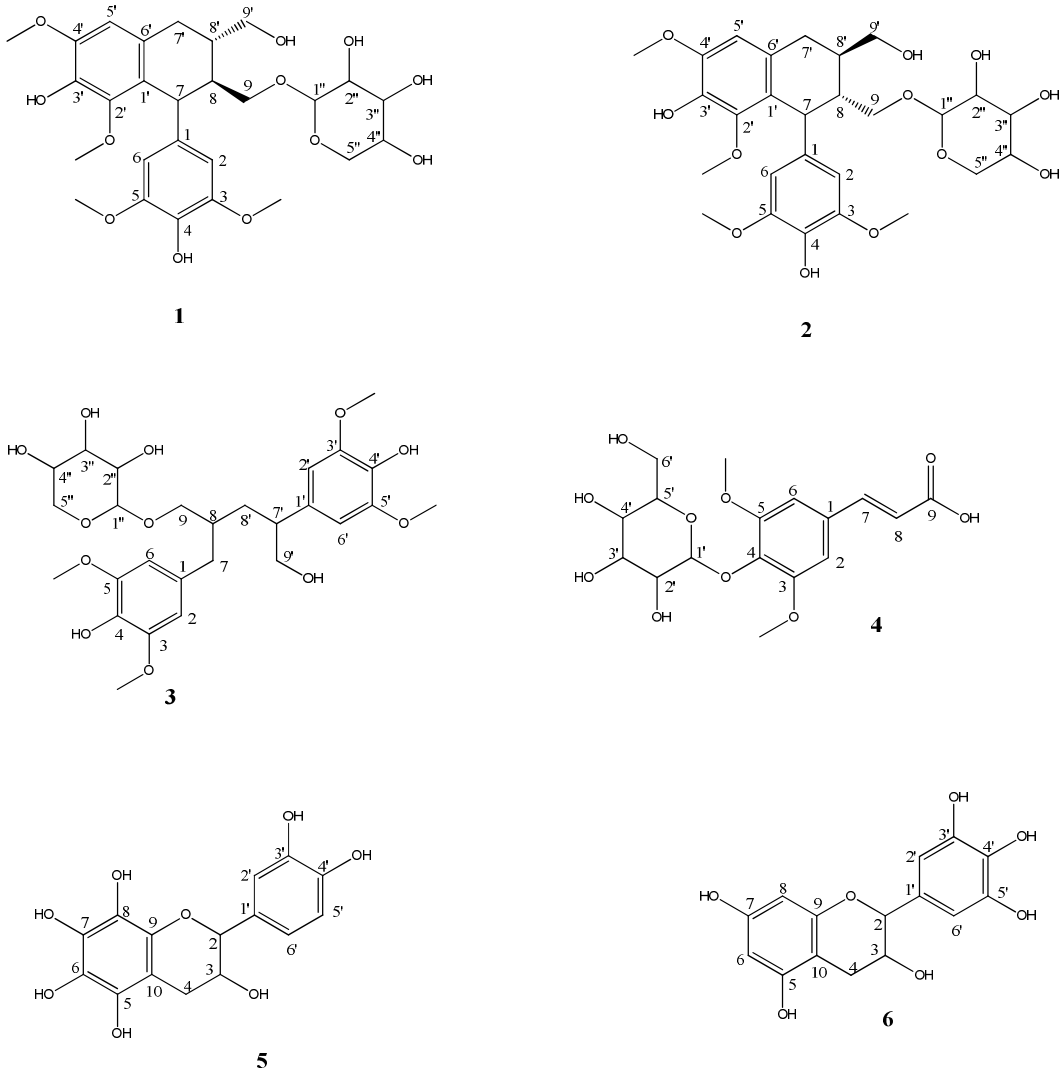

Figure 5. The chemical structures of the isolated compounds: lyoniside (1); nudiposide (2); ssioriside (3); sinapic acid glycoside (4); dulcisflavan (5); and epigallocatechin (6)

