

Evaluation of the Antioxidant and Anti-inflammatory Activity of Extracts and Flavonol Glycosides Isolated from the Seed Coats of Coloured Beans (*Phaseolus vulgaris* L.)

by

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ABSTRACT

The seed coat of the common bean (*Phaseolus vulgaris* L.) can be considered an important source of flavonoids. Flavonoids are well known for their beneficial effects on health long before they were isolated as effective compounds. Dry beans are typically processed and the seed coats may be removed and discarded prior to consumption. Therefore, a better understanding of the antioxidant and anti-inflammatory activity of coloured dry bean seed coats would be beneficial in determining their potential use as an ingredient in the functional food and nutraceutical industry. Flavonol glycosides were identified from acetone extracts of seed coats of black beans, pinto beans, and dark and light red kidney beans, representing nine varieties grown in Manitoba. Based on HPLC-MS/MS, black beans contained the 3-*O*-glycosides of kaempferol, quercetin, and myricetin. Pinto beans contained kaempferol 3-*O*-glycosides, while light and dark red kidney beans contained quercetin 3-*O*-glycosides. In addition, we reported the presence of a flavonol triglycoside for the first time in dry bean seed coats. Concentrations of kaempferol-3-*O*-glucoside were the greatest varying from 0.44 to 7.08 mg/g of dried seed coat weight, followed by quercetin-3-*O*-glucoside varying from 0.91 to 3.84 mg/g of dried seed coat weight, and then rutin varying from 0.13 to 0.22 mg/g of dried seed coat weight. The DPPH method demonstrated seed coat crude extracts of Eclipse, a black bean, and Windbreaker, a pinto bean, to have the highest antioxidant activities among the samples. Eclipse, with the maximum concentration of total phenolic compounds, exhibited an antioxidant activity of 57,816 μmol trolox equivalent/100g of dried seed coat weight and Windbreaker, with the maximum concentration of total flavonoid compounds, exhibited an antioxidant activity of 57,451 μmol trolox equivalent/100g of dried seed coat

weight. Cellular measures of anti-inflammatory activity of seed coat crude extracts in LPS-induced murine macrophage RAW 264.7 cells showed both anti- and pro-inflammatory effects. Extracts of Windbreaker and Eclipse decreased TNF- α levels, suggesting anti-inflammatory properties, while other varieties showed increased levels of TNF- α production, or pro-inflammatory activity. The results indicate seed coats of Windbreaker and Eclipse may have the potential to be used as a functional food ingredient and possibly prevent disease and enhance human health.

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
amu	Atomic mass unit
ATCC	American Type Culture Collection
DAD	Diode Array Detector
DMEM	Dulbecco's modified Eagles media
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffer solution
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
FAE	Ferulic Acid Equivalent
FBS	Fetal bovine serum
HPLC	High-performance liquid chromatography
IFN	Interferon
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-12	Interleukin-12
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
K3G	Kaempferol-3- <i>O</i> -glucoside

LPS	Lipopolysaccharide
MAFRI	Manitoba Agriculture, Food and Rural Initiatives
MS	Mass Spectrometer (or Mass Spectrometry)
MCAF	Macrophage chemotactic and activating factor
MTT	(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)
NMR	Nuclear magnetic resonance
NO	Nitric oxide
Q3G	Quercetin-3- <i>O</i> -glucoside
QTOF	Quadrupole time-of-flight
PBS	Phosphate Buffered Saline
P/S	Penicillin-streptomycin solution
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
TE	Trolox Equivalents
TNF- α	Tumor Necrosis Factor alpha
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
t_R	Retention time
USDA	United States Department of Agriculture
UV-Vis	Ultraviolet-Visible

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CHAPTER 1: INTRODUCTION

Dry bean (*Phaseolus vulgaris* L.), commonly known as the common bean, is one of the most widely produced legume species in the world. The main types of dry beans produced in Canada are white and coloured beans. The different market classes of coloured beans include pinto, brown, cranberry, azuki, dark and light red kidney, small red, pink, and black. In 2010, Canada produced 254 thousand tonnes of dry beans primarily in Manitoba, Alberta, and southern Ontario with Manitoba representing 33 % of production (MAFRI, 2010). World demand for dry beans is increasing. Dry beans are a significant source of nutritional and non-nutrient compounds. Bioactive non-nutrient plant substances, including polyphenolic compounds, are mainly found in the seed coat of coloured dry beans (Aparicio-Fernandez et al., 2005; Oomah, et al. 2005). These polyphenolic compounds, specifically flavonols, have been suggested to exert beneficial effects in diseases such as cardiovascular disease (Perez-Vizcaino et al., 2006), some forms of cancer (Yang et al., 2001), and Parkinson's and Alzheimer's diseases (Weinreb et al., 2004). Prior to consumption, dry beans are typically processed and the seed coat for coloured cultivars can be removed and discarded. The coloured seed coats are a dietary source of flavonol compounds, which can potentially prevent disease and enhance human health. Therefore, an understanding of the flavonols, specifically flavonol glycosides, and their contents in coloured dry bean seed coats, as well as their chemical antioxidant activity and cellular anti-inflammatory activity, will assist in their further utilization as flavonoid resource materials.

Hypothesis:

Coloured dry bean seed coat extracts will show potent chemical antioxidant activity and cellular anti-inflammatory activity, which reflect their potential to be used as a functional food or nutraceutical ingredients.

Objectives:

The main objective of the study is to investigate the potential of coloured dry bean seed coat extracts as a functional food or nutraceutical ingredient. The specific objectives are:

1. To identify and quantify the main flavonoids, specifically flavonol glycosides, in the extracts of coloured dry bean seed coats
2. To investigate the chemical antioxidant and cellular anti-inflammatory effects of the extracts.

CHAPTER 2: LITERATURE REVIEW

2.1 Dry beans (*Phaseolus vulgaris* L.)

The common bean, or dry bean, belongs to the family Leguminosae, subfamily Papilionoideae. Among all food legumes, the common bean is the most important in the world after soybeans and peanuts (Singh, 1999). It is an ancient crop native to South America, Central America, and Mexico, which has expanded to North America and other parts of the world (Miklas and Singh, 2007). The common bean has been developed into many hundreds of varieties of different sizes, shapes, seed coat colors and patterns, and flavors. The beans are grown as dry seeds (dry beans) and harvested once the seeds are fully mature. Dry beans are a warm-season crop and sensitive to frost. The quality of dry beans is evaluated on the basis of size, shape, and color. The color of dry bean seed coats varies greatly among cultivars, with extensive genetic variation. Breeders are concerned with seed coat color in their breeding programs because of its commercial value. The dry bean seed consists of three distinctive parts: the cotyledon, the seed coat (hull), and the embryonic axis (germ), which represents, on average, 89, 10, and 1% respectively, of the total seed weight (Duenas et al., 2006).

2.1.1. Dry bean production

The total world production of dry beans exceeds 20 million metric tonnes (FAO, 2009). Bean production was greatest in Asia with 9 million metric tonnes, followed by

the Americas with 7 million metric tonnes. Of the total bean production in North America, Canada represents 5%. In Canada, the dry bean is an important crop, with production over 224 thousand tonnes in 2009 (AAFC, 2011). In 2010, total production increased to 254 thousand tonnes (AAFC, 2011). Dry beans in Canada are primarily produced in Manitoba, Alberta, and Ontario, with a small amount of production in Quebec and Saskatchewan (AAFC, 2005). In 2010, Manitoba produced 83.2 thousand tonnes of dry beans representing 33 % of those produced in Canada (MAFRI, 2010). A wide variety of market classes are available. The main types of dry beans produced in Canada are white and colored. The market classes for both white and coloured beans produced include navy (white), pinto, kidney (light red, dark red and white), cranberry, black, small Red, brown, ink and great northern.

2.1.2. Consumption of dry beans

The dry beans produced are primarily used for human consumption with a small portion of weather-damaged, low quality beans used for livestock feed. To be suitable for consumption, different processing methods are required and prior to certain methods, the seed coats or hulls are removed (Deshpande et al., 1982). Dry beans are either canned, packaged dry for retail sale or further processed into other products. In the preparation of protein concentrates and flours, dry beans are commonly dehulled and milled (Boye et al., 2010). Flours produced with dehulled beans have higher protein contents and the coloured seed coats are known to contain antinutritional compounds, which interfere with the bioavailability of nutrients (Barampama and Simard, 1993; Deshpande and Cheryan,

1984). The seed coat for colored beans are also removed for flour processing to deliver an acceptable end product to consumers. Abrasive dehulling and pre-soaking techniques are used to separate the seed coats from embryos of dry seed and the removed seed coats are typically discarded (Deshpande et al., 1982).

2.1.3. General health benefits

Dry beans have many health benefits. Dry beans are an excellent source of complex carbohydrates, protein, fiber, vitamins and minerals and are low in fat (Costa et al., 2006; Feregrino-Perez et al., 2008; Reyes-Moreno and Paredes-Lopez, 1993). On a dry weight basis, the beans contain approximately 5% crude fibre, 4-6% ash, up to 60% total carbohydrate, and less than 2% lipid (Van Der Poel, 1990). The major component of the carbohydrate fraction is starch. The high complex carbohydrates and high fiber content of dry bean elicits a low glycemic response, which reduces the risk of developing diabetes (Anderson et al., 1999; Anderson et al., 1994; Rizkalla et al., 2002). The soluble fiber in beans lowers blood cholesterol, which has a role in the prevention of cardiovascular disease (Anderson and Gustafson, 1988). Also, the available minerals in beans are important in reducing the risk of osteoporosis (Dawson-Hughes et al., 1990) and hypertension (Appel et al., 1997). Beans are an excellent source of folate, which in addition to being an essential nutrient is assumed to reduce the risk of neural tube defects (Daly et al., 1995). In addition to nutritional components, dry beans are a source of several non-nutrient plant substances, particularly plant polyphenols, which also exert beneficial health effects (Champ, 2002).

2.2 Phenolic compounds

Phenolic compounds are chemical compounds possessing a six-carbon (C_6) aromatic ring with one or more hydroxyl groups. Phenolic compounds are one of the most numerous and widely distributed groups of substances occurring in nature. They range from simple molecules to highly polymerized compounds and have different structures and chemical properties. Phenolic compounds can be classified according to the number of carbons in their basic skeleton (Table 1).

2.2.1. Total Phenolic Content (TPC)

Total phenolic content in food products is normally determined by the Folin-Ciocalteu method. The Folin-Ciocalteu reagent is a yellow acidic solution containing phosphotungstic and phosphomolybdic acids, which undergo a complex redox reaction with phenolic compounds (Singleton and Rossi, 1965). Phenolic compounds in alkaline medium transfer electrons to the acid complexes to form blue complexes determined spectrophotometrically at approximately 725 nm (Singleton and Rossi, 1965). The intensity of light absorption at that wavelength is proportional to the concentration of phenolics. Studies reporting total phenolic content of dry beans have shown dry beans possessing darker coloured seed coats to have relatively higher total phenolics in comparison to those with lighter coloured seed coats (Barampama and Simard, 1993).

Table 1. Basic skeleton and classes of phenolics*

No of carbon atoms	Basic skeleton	Class
6	C ₆	Simple phenols, Benzoquinones
7	C ₆ -C ₁	Phenolic acids
8	C ₆ -C ₂	Acetophenones, Phenylacetic acids
9	C ₆ -C ₃	Hydroxycinnamic acids, Phenylpropenes, Coumarins, Isocoumarins, Chromones
10	C ₆ -C ₄	Naphthoquinones
13	C ₆ -C ₁ -C ₆	Xanthones
14	C ₆ -C ₂ -C ₆	Stilbenes
15	C ₆ -C ₃ -C ₆	Flavonoids
18	(C ₆ -C ₃) ₂	Lignans
30	(C ₆ -C ₃ -C ₆) ₂	Biflavonoids
n	(C ₆ -C ₃) _n	Lignins
36	(C ₆) ₆	Catechol melanins
n	(C ₆ -C ₃ -C ₆) _n	Condensed tannins

*Adapted from Robards and Antolovich (1997).

2.3 Flavonoids

Flavonoids were discovered in the 1930s by a Hungarian scientist, Albert Szent-Gyorgyi. They were first identified as “vitamin p” and demonstrated the ability to strengthen capillary walls of vascular purpura patients (Kugelmass, 1940). Since then,

extensive research has shown flavonoids to exert numerous beneficial health effects. Flavonoids are the largest group of polyphenols distributed among the plant kingdom with over 9000 different individual flavonoid compounds identified (Champ, 2002; Pietta, 2000). Flavonoids are found in most of all vascular plants, including plant-based foods such as vegetables, fruits, nuts, and seeds. The content of flavonoids in plants varies and is influenced by a range of factors, including light, temperature, mineral nutrition, pathogens, mechanical damage, and plant growth regulators (Dinelli et al., 2006). Flavonoids are the products of the secondary metabolism, meaning they are chemicals produced by plants that do not deliver energy and are not classified as essential nutrients but have health-promoting properties. In plants, flavonoids have a variety of functions. These naturally occurring compounds have vital roles in attracting animals for pollination and seed dispersion, in signaling among plants and microbes, in defense as antimicrobial agents and feeding deterrents, and in protection against UV damage (Bravo and Mateos, 2008; Winkel-Shirley, 2001).

2.3.1. Flavonoid Biosynthesis

Flavonoid biosynthesis has been extensively studied in numerous plant species. Several different pathways, including the glycolytic pathway, the pentose phosphate pathway, the shikimate pathway, the phenylpropanoid metabolism, and the specific individual flavonoid pathways, are required for flavonoid synthesis (Atmani et al., 2009). Biosynthesis starts with phenylalanine, which is converted to coumaroyl-CoA by chalcone synthase, phenylalanine ammonia-lyase, and 4-coumaroyl:CoA-ligase (Figure

1). Chalcone synthase uses coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin (Woo et al., 2005). Glycosylation, acylation, and methylation of naringenin results in the formation of substituted flavonoid compounds.

2.3.2. Total Flavonoid Content (TFC)

A simple method to determine the flavonoid content in a sample extract/fraction is the colorimetric aluminum chloride method. In this method, flavonols and flavones react with sodium nitrite followed by aluminum chloride to produce acid stable complexes. Aluminum chloride forms complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group (Chang et al., 2002). Acid stable complexes are also produced with ortho-dihydroxyl groups in the A- or B-ring of flavonoid compounds (Chang et al., 2002). The coloured aluminum complex is monitored spectrophotometrically with a standard, typically expressed as quercetin or rutin equivalents, at a maximum wavelength of 510 nm (Abu Bakar et al., 2009). According to the limited research on the total flavonoid content of dry bean seed coats, those with darker coloured seed coats to have higher total flavonoid contents compared to those with lighter coloured seed coats (Oomah et al., 2010).

2.3.3. Flavonoid structure

The flavonoid compounds have a common basic chemical structure, the flavan nucleus, consisting of a carbon skeleton arranged in three rings (C₆-C₃-C₆) (Figure 2). The typical

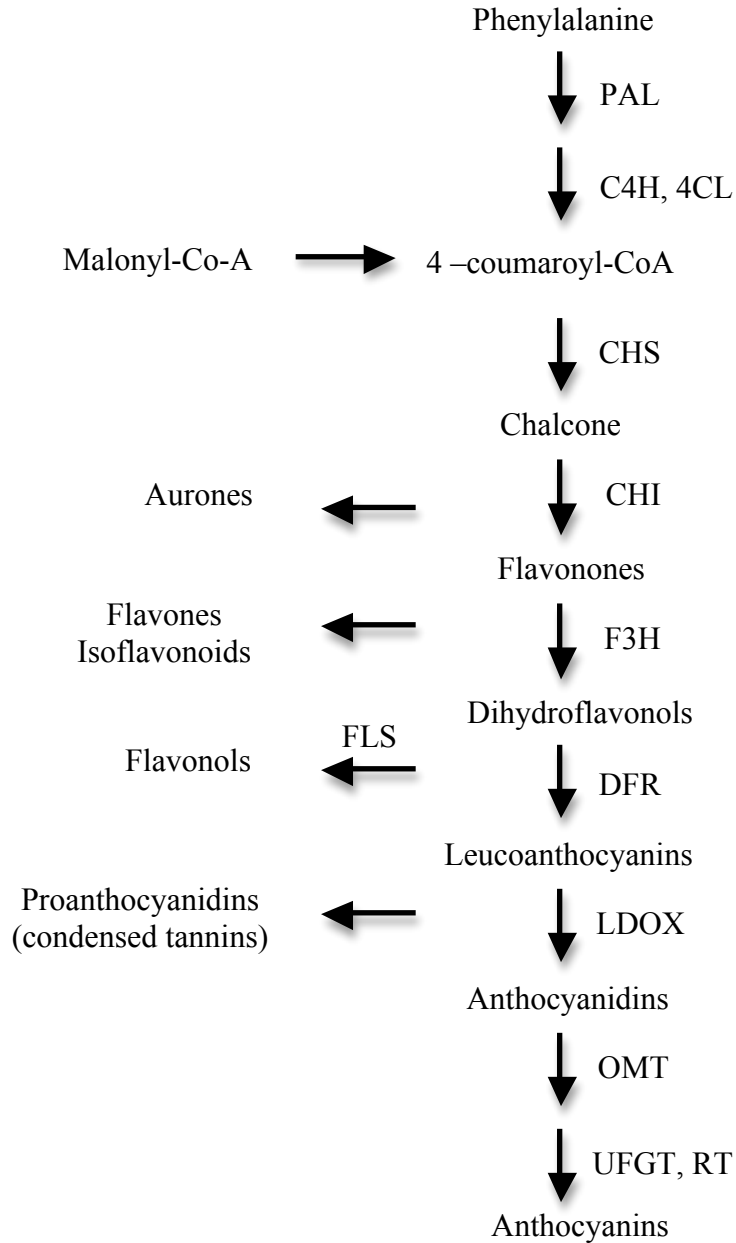


Figure 1. Schematic of main pathways in flavonoid biosynthesis. Enzymes are abbreviated as follows: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone sintase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), leucoanthocyanidin dioxygenase (LDOX), O-methyltransferase (OMT), Phe ammonia-lyase (PAL), rhamnosyl transferase (RT), and UDPG- flavonoid glucosyl transferase (UFGT). Adapted from Winkel-Shirley (2001).

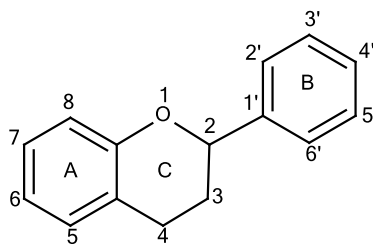


Figure 2. Flavan nucleus. Adapted from Pietta (2000).

substituents on the ring structures are hydroxyl (-OH) groups. At various stages along the biosynthetic pathway, the different flavonoid compounds are formed.

Flavonoids can be grouped into 7 different classes (Table 2). The flavonoids are divided into classes based on the connection of the B-ring to the C-ring, as well as the oxidation state and functional groups of the C-ring. Compounds within a class vary in the pattern of substitution on the A- and B- rings (Pietta, 2000). Flavonoids occur as aglycones (free form) but they are normally found in plants as glycosides (bonded to a sugar). Glycosylation (*O*- or *C*-glycosides) is important because it increases the polarity of flavonoid molecules, which is required for storage in plant cells (Justesen et al., 1998). The typical glycosides are the sugars glucose, galactose, rhamnose, arabinose, xylose, and rutinose, among which glucose predominates. Monosaccharides, disaccharides, and trisaccharides are possible glycosides and glycosides may be substituted by a methyl, malonyl, or acyl group. Among the various classes of flavonoids, those of dietary significance include flavones, flavanones, flavanols, anthocyanidins, isoflavones, and flavonols.

Table 2. Various classes of flavonoids and their substitution patterns*

Class	Name	Substitutions
Flavone	Chrysin	5, 7 - OH
	Apigenin	5, 7, 4' - OH
Flavonone	Naringin	5, 4', - OH; 7 - rhamnoglucose
	Naringenin	5, 7, 4' - OH
	Taxifolin	3, 5, 7, 3', 4' - OH
	Hesperidin	3, 5, 3' - OH, - OMe; 7 - rutinose
Flavonol	Kaempferol	3, 5, 7, 4' - OH
	Quercetin	3, 5, 7, 3', 4' - OH
	Myricetin	3, 5, 7, 3', 4', 5' - OH
Flavononol	Engeletin	3, 5, 7, 4' - OH; 3 - O-rhamnose
	Genistin	5, 4' - OH; 7 - glucose
Isoflavone	Genistein	5, 7, 4' - OH
	Daidzin	4' - OH, 7 - glucose
	Daidzein	4', 7 - OH,
Flavanol	(+)-Catechin	3, 5, 7, 3', 4' - OH
	(-)-Epicatechin	3, 5, 7, 5', 4' - OH
	(-)-Epigallocatechin	3, 5, 7, 3', 4' - OH
Anthocyanidin	Cyanidin	3, 5, 7, 4' - OH; 3, 5 - OMe
	Delphinium	3, 5, 7, 3', 4', 5' - OH
	Pelargonidin	3, 5, 7, 4' - OH

*Adapted from Naczka and Shahidi (2004).

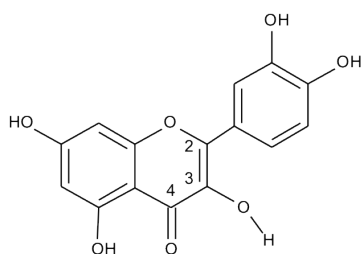
2.3.4. Flavonols

The flavonols are one of the most important flavonoids. They are the most ancient and widespread class of flavonoids in the plant kingdom, synthesized even in mosses and ferns, and have a wide range of potent physiological activities (Stafford, 1991). More than 200 flavonol aglycones have been identified in plants (Robards and Antolovich, 1997). Among the flavonols identified, quercetin (3,5,7,3',4'-pentahydroxyflavone), kaempferol (3,5,7,4'-tetrahydroxyflavone), myricetin (3,5,7,3',4',5'-hexahydroxyflavone), and isorhamnetin (3,5,7,4'-tetrahydroxy-3'-methoxy flavone) are the most abundant in foods (Hollman and Arts, 2000). The structure of these particular flavonols differs by the number and position of hydroxyl groups on the B ring (Figure 3). In the edible portions of plant based foods, they occur in the form of glycosides. For glycosides, the bonding site of the sugar is mainly at the 3-position, less frequently the 7-position, and rarely at the 4'-, 3'- and 5- positions (Hollman and Arts, 2000). With many possible substituents, a large number of flavonol glycosides (approximately 400) have been identified (Bravo and Mateos, 2008). The formation of flavonol glycosides typically depends on light with the highest levels of these compounds located in the leaves and outer parts of plants.

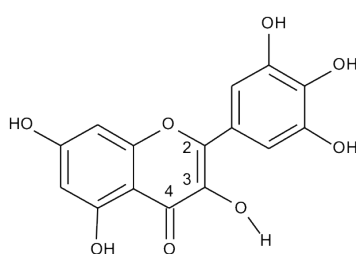
2.3.5. Flavonoids in plant based foods

Flavonoids are widely distributed in plant-based foods. Foods such as apples, blueberries, blackberries, cacao beans, and onion are rich sources of flavonoids (USDA, 2007). There are numerous studies on the flavonoid content of foods. In legumes, several

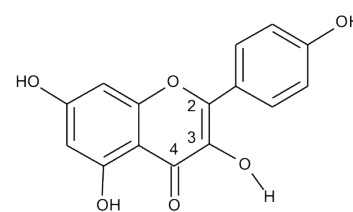
(a)



Quercetin

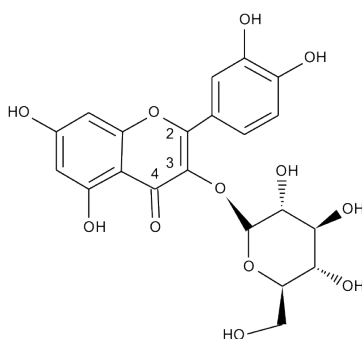


Myricetin

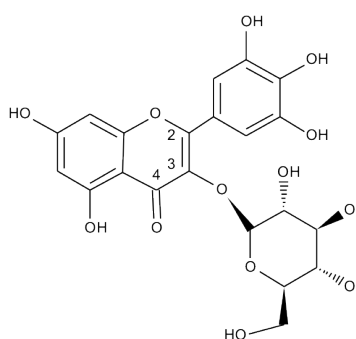


Kaempferol

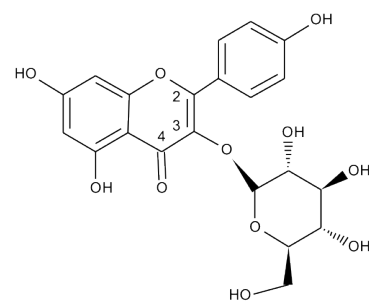
(b)



Quercetin-3-*O*-glucoside



Myricetin-3-*O*-glucoside



Kaempferol-3-*O*-glucoside

Figure 3. Structure of common flavonols as aglycones (a) and glycosides (b). For glycosylated flavonols, the sugar can be a common sugar, such as glucose, galactose, rhamnose, arabinose, and xylose, a disaccharide, such as rutinose, or an oligosaccharide.

studies have shown that the amounts of these plant metabolites can vary as a result of variations in plant cultivar, maturity, color, size and growth conditions. The reported flavonoid content in the literature is often inconsistent since several factors affect content in foods and different methods have been used to analyze the flavonoid contents. Among the flavonoids though, the flavonol quercetin is the most frequently occurring compound

in foods (Ross and Kasum, 2002). Other commonly found flavonoids are kaempferol, myricetin, and the flavones apigenin and luteolin (Ross and Kasum, 2002).

2.3.6. *Flavonoids in dry beans (P. vulgaris L.)*

The natural flavonoids present in dry beans were first isolated by Feenstra (1960). Feenstra (1960), who was attempting to determine the relationship between genes and pigment synthesis in dry beans, isolated 18 different coloured compounds. The coloured compounds were identified as anthocyanins, flavonol glycosides, and leucoanthocyanidins. Numerous studies thereafter have also demonstrated that seed coat colour is due to the presence of flavonols, as well as other phenolic compounds, anthocyanins and tannins (Beninger and Hosfield, 1998; 1999; 2003; Beninger et al., 1999; Takeoka et al., 1997). Phenolic compounds are not distributed uniformly in plants. At the tissue level, the outer layers of plants or plant-based foods contain higher levels of phenolics than those located in the inner parts (Naczka and Shahidi, 2004). The flavonols, in particular, are mainly concentrated in the seed coat or hull (Aparicio-Fernandez et al., 2005; Cardador-Martinez et al., 2002; Oomah et al., 2005). Beans with white seed coats (Navy beans) do not contain flavonol compounds (Beninger and Hosfield, 2003).

Several investigators have characterized different flavonol compounds from diverse dry bean varieties possessing various coloured seed coats. Among pinto bean seed coats, the identified flavonol compounds included kaempferol, kaempferol glucoside derivatives with acetyl, xylose, and malonyl groups, and a quercetin 3-*O*-glucoside (Beninger et al., 2005; Lin et al., 2008). For light and dark red kidney beans, similar

compounds have been detected. These included both kaempferol and quercetin glucoside derivatives with acetyl, xylose, and malonyl groups and flavonoid profiles have shown slight differences when comparing light and dark varieties (Beninger and Hosfield, 1999; 2003; Lin et al., 2008). In the case of black bean seed coats, the only difference in the flavonol glycoside profile from pinto and light and dark red kidney beans was the presence of myricetin 3-*O*-glycoside (Aparicio-Fernandez et al., 2005; Dong et al., 2007; Romani et al., 2004). Aparicio-Fernandez et al. (2005) was the first and only report of a myricetin 3-*O*-glycoside in dry beans.

The content of these flavonoids in dry beans has been determined in some of the available literature (Beninger and Hosfield, 1998; Romani et al., 2004; Beninger et al., 2005; Dinelli et al., 2006). Compared to isoflavones and anthocyanins, flavonols represented the most abundant class of flavonoids in different colored seed coats of *P. vulgaris* L. (Romani et al., 2004). For flavonols, the content of individual compounds varies in seed coats, ranging from trace amounts to 585 mg/kg, and among the individual flavonol compounds quantified, kaempferol 3-*O*-glucoside is generally the main flavonol found in all various colored seed coats of *P. vulgaris* L.

2.3.7. *Flavonoids as antioxidants*

The growing interest in flavonoids, either as a non-nutrient in a normal diet, as dietary supplements, or as a potential pharmacological agent, is based on their free radical scavenging antioxidant actions. Free radicals are typically produced through ionization of oxygen and give rise to reactive oxygen species (ROS). ROS include

superoxide (O_2^-), hydroxyl (OH), nitric oxide (NO), alkoxy (RO), peroxy (ROO) and non-radical molecules (hydrogen peroxide and singlet oxygen) (Potapovich and Kostyuk, 2003; Pietta, 2000). These compounds are produced regularly by cells during normal human organ metabolism and are required for certain biological functions of the body; however when in excess, ROS can exert harmful effects. When ROS are overproduced, an imbalance occurs between natural defense systems and ROS, resulting in oxidative damage, which can cause membrane damage, protein and enzyme modification, and damage to DNA (Pietta, 2000). Oxidative damage by free radicals has also been associated with many diseases, including cancer, cardiovascular diseases, neurodegenerative, and inflammation diseases (Emerit et al., 2004; Heistad et al., 2009; Klaunig and Kamendulis, 2004; Tak et al., 2000). To prevent the effects of oxidative damage, exogenous antioxidants, such as flavonoids, are required.

2.3.8. Flavonol antioxidant activity and structure

The antioxidant capacity of phenolic compounds, including flavonoids, is determined by their structural moiety and the substituents on the rings (Bors et al., 1990). Bors et al. (1990) suggested the following structural elements should be responsible for effective radical scavenging by flavonoids: (1) the ortho-dihydroxy or catechol group in the B-ring, which gives a high stability to the radical formed; (2) the conjugation of the B-ring to the 4-oxo group via the 2,3-double bond, which ensures the electron delocalization from the B-ring and (3) the 3- and 5- OH groups with the 4-oxo group, which allows electron delocalization from the 4-oxo group to both substituents (Figure

4). Greater electron delocalization, and therefore antioxidant activity, is enabled with a combination of all these structural features.

Antioxidant activity has been reported in the seed coats of various coloured dry beans (Cardador-Martinez et al., 2002; Ranilla et al., 2007). It has been suggested that the antioxidant activity is associated with the presence of phenolic compounds, including flavonoids, which as stated previously give rise to the seed coat colour (Beninger and Hosfield, 2003). In a study examining more than 100 common dietary fruits and vegetables, dry beans with a red seed coat were identified as possessing one of the highest antioxidant activities (Wu et al., 2004). This implies extracts of flavonoids from the seed coats of coloured dry beans should possess high antioxidant activity.

2.3.9. DPPH

Among the methods used to measure antioxidant activity, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a common assay in natural antioxidant studies. In this method, based on the work by Brand-Williams et al. (1995), the stable free radical, DPPH, is characterized by a deep purple colour and a maximum absorbance at 515 nm. In the presence of an antioxidant (proton donator) the free radical becomes paired off and the absorption strength decreases, resulting in discolouration of the radical solution. Discolourization is stoichiometric with respect to the number of electrons captured (Molyneux, 2004). The amount of DPPH radical in the antioxidant test system is typically monitored with a UV spectrometer because of its simplicity and accuracy (Moon and Shibamoto, 2009). To standardize the results from various studies, Trolox

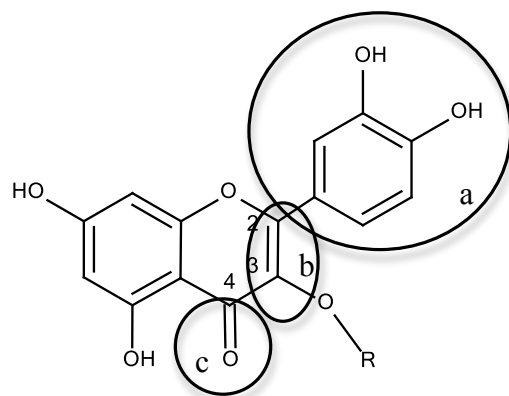


Figure 4. Structural elements related to the antioxidant and radical scavenging activity of flavonoid molecules. The ortho-dihydroxy or catechol group in the B-ring (a), the conjugation of the B-ring to the 4-oxo group via the 2,3-double bond (b), and the 4-oxo group (c).

equivalents (TE) are typically used. Trolox is a commercial water-soluble vitamin E (Moon and Shibamoto, 2009). Antioxidant activity of a sample is then expressed in terms of micromoles of Trolox equivalents per 100 g of sample (TE/100 g) (Moon and Shibamoto, 2009). Results can also be reported as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50% (EC_{50}). In recent literature, only one study has reported antioxidant activities of dry bean seed coats using the DPPH method (Ranilla et al., 2007). Lighter coloured seed coats had greater antioxidant activity compared to darker coloured seed coats.

2.4 Inflammation and inflammatory mediators

Inflammation plays an important role in various diseases. It is a normal response of the body to tissue injury, the presence of a pathogen, or chemical irritation. During

inflammation, inflammatory cells, including neutrophils and macrophages, are activated. Activated macrophages stimulate the expression of a series of genes involved in host defense, which results in the release of different inflammatory mediators, such as pro-inflammatory cytokines and NO.

2.4.1. Pro-inflammatory cytokines

Cytokines are cell-produced proteins that function as intercellular signals and regulate cell function (Standiford, 2000). Cytokines are produced predominantly by stimulated macrophages and lymphocytes and mediate many inflammatory processes (Brennan and Feldman, 1996). The pro-inflammatory cytokines include tumor necrosis factor alpha (TNF- α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), and chemokines, such as interleukin-8 [IL-8] and macrophage chemotactic and activating factor [MCAF] (Standiford, 2000; Yang et al., 1998). These cytokines are important factors in the inflammatory process, although TNF- α appears to be central to the inflammatory cascade. Several studies have demonstrated TNF- α levels increase after acute inflammation and injury decreases when TNF- α activity is blocked (Beutler and Cerami 1986; Machleidt et al., 1996; Pfeffer et al., 1993; Tracey et al., 1987). TNF- α is a critical molecule and therapeutic target in chronic inflammatory diseases.

TNF- α exists as a transmembrane protein (Perez et al., 1990) and is produced by different types of cells of the immune system, including macrophages, monocytes, keratinocytes, and activated T cells (Standiford, 2000). Production of TNF- α by cells is increased or stimulated following exposure to bacterial products and antigens

(Kumazawa et al., 2006). *In vitro* studies have indicated activated macrophages synthesize the highest levels of TNF- α . In macrophages, bacterial lipopolysaccharide (LPS) or in combination with interferon (IFN)- γ , are the best stimuli considered to stimulate cytokines. Stimulated TNF- α in macrophages can bind directly to two receptors, TNF- α receptor type I and TNF- α receptor type II (Standiford, 2000). TNF- α can also undergo cleavage and bind to these receptors in its soluble form. Activation of macrophages initiates phagocytosis and potentially leads to further TNF- α release, stimulating a self-amplifying reaction. TNF- α has a great effect on tissue remodeling, repair, and inflammation by coordinating the activities of many other cells, including endothelial cells, granulocytes, osteoclasts, fibroblasts, hematopoietic cells, and lymphoid cells (Kumazawa et al., 2006). At low levels, TNF- α has beneficial effects by inducing host defense, however the more amplified and prolonged the release of TNF- α , the more ROS are produced by cells, and without sufficient antioxidant protection, the more cells producing TNF- α are activated. Cells overproducing TNF- α also stimulate further production of other inflammatory cytokines, such as IL-1, and chemokines. Excessive inflammatory cytokines, chemokines, and ROS in the body result in extensive inflammation and the depletion of endogenous antioxidants used by cells, which can lead to inflammatory disorders. Elevated TNF- α levels have been associated with the development of rheumatoid arthritis (RA), Crohn's disease, inflammatory bowel disease, multiple sclerosis, Parkinson's disease, and Alzheimer's disease (Kumazawa et al., 2006). Therefore, inhibiting TNF- α production under physiological conditions is crucial in the prevention of harmful health effects.

2.4.2. Effect of flavonoids on pro-inflammatory cytokines

Since the identification of TNF- α in the mid-1970s, a large amount of research has been conducted to determine the precise biological action and to develop drugs to treat inflammatory diseases mediated by TNF- α (Paul et al., 2006). More recently, interest in the use of compounds from natural sources has become important. Flavonoids are among the natural compounds that have been found to interfere with pro-inflammatory mediators, such as TNF- α . Herath et al. (2003) reported flavones, flavonols, and chalcone are the most potent inhibitors of production of TNF- α by LPS-stimulated cells. Flavonoids were classified into four groups, based on a strong-acting group (flavones, flavonols and chalcones), a moderate-acting group (flavonones, naringenin, anthocyanidin, and pelargonidin), a weak-acting group (isoflavone genistein) and an inactive group (eriodictyol flavanone). The authors also reported that for flavonoids the presence of the double bond between carbon atoms 2 and 3 and the ketone group at position 4 are required for a strong TNF- α inhibitory effect.

Several studies have examined the effects of the flavonols quercetin, kaempferol, and myricetin and their derivatives on the production of TNF- α in macrophages; however the majority of the research has been focused on flavonol aglycones rather than flavonol glycosides. Among several flavonoids, quercetin has been the most efficacious inhibitor in LPS-induced TNF- α in RAW 264.7 mouse macrophages (Manjeet and Ghosh, 1999; Nakamura and Omura, 2008). However, according to Wadsworth and Koop (1999) and Wang and Mazza (2002), quercetin at low concentrations induced TNF- α production. Kaempferol has been reported to induce TNF- α production at low concentrations in LPS-

stimulated and IFN- γ -activated RAW 264.7 macrophages (Wang and Mazza, 2002) and show no activity toward TNF- α production (Ueda et al., 2004). Myricetin has demonstrated both the release of TNF- α with increasing concentration (Wang and Mazza, 2002) and to have no effect on LPS-induced TNF- α release from RAW 264.7 mouse macrophages (Xagorari et al., 2001). All of these results suggest quercetin and perhaps kaempferol and myricetin, have a positive effect on improving clinical symptoms of inflammatory diseases. Evidence suggests that flavonoids are able to inhibit the production of TNF- α by suppressing the activation of various enzymes involved in the inflammatory process (Gonzalez-Gallego et al., 2007).

To our knowledge, no research has been conducted on extracts or pure flavonoids from dry beans or their seed coats, but the effects of some flavonol glycosides isolated from berries, including quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, and rutin, on TNF- α production in LPS-stimulated RAW 264.7 macrophages has been reported (Wang and Mazza, 2002). When comparing the inhibitory effect of aglycones and glycosides of flavonols on TNF- α production, aglycones were found to have a greater inhibitory effect than that of the corresponding glycosides, however flavonol glycosides did appear to reduce TNF- α production activities in macrophages (Wang and Mazza, 2002). Therefore pure flavonol glycosides and potentially extracts from other natural sources, such as dry bean seed coats, which contain these flavonoid derivatives, should also possess an inhibitory effect on TNF- α production.

2.4.3. Nitric oxide

NO is a short-lived free radical that can diffuse freely through cells and acts as a multi-functional mediator regulating different biological functions in the body. NO is produced by a family of enzymes termed nitric oxide synthase, which include inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS) (Nathan, 1992). It is an important molecule involved simultaneously in both cell viability and cell death. Among protective effects, NO contributes to the antimicrobial and tumoricidal activity of the macrophages (Faris-Eisner et al., 1994; MacMicking et al., 1995) and demonstrates cytoprotective properties towards hepatocytes and endothelial cells, among other cells (Kim et al., 1997). However, NO can also exert harmful effects. NO acts as a secondary mediator of some actions of pro-inflammatory cytokines and overproduction of NO can be directly cytotoxic. NO can destroy DNA, lipids, proteins, and carbohydrates, leading to impaired cellular functions and enhanced inflammatory reactions (Wang et al., 2006). NO can also react with O_2^- and form peroxynitrite (ONOO⁻), which is the most reactive nitrogen species (RNS). In addition, excessive NO production has been associated with the pathogenesis of various diseases which include vascular, cancer, immunological disorders, and inflammation diseases (Wolfe and Dasta, 1995). In rheumatoid arthritis, an inflammatory disease, excessive NO production by activated macrophages has been observed (Gryglewski, 1981). Therefore, similar to TNF- α , harmful physiological effects can be prevented by inhibition of NO production.

2.4.4. Effect of flavonoids on nitric oxide

Flavonols, including the aglycones quercetin, kaempferol, and myricetin, have demonstrated an inhibitory effect on NO production from activated macrophages. The majority of the research on NO inhibition has been conducted with quercetin, showing significant inhibition of NO release in murine macrophages (Manjeet and Ghosh, 1999; Rao et al., 2005; Wadsworth and Koop, 1999; Wang and Mazza, 2002; Xagorari et al., 2001). Kaempferol has demonstrated more potent inhibitory effect on NO release in LPS-stimulated RAW 264.7 macrophages, followed by quercetin, and then myricetin (Wang et al., 2006). Little research has been conducted on inhibition of NO production with flavonol glycosides, only kaempferol-3-*O*-glucoside, isolated from the tree *Bauhinia variegata*, has shown to inhibit NO production with LPS/IFN- γ stimulated macrophages (Rao et al., 2005), and no research exists on the inhibition of NO production with extracts of dry beans or their seed coats.

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals and standards

Acetone (HPLC grade), acetic acid, phosphoric acid (H_3PO_4), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), Folin-Ciocalteu reagent, ferulic acid, sodium carbonate, and standards quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, and rutin, were from Sigma (Toronto, ON, Canada). Acetonitrile (HPLC grade) and sodium hydroxide were from Fisher Scientific Co. (Nepean, ON, Canada). Sodium carbonate (anhydrous) was obtained from Mallinckrodt Laboratory Chemicals (Phillipsburg, NJ, USA). Sodium nitrite and aluminum chloride were provided by the University of Manitoba Chemistry Department (Winnipeg, MB, Canada)

3.2 Cell culture materials

The murine macrophage (RAW 264.7) cell line, Dulbecco's modified Eagles media (DMEM), heat inactivated fetal bovine serum (FBS), penicillin-streptomycin solution (P/S), Trypsin/EDTA solution, and Dulbecco's phosphate buffer solution (DPBS) were from the American Type Culture Collection (ATCC). Sterile 96-well tissue culture plates and Cryogenic freezing vials were obtained from VWR International Inc. (Mississauga, ON, Canada). 25 cm² and 75 cm² tissue culture flasks were obtained from Fisher Scientific Co. (Ottawa, ON, Canada). For the enzyme-linked immunosorbent assay

(ELISA), the TNF α ELISA kits were obtained from e-Bioscience, Inc. (Cincinnati, OH, USA). The Costar (Corning) EIA/RIA 96-well easy wash high binding polystyrene plates were obtained from Fisher. Wash buffer (phosphate buffer saline (PBS) with Tween 20), dimethyl sulfoxide, D-(–)-salicin, lipopolysaccharide (LPS) from *E. coli* 0111:B4, and Griess reagent were all obtained from Sigma (Toronto, ON, Canada).

3.3 Plant material

Dry seeds of *P. vulgaris* L. grown and harvested in southern Manitoba in 2008 were provided by Parent Seed Farms Ltd. (Saint Joseph, Manitoba). Three groups of different colored beans examined in this study were black (cv. Eclipse, Black violet), pinto (cv. Windbreaker, Maverick, Buster), and light and dark red kidney (cv. Pink panther, ROG802, Montcalm, Red hawk). Beans were provided as samples according to Table 3. Beans were stored in sealed plastic bags in a dark cold room (4-7 °C) to preserve freshness until sample preparation.

3.3.1. Sample preparation

For each sample, approximately 200 g of beans were placed in a 1 L beaker, covered with distilled water (just enough to cover the beans), and allowed to soak overnight in the dark at 4-7 °C. The seed coats were manually separated from the seeds with the aid of tweezers. The seed coats were lyophilized and stored in the dark at -20 °C until extraction and further analysis.

Table 3. List of collected bean samples

Bean market class	Sample	Cultivars
Pinto	1	Windbreaker
	2	Maverick/Buster
Light and dark red kidney	3	Pink panther
	4	ROG802/Montcalm/Redhawk
Black	5	Eclipse
	6	Black violet

3.4 Extraction of flavonoids

Lyophilized bean seed coats were manually ground with a mortar and pestle to a fine powder. The ground sample was able to pass through a number 40 mesh US Standard sieve. Samples (200 mg) of ground seed coats were added to 10 mL of 80% (v/v) aqueous acetone in an amber glass container to protect the extract from light. The container was capped and the mixture was sonicated (Branson 3200, Branson ultrasonic cleaner, 50/60 Hz, 117 volts, 2.5 Amps, Branson Cleaning equipment company, Shelton, CT, USA) for 1 hour at room temperature. This extraction procedure was determined based on preliminary experiments, using the total flavonoid content assay. Different extraction methods (stirring for two hours and sonication for one hour) and solvents (50% and 80% ethanol, 50% and 80% methanol, and 50% and 80% acetone) on selected dry bean samples were evaluated to determine the optimal extraction procedure. Based on the results (data not shown) and literature (Xu and Chang, 2007), the optimal extraction

procedure was 80% acetone with sonication for one hour. The solid was removed from the supernatant by filtering with Whatman filter paper no. 5.