Table 1. δ_H and δ_C (400MHz) NMR Spectra Data of lyoniside (**1**) and nudiposide (**2**) (CD_3OD) (δ in ppm)

	1		**2**	
Position	δ_H	δ_C	δ_H	δ_C
1		139.3		139.0
2,6	6.43,6.42 s	106.9	6.42, s, 2H	107.0
3,5		149.0		149.1
4		134.5		134.0
7	4.38, t	43.2	4.23, t, J= 7.2	43.5
8	2.1, m	46.9	2.02, m	47.0
9	3.83, 2H, m	71.0	3.83, 3.5, m	71.1
1'		126.5		126.0
2'		147.7		147.8
3'		139.1		139.0
4'		148.8		147.8
5'	6.58, s	107.9	6.58, s	107.8
6'		130.2		130.2
7'	2.7, 2H, m	34.1	2.7, 2H, m	34.2
8'	1.75, m	40.5	1.72, m	40.8
9'	3.65, 3.58, m	66.1	3.8, 3.5, m	66.1
OCH_3		60.1		60.1
$3xOCH_3$		56.6		56.6
1"	4.23, d, J= 7.2	105.7	4.10, d, J= 7.2	105.7
2"	3.25, m	75.1	3.25, m	75.1
3"	3.35, m	78.2	3.35, m	78.1
4"	3.50, m	71	3.50, m	71.4
5"	3.5, 3.83, m	67.1	3.5, 3.80, m	67.2

Table 2. δ_H and δ_C (400MHz) NMR Spectra Data of ssioriside (3) and sinapic acid glycoside (4) (CD_3OD) (δ in ppm)

	3		**4**	
position	δ_H	δ_C	δ_H	δ_C
1		133.1		130.8
2	6.35 or 6.33, s	107.2	6.58, s, J= 16.5	105.8
3		149.0		153.1
4		133.3		144.7
5	4.38, t	149.0		153.1
6	6.35 or 6.33 s	107.1	6.58, s, J= 16.5	105.8
7	2.56, m	36.2	6.36, d, J= 16.5	108.1
8	2.06, m	44.2	7.53, d, J= 16.5	117.0
9	3.55, 3.86, m	70.96		68.9
10				
1'		133.1	4.89, d, J= 7.5 Hz	103.4
2'	6.35, s	107.1	3.17-3.4, m	77.3
3'		149.0	3.17-3.4, m	76.4
4'		133.3	3.17-3.4, m	74.3
5'		149.0	3.17-3.4, m	69.9
6'	6.35, s	107.1	3.57 and 3.66, d	61.1
7'	2.56, 2.62, m	36.4		
8'	1.94, m	41.47		
9'	3.98, 3.47, m	62.8		
OCH_3	3.75, s	56.7	3.79, s	55.7
OCH_3	3.75, s	56.7	3.79, s	55.7
OCH_3	3.75, s	56.7		
OCH_3	3.75, s	56.7		
1"	4.19, d, J= 7.2	105.5		
2"	3.2-3.8, m	75.2		
3"	3.2-3.8, m	78.1		
4"	3.2-3.8, m	71.4		
5"	3.2-3.8, m	67.2		
6"				
7"				

Table 3. δH (400MHz) NMR Spectra Data of dulcisflavan (**5**) and epigallocatechin (**6**) (CD_3OD) (δ in ppm)

	5	**6**
Position	δ_H	δ_H
1		
2	4.82, s	4.4, s
3	4.18, br, s	3.4, br, s
4	2.88, d, J=17.0, 5.0 2.75 dd, J=17.0, 3.0	2.8, d, 2.7, d
5		
6		5.9
7		
8		5.9
9		
1'		
2'	6.97, d, J= 2.0	6.5, s
3'		
4'		
5'	6.76, d, J=8.0	
6'	6.81, dd, J=8.0, 2.0	6.5,s

3. Results and Discussion:

3.1 Isolation and Structural Elucidation of Compounds

Six phenolic compounds (chemical structures shown in Figure 5) were isolated from the roots of the highbush blueberry plant using a variety of chromatographic techniques (see Figure 4). The compounds were identified by using NMR and Mass spectral data and then compared to previous literature. The ^{13}C NMR data for compounds **1-2** and **3-4** are provided in Table 1 and 2, respectively. The ^{1}H NMR data

for compounds; **1-2**, **3-4**, **5-6** are provided in Table 1, 2 and 3, respectively. For ease of discussion, the isolates are grouped into three phenolic sub-classes as follows.