3.5 Analytical techniques and equipment

3.5.1. Total Flavonoid Content

To estimate the flavonoid content of the bean samples, a colorimetric method was adapted from Subhasree et al. (2009). Appropriately diluted crude extract (0.25 mL) was diluted with 1.5 mL of distilled water in a test tube and 0.15 mL of a 5 % (w/v) sodium nitrite solution was added. The solution was mixed well and after standing for 5 minutes at room temperature, 0.3 mL of a 10 % (w/v) aluminum chloride solution was added. The solution was mixed well and allowed to stand for another 6 minutes at room temperature before adding 1.0 mL of 1 M sodium hydroxide. The solution was made up to 5 mL with distilled water and mixed well. A UV-Vis spectrophotometer (Ultrospec 1100 pro, Biochrom, England) at 510 nm was used to measure the absorbance against 80% (v/v) aqueous acetone as a blank. The results were calculated and expressed as micrograms of rutin equivalents (mg of rutin/g sample) using a calibration curve of rutin. Linearity range of the calibration curve was 20 to 100 $\mu\text{g/mL}$ ($r = 0.99$). The extraction was conducted in triplicate.

3.5.2. Total Phenolic Content

To determine the phenolic content, the Folin–Ciocalteu method of Singleton and Rossi (1965) was used as modified by Anton et al. (2009). Appropriately diluted crude extract (0.2 mL) was added to freshly prepared 10-fold-diluted Folin-Ciocalteu reagent (1.5 mL). The mixture was allowed to stand for 5 minutes at room temperature. Then a 1.5 mL dose of sodium carbonate solution (60 g/L) was added. The solution was mixed well and allowed to stand for 90 minutes at room temperature. The absorbance was measured at 725 nm with a UV-Vis spectrophotometer against 80% (v/v) aqueous acetone as a blank. The results were calculated and expressed as micrograms of ferulic acid equivalents (μg of ferulic acid /g sample) using a calibration curve of ferulic acid. Linearity range of the calibration curve was 40 to 200 $\mu\text{g}/\text{mL}$ ($r = 0.99$). The extraction was conducted in triplicate.

3.5.3. Antioxidant activity (DPPH method)

The DPPH (2,2-diphenyl-1-picrylhydrazyl)-free radical scavenging capacity of seed coat extracts was evaluated according to the method of Brand-Williams, Cuvelier, and Berset (1995) as modified by Maskus, (2008). Appropriately diluted crude extract (0.1 mL) or standard was added with 3.9 mL of an 80% (v/v) acetone solution of DPPH radical (60 $\mu\text{mol}/\text{L}$). A UV-Vis spectrophotometer at 515 nm was used to measure the absorbance at $t=0$ minutes (immediately after the addition of DPPH) and at $t=30$ minutes (30 minutes after the addition of DPPH). The mixture was allowed to stand in the dark for 30 minutes at room temperature. Aqueous acetone (80%, v/v) was used as a blank.

The percent of DPPH radical discoloration of the sample was calculated according to the equation:

$$(\%) \text{ discoloration} = [1 - (\text{Absorbance at } t=30 / \text{Absorbance at } t=0)] \times 100$$

The free radical scavenging activity of bean extracts was expressed as mean micromoles of Trolox equivalent per gram of sample ($\mu\text{mol TE/g sample}$) using a calibration curve of Trolox. Linearity range of the calibration curve was 250 to 1000 $\mu\text{mol/L}$ ($r = 0.99$). The extraction was conducted in triplicate.

3.5.4. HPLC-MS/MS analysis

For identification and characterization of flavonol glycosides the solvent from the aqueous acetone extract was removed under reduced pressure in a rotary evaporator (model RE-51, Yamato Scientific America Inc., Santa Clara, CA, USA) at 30 °C. The dried extract was then dissolved in 1.0 mL of acetonitrile/water (25:75, v/v) and filtered through a 0.45 μm syringe filter (PTFE). The HPLC (Waters 2695 Alliance Separation Module) was equipped with an autosampler (Waters 717 Plus) and diode array detector (Waters 996). A quadrupole time-of-flight (QTOF) mass spectrometer (MS) (Waters, Milford, MA, USA) was used. Waters MassLynx 4.1 Software was used for data processing. A reversed phase C18 Gemini column (150 \times 4.6 mm; particle size 5 μm) (Phenomenex, Torrance, CA, USA) with a 20 \times 3.9 i.d. was used at a flow rate of 0.5 mL/min for separation of flavonoids. Sample volumes of 10 μL were used for injection.

The column temperature was set at 30 °C. The mobile phase, as modified by Beninger and Hosfield (1999), consisted of a combination of A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile). Separations were performed with a linear gradient as follows: 0-30 minutes, 10-70% B; 30-31 minutes, 70-10% B; 31-35 minutes, 10% B. Detection was carried out at 355 nm, with peak scanning between 200 and 400 nm. Spectra were recorded in negative ion mode. The mass spectrometer operating conditions were: capillary voltage 1.8 kV, cone voltage 35 V, desolvation temperature 350 °C, source temperature 150 °C, mass range 100-1000 amu. The MS/MS spectra were acquired with a collision energy of 10, 20, and 30 V. Flavonoid compounds were identified by comparison of mass fragmentation patterns with the available literature.

3.5.5. HPLC analysis

For quantification of flavonol glycosides the solvent from the aqueous acetone extract was removed under reduced pressure in a rotary evaporator at 30 °C. The dried extract was then dissolved in 1.0 mL of acetonitrile/water (25:75, v/v) and filtered through a 0.45µm syringe filter (PTFE). The HPLC (Waters 2695, Waters Corp., Milford, MA, USA) was equipped with a diode array detector (DAD) (Waters 2996). The pumps, autosampler (Waters 717 Plus), and detector were controlled and the data were processed by Millennium 32 Chromatography Data software program. The analytical column was a reversed phase C18 Gemini column (150 × 4.6 mm; particle size 5 µm) (Phenomenex, Torrance, CA, USA), protected by a C18 guard column (4 × 3.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase, as modified by Beninger and Hosfield (1999),

consisted of a combination of A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile). Separations were performed with a linear gradient as follows: 0-30 minutes, 10-30% B; 30-35 minutes, 30-90% B; 35-40 minutes, 90-10% B. 40-45 minutes, 10% B. The flow rate was 1.0 mL/min and detection at 355 nm. Sample and standard volumes of 10 μ L were used for injection. Flavonoid compounds were quantified with external standards. Comparison of retention times and UV spectra of the samples with the standards allowed for flavonoid quantification. The standards were prepared in acetonitrile/water (25:75, v/v). For kaempferol 3-*O*-glucoside, the equation for the calibration curve (Appendix A) was peak area = $1E+07(\text{concentration}) + 1E+06$ ($r = 0.99$) and linearity range of the calibration curve was 0.2 to 1.0 mg/mL. For quercetin 3-*O*-glucoside, the equation for the calibration curve (Appendix A) was peak area = $2E+07(\text{concentration}) - 671076$ ($r = 0.99$) and linearity range of the calibration curve was 0.1 to 0.5 mg/mL. For rutin, the equation for the calibration curve (Appendix A) was peak area = $1E+07(\text{concentration}) + 9221.2$ ($r = 0.99$) and linearity range of the calibration curve was 0.02 to 0.1 mg/mL.

3.5.6. Cell culture

The murine macrophage cell line RAW 264.7 was cultured in DMEM supplemented with 10% FBS and 1% P/S. Cells were grown in an incubator with 5% CO₂ at 37 °C and the media was changed every 3 days. Cells were grown to 80-85 % confluence in 75 cm² cell culture flasks and gently detached using a scraper. Cells were subcultured. For cytokine screening, cells were grown to 80-85 % confluence and

trypsinized. Cell number was assessed using a haemocytometer. The cells were centrifuged at 133 x g to separate cells from the media. The media was removed and fresh media was added. Cells were plated at a density of 5×10^4 cells/well in a 96-well tissue culture plate and placed in the incubator for 24 hours. A preliminary experiment was conducted with concentration of 50, 100, 200, and 500 $\mu\text{g/mL}$ for dried crude acetone extracts and pure flavonoids. Dried extracts and standards to be tested in a preliminary experiment (no LPS added) were diluted in media to the following dilutions: 50, 100, 200, and 500 $\mu\text{g/mL}$. The cells were incubated overnight and supernatants were then collected and frozen for future analysis. In subsequent experiments, dried extracts and standards to be tested were diluted in media to the following dilutions: 20, 100, 500 and 1000 $\mu\text{g/mL}$. Cells were supplemented with the samples and standards for 5 hours before stimulation with 1 $\mu\text{g/mL}$ LPS. The activated cells were and incubated overnight to stimulate the inflammatory response. Supernatants were then collected and frozen for future analysis.

It should be noted that all the cell culture work, including subsequent analysis, was conducted at the Richardson Centre for Functional Foods and Nutraceuticals with a great deal of input from technical staff. However, this created a number of challenges including long storage times (sometimes > 6 months) for extracts under unknown conditions, lack of control over experimental protocol (e.g. a data set was completely lost as all absorbance values were the same at maximum possible for the plate ready, possibly due to the incorrect wavelength being used), as well as equipment problems and limited access to cell cultures that prevented further evaluation of additional extracts and validation of the results obtained. What is presented is the data that was obtained;

however, it is recommended that further work be done in this area to confirm some of the observations reported herein.

3.5.7. Cell viability

The growth inhibitory effect of dried crude acetone extracts or standards on RAW 264.7 cells were evaluated by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay, as described earlier (Van et al., 2009). Cells were treated with 10 μ L MTT solution and were incubated for 24 hours at 37 °C. The medium was aspirated and the resulting purple formazan crystals were dissolved in detergent solution (200 μ L). The absorbance was measured at 570 nm to quantify the reduction of MTT as cell viability.

3.5.8. Cytokine measurement

For the anti-inflammatory effects of dried crude seed coat extracts and standards, the enzyme-linked immunosorbent assay (ELISA, e-Bioscience, Inc., Cincinnati, OH, USA) was carried out as specified by the manufacture. For the assay, a 96-well tissue culture plate was coated with 100 μ L/well of diluted capture antibody and incubated overnight at 4 °C. Wells were washed five times with wash buffer (PBS with 0.05% Tween-20) to remove unbound antibodies and then incubated with 200 μ L/well of diluted 1X Assay Diluent at room temperature for 1 hour to block nonspecific protein binding sites. After washing five times with wash buffer, standard murine TNF- α (25, 50, 100, 200, 500, and 1000 pg/mL) or 100 μ L of crude extract supernatants were added to

appropriate wells and incubated overnight at 4 °C. Any TNF- α molecules present in the standard or supernatants will bind to the capture antibodies. Plates were washed five times with wash buffer. Approximately 100 μ L of diluted detection antibody (Avidin-horseradish peroxidase) was added to each well and the plate was incubated at room temperature for 30 minutes. The TNF- α molecules react with both capture antibody (solid-phase) and detection antibody (enzyme-linked). Plates were washed seven times with wash buffer to remove unbound-labeled antibodies and wells were soaked for 1 to 2 minutes prior to aspiration. Substrate solution (100 μ L/well) was added and the plates were incubated at room temperature. Addition of the substrate solution results in the development of a blue color. Color development was stopped after 15 minutes by addition of Stop Solution (1 M phosphoric acid [H₃PO₄]). The Stop Solution results in a colour change from blue to yellow. The concentration of TNF- α is directly proportional to the colour intensity of the test sample. Optical density readings were taken at 450 nm within 10 min of stopping reaction on a microplate reader.

3.5.9. Nitrite measurement

NO secretion in cultured macrophages was measured by a micro-plate assay method involving the Griess reaction, as described earlier (Van et al., 2009). The detection of NO is difficult as a result of its extremely short half-life, however, as NO is metabolized to nitrate and nitrite (NO₂⁻) in the cell, quantification of NO₂⁻ can be used to measure the amount of NO that was originally present in a sample. To measure NO, 100 μ L of macrophage culture supernatant was mixed with an equal volume of Griess reagent

(1:1 mixture of 1 % sulfanilamide in 5 % phosphoric acid, and 0.1 % and α -naphthylamine in distilled water) and incubated for 10 min at room temperature. Griess reagent is used to convert NO_2^- into a purple-coloured azo compound, which is quantified spectrophotometrically. Nitrite concentration was determined by measuring the absorbance at 540 nm and referred to a sodium nitrite (NaNO_2) standard curve (0 to 100 g/L). No LPS and no control were used for this assay.

3.6 Statistical analysis

The results show mean \pm standard deviation for the number of experiments indicated (n). Statistical significance was performed using SAS (version 9.2) using PROC GLM followed by Tukey's test. In all cases, a probability (p) value of the significant difference was set at $p < 0.05$.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Total Flavonoid Content

The colorimetric determination of flavonoid content in plant material, based on the color reaction with AlCl_3 reagent, is a well-known method to determine total flavonoid content (Zhishen et al., 1999). The color produced is proportional to the flavonoid concentration. The total flavonoid content of dry bean seed coat extracts, expressed as mg rutin equivalents/g sample, is presented in Table 4. The content for total flavonoids among all dry bean seed coat extracts ranged from 121.4 to 190.8 mg rutin equivalents/g sample. Significant differences ($p < 0.05$) in total flavonoid content were found among the different cultivars. Windbreaker was significantly higher than all other cultivars with 190.8 mg rutin equivalents/g sample. The content of total flavonoids in Black violet was the lowest with 121.4 mg rutin equivalents/g sample. When comparing market classes in general, pinto bean seed coats had the greatest flavonoid content followed by dark and light red bean seed coats, and then black bean seed coats. When comparing cultivars within a market class, the results showed that flavonoid concentrations were significantly different between Windbreaker and Maverick/Buster (pinto) and Eclipse and Black violet (black), however no significant differences were found between Pink panther and ROG802/Montcalm/Redhawk (dark and light red kidney). The results of the present work suggest that specific groups of coloured seed coats, such the brown and red groups contain greater levels of total flavonoids than black

Table 4. Measured total flavonoid content of dry bean seed coats using a colorimetric method

Sample	mg rutin equivalents/g sample
Windbreaker	190.8 ± 11.9 ^a
Maverick/Buster	172.7 ± 7.3 ^b
Pink panther	166.8 ± 2.9 ^b
ROG802/Montcalm/Red hawk	166.7 ± 7.9 ^b
Eclipse	134.6 ± 7.9 ^c
Black violet	121.4 ± 3.5 ^d

Results are presented as a means ± standard deviations (n=6). Different letters within the same column represent significant differences. ($p < 0.05$)

groups. Several studies have shown that the amounts of flavonoids can vary as a result of variations in plant cultivar, maturity, color, size, and growth conditions.

Most of the research based on the total flavonoid content of dry beans has been conducted on the whole bean seed (Akillioglu and Karakaya, 2010; Boateng et al., 2008; Oomah et al., 2005; Thompson et al., 2009; Xu and Chang, 2007; Xu and Chang, 2009; Xu et al., 2007) with limited research conducted only on dry bean seed coats. According to the available literature, market classes of dry beans differing in seed coat color have contained different flavonoid contents. Oomah et al. (2010) found significant differences in flavonoid concentration among black and pinto bean hull extracts. Based on a total flavonol assay using 2 % hydrochloric acid and 70% acetone to extract flavonoids, Black violet beans, possessing a black seed coat color, contained 3.48 mg quercetin equivalents/g sample and Othello, a pinto bean, possessing a beige or brown seed coat color, contained 3.04 mg quercetin equivalents/g. These reported results are much lower

compared to the results of the present work and the results are not in agreement with the present work, which show pinto bean seed coats with higher total flavonoid contents than black bean seed coats. The higher values obtained for the dry bean seed coats in the present work may be the result of the flavonoid assay method and/or the solvent used for extraction. It has been reported that the extraction yield of flavonoid content is greatly dependant on the solvent polarity (Xu and Chang, 2007). Higher amounts of flavonoids were reported in acetone extracts than in ethanol and methanol extracts.

4.2 Total Phenolic Content

The total phenolic content assay using Folin-Ciocalteu reagent has been widely used to investigate the content of phenolics in plant materials. Data reported in the literature has indicated the presence of ferulic acid in both bean cotyledons and seed coats (Ranilla et al., 2007) and therefore the total phenolic contents were expressed as ferulic acid equivalents (FAE). The total phenolic content of the extracts from selected dry bean seed coats are presented in Table 5. The content for total phenolics among all extracts ranged from 91.4 mg FAE/g sample in red kidney beans to 124.8 mg FAE/g sample in black beans. Significant differences ($p < 0.05$) in total phenolic content were found among the different cultivars. Eclipse and Black violet were significantly higher than all other cultivars with 124.8 and 112.7 mg FAE/g sample, respectively. The content of total phenolics was the lowest in ROG802/Montcalm/Redhawk with 91.4 mg FAE/g sample. In general, black bean seed coats had the greatest phenolic content followed by pinto

bean seed coats and dark and light red bean seed coats, which were not significantly different. Among market classes, no significant differences were observed between

Table 5. Measured total phenolic content of dry bean seed coats using a the Folin-Ciocalteu method

Sample	mg FAE/g sample
Windbreaker	103.2 ± 7.6 ^{bc}
Maverick/Buster	99.5 ± 5.5 ^c
Pink panther	96.9 ± 7.4 ^c
ROG802/Montcalm/Red hawk	91.4 ± 7.1 ^c
Eclipse	124.8 ± 5.8 ^a
Black violet	112.7 ± 9.1 ^{ab}

Results are presented as a means ± standard deviations (n=6). Different letters within the same column represent significant differences. ($p < 0.05$)

cultivars. These results indicate that dry beans possessing dark seed coats, such as black beans, have relatively higher total phenolics compared to those possessing lighter colored seed coats, as seen with pinto and dark and light red kidney beans.

The results in the present work are similar, higher, and lower to those previously reported for dry bean seed coats. The total phenolic contents of extracted seed coats in the present work were similar to those of black (cv. AC Black Violet) and pinto (cv. Othello) beans using a modified version of the Glories' method (Oomah et al., 2010). This assay, developed as an indicator of phenolic ripeness in grapes, uses strong acids to extract and solubilize phenolics in grapes. Black violet seed coats contained 108.68 mg (+)-catechin equivalents/g seed coat and Othello seed coats contained 158.20 mg (+)-catechin

equivalents/g seed coat extracted with 70% aqueous acetone. Ranilla, et al. (2007), who extracted seed coats with 70% aqueous methanol, reported lower results for total phenolic content of black, red, and light brown seed coats of Brazilian and Peruvian bean cultivars ranging from 49 to 73, 64 to 76, and 57 to 69 catechin equivalents/g seed coat (fresh weight), respectively. Madhujith et al. (2004), reported greater results for total phenolic contents of 80% aqueous acetone extracts of red, brown, and black bean hulls compared to the present work. Extracts of red, brown, and black bean hulls were 223.5, 253.2, and 270.0 mg catechin equivalents/g of extract, respectively. The different results obtained in the present work may be the result of the type of standard used or as a result of variations in plant cultivar, maturity, color, size, and growth conditions. Despite the different results reported, the present work and other studies suggest that dry beans possessing darker colored seed coats have relatively higher total phenolics in comparison to those with lighter colored seed coats (Barampama and Simard, 1993).

4.3 Antioxidant activity (DPPH method)

The DPPH assay is a common assay in natural antioxidant studies. One of the reasons is that this method is simple and highly sensitive. DPPH is a stable free radical at room temperature. Reduction of DPPH by antioxidants results in a decrease in absorbance and the degree of discoloration of the solution shows the scavenging efficiency of the samples. Higher values for antiradical activity by the DPPH method indicate a higher concentration of DPPH needed to react with each molar of compound and, therefore, higher potential antioxidant activity. Table 6 reports the free radical

scavenging activity of the dry bean extracts tested using the DPPH method. DPPH values ranged from 42275.4 μmol of TE/100g in ROG802/Montcalm/Red hawk to 57816.8 μmol of TE/100g in Eclipse. Extracts of Eclipse, a black bean, and Windbreaker, a pinto bean, were the most active. They were significantly higher in activity compared to the other samples and did not differ from each other significantly. The remaining samples, including Black violet, ROG802/Montcalm/Redhawk, Pink panther, and Maverick/Buster, possessing black, red, and beige or brown coloured seed coats, all had other samples and did not differ from each other significantly. The remaining samples, including Black violet, ROG802/Montcalm/Redhawk, Pink panther, and Maverick/Buster, possessing black, red, and beige or brown coloured seed coats, all had similar activity and no significant differences were found among these dry beans. Among market classes, significant differences were observed between cultivars. Results are similar to those observed with total flavonoid content (Table 4) where flavonoid concentrations were significantly different between Windbreaker and Maverick/Buster (pinto) and Eclipse and Black violet (black), however no significant differences were found between Pink panther and ROG802/Montcalm/Red hawk (dark and light red kidney). The results of the present work suggest that specific groups of coloured seed coats, such the pinto and black groups possess greater antioxidant activity than light and dark red kidney groups.

Only one study has reported antioxidant activities of dry bean seed coats in the recent literature and the results were in agreement with the present work. Ranilla et al. (2007) reported a similar trend in antioxidant capacity for black, red, and light brown seed coats of Brazilian and Peruvian bean cultivars. In general, light brown seed

Table 6. Measured antioxidant activity of dry bean seed coats using the DPPH method

Sample	Seed coat antioxidant activity ($\mu\text{mol TE}/100\text{g}$)
Windbreaker	57451.0 ± 2639.5^a
Maverick/Buster	46805.2 ± 2024.1^b
Pink panther	47587.5 ± 3545.4^b
ROG802/Montcalm/Red hawk	42275.4 ± 1205.4^b
Eclipse	57816.8 ± 5056.4^a
Black violet	45195.7 ± 3039.6^b

Results are presented as means \pm standard deviations (n=6). Different letters within the same column represent significant differences. ($p < 0.05$)

coats (32400-43800 μmol of TE/100g seed coat), were greater or similar in antioxidant activity to black seed coats (24900-32700 μmol of TE/100g seed coat), which were greater in antioxidant activity to red seed coats (14700-19800 μmol of TE/100g seed coat) using the DPPH method. The results reported in the present work were much greater than the literature. In the literature, it is very difficult to compare the antioxidant activity when complex extracts are analyzed.

As flavonoid compounds have been shown to possess antioxidant activity, two flavonol glycosides, quercetin-3-*O*-glucoside and rutin, which were identified in the samples (results appear later) were tested in the antioxidant assay. Kaempferol 3-*O*-glucoside was not tested as not enough of the compound was available for the assay. Of the pure compounds tested, quercetin-3-*O*-glucoside had significantly greater activity, showing almost 2-fold the activity of rutin (Table 7).

Table 7. Measured antioxidant activity of pure flavonoids identified in dry bean seed coats using the DPPH method

Standard	Standard antioxidant activity ($\mu\text{mol TE}/100\text{g}$)
Quercetin 3- <i>O</i> -glucoside	172852.4 \pm 7118.8 ^a
Rutin	99880.9 \pm 1698.7 ^b

Results are presented as means \pm standard deviations (n=6). Different letters within the same column represent significant differences. ($p < 0.05$)

Structurally quercetin-3-*O*-glucoside and rutin are both glycosides of quercetin but differ in that rutin is composed of a disaccharide, rutinose, consisting of glucose and rhamnose. The sugar moiety, glucose or rutinose, affects the antioxidant activity of flavonoids (Heim et al., 2002). Flavonoids with additional sugar substituents, such as with rutin compared to quercetin-3-*O*-glucoside, occupy free OH groups that are required for hydrogen abstraction and radical scavenging (Heim et al., 2002). Therefore, rutin would be expected to have a lower antioxidant activity. An assay based on the relative ability of antioxidants to scavenge the radical cation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) generated by interaction with activated metmyoglobin and hydrogen peroxide, has demonstrated that quercetin-3-*O*-glucoside has significantly greater antioxidant activity compared to rutin (Williamson et al., 1996).

4.3.1. Correlation analyses between phenolic and flavonoid content and antioxidant activities

Correlation analyses between phenolic and antioxidant activities among the sample extracts were performed. The correlation coefficient (r) for the relationship

between the scavenging activity obtained from the DPPH assay and the total phenolic content was moderate with a value of 0.6198 (Figure 5). According to Ranilla et al., (2007), total phenolics had a significant correlation with antioxidant activity in dry bean seed coats and the phenolic compounds that were suggested as responsible for the antioxidant capacity were condensed tannins. Condensed tannins ranged from to 11.5 to 449 mg catechin equivalents/g seed coat. Good correlations were not observed in the present work, however in general, extracts with a high antioxidant activity showed a high phenolic content. The moderate correlation coefficient could be explained by the low number of samples used in the analysis. Also important is a variation in the response of different phenolic compounds to the Folin-Ciocalteu reagent (Kahkonen et al., 1999).

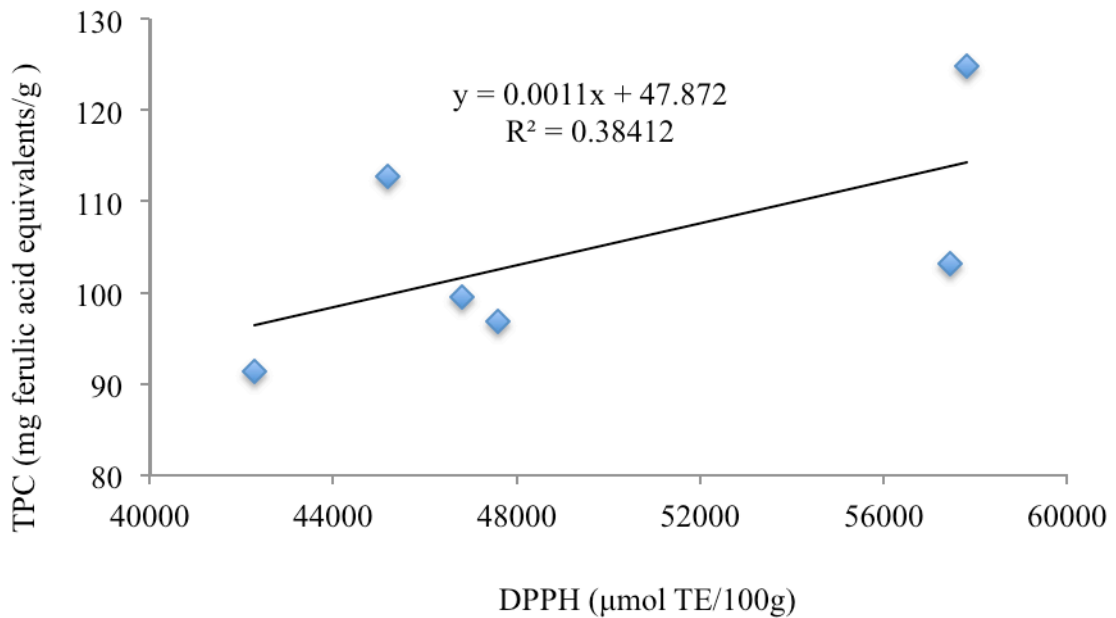


Figure 5. Correlation of total phenolic content with the antioxidant activity of samples as measured by the DPPH method.

Correlation analyses between flavonoid and antioxidant activities among the sample extracts were performed. The correlation coefficient between the scavenging activity obtained from the DPPH assay and the total flavonoid content was low with a value of 0.1236 (Figure 6). The lack of correlation with total flavonoid content is in agreement with other reports on correlation of total flavonoid content with antioxidant activity in seed coats of dry beans (Ranilla et al., 2007) and other legumes (Malencic et al., 2008; Nickavar et al., 2007). As previously stated, only certain flavonoids with a structure possessing hydroxyl groups at specific positions in the molecule may act as proton donating and demonstrate antioxidant activity. Therefore a high level of flavonoids does not necessarily result in high antioxidant activity. It is also possible that scavenging effects are not limited to phenolic and flavonoid compounds. The activity may also result

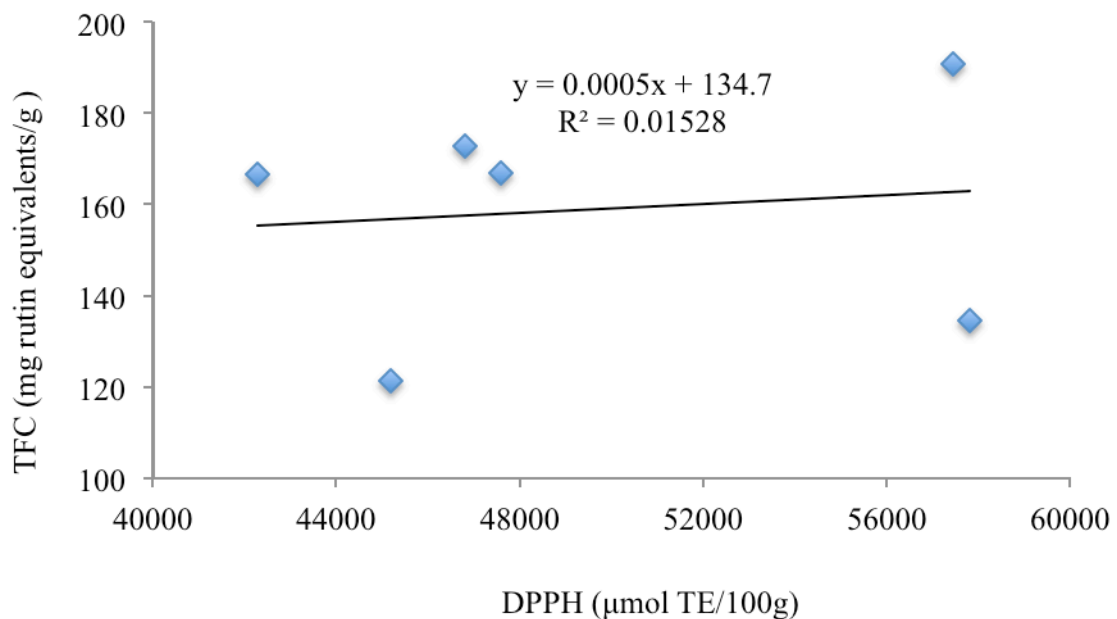


Figure 6. Correlation of total flavonoid content with the antioxidant activity of samples as measured by the DPPH method.

from the presence of other antioxidant secondary metabolites in the crude extracts, which directly or indirectly contribute to the antioxidant activity.

4.4 HPLC-MS/MS analysis for identification and characterization of flavonoids

To identify flavonoid compounds, HPLC-MS/MS in negative ion mode was carried out on the six crude dry bean seed coat extracts. The negative ion mode was used for flavonoid analysis, as it is more sensitive than positive ion mode (Cuyckens and Claeys, 2004). Initial identification of compounds in crude extracts was achieved by comparison of the sample data with data obtained for materials with compounds previously identified in the published literature.

In general, more polar phenolic compounds elute first under reverse phase chromatographic conditions. Glycosylation of flavonoids increases their polarity and therefore triglycosides elute before diglycosides, followed by monoglycosides and aglycones. For flavonoid aglycones, the elution order is typically dependent on the number of polar hydroxyl groups and therefore the expected elution order for flavonols is myricetin, followed by quercetin, and kaempferol. Acylation and methylation of flavonoids have the opposite effect. Acylation and methylation increase the retention times for flavonoids under reverse phase chromatographic conditions (Cuyckens and Claeys, 2004).

Flavonol glycosides were further identified in all crude extracts of dry bean seed coat samples based on chromatograms showing MS² fragmentation patterns (Appendix A). Based on the MS² fragmentation, the fragment ions for flavonol aglycones identified

in beans were quercetin (m/z 301/300), kaempferol (m/z 285/284), and myricetin (m/z 315). In the MS² fragmentation, the aglycone-related anion was observed as the commonly deprotonated molecular ([Aglycone-H]⁻). Although for certain compounds, the loss of two protons from the aglycone-related anion ([Aglycone-2H]⁻) was detected, which was possibly the result of deprotonation during analysis. This was observed only with quercetin and kaempferol derivatives. The aglycone and molecular ions were clearly observed for all the glycosylated flavonols. The type of sugar was determined based on the mass differences between the aglycones and the glycosides, for example, a difference of 132 amu for pentose, 162 amu for hexose, 248 amu for malonylhexose, and 308 amu for deoxyhexosylhexose (Cuyckens and Claeys, 2004).

4.4.1. Beige/brown coloured seed coats

In Windbreaker (Figure 7 and Table 8), three kaempferol glycosides (A1-A3) were identified. Peak A1 ($t_R = 13.90$) produced two important ion peaks at m/z 579 and 284. The peaks at m/z 579 and 284 correspond respectively to the quasi-molecular ion of kaempferol 3-*O*-xylosylglucoside or kaempferol 3-*O*-glucosylxyloside [M - H]⁻ and to the kaempferol aglycone [M - 2H - 294]⁻. The kaempferol aglycone ion or base peak was most likely obtained as a result of the loss of a hexose (162 amu), a glucosyl radical, and a pentose (132 amu), a xylosyl radical. Another ion in the MS² experiment on the [M - H]⁻ with an important relative abundance was a peak at m/z 408 and the mass spectra did not give any other fragmentation patterns to identify the sugar residues of different mass. This compound was tentatively identified as kaempferol 3-*O*-

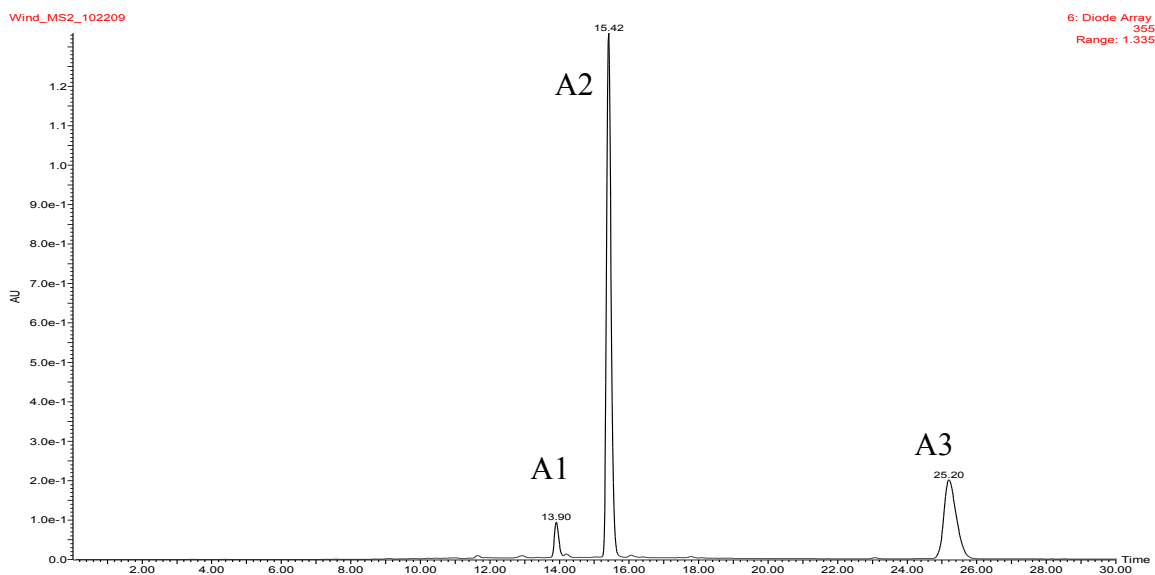


Figure 7. Chromatogram (355 nm) of the flavonol glycosides in Windbreaker seed coat crude extract.

Table 8. Peak assignment for the identification of flavonol glycosides of the seed coat of Windbreaker

Peak	t_R (min)	MS [M-H] ⁻ (m/z)	-MS ² [M-H] ⁻ (m/z)*	Tentative identification
A1	13.90	579	284	Kaempferol 3- <i>O</i> -xylosylglucoside
A2	15.42	447	284	Kaempferol 3- <i>O</i> -glucoside
A3	25.20	533	489, 284	Kaempferol 3- <i>O</i> -(malonyl)glucoside

*Based on MS² chromatograms of individual peaks.

xylosylglucoside or kaempferol 3-*O*-glucosylxyloside. Further structural analysis by NMR is required to identify the exact attachment points of the glucosyl and xylosyl groups. Kaempferol 3-*O*-xylosylglucoside has been previously reported in Italian bean varieties possessing a yellow coloured seed coat (Romani et al., 2004). Peak A2 (t_R =

15.24) showed signals at m/z 447 and 284 corresponding respectively to the quasi-molecular ion of kaempferol 3-*O*-glucoside $[M - H]^-$ and to the fragment after the loss of a glucose moiety $[M - 2H - 162]^-$. The literature shows this kaempferol glucoside was previously reported in pinto beans (Beninger et al., 2005; Lin et al., 2008). The fragmentation pattern of peak A3 ($t_R = 25.20$) produced peaks at m/z 533, 489, and 284 corresponding respectively to the quasi-molecular ion of kaempferol 3-*O*-malonylglucoside $[M - H]^-$, to the fragment after the loss of a CO₂ group $[M - H - 44]^-$, and to the kaempferol aglycone $[M - H - 248]^-$, respectively. The loss of a CO₂ group (44 amu) is characteristic for many flavonoid malonylglucosides because they have a COOH in their malonyl group (Lin et al., 2008) and the kaempferol aglycone-related ion is 248 amu less than the molecular ion, which corresponds to a malonyl group (OCCH₂COOH) and a glucosyl group. Therefore peak A3 was identified as kaempferol 3-*O*-malonylglucoside, which has been previously detected in pinto beans (Lin et al., 2008).

In Maverick/Buster (Figure 8 and Table 9), three kaempferol glycosides (B1-B3) were identified. Peak B1 ($t_R = 14.15$) produced two important ion peaks at m/z 579 and 284. Based on the previous identification of this peak in Windbreaker, the peak was proposed to be kaempferol 3-*O*-xylosylglucoside or kaempferol 3-*O*-glucosylxyloside. Peak B2 ($t_R = 15.62$) showed signals at m/z 447 and 284 and based on the previous identification of this peak in Windbreaker, the peak was identified as kaempferol 3-*O*-glucoside. Peak B3 ($t_R = 23.05$) showed signals at m/z 489 and 285 corresponding respectively to the quasi-molecular ion of kaempferol 3-*O*-acetylglucoside $[M - H]^-$ and

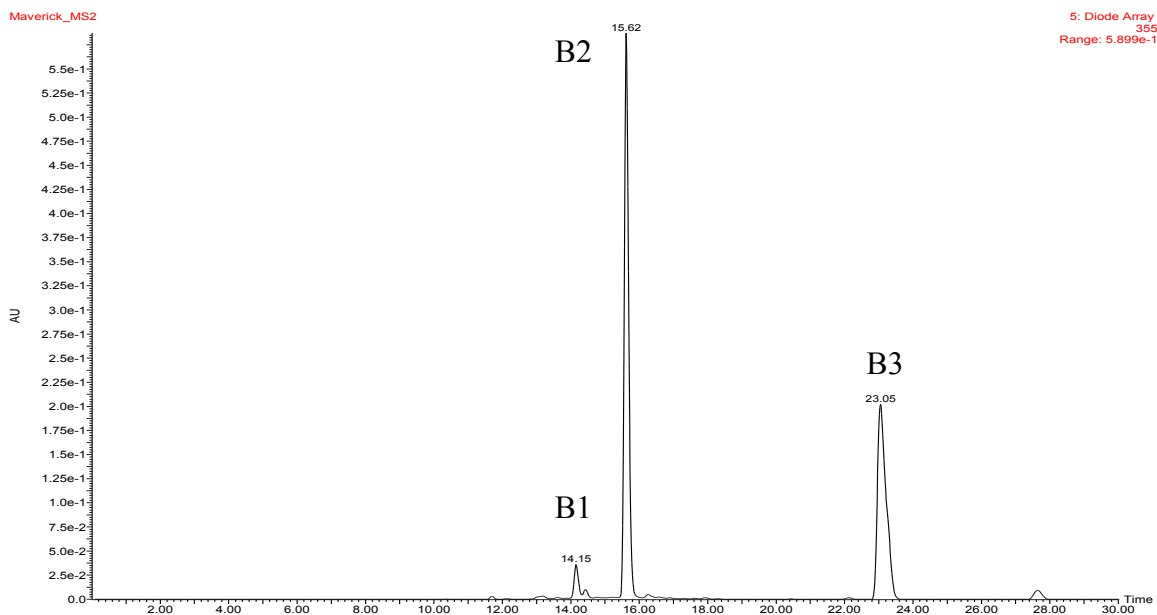


Figure 8. Chromatogram (355 nm) of the flavonol glycosides in Maverick/Buster seed coat crude extract.

Table 9. Peak assignment for the identification of flavonol glycosides of the seed coat of Maverick/Buster

Peak	t_R (min)	MS [M-H] ⁻ (m/z)	-MS ² [M-H] ⁻ (m/z)*	Tentative identification
B1	14.15	579	429, 284	Kaempferol 3- <i>O</i> -xylosylglucoside
B2	15.62	447	284	Kaempferol 3- <i>O</i> -glucoside
B3	23.05	489	285	Kaempferol 3- <i>O</i> -acetylglucoside

*Based on MS² chromatograms of individual peaks.

to the fragment after the loss of a acetylhexose moiety [M – H – 204]⁻. The mass spectra did not give any other fragmentation patterns to identify the residues of different mass. Kaempferol 3-*O*-acetylglucoside has been previously identified in *P. vulgaris* with yellow and brown seed coat colours on the basis of MS data (Romani et al., 2004;

Beninger et al., 2005).

4.4.2. Red coloured seed coats

For dark red kidney bean varieties (Figure 9 and Table 10), the compounds identified were all various quercetin glycosides (C1-C5). In ROG802/Montcalm/Redhawk, peak C1 ($t_R = 11.60$) produced a quasi-molecular ion $[M - H]^-$ at m/z 741 and a fragmentation peak m/z 300 corresponding to a quercetin aglycone $[M - 2H - 440]^-$. The loss of 440 amu from the $[M - 2H]^-$ suggests the possible presence of three sugar residues, a pentose (132 amu), a rhamnose (146 amu), and a hexose (162 amu). Peak C1 was tentatively identified as a quercetin triglycoside [quercetin (301 amu) + pentose (132 amu) + rhamnose (146 amu) + hexose (162 amu) = 741]. Besides the peaks at m/z 741 and 300, no other important fragmentation peaks were observed on the mass spectra to identify the sugar residues of different mass or the order of the sugar residues. The identification of this compound as a flavonol triglycoside is also based on the systematic consideration of the information from HPLC- MS. Under reverse phase chromatographic conditions it is known that triglycosides elute before diglycosides, followed by monoglycosides. The peaks eluted after peak C1 all appear to be flavonol diglycosides and monoglycosides. A similar compound has been identified using MS in seeds of tomato (Ferrerres et al., 2010) and MS and NMR in green bean (Price et al., 1998). At the present time, flavonol triglycosides have not been identified in the seed coats of *P. vulgaris*. Peak C2 ($t_R = 12.50$) showed a $[M - H]^-$ ion at m/z 595.

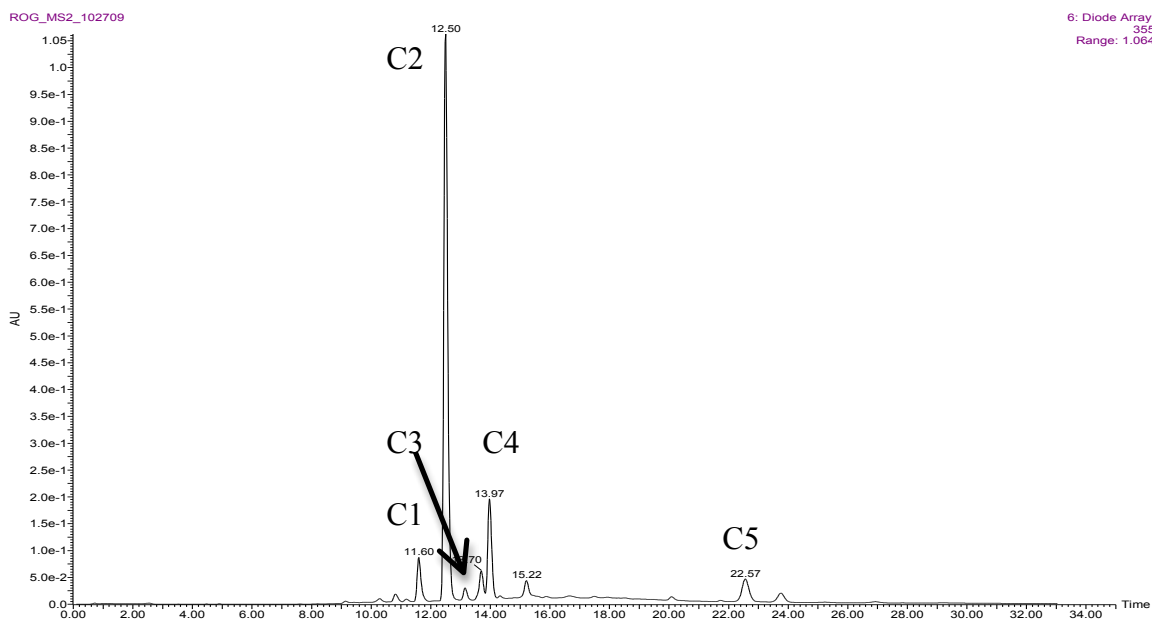


Figure 9. Chromatogram (355 nm) of the flavonol glycosides in ROG802/Montcalm/Redhawk seed coat crude extract.