3.2 Lignans

The blueberry roots isolates included lignans and were identified as lyoniside (**1**), nudiposide (**2**), ssiorside (**3**). Lyoniside was obtained from the European blueberry known as the bilberry (Szakiel, Voutquenne-Nazabadioko, & Henry, 2011). However; this is the first report of compounds, **2** and **3** being isolated from the blueberry plant.

3.3 Phenolic acid

One phenolic acid was isolated from the highbush blueberry roots and identified as sinapic acid glycoside (**4**). This is the first report of sinapic acid glycoside being isolated from the blueberry plant.

3.4 Catechins

Two catechins were isolated from the highbush blueberry roots and were identified as dulcisflavan (**5**) and epigallocatechin (**6**). This is the first report of compound **5** from the genus *Vaccinium*.

3.5 Antioxidant Activity

Blueberry fruits are a good source of antioxidants. Thus, a preliminary study was conducted to test the crude extract of the blueberry roots. It was observed that the roots had free-radical scavenging activity *in vitro*.

3.6 α-glucosidase enzyme Inhibitory Activity

The number of people with diabetes is increasing by 4-5% each year and through its long-term effect it is the cause of the of the highest morbidity rate around the globe. Wan *et al*. showed that the flavonol glycosides and phenylpropanoid-

substituted catechins isolated from the flowers of the highbush blueberry showed superior α-glucosidase inhibitory activity compared to the positive control, acarbose (Wan, Yuan, Cirello, & Seeram, 2012). Moghe et al. showed that blueberry polyphenols suppressed adipocyte differentiation in the cell lines at three doses. The higher doses had a stronger effect against mouse 3T3-F442A preadipocyte cell lines (Moghe, Juma, Imrhan, & Vijayagopal, 2012). With much effort focused on diabetes research and the positive results from the study of the blueberry flowers, it is worthwhile to investigate the pure compounds of the roots for their α-glucosidase inhibitory activity. Of the six compounds evaluated, ssioriside was a moderate inhibitor of α-glucosidase (IC_{50} = 650 μM).

3.7 Tyrosinase enzyme Inhibitory Activities

Melanin's main function in humans is to protect the skin from harmful effects of UV radiation from the sun. Melanin is produced through a mechanism in which tyrosinase plays a major role. Tyrosinase is a multifunctional enzyme which catalyzes the first two steps in the formation of melanin. It is also responsible for enzymatic browning of fruit after harvesting during the handling and processing. Although, melanin is important in protecting the skin an accumulation of abnormal amounts of melanin can lead to cancer (Matsuura, Ukeda, & Sawamura, 2006). Therefore, it is important to look into potent tyrosinase inhibitors. Of the compounds tested the only compound to show inhibitory effects was epigallocatechin (IC_{50} = 2001 μM).

Table 4

α-glucosidase & tyrosinase enzyme inhibitory activities of compounds **1-7**. Antioxidant activity of crude root extract