Table 10. Peak assignment for the identification of flavonol glycosides of the seed coat of ROG802/Montcalm/Redhawk

Peak	t_R (min)	MS [M-H] ⁻ (m/z)	-MS ² [M-H] ⁻ (m/z)*	Tentative identification
C1	11.60	741	300	Quercetin triglycoside
C2	12.50	595	300	Quercetin 3- <i>O</i> -xylosylglucoside
C3	13.19	609	343, 300	Quercetin 3- <i>O</i> -rutinoside (rutin)
C4	13.97	463	300	Quercetin 3- <i>O</i> -glucoside
C5	22.57	505	463, 300	Quercetin 3- <i>O</i> -acetylglucoside

*Based on MS² chromatograms of individual peaks.

The MS² fragmentation of this peak resulted in an ion with m/z 300, corresponding to the radical quercetin aglycone anion $[M - 2H - 294]^-$. The loss of 294 amu from the $[M - H]^-$ suggests the presence of a glucosyl (162 amu) and xylosyl (132 amu) group. This compound was tentatively identified as quercetin 3-*O*-xylosylglucoside, which has been previously detected in dark and light red kidney beans (Beninger and Hosfield, 2003; Lin et al., 2008). The fragmentation pattern of peak C3 ($t_R = 13.19$) showed signals at m/z 609 and 300, corresponding respectively to the quasi-molecular ion of quercetin 3-*O*-rutinoside $[M - H]^-$ and to the quercetin aglycone $[M - 2H - 308]^-$. The quercetin aglycone ion or base peak was most likely formed as a result of the loss of a deoxyhexosylhexose moiety consisting of rhamnose (146 amu) and glucose (162 amu). In the literature, Redhawk kidney beans did not contain quercetin 3-*O*-rutinoside (rutin) (Lin et al., 2008), however this is a mixture of dark red kidney bean varieties and the other varieties may be the ones containing rutin. Peak C4 ($t_R = 13.97$) produced peaks at m/z 463 and 300 corresponding respectively to the quasi molecular ion of quercetin 3-*O*-glucoside $[M - H]^-$ and to the fragment after the loss of a glucose moiety $[M - 2H - 162]^-$. This peak was identified as quercetin 3-*O*-glucoside, which has also been previously reported in dark and light red kidney beans (Beninger and Hosfield, 1999; Beninger and Hosfield, 2003; Lin et al., 2008). Peak C5 ($t_R = 22.57$) produced three important ion peaks at m/z 505, 463, and 300. The peaks at m/z 505 and 463 correspond respectively to the quasi-molecular ion of quercetin 3-*O*-acetylglucoside $[M - H]^-$ and to quercetin 3-*O*-glucoside $[M - H - 42]^-$. The quercetin 3-*O*-glucoside ion was most likely obtained as a result of the loss of an acetyl group (42 amu). This compound was

tentatively identified as quercetin 3-*O*-acetylglucoside. This particular flavonol glycoside has not been previously identified in the seed coats of *P. vulgaris* L.

For light red kidney bean varieties (Figure 10 and Table 11), the compounds identified were all various quercetin glycosides (D1-D3). In Pink panther, the fragmentation pattern of peak D1 ($t_R = 13.23$) showed signals at m/z 609 and 300, corresponding respectively to the quasi-molecular ion of quercetin 3-*O*-rutinoside $[M - H]^-$ and to the quercetin aglycone $[M - 2H - 308]^-$. The literature shows quercetin 3-*O*-rutinoside (rutin) was previously reported in light red kidney beans (Lin et al., 2008). Peak D2 ($t_R = 14.05$) produced a quasi-molecular ion $[M - H]^-$ at m/z 463 and a fragmentation peak m/z 300 corresponding to a quercetin aglycone $[M - 2H - 162]^-$. The loss of 162 amu from the $[M - H]^-$ suggests the presence of a glucosyl (162 amu) group. This compound was identified as quercetin 3-*O*-glucoside, which was also identified in crude extracts of ROG802/Montcalm/RedHawk. Another quercetin glycoside that was identified in ROG802/Montcalm/RedHawk was peak D3 ($t_R = 22.82$). The MS² fragmentation of this peak showed signals at m/z 505 and 463, corresponding respectively to the quasi-molecular ion of quercetin 3-*O*-acetylglucoside $[M - H]^-$ and to quercetin 3-*O*-glucoside $[M - H - 42]^-$. Again, this compound was tentatively identified as quercetin 3-*O*-acetylglucoside and has not been previously identified in the seed coats of *P. vulgaris*.

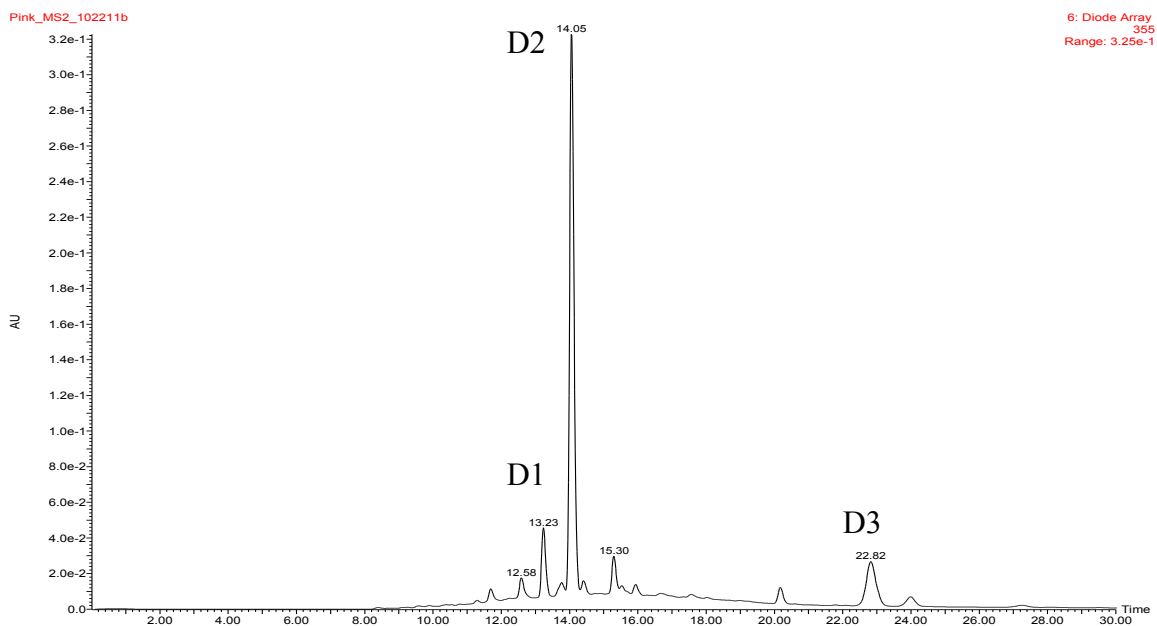


Figure 10. Chromatogram (355 nm) of the flavonol glycosides in Pink panther seed coat crude extract.

Table 11. Peak assignment for the identification of flavonol glycosides of the seed coat of Pink panther

Peak	t_R (min)	MS [M-H] ⁻ (m/z)	-MS ² [M-H] ⁻ (m/z)*	Tentative identification
D1	13.23	609	343, 300	Quercetin 3- <i>O</i> -rutinoside (rutin)
D2	14.05	463	300	Quercetin 3- <i>O</i> -glucoside
D3	22.82	505	463, 300	Quercetin 3- <i>O</i> -acetylglucoside

*Based on MS² chromatograms of individual peaks.

4.4.3. Black coloured seed coats

For Eclipse dry beans (Figure 11 and Table 12), peaks E1 ($t_R = 8.45$), E2 ($t_R =$

10.35), and E3 ($t_R = 12.10$) could not be identified as flavonol glycosides based on the acquired spectra. Peak E4 ($t_R = 12.58$) produced peaks at m/z 479 and 316 corresponding respectively to the quasi molecular ion of myricetin 3-*O*-glucoside $[M - H]^-$ and to the fragment after the loss of a glucose moiety $[M - 2H - 162]^-$. This compound was tentatively identified as myricetin 3-*O*-glucoside. This particular flavonol glycoside has been previously identified only in black bean varieties of *P. vulgaris* L. (Aparicio-Fernandez et al., 2005; Lin et al., 2008). Peak E5 ($t_R = 14.07$) produced a quasi-molecular ion $[M - H]^-$ at m/z 463 and a fragmentation peak m/z 300 corresponding to a quercetin aglycone $[M - 2H - 162]^-$. The loss of 162 amu from the $[M - H]^-$ suggests the presence of a glucosyl (162 amu) group. This compound was identified as quercetin 3-*O*-glucoside, which has been previously reported in black beans (Aparicio-Fernandez et al., 2005; Lin et al., 2008; Romani et al., 2004) and identified in the red dry bean crude extract samples. Peak E6 ($t_R = 15.32$) showed signals at m/z 447 and 284 corresponding respectively to the quasi-molecular ion of kaempferol 3-*O*-glucoside $[M - H]^-$ and to the fragment after the loss of a glucose moiety $[M - 2H - 162]^-$. Kaempferol 3-*O*-glucoside has been detected previously in different varieties of black dry beans (Dong et al., 2007; Lin et al., 2008; Romani et al., 2004).

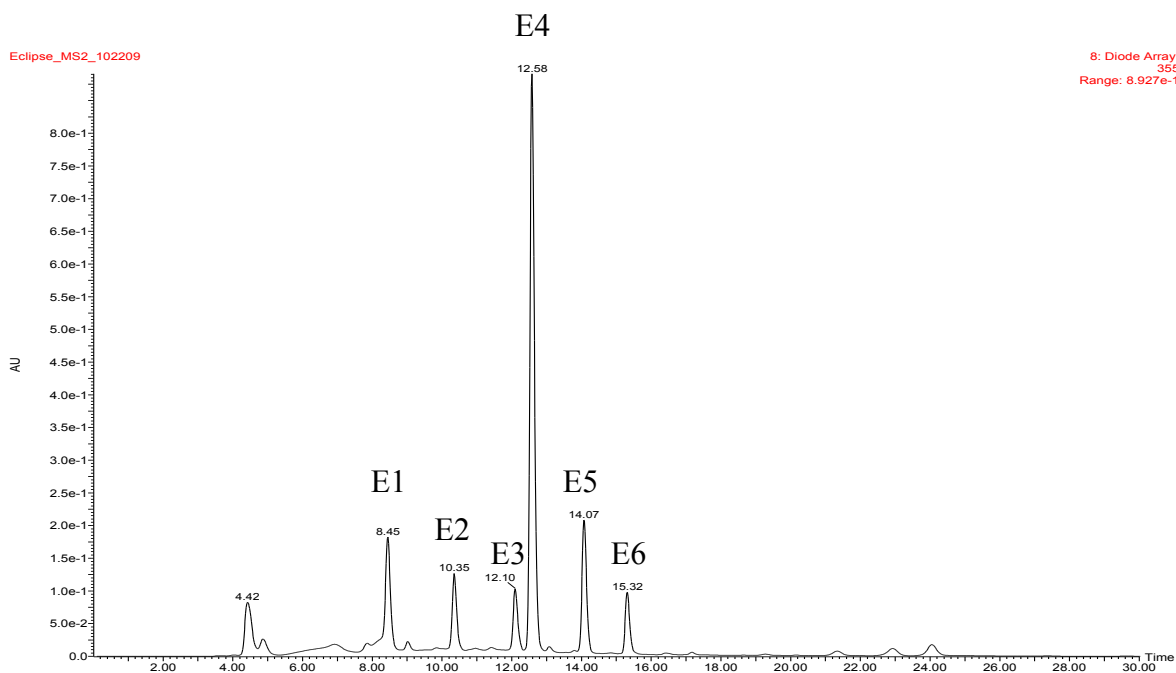


Figure 11. Chromatogram (355 nm) of the flavonol glycosides in Eclipse seed coat crude extract.

Table 12. Peak assignment for the identification of flavonol glycosides of the seed coat of Eclipse

Peak	t_R (min)	MS [M-H] ⁻ (m/z)	-MS ² [M-H] ⁻ (m/z)*	Tentative identification
E1	8.45	481	329	Not identified
E2	10.35	495	315	Not identified
E3	12.10	509	329	Not identified
E4	12.58	479	316	Myricetin 3- <i>O</i> -glucoside
E5	14.07	463	300	Quercetin 3- <i>O</i> -glucoside
E6	15.32	447	284	Kaempferol 3- <i>O</i> -glucoside

*Based on MS² chromatograms of individual peaks.

In Black violet (Figure 12 and Table 13), peaks F1 ($t_R = 8.32$), F2 ($t_R = 10.28$), and F3 ($t_R = 12.05$) could not be identified as flavonol glycosides based on the acquired spectra. The fragmentation pattern of peak F4 ($t_R = 12.53$) showed signals at m/z 479 and 316 corresponding respectively to the quasi-molecular ion of myricetin 3-*O*-glucoside $[M - H]^-$ and to the myricetin aglycone $[M - 2H - 162]^-$. This compound was tentatively identified as myricetin 3-*O*-glucoside, which was also tentatively identified in Eclipse crude extracts. Again, myricetin 3-*O*-glucoside has been previously reported in black beans (Aparicio-Fernandez et al., 2005; Lin et al., 2008; Romani et al., 2004). Peak F5 ($t_R = 14.05$) produced a quasi-molecular ion $[M - H]^-$ at m/z 463 and a fragmentation peak m/z 300 corresponding to a quercetin aglycone $[M - 2H - 162]^-$. This compound was identified as quercetin 3-*O*-glucoside, which has been previously reported in black beans (Aparicio-Fernandez et al., 2005; Lin et al., 2008; Romani et al., 2004) and identified in the red dry bean crude extract samples. The fragmentation pattern of peak F6 ($t_R = 15.28$) showed signals at m/z 447 and 284, corresponding respectively to the quasi-molecular ion of kaempferol 3-*O*-glucoside $[M - H]^-$ and to the kaempferol aglycone $[M - 2H - 162]^-$. This compound was identified as kaempferol 3-*O*-glucoside, which has been previously reported in black beans (Dong et al., 2007; Lin et al., 2008; Romani et al., 2004). Peak F7 ($t_R = 24.07$) showed a $[M - H]^-$ ion at m/z 489. The MS² fragmentation of this peak resulted in ions with m/z 327 and 285. The peaks at m/z 327 and 285 correspond respectively to the loss of a glucosyl (162 amu) group and the loss of an acetyl (42 amu) group from the quasi-molecular ion of kaempferol 3-*O*-acetylglucoside $[M - H]^-$. This compound was tentatively identified as kaempferol 3-*O*

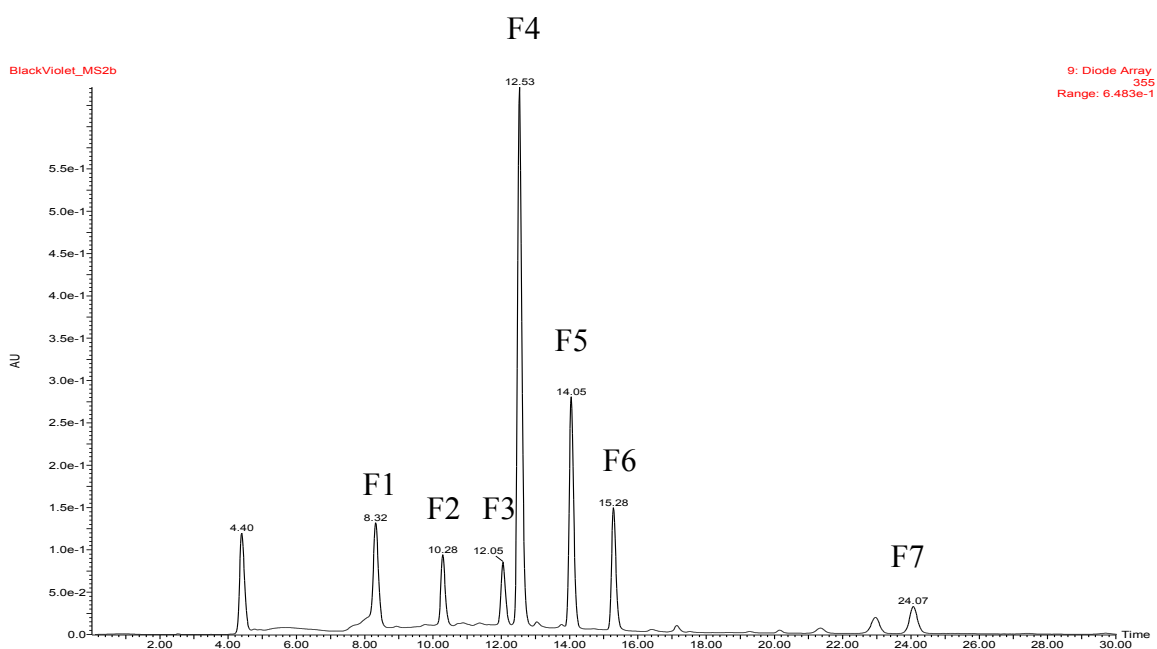


Figure 12. Chromatogram (355 nm) of the flavonol glycosides in Black violet seed coat crude extract.

Table 13. Peak assignment for the identification of flavonol glycosides of the seed coat of Black violet

Peak	t_R (min)	MS [M-H] ⁻ (m/z)	-MS ² [M-H] ⁻ (m/z)*	Tentative identification
F1	8.32	481	329	Not identified
F2	10.28	495	315	Not identified
F3	12.05	509	329	Not identified
F4	12.53	479	316	Myricetin 3- <i>O</i> -glucoside
F5	14.05	463	300	Quercetin 3- <i>O</i> -glucoside
F6	15.28	447	284	Kaempferol 3- <i>O</i> -glucoside
F7	24.07	489	327, 285	Kaempferol 3- <i>O</i> -acetylglucoside

*Based on MS² chromatograms of individual peaks.

acetylglucoside. Black Italian bean varieties have shown to contain kaempferol 3-*O*-acetylglucoside (Romani et al., 2004).

Based on the identification of the flavonol glycosides and their antioxidant activity, as determined by the DPPH method, crude seed coat extracts containing quercetin-3-*O*-glucoside were expected to have a significant amount of activity compared to those containing rutin. However, for those samples identified as containing quercetin-3-*O*-glucoside, including Eclipse, Black violet, ROG802/Montcalm/Redhawk, and Pink panther, only Eclipse had significantly greater antioxidant activity. It is possible that there may be other compounds, such as condensed tannins or other flavonoids or polyphenolics, responsible for some of the antioxidant activity in the acetone extracts. Also, the concentration of quercetin-3-*O*-glucoside in the samples containing quercetin-3-*O*-glucoside may actually be low or in trace amounts. This would expect to give low antioxidant activity for these samples.

4.5 HPLC analysis for quantification of flavonol glycosides

The content of flavonol glycosides in crude dry bean extracts was determined by the separation method of HPLC. Quantification was carried out using the commercially available standards, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, and rutin. Among previous reports (Aparicio-Fernandez et al., 2005; Diaz-Batalla, et al., 2006; Espinosa-Alonso et al., 2006; Romani et al., 2004), flavonol glycosides were the most common flavonoids identified in the majority of dry bean samples. The quantitative data

of flavonol glycosides for the crude extracts of dry bean seed coats varying in colour are reported (Table 14). Among all the pure flavonol glycosides evaluated, kaempferol 3-*O*-

Table 14. Quantification of individual flavonol glycosides in dry bean seed coats by HPLC

Sample	Q3G (mg/g sample)	K3G (mg/g sample)	Rutin (mg/g sample)
Windbreaker	nd	7.08 ± 0.30 ^a	nd
Maverick/Buster	nd	2.80 ± 0.07 ^b	nd
Pink panther	1.23 ± 0.18 ^b	nd	0.22 ± 0.01 ^a
ROG802/Montcalm/Redhawk	3.84 ± 0.17 ^a	nd	0.13 ± 0.01 ^b
Eclipse	0.91 ± 0.09 ^c	0.44 ± 0.03 ^c	nd
Black violet	0.92 ± 0.05 ^c	0.54 ± 0.03 ^c	nd

Results are presented as a means ± standard deviations (n=6). Different letters within the same column represent significant differences. ($p < 0.05$). Abbreviations: Q3G, quercetin 3-*O*-glucoside; K3G, kaempferol 3-*O*-glucoside; nd, not detected.

glucoside was the most prevalent among all the crude extracts, followed by quercetin 3-*O*-glucoside, and then rutin. Extracts of pinto bean seed coats contained the highest amount of kaempferol 3-*O*-glucoside (7.08 and 2.08 mg/g sample for Windbreaker and Maverick/Buster, respectively) whereas extracts of seed coats of black beans contained the lowest content (0.54 and 0.44 mg/g sample for Black violet and Eclipse, respectively). For quercetin 3-*O*-glucoside, red bean seed coat extracts were significantly higher (3.84 and 1.23 mg/g sample for ROG802/Montcalm/Redhawk and Pink panther, respectively) compared with black bean extract content (0.92 and 0.91 mg/g sample for Black violet and Eclipse, respectively). Rutin was only detected in red bean seed coat

extracts at 0.22 and 0.13 mg/g sample for ROG802/Montcalm/Redhawk and Pink panther, respectively. Within the different seed coat colour groups there were significant differences in flavonol glycoside contents among varieties except for black beans. No significant differences were observed among contents of kaempferol 3-*O*-glucoside and quercetin 3-*O*-glucoside in Black violet and Eclipse seed coat extracts.

The content of flavonol glycosides in dry bean seed coats determined in the present work is generally greater compared to the reported content of these or similar compounds in the literature. For pinto beans, Beninger et al. (2005) reported the kaempferol 3-*O*-glucoside concentration ranged from 0.054 to 0.492 mg/g of seed coat. Lower concentrations in dry bean seed coats possessing a similar colour to pinto beans have also been reported. A genetic stock of dry bean possessing a mineral brown seed coat colour contained 0.23 mg kaempferol 3-*O*-glucoside/g of seed coat (Beninger et al., 1999). The content of pure flavonol glycosides in the seed coats of red or black dry beans has yet to be reported in the literature. The other studies that have reported the content of these particular flavonol derivatives in dry beans have evaluated whole dry bean seeds rather than seed coats alone (Beninger and Hosfield, 1998; Dinelli et al., 2006; Romani et al., 2004). Beninger and Hosfield (1998) reported the kaempferol 3-*O*-glucoside concentration as 0.499 mg/g of fresh whole bean weight in dry beans possessing a yellow coloured seed coat. Dinelli et al. (2006) reported the concentration of kaempferol 3-*O*-glucoside varied from 0.21 to 0.52 mg/g in whole Italian dry bean seed ecotypes of different seed coat colours. In similar Italian type dry beans with yellow, black, and tobacco coloured seed coats, Romani et al. (2004) reported the concentration of kaempferol 3-*O*-glucoside varied from 0.066 to 0.545 mg/g of fresh weight of seed.