Compounds	IC_{50} (μM)[a]		
	α-glucosidase	tyrosinase	Antioxidant
lyoniside	n.d.	n.d.	
nudiposide	n.d.	n.d.	
ssioriside	650	n.d.	
sinapic acid glycoside	n.d.	n.d.	
dulcisflavan	n.d.	n.d.	
epigallocatechin	n.d.	2001	
Crude root extract	-	-	42 (μg/mL)
BHT[b]	-	-	
Vitamin C[b]	-	-	
Kojic acid[b]	-	830	-
Acarbose[b]	170	-	-

n.d. = not detected

[a] IC_{50} values

[b] Positive control

4. Conclusion:

In summary, six phenolics were isolated from highbush blueberry roots. This is the first report of nudiposide, ssioriside, sinapic acid glycoside, and dulcisflavan to be isolated from this genus. The structural elucidation of the phenolic compounds from a non-consumed plant part could help to further understand the potential health benefits of secondary metabolites of natural products. This supports the data suggesting that phenolic compounds are known to have potential nutraceutical applications. Non-

consumed plant parts from food plants should be exploited as potential sources of bioactive compounds.

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CONCLUSION

In this phytochemical study, the focus was on Northern Highbush blueberry (*Vaccinum corymbosum*) constituents obtained from the roots. Previous studies reported that blueberry fruit contain phenolic compounds which are praised for having positive health benefits which led to further examination of the plants' composition beyond the fruit.

Blueberries are known not only to be packed with antioxidants but also to aid in inhibiting cell proliferation and adipogensis and to help promote urinary tract health and digestion (Moghe *et al.* 2012; Wan *et al*. 2012). Along with being naturally sweet, blueberries contain 14mg of vitamin C in one serving, which is 25% of one's recommended daily requirement. They are also an excellent source of manganese, which plays an important role in bone development and conversion of proteins, carbohydrates and fats into energy. Blueberry producers convert the fruit into many consumable products including beverages, cakes, jams and dried fruits. Scientific studies have shown that the fruit of blueberries possess a range of pharmacological properties and are leaders in antioxidant activity (Su, 2012).

The compounds isolated from the roots have been previously reported in other plant species and their plant parts. All compounds besides sinapic acid glycoside have been tested and reported to have biological activity which may contribute to human health(Matsuda, Ishikado, Nishida, Ninomiya, Fujwara, Kobayashi, et al., 1998; Szakiel & Henry, 2007; Szakiel, Voutquenne-Nazabadioko, & Henry, 2011; Yang, Chang, Chen, & Wang, 2003).

Nudiposide was isolated from the root barks of *Ulmus davidiana* var. *japonica* by Zeng *et al*. in 2011. Zeng *et al*. reported that nudiposide exhibited the most potent protective effect against sepsis *in vivo*. Sepsis is a clinical syndrome defined by the presence of both infection and systemic inflammatory response. It is often life-threatening, especially in people with a weakened immune system or with chronic illness. Zeng *et al*. also reported that nudiposide decreased the plasma levels of TNF-α, IL-10 and ALT activity (Zeng et al. 2011).

Matsuda *et al.* conducted a preliminary study of 50% aqueous methanolic extract from the bark of *Betula platyphylla* which showed potent inhibiting activity against liver injury induced by CCl_4 (Matsuda, et al., 1998). They then isolated nudiposide and lyoniside and revealed that these compounds had a positive effect on lipid peroxidation. Due to their antioxidative activity, it is believed these compounds are responsible for the potent inhibitory effect upon liver injury (Matsuda, et al., 1998).

Lyoniside was also isolated from the inner bark of *Betula pendula* by Smithe *et al.* in 1995 (Smite, Pan, & Lundgren, 1995). The compound was then re-isolated from the rhizomes and stems of *Vaccinium myrtillus* in 2011 by Szakiel *et al*. Lyoniside showed significant radical scavenging properties in a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The group also reported that lyoniside exerted strong antifungal activity (Szakiel, Voutquenne-Nazabadioko, & Henry, 2011).

Ssioriside was isolated from the root bark of *Ulmus davidiana* that is able to inhibit cellular senescence in HDFs and human umbilical vein endothelial cells. Twenty-two compounds from the root bark of *Ulmus davidiana* were isolated and

screened for their inhibitory effects on adriamycin-induced cellular senescence by measuring senescence-associated β-galatosidase activity. Among those twenty-two compounds isolated, ssioriside had shown significant inhibitory effects on adriamycin-induced cellular senescence in HDFs (Yang 2010).

A plethora of data has been reported on epigallocatechin. This compound possesses two epimers. The most common is (-)-epigallocatechin, most commonly found in green tea. This plant is used as folk medicine for stomach disorders and diarrhea. Catechins are known for their health-promoting effects (Ruidavets, Teissedre, Ferrieres, Carando, Bougard, & Cabanis, 2000). These include the slowing of age-related cognitive decline, according to Kuriyama *et al*. in 2006, and protection against obesity (Kao, Hiipakka, & Liao, 2000; Kuriyama, Hozawa, Ohmori, Shimazu, Matsui, Ebihara, et al., 2006). At the cellular level, catechins induce anti-inflammatory (Tedeschi, Suzuki, & Menegazzi).

Ruidavets *et al.* in 2000 reported which type of diet contributes most to plasma concentration of (+)-catechin by testing 180 subjects (Ruidavets, Teissedre, Ferrieres, Carando, Bougard, & Cabanis, 2000). A blood sample was collected after a fasting period, and (+)-catechin measurement in plasma was performed by HPLC method using fluorescence detection. Dietary consumption of the last evening meal was assessed by a dietary recall method. Taking fruit, vegetable and wine consumption into account, four types of diets were identified. After adjustment for confounding factors, concentration of (+)-catechin in plasma was three-fold higher in diet with fruit and vegetable but without wine (449.5 mg/l), and four-fold higher in diet with wine but without vegetable and fruit (598.5 mg/l) in comparison to diet without fruit,

vegetable and wine (131.6 mg/l). When the consumption of vegetable, fruit and wine was combined, the concentration was the highest (637.1 mg/l). Vegetable, fruit and wine were the major determinants of plasma (+)-catechin concentration. This study demonstrates that the highest plasma concentration of (+)catechin was observed in subjects consuming fruit, vegetable and wine, and its antioxidant and antiaggregant activity may be able to explain the relative protection against coronary heart disease (Ruidavets, Teissedre, Ferrieres, Carando, Bougard, & Cabanis, 2000).

Catechins have also been associated with a lower prevalence of cognitive impairment in humans. Kuriyama *et al.* examined the association between green tea consumption and cognitive function in humans. They analyzed data from a community-based Comprehensive Geriatric Assessment conducted in 2002. The subjects were 1,003 Japanese subjects aged less than 70 years old. They completed a self-administered questionnaire that included questions about the frequency of green tea consumption and evaluated cognitive function by using the Mini-Mental State Examination. Higher consumption of green tea was associated with a lower incidence of cognitive impairment (Kuriyama, et al., 2006).

Dulcisflavan was previously isolated from *Garcinia dulcis* Kurz (Deachathai *et al*. 2005) and *Spatholobus suberectus* (Ren-Neng *et al*. 2012). It is a medicinal plant used in Oriental folk medicine. In Thailand, its bark has been used as an anti-inflammatory agent; and in other traditional medicines, the fruit juice has been used as an expectorant (Deachathai, Mahabusarakam, Phongpaichit, & Taylor, 2005). Deachathai *et al.* reported on the antibacterial and radical scavenging abilities of this compound which had promising results. Dulcisflavan gave a reduction greater than

that produced by the positive control which was10µM BHT (Deachathai, Mahabusarakam, Phongpaichit, & Taylor, 2005).

Part I reviews the background of natural products while part II is the review of isolation and elucidation of phytochemicals extracted from the roots of the Northern Highbush blueberry which yielded six pure compounds: ssisoriside, lyoniside, nudiposide, sinapic acid glucoside, epigallocatechin and dulcisflavan. These compounds were elucidated using spectroscopic techniques including 1D- and 2D-NMR and mass spectrometry. The NMR data was confirmed by comparing it to previously published literature. The isolated compounds were tested for α-glucosidase and tyrosinase inhibitory activity.

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