Based on these results, the contents of specific flavonol glycosides, such as kaempferol 3-*O*-glucoside, appear to be much lower in whole seeds compared to seed coats.

It should be noted that seed coat concentrations of flavonol glycosides in the present work were probably actually greater than reported because the seeds were soaked in distilled water to help the manual removal of seed coats. Soaking leads to softening of cell wall tissues, which usually results in the solubilization of bound polyphenols (Boateng et al., 2008).

4.6 Cell culture

4.6.1. Preliminary cytotoxic effect of crude extracts and pure flavonol glycosides

To investigate the anti-inflammatory properties of the six crude dry bean seed coat extracts and pure flavonol glycosides, RAW 264.7 murine macrophages were used to produce TNF- α . In the macrophages, the potential toxicity of samples was assessed by the MTT assay. The results of preliminary cytotoxicity effects were illustrated in Figure 13. Preliminary effects were investigated without the addition of LPS, a known potent macrophage activator. The cells were treated with crude extracts at various doses of 50-500 $\mu\text{g}/\text{mL}$ and pure flavonoids at various doses of 50-500 $\mu\text{g}/\text{mL}$ for 24 hours. After the incubation period, all samples did not demonstrate strong cytotoxicity at the tested concentrations. Windbreaker at 200 and 500 $\mu\text{g}/\text{mL}$ reduced cell viability to 95 and 89 %, of control levels respectively, while Black violet at 100 $\mu\text{g}/\text{mL}$ reduced cell viability to 97 % of control levels. Maverick/Buster, Pink panther, and ROG802/Montcalm/

Redhawk at 500 µg/mL reduced cell viability to 97, 89, and 81 % of control levels, respectively. Based on review of the literature, more than a 10 % percent reduction in cell viability appears to be cytotoxic. Considering the high % reduction in cell viability for ROG802/Montcalm/ Redhawk, the crude extract at 500 µg/mL appears to be cytotoxic. The remaining extracts did appear not to exhibit cytotoxicity as reduced cell viability was not in a dose-dependent manner. The pure flavonol glycosides showed a reduction in cell viability compared to the control but not in a dose-dependent manner. Most doses of pure flavonoids in resulted in a slight reduction in cell viability. Quercetin-3-*O*-glucoside and rutin showed the greatest reduction at 500 µg/mL to 90 % of control levels. Reductions in cell viability varied ≤ 10 % and therefore appear not to be cytotoxic. Based on the results, dry bean seed coat extracts and pure flavonol glycosides appeared not to strongly inhibit RAW 264.7 macrophage cell viability at the concentrations under investigation and these concentrations were chosen in the subsequent experiment for inhibition of cytokine production.

4.6.2. Preliminary inhibition of cytokine TNF- α production

RAW 264.7 cells release low levels of TNF- α (Xagorari et al., 2001). To determine the inhibitory effect of the various crude seed coat extracts and pure flavonoids at 50, 100, 200 and 500 µg/mL on pro-inflammatory cytokine release, the TNF- α levels were quantified by performing ELISA (Figure 14). For the pure flavonoids, the doses of 50, 100, 200, and 500 µg/ml translate into the following concentrations: 166, 332, 664, and 1661 µM for quercetin-3-*O*-glucoside, 175, 351, 702, and 1754 µM for kaempferol-

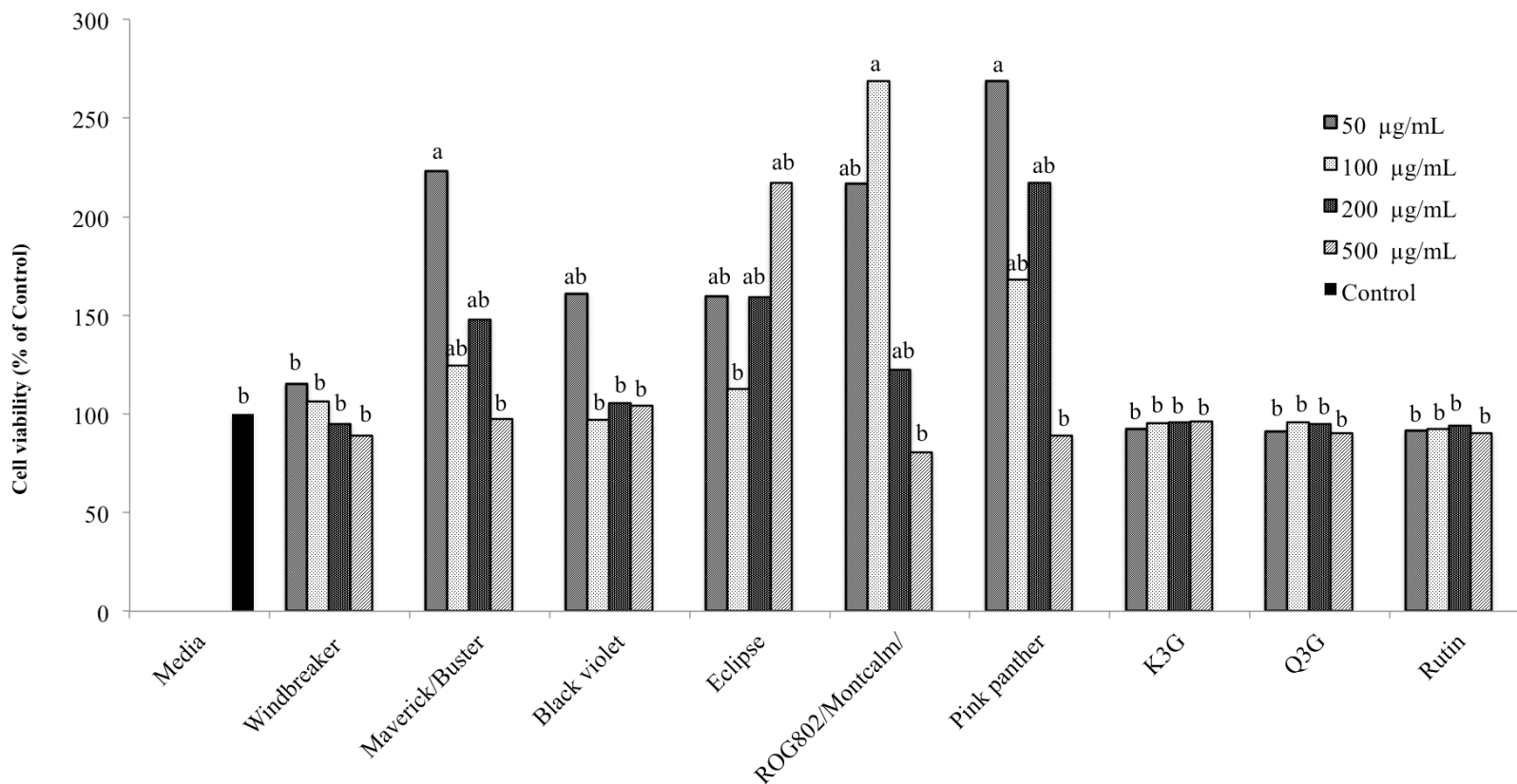


Figure 13. Preliminary cytotoxic effects of the crude seed coat extracts and pure flavonoids without the addition of LPS. Cytotoxic effects were determined by analyzing the mitochondrial-dependent reduction of MTT to formazan in living cells. RAW 264.7 cells were incubated with media (control) and different concentrations of crude extracts (50, 100, 200 and 500 µg/mL) and pure flavonoid glycosides (50, 100, 200 and 500 µg/mL). The results are expressed as means of triplicates. Abbreviations: Q3G, quercetin 3-*O*-glucoside; K3G, kaempferol 3-*O*-glucoside.

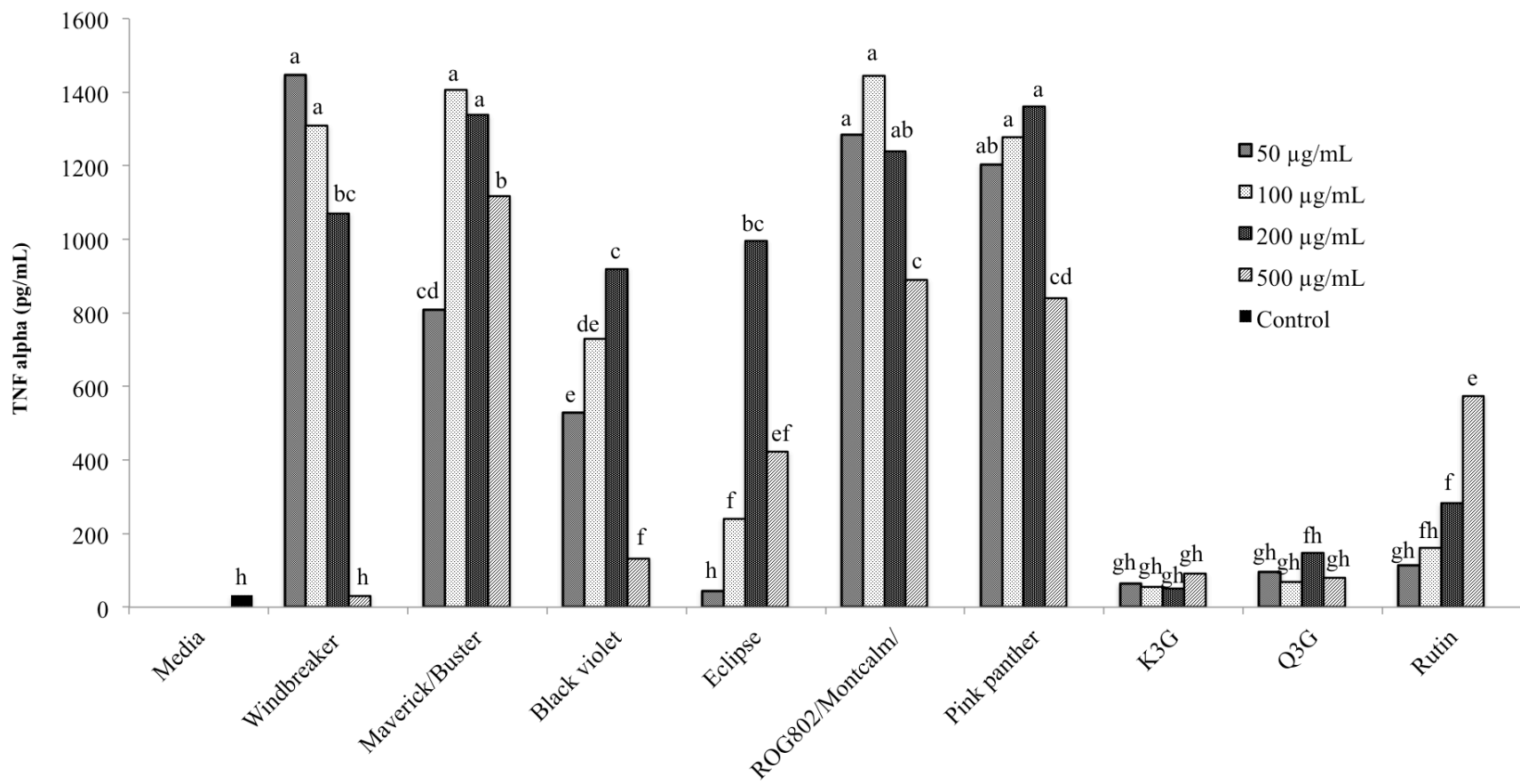


Figure 14. Effect of the crude seed coat extracts and pure flavonoids on the production of TNF- α without the addition of LPS. RAW 264.7 cells were incubated with different concentrations of crude extracts (50, 100, 200 and 500 $\mu\text{g/mL}$) and pure flavonol glycosides (50, 100, 200 and 500 $\mu\text{g/mL}$). Supernatants were collected after 24 hours. The results are expressed as means of triplicates. Abbreviations: Q3G, quercetin 3-*O*-glucoside; K3G, kaempferol 3-*O*-glucoside

3-O-glucoside, and 82, 164, 328, and 820 μM for rutin. At the doses tested, the majority of the crude extracts showed a significant ($p < 0.05$) increase in TNF- α production compared to the control (media alone). Since these are crude extracts, there are potentially other polyphenolic compounds present that were extracted from the seed coats, which induced the production of the cytokine TNF- α . The seed coats of coloured beans, specifically black and red seed coats, are known to possess anthocyanins (Beninger and Hosfield, 1999; 2003; Beninger et al., 1999; Romani et al, 2004; Takeoka et al., 1997) and anthocyanins have demonstrated to induce TNF- α production in RAW 264.7 cells (Wang and Mazza, 2002). Only a couple samples demonstrated potential anti-inflammatory activity. Extracts showing potential activity in inhibiting TNF- α production were Windbreaker, a pinto bean, at 500 $\mu\text{g}/\text{mL}$ and Eclipse, a black bean, at 50 $\mu\text{g}/\text{mL}$. These crude seed coat extracts had TNF- α levels relative to the control, with only Windbreaker showing a very slight inhibitory effect on TNF- α production.

For the pure flavonoids tested, no inhibitory effect on TNF- α production was observed. However, kaempferol-3-*O*-glucoside showed the most potential activity in inhibiting TNF- α production. The TNF- α levels were only slightly greater than the control at 50, 100, and 200 $\mu\text{g}/\text{mL}$. Kaempferol-3-*O*-glucoside was the main flavonol glycoside in Windbreaker and was also present in Eclipse in the present work. However, other crude extracts that were found to possess kaempferol-3-*O*-glucoside, such as Maverick/Buster, did not appear to exhibit any potential inhibitory effect. Quercetin-3-*O*-glucoside and rutin slightly induced the release of TNF- α , but to a lesser extent compared to the crude extracts, and rutin significantly increased TNF- α levels in a dose-dependent manner. It has been reported that flavonoids, such as quercetin (200 μM) alone, have no

effect on basal levels of TNF- α (Wadsworth et al., 2001) and that an effect is typically observed with the addition of a stimulant for TNF- α production. Based on these results, extracts of Windbreaker and Eclipse, and the pure flavonoids kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside, and rutin were tested using stimulated RAW 264.7 cells.

4.6.3. Cytotoxic effect of crude extracts and pure flavonol glycosides with LPS

Exposure of cells in culture to bacterial LPS triggers gene induction and the generation of ROS by macrophages. The inducible genes encode pro-inflammatory cytokines, such as TNF- α . We further evaluated the cytotoxic effects of crude extracts of Windbreaker, a pinto bean, and Eclipse, a black bean, and pure flavonoids kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside and rutin in the presence of LPS (1 μ g/mL) by the MTT assay (Figure 15). LPS (1 μ g/mL), was used as a positive control and salicin (500 μ M), a known anti-inflammatory compound, was used as a negative control. In the experiments using LPS, the doses of selected samples were chosen to cover a wider range (20, 100, 500 and 1000 μ g/mL) than previous experiments without LPS. When the cell were treated with crude extracts and pure flavonoids at various doses of 20-1000 μ g/mL for 24 hours, cell viability was not affected. All samples were not strongly cytotoxic at the tested concentrations. Salicin reduced cell viability to 96 % of control (media alone) levels. Among dry bean seed coat crude extracts, a reduction in cell viability was only observed with Windbreaker. The Windbreaker extract at 20 and 500 μ g/mL reduced cell viability to 99 and 91 % of control (media alone) levels, respectively. Reductions in cell viability were also observed for pure flavonol glycosides. Kaempferol-3-*O*-glucoside

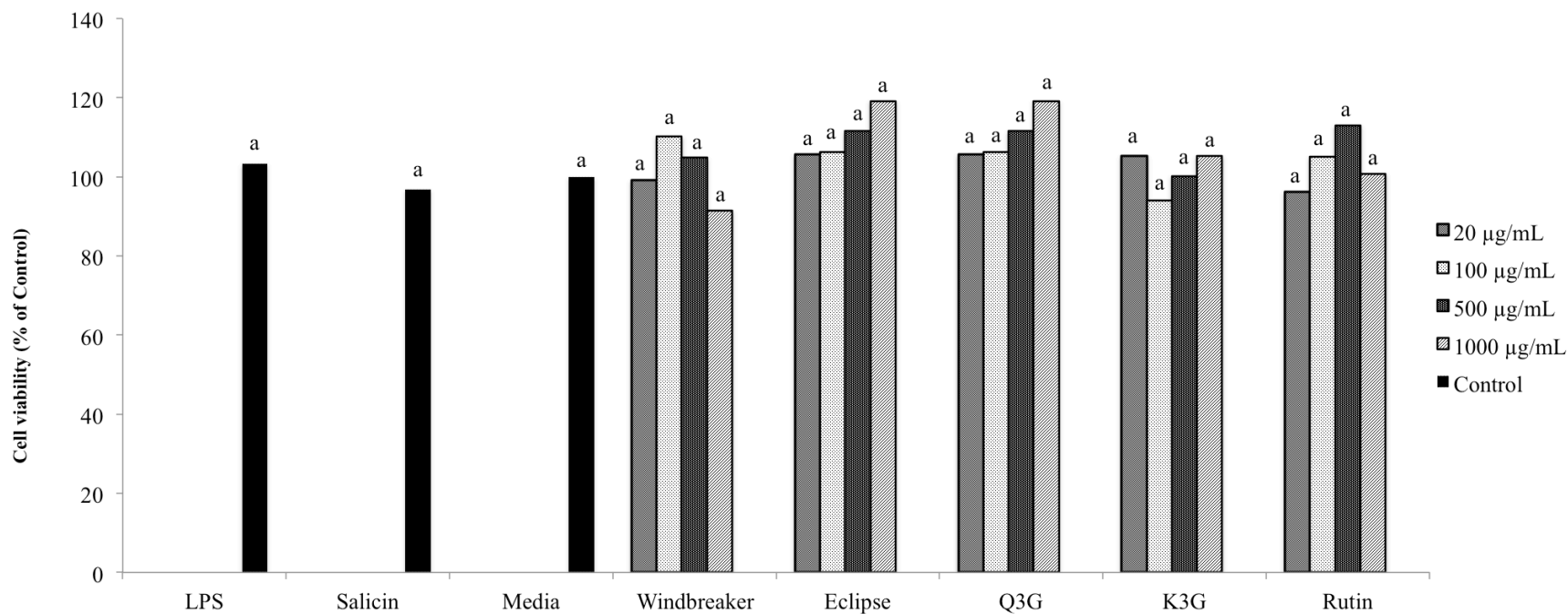


Figure 15. Cytotoxicity of the crude seed coat extracts and pure flavonoids with the addition of LPS. Cytotoxic effects were determined by analyzing its effects on the mitochondrial-dependent reduction of MTT to formazan in living cells. RAW 264.7 cells were incubated with controls (LPS [1µg/mL], Salicin [500 µM], and media), and different concentrations of crude extracts

reduced cell viability to 94 and 96 % of control (media alone) levels at 100 and 20 $\mu\text{g}/\text{ml}$, respectively, while rutin reduced cell viability to 96 % of control (media alone) levels at 20 $\mu\text{g}/\text{mL}$. The results did not show dry bean seed coat extracts and pure flavonol glycosides in the presence of LPS to strongly inhibit RAW 264.7 macrophage cell viability at the tested concentrations. These concentrations were chosen for the subsequent experiment to determine inhibition of cytokine production with LPS.

4.6.4. Inhibition of cytokine TNF- α production with LPS

The inhibitory effects of crude dry bean seed coat extracts and pure flavonol glycosides on the production of TNF- α in LPS treated RAW 264.7 cells were evaluated (Figure 16). Macrophages were incubated in the presence of crude extracts and flavonol glycosides at various concentrations (20, 100, 500 and 1000 $\mu\text{g}/\text{mL}$) and the quantity of TNF- α secreted into culture supernatants was then monitored by ELISA. For the pure flavonoids, the doses of 20, 100, 500 and 1000 $\mu\text{g}/\text{mL}$ translate into the following concentrations: 66, 332, 1661, and 3322 μM for quercetin-3-*O*-glucoside, 70, 351, 1754, and 3509 μM for kaempferol-3-*O*-glucoside, and 33, 164, 820, and 1639 μM for rutin. LPS (1 $\mu\text{g}/\text{mL}$), was used as a positive control, salicin (500 μM), a known anti-inflammatory compound, was used as a negative control, and media contained no LPS. For non-stimulated macrophages (media alone), only small amounts of TNF- α were secreted into the medium. However, when macrophages were stimulated with LPS, a significant ($p < 0.05$) increase in TNF- α was observed. Pretreatment of stimulated cells with salicin

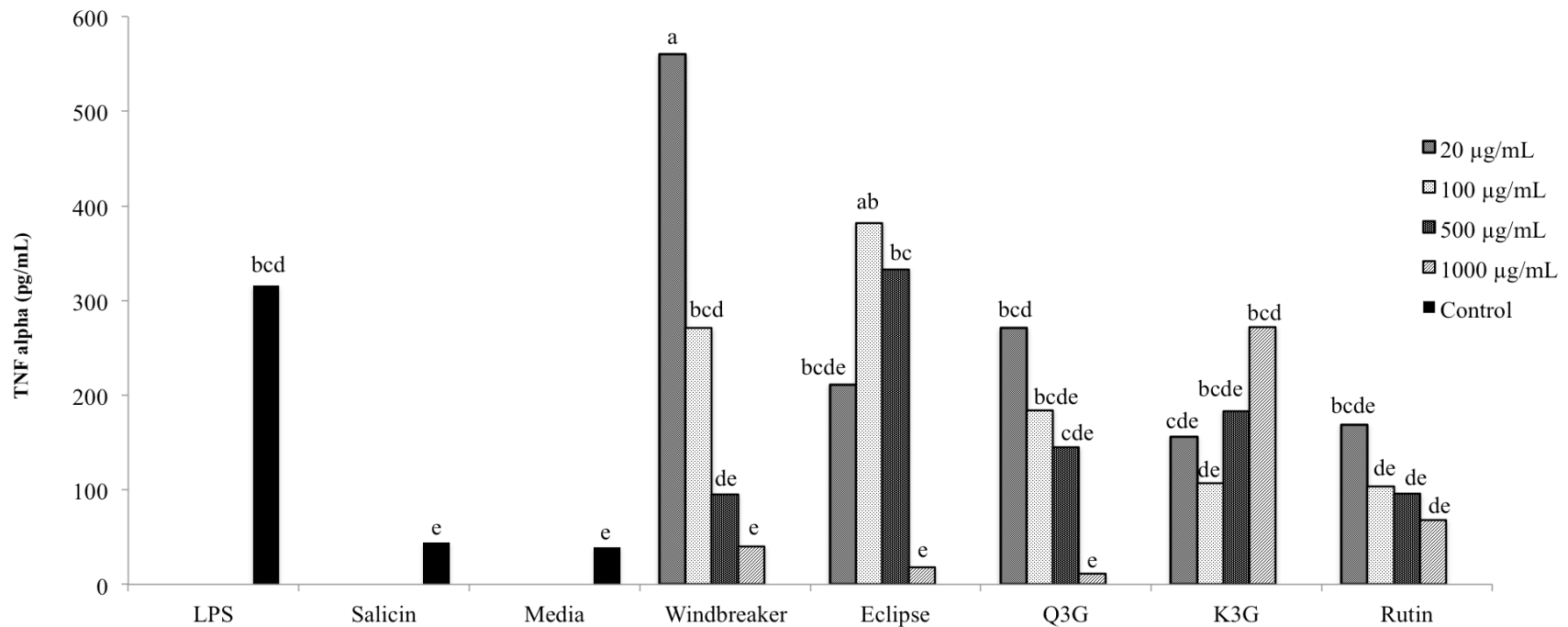


Figure 16. Effect of the crude seed coat extracts and pure flavonoids on the production of TNF- α in LPS treated RAW 264.7 cells. Cells were incubated with controls (LPS [1 μ g/mL], Salicin [500 μ M], and media), and different concentrations of crude extracts (20, 100, 500 and 1000 μ g/mL) and pure flavonol glycosides (20, 100, 500 and 1000 μ g/mL). Supernatants were collected after 24 hours. The results are expressed as means of triplicates. Abbreviations: LPS, lipopolysaccharide; Q3G, quercetin 3-*O*-glucoside; K3G, kaempferol 3-*O*-glucoside

resulted in a significant decrease in TNF- α levels compared to macrophages treated with LPS alone and the level of salicin was similar to non-stimulated macrophages (media only). When the LPS stimulated cells were treated with crude extracts of dry bean seed coat, TNF- α production was inhibited in a dose dependent manner. At 1000 $\mu\text{g}/\text{mL}$, Windbreaker and Eclipse significantly reduced TNF- α levels to 13 and 6 % of the positive control, respectively. The TNF- α levels for these extracts were relative to non-stimulated macrophages (media alone) and salicin.

Pretreatment of stimulated cells with the pure flavonoids, quercetin-3-*O*-glucoside and rutin, inhibited TNF- α production in a manner similar to that reported above for crude extracts. These pure flavonoids reduced TNF- α levels in a dose-dependent manner and appeared to be more potent than the crude extracts. Among quercetin-3-*O*-glucoside and rutin, only quercetin-3-*O*-glucoside demonstrated significant inhibition. Inhibition was significant at 1000 $\mu\text{g}/\text{mL}$ with TNF- α levels reduced to 3 % of the positive control. The inhibitory effect observed with quercetin-3-*O*-glucoside was not in agreement with previous results (Wang and Mazza, 2002), where quercetin-3-*O*-glucoside, at concentrations from 16 to 500 μM , showed no significant reduction in activated RAW 264.7 macrophages. The TNF- α reduction in a dose-dependent manner demonstrated with rutin was also no in agreement with previous results (Wang and Mazza, 2002), where rutin, at concentrations from 16 to 500 μM , showed only a slight reduction in activated RAW 264.7 macrophages and the reductions were not in a dose-dependent manner. The RAW 264.7 macrophages in the work of Wang and Mazza (2002) were activated using LPS/IFN- γ and not just LPS.

Pretreatment of stimulated cells with kaempferol-3-*O*-glucoside appeared to show an opposing effect compared to quercetin-3-*O*-glucoside and rutin, in that increasing concentrations resulted in increasing levels of TNF- α production. Although kaempferol-3-*O*-glucoside appeared to induce TNF- α production, the TNF- α levels at all doses were lower than the positive control. The TNF- α -induced effect observed with kaempferol-3-*O*-glucoside was not in agreement with previous reported results. In LPS/IFN- γ activated RAW 264.7 macrophages, kaempferol-3-*O*-glucoside at 16 μ M induced TNF- α production to a level greater than the positive control and at concentrations from 31 to 500 μ M significant TNF- α reductions were reported (Wang and Mazza, 2002). Also, kaempferol-3-*O*-glucoside (25 to 200 μ M), isolated from *Bauhinia variegata*, inhibited TNF- α production in a concentration-dependent manner with LPS/IFN- γ stimulated peritoneal macrophage cultures (Rao et al., 2005).

When examining the results obtained with stimulated RAW 264.7 macrophages and those of non-stimulated RAW 264.7 macrophages, some inconsistencies were observed. The effect of rutin on TNF- α production showed opposing effects. With LPS-stimulated macrophages, rutin showed inhibition in a dose dependent manner and with non-stimulated macrophages, rutin induced TNF- α production in a dose dependent manner. A similar result was observed with kaempferol-3-*O*-glucoside. With LPS-stimulated macrophages, kaempferol-3-*O*-glucoside induced TNF- α production and with non-stimulated macrophages, kaempferol-3-*O*-glucoside appeared to inhibit the release of TNF- α . Also, the TNF- α levels obtained with non-stimulated macrophages appear to be more elevated relative to the TNF- α levels obtained with LPS-stimulated macrophages. These discrepancies could be due to the fact that the ELISA assay for non-macrophages

was conducted first and the ELISA assay for LPS-stimulated macrophages was conducted approximately 6 months later and materials may have not been stored appropriately, as noted in the materials and methods. It was not possible to repeat the assay to confirm these results.

The results of the present work demonstrated both Windbreaker and Eclipse dry bean seed coat extracts, up to doses of 1000 µg/ml, appear to demonstrate considerable effects on TNF- α induction without causing cytotoxicity to cells. The other seed coat extracts, however, did not appear to possess this property. The present work also provides further evidence that pure flavonol glycosides, particularly quercetin 3-*O*-glucoside, are able to reduce the production of TNF- α in stimulated macrophages. Since quercetin 3-*O*-glucoside appeared to show the most potent inhibitory activity, it was expected that crude extracts containing primarily quercetin 3-*O*-glucoside (the light and dark red kidney beans) would have a significant amount of activity. However, the light and dark red kidney beans, ROG802/Montcalm/Redhawk and Pink panther, demonstrated pro-inflammatory properties with non-stimulated RAW 264.7 macrophages. One explanation is that the content of quercetin 3-*O*-glucoside in these crude extracts was too low to show an effect. Also, these extracts, in addition to Maverick/Buster and Black violet, were not tested with LPS-stimulated macrophages and an effect may be apparent only when tested under stimulated conditions. For Windbreaker and Eclipse, these samples both contained kaempferol 3-*O*-glucoside, which appeared to induce TNF- α production in LPS-stimulated macrophages in the present work. It was expected that crude extracts containing primarily kaempferol 3-*O*-glucoside would demonstrate pro-inflammatory properties. One explanation is that the content of kaempferol 3-*O*-glucoside in these

crude extracts was too low and that perhaps there are other phytochemicals, such as anthocyanins, present in crude extracts responsible for the considerable effects on TNF- α induction. In any case, the present cell culture work was not validated and further work is necessary to confirm these observations.

4.6.5. *NO production*

To further characterize the effects of crude dry bean seed coat extracts and pure flavonol glycosides on pro-inflammatory molecule expression, the ability of these samples to inhibit nitrite accumulation in cells was tested. The effects of extracts and flavonols on NO production in RAW 264.7 macrophages are shown in Figure 17. Cells were not stimulated with LPS and the control (media alone) had a low NO basal level (0.16 mg/mL). The low basal level may be the result of issues with equipment or the cells may have been stored incorrectly. The Griess reaction revealed that none of the crude extracts significantly decreased nitrite release compared to the NO basal level; however decreases from the basal level were observed with Windbreaker at 500 μ g/mL and Eclipse at 50 and 100 μ g/mL. These crude seed coat extracts had NO levels lower than the control, but they were not significantly different ($p < 0.05$). It is worth noting that the effects of these particular crude extracts on both NO and TNF- α production showed the same trend.

For pure flavonol glycosides, decreases in NO production from the basal level were demonstrated with quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside at all doses and with rutin at 50 and 200 μ g/mL. However, none of the compounds significantly

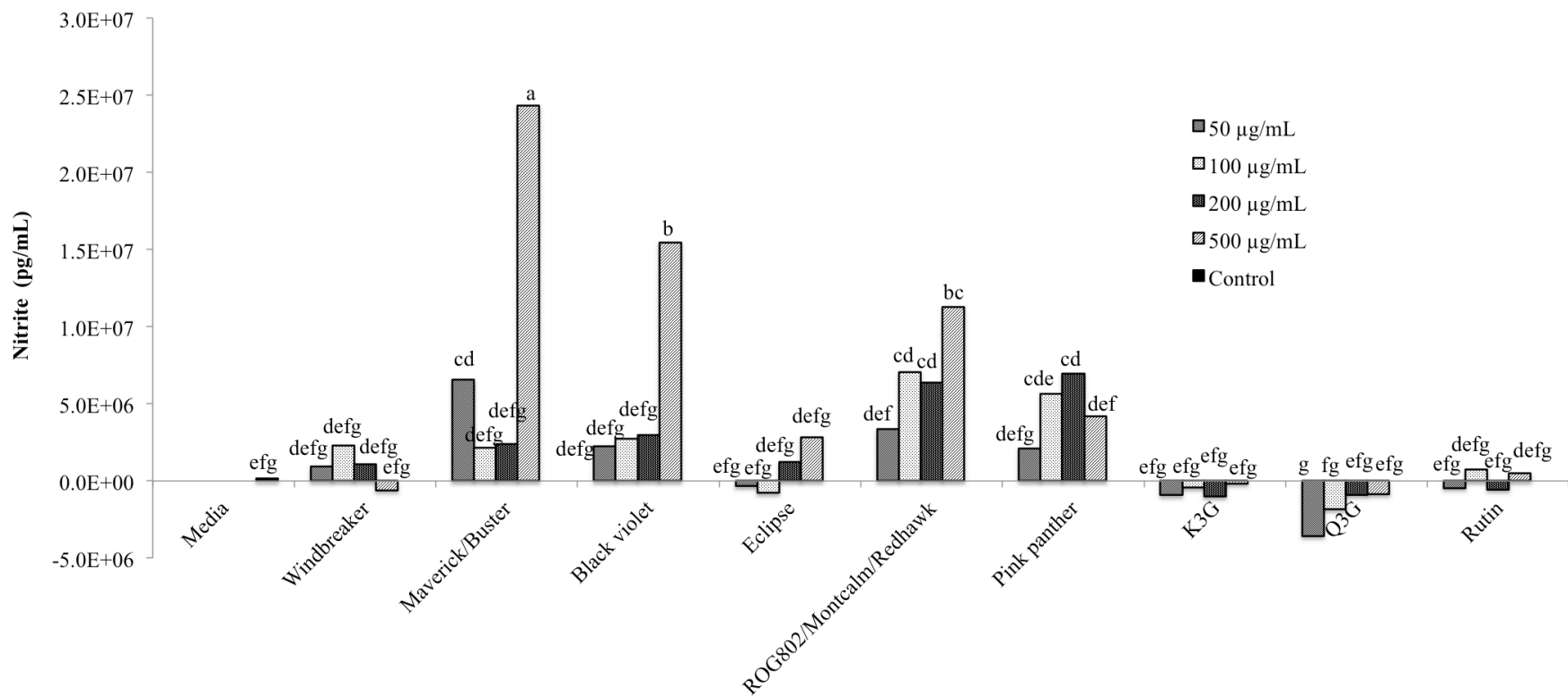


Figure 17. Inhibition of NO production by crude seed coat extracts and pure flavonoids without the addition of LPS. RAW 264.7 cells were incubated with different concentrations of crude extracts (50, 100, 200 and 500 µg/mL) and pure flavonol glycosides (50, 100, 200 and 500 µg/mL). NO levels were measured as described in Methods and materials. The results are expressed as means of triplicates. Abbreviations: Q3G, quercetin 3-*O*-glucoside; K3G, kaempferol 3-*O*-glucoside.

decreased nitrite release compared to the NO basal level. Among the pure flavonoids, quercetin-3-*O*-glucoside appeared to be the most active, followed by and kaempferol-3-*O*-glucoside, and then rutin. For the inhibitory effects on NO production, this suggests that for flavonol 3-*O*-glucosides 3'-OH is beneficial for activity and that rutinosides, or disaccharides, of flavonols are deleterious for activity compared to glucosides of flavonols. Previous research on NO inhibition by the flavonol glycosides used in the present work has only been reported with kaempferol-3-*O*-glucoside. Kaempferol-3-*O*-glucoside (25 to 200 μ M), isolated from *Bauhinia variegata*, inhibited NO production in a concentration-dependent manner with LPS/IFN- γ stimulated peritoneal macrophage cultures (Rao et al., 2005).

CHAPTER 5: CONCLUSIONS AND FUTURE RESEARCH SUGGESTIONS

5.1 Conclusion

In conclusion, it appears the seed coats from specific dry beans have the potential to be used as a functional food or nutraceutical ingredients. In the present work, flavonol glycosides were identified and quantified in the seed coats, with pinto beans, beige/brown in colour, possessing kaempferol glycosides, light and dark red kidney beans possessing quercetin glycosides, and black beans possessing kaempferol, quercetin, and myricetin glycosides. In addition, a flavonol triglycoside was tentatively identified for the first time in the seed coats of dry beans. In terms of quantification, the kaempferol glycoside was greatest in Windbreaker, and both the quercetin glycosides were greatest in light and dark red kidney seed coats. The chemical analyses revealed that coloured seed coats are an excellent source of natural antioxidants, with Windbreaker and Eclipse demonstrating significantly high antioxidant activity. The same extracts also showed potential anti-inflammatory activity. When applied to LPS treated murine macrophages at a heightened state of inflammation, Windbreaker and Eclipse appeared to significantly decrease pro-inflammatory cytokines, although it was not confirmed that the compounds responsible for the effect are flavonol glycosides as there are potentially other phytochemicals in the crude extracts inhibiting pro-inflammatory cytokines. Also, the cell culture data was not validated and further work is necessary to confirm these observations. Overall, Windbreaker and Eclipse seed coats were the only samples to

show potential use as a functional food ingredient and possibly prevent disease and enhance human health, but this can only be possible with additional research in this area.

5.2 Future research suggestions

Based on the results obtained for this work, there is potential that the seed coats from dry beans can be used as a functional food or nutraceutical ingredients, however additional research is required. A complete polyphenolic profile of coloured seed coats from dry beans would be a valuable tool. With a complete profile, each compound could be further tested to determine the exact compound(s) responsible for anti-inflammatory activity. Also, it is necessary to confirm cellular anti-inflammatory activity of seed coat extracts with TNF- α , and it would be beneficial also to evaluate other key inflammatory markers, such as IL-1, IL-6, and IL-12. With this information, incorporation of coloured dry bean seed coats into foods as an ingredient could be possible.

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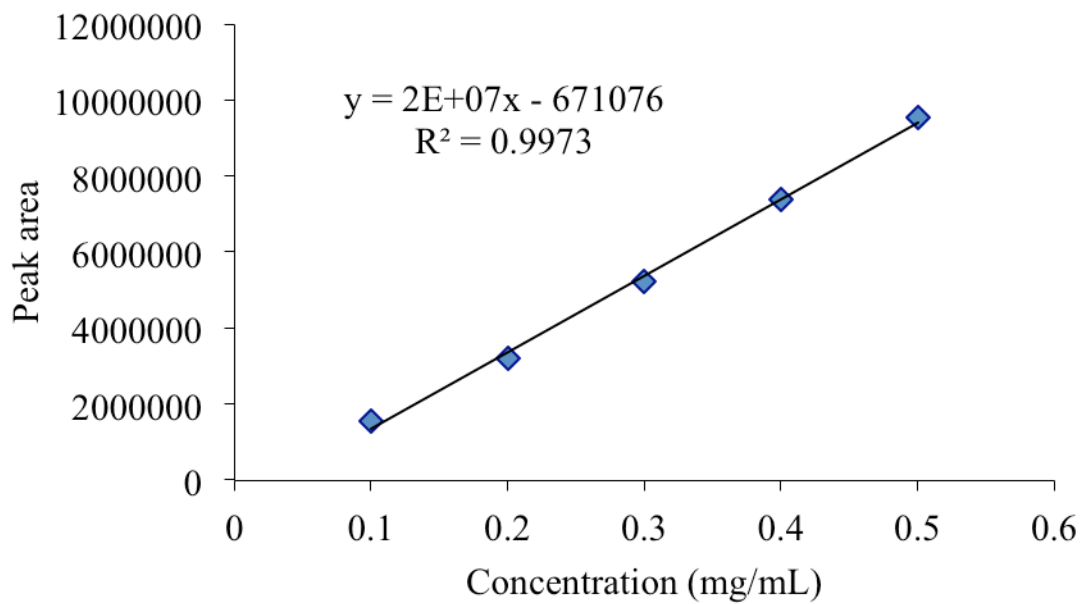
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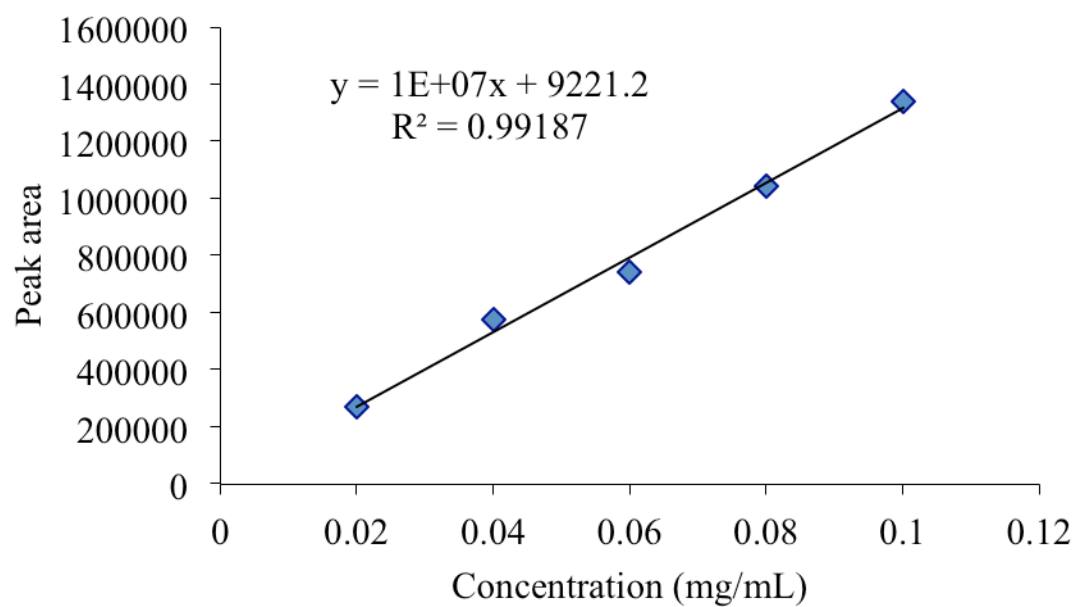
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Appendix A

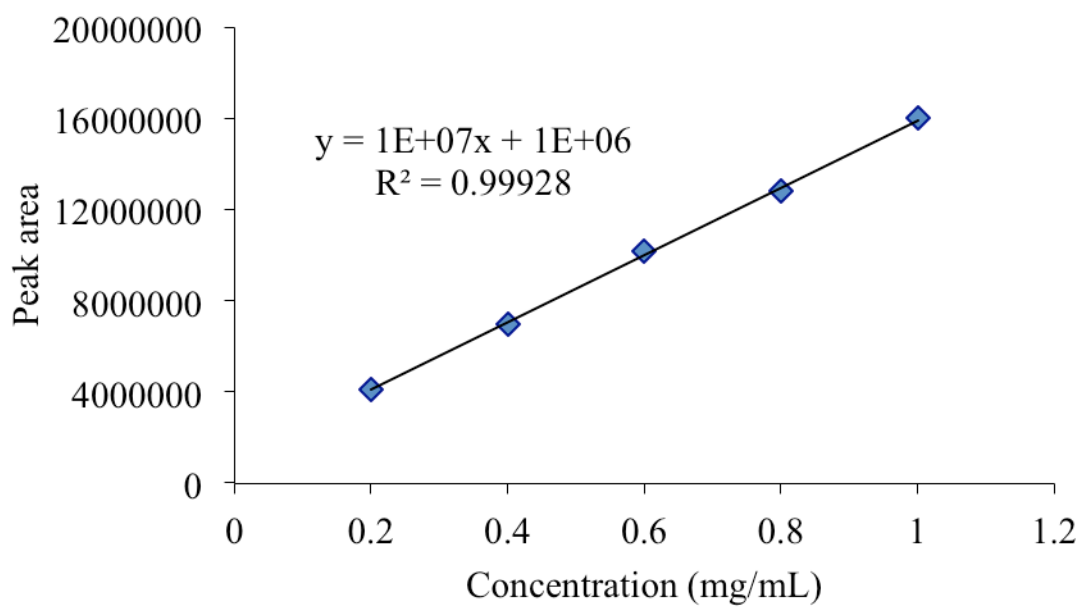
Calibration curves for quantification of standards in seed coat extracts



Calibration curve for the quantification of quercetin 3-*O*-glucoside in seed coat extracts by HPLC.



Calibration curve for the quantification of rutin in seed coat extracts by HPLC.



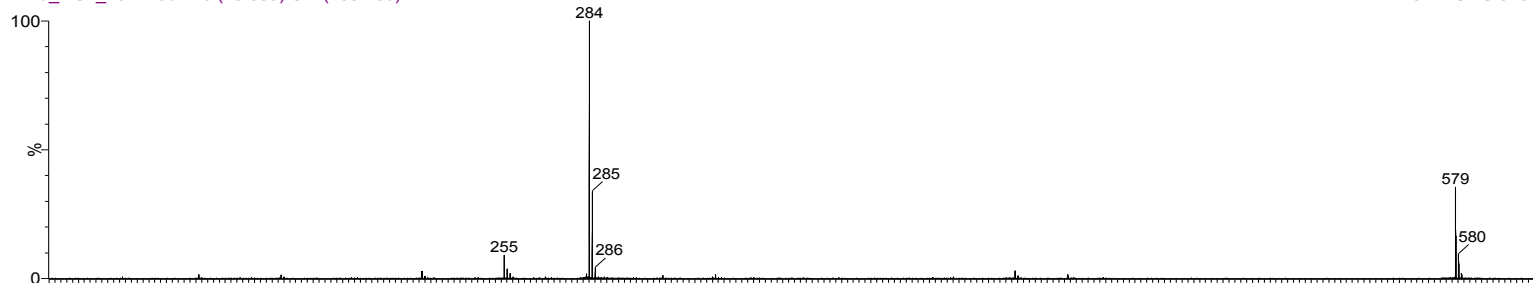
Calibration curve for the quantification of kaempferol 3-*O*-glucoside in seed coat extracts by HPLC.

Appendix B

Chromatograms of extracts showing MS² fragmentation patterns

Wind_MS2_102210b 220 (13.883) Cm (209:236)

2: TOF MSMS 579.00ES-
1.10e3



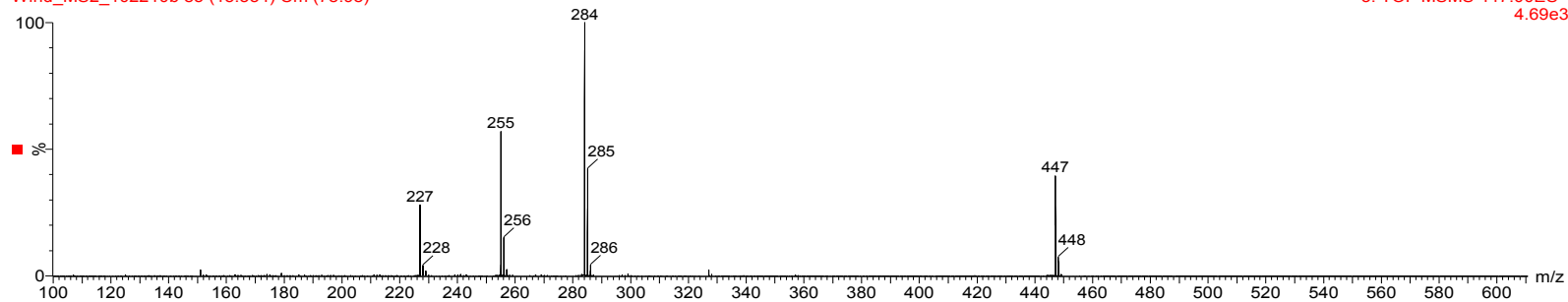
Wind_MS2_102210b 49 (23.906) Cm (36:66)

4: TOF MSMS 533.00ES-
2.90e3



Wind_MS2_102210b 85 (15.354) Cm (78:98)

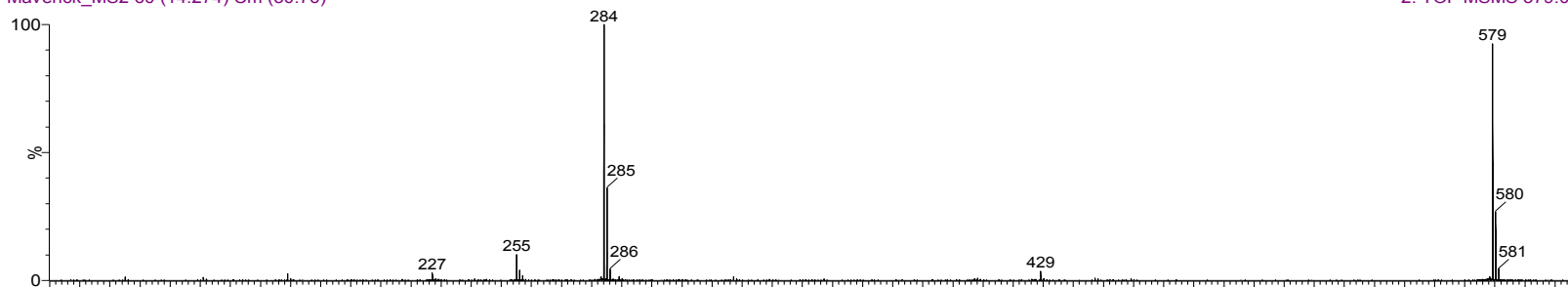
3: TOF MSMS 447.00ES-
4.69e3



MS² chromatograms acquired in negative ion mode for Windbreaker crude extracts of dry bean seed coat.

Maverick_MS2 60 (14.274) Cm (50:76)

2: TOF MSMS 579.00ES-626



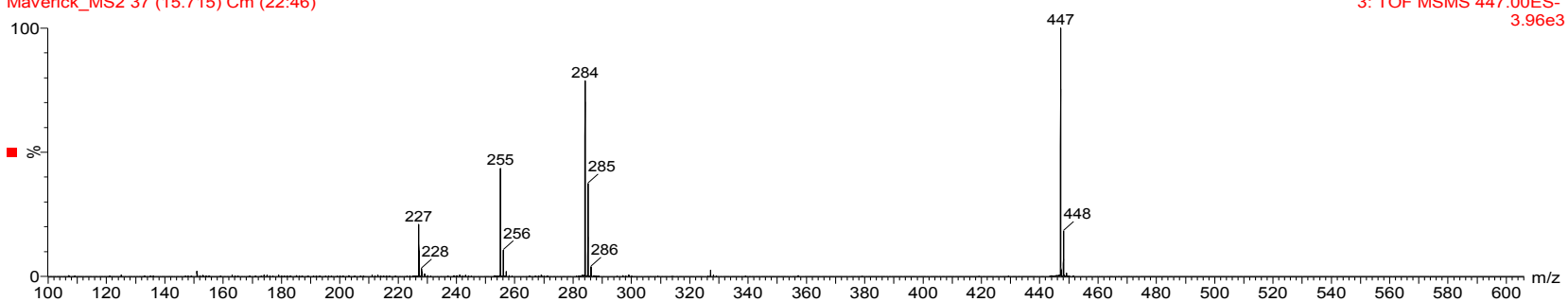
Maverick_MS2 34 (23.003) Cm (16:34)

4: TOF MSMS 489.00ES-707

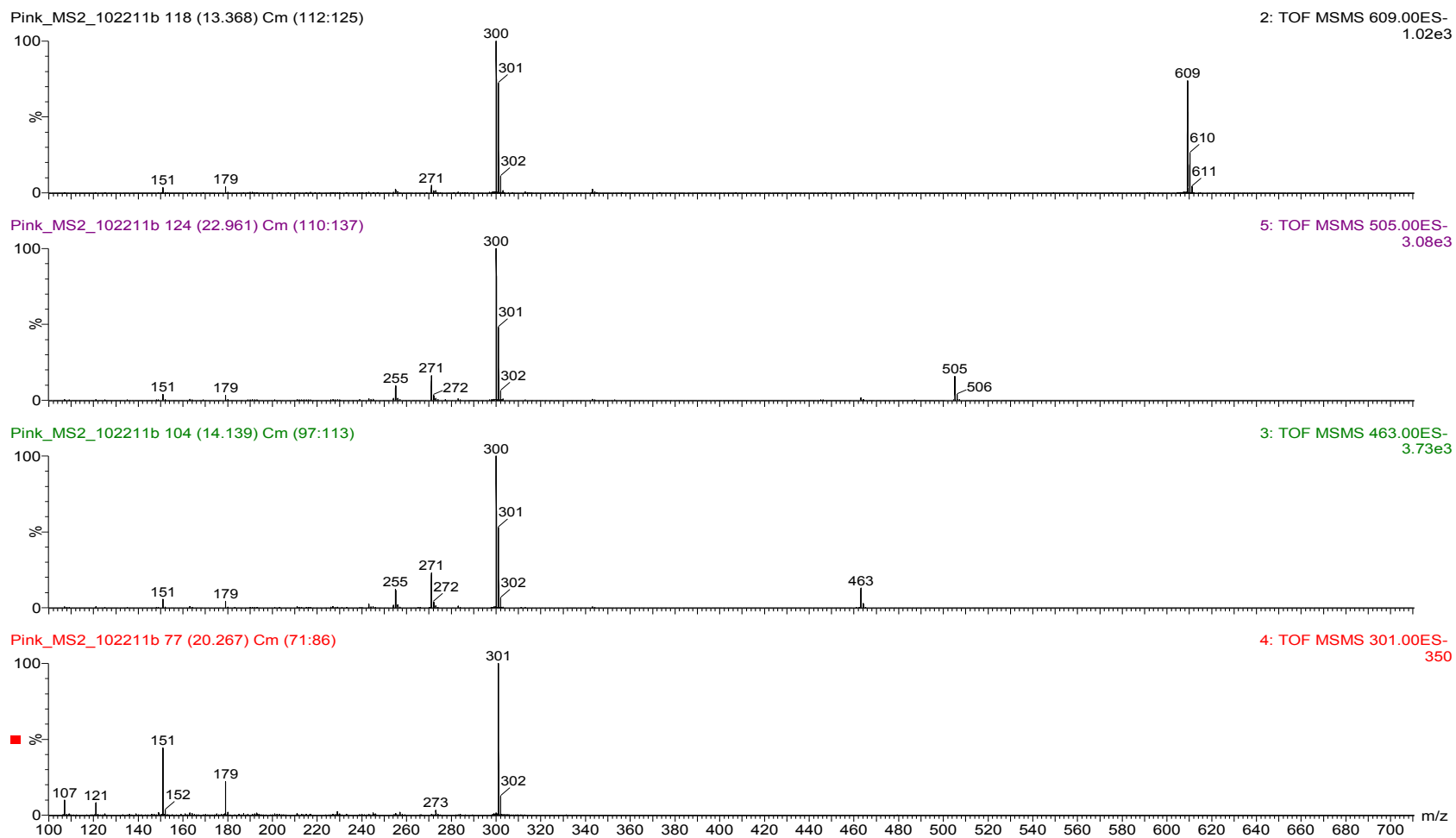


Maverick_MS2 37 (15.715) Cm (22:46)

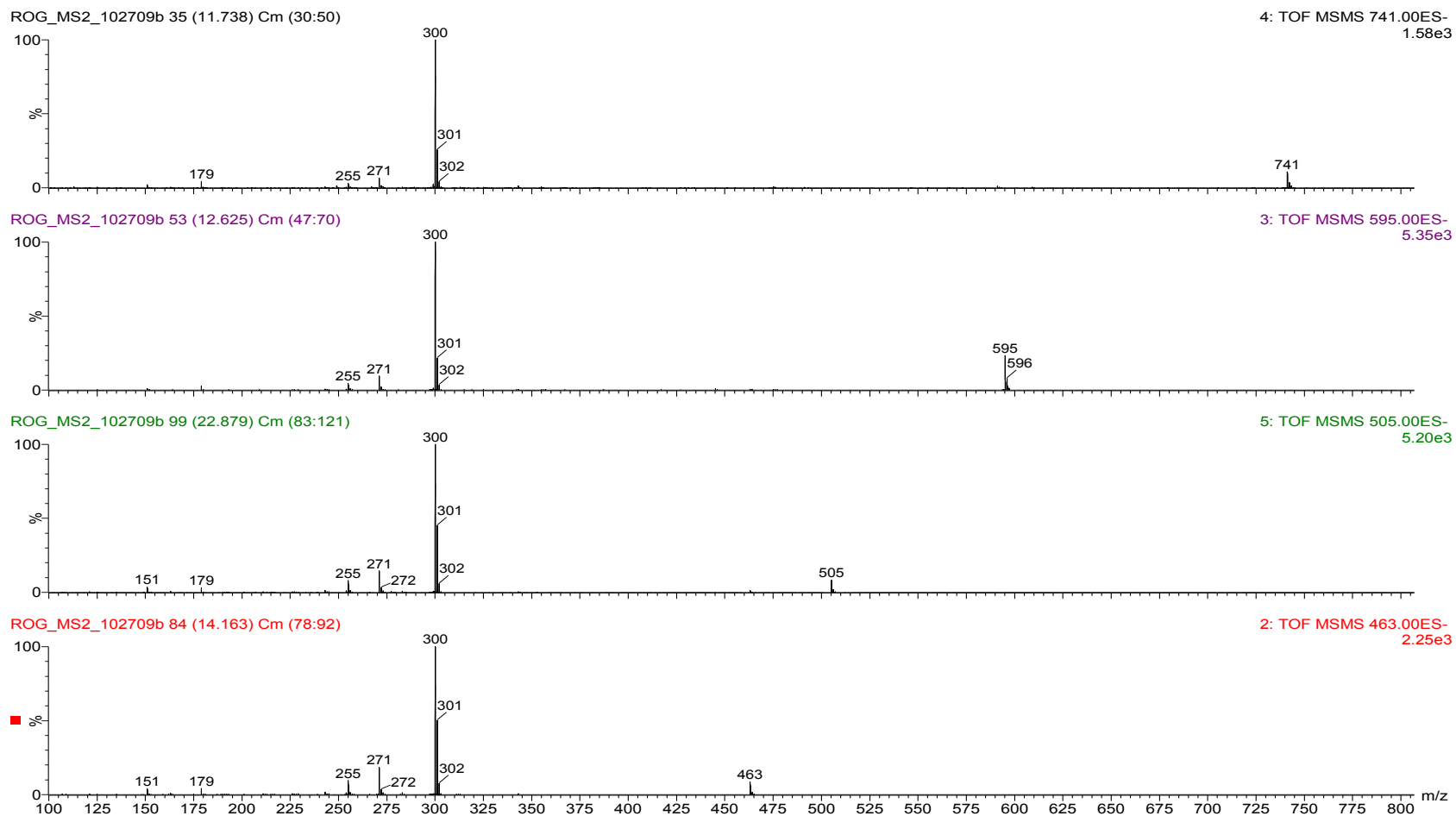
3: TOF MSMS 447.00ES-3.96e3



MS² chromatograms acquired in negative ion mode for Maverick/Buster crude extracts of dry bean seed coat.



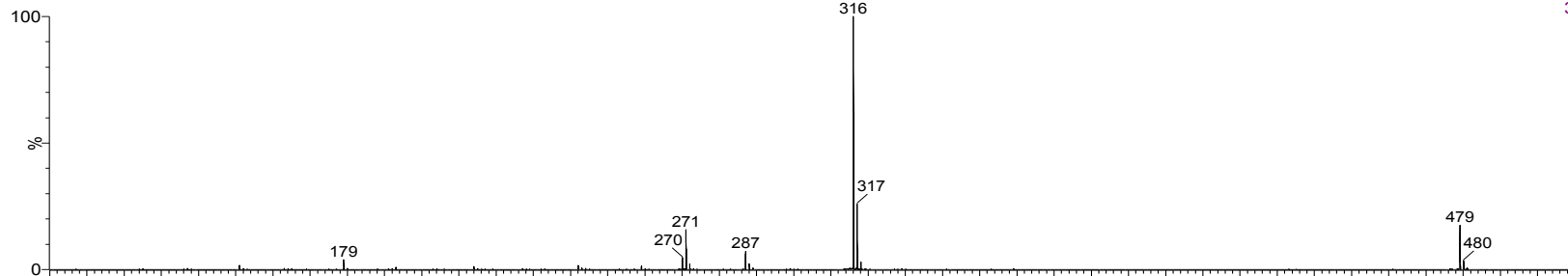
MS² chromatograms acquired in negative ion mode for Pink panther crude extracts of dry bean seed coat.



MS² chromatograms acquired in negative ion mode for ROG802/Montcalm/Redhawk crude extracts of dry bean seed coat.

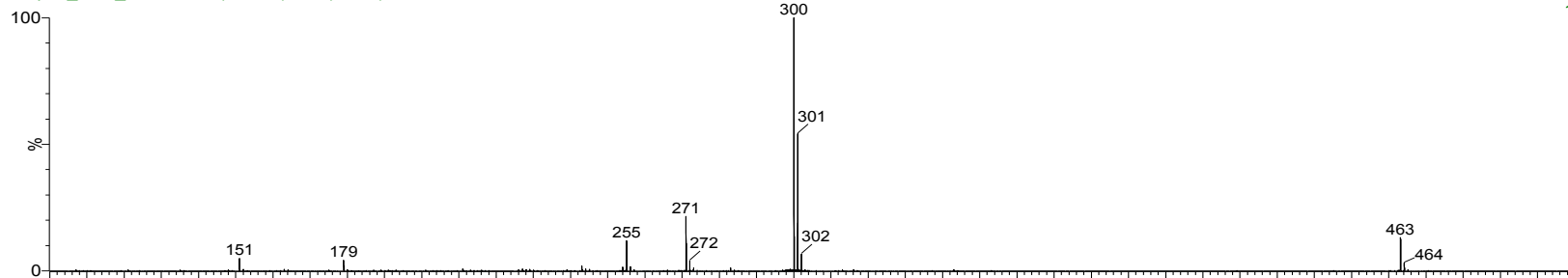
Eclipse_MS2_102209 36 (12.663) Cm (32:43)

5: TOF MSMS 479.00ES-
3.23e3



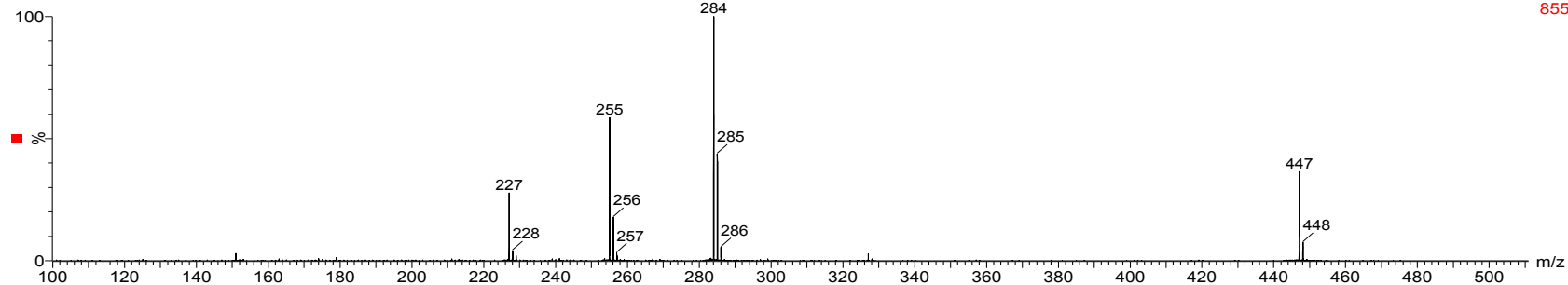
Eclipse_MS2_102209 57 (14.167) Cm (52:64)

6: TOF MSMS 463.00ES-
1.78e3

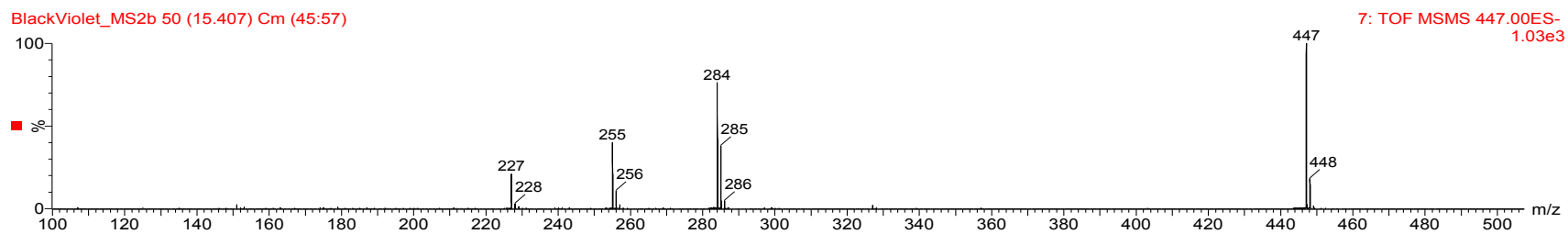
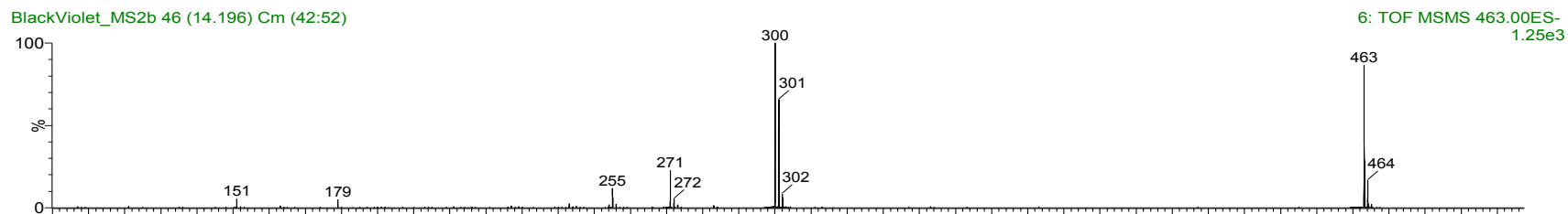
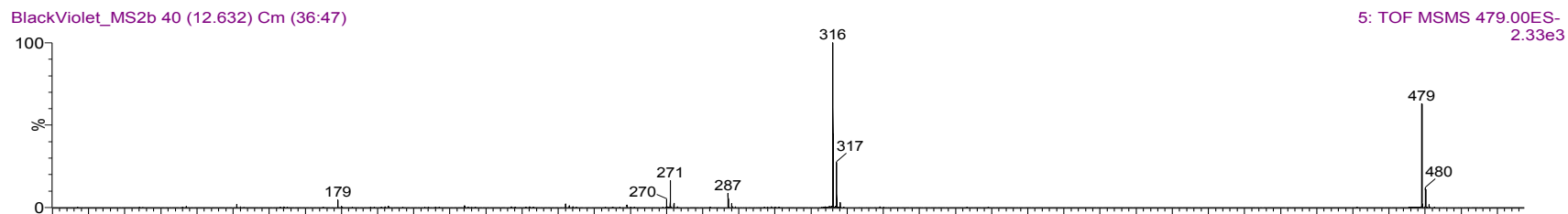
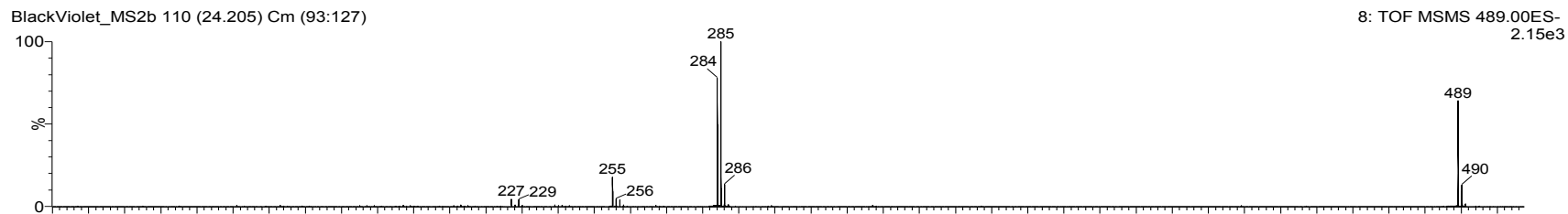


Eclipse_MS2_102209 61 (15.398) Cm (57:67)

7: TOF MSMS 447.00ES-
855



MS² chromatograms acquired in negative ion mode for Eclipse crude extracts of dry bean seed coat.



MS² chromatograms acquired in negative ion mode for Black violet crude extracts of dry bean seed coat.