This is to certify that the
dissertation entitled

BIOACTIVE CONSTITUENTS IN *AMELANCHIER* FRUITS

presented by

Devi Prasad Adhikari

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Horticulture

Major Professor's Signature

5/13/04

Date

BIOACTIVE CONSTITIUENTS IN *AMELANCHIER* FRUITS

By

Devi Prasad Adhikari

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Submitted to
Michigan State University
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ABSTRACT

BIOACTIVE CONSTITUENTS IN *AMELANCHIER* FRUITS

By

Devi Prasad Adhikari

Despite the long-standing use of *Amelanchier* fruits in juice, jelly and pies, very little work has been done on the isolation and characterization of bioactive compounds present in *Amelanchier arborea* and *Amelanchier canadensis* fruits. We have investigated bioactive compounds in Michigan grown *Amelanchier alnifolia*, *Amelanchier arborea* and *Amelanchier canadensis*. Bioassay-directed isolation and purification of the ethyl acetate extract of *A. canadensis* resulted in 1,3-dilinoleoyl 2-olein (**4**), 1,3-dioleoyl 2-linolein (**5**), and β-sitosterol (**6**). Similarly, the methanol extract of *A. canadensis* fruits yielded cyaninidn-3-*O*- β-galactopyranoside (**1**), cyaninidn-3-*O*-β-galactopyranoside (**2**), cyaninidn (**3**), 5-hydroxymethyl-2-furfural (**7**), 5-(sorbitoloxymethyl)-furan-2-carboxaldehyde (**8**), 5-(mannitoloxymethyl)-furan-2-carboxaldehyde (**9**), 5-(α-D-glucopyranosyloxymethyl)furan-2-carboxaldehyde (**10**), and 5-(β-D-glucopyranosyloxymethyl)furan-2-carboxaldehyde (**11**). The concentration of anthocyanins, **1** and **2**, in *A. alnifolia*, *A. arborea* and *A. canadensis* were 155 and 54, 390 and 0, and 165 and 48 mg in 100 g of fresh fruits, respectively. Compounds **7-11** were isolated for the first time from *A. canadensis* fruits. The structures of these compounds were determined by various spectral techniques such as ^{1}H- and ^{13}C-NMR DEPT, HMQC, HMBC, MS and chemical methods.

A. arborea fruits were sequentially extracted with hexane, ethyl acetate and methanol. The separation and purification of ethyl acetate extract of *A. arborea* fruits yielded β-sitosterol-3-glucoside (**12**), oleanolic acid (**13**), ursolic acid (**14**), kaempferol-3-*O*-α-L-rhamnopyranosyl (1←2)rhmnopyranoside (**15**) and kaempferol-3-*O*-α-L-rhamnopyranoside (**16**).

Anthocyanins **1-3** at 10 ppm exhibited 70, 75, 78 % of lipid peroxidation inhibitory activity, respectively. Compounds **8-11**, and 13-16 at 100 ppm exhibited 83, 26, 76, 85, and 83 % lipid peroxidation inhibitory activity, respectively. Commercial antioxidant butylated hydroxy toluene (BHT) at 2.2 ppm gave 87 % lipid peroxidation inhibitory activity. In cyclooxygenase enzyme inhibitory assay, compounds **1-3** at 100 ppm showed 50 and 18; 46 and 18; and 96 and 75 % COX-1 and COX-2 enzyme inhibitory activity, respectively. Non steroidal anti-inflammatory drugs (NSAIDs) aspirin, celebrex and vioxx at 180, 1.67 and 1.67 ppm showed 74 and 69; 5 and 82; and 0 and 85 % COX-1 and COX-2 inhibitory activity, respectively. Compound **4**, mixtures of compounds **8** and **9** and **10** and **11** at 100 ppm gave 39 and 0; 68 and 49; 63 and 45 % COX-1 and COX-2 inhibitory activity, respectively. Mixtures of compounds **13** and **14**, and compound **15** at 100 ppm showed 4 and 6; and 16 and 15 of COX-1 and COX-2 enzyme inhibitory activities, respectively.

Lipid peroxidation inhibitory and COX enzyme inhibitory activities of the compounds isolated from *Amelanchier* fruits showed that the fruits contained many bioactive compounds. These results suggest that consumptions of *Amelanchier* fruits might be beneficial to human health.

ACKNOWLEDGEMENTS

I am grateful and thankful to my major advisor Prof. Muraleedharan G. Nair for his supervision, continuous support, useful criticism, and the final shaping of this dissertation. The timely completion of this thesis would not have been possible without his support, mentoring, and patience.

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I thankfully acknowledge Tribhuvan University, Nepal for granting me the study leave during the course of this study.

I have been indebted by my parents, brothers and sisters for their love, compassion, motivation, moral support and blessings. Finally, I must convey deep love and gratitude to my wife Kalpana and our daughters Rashmi and Ranju for their endless support and understandings during the time-consuming nights absent from them, and for their sacrifices, self-reliance, back up, eagerness and moral support.

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LIST OF ABBREVIATIONS

BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
$CHCl_3$	Chloroform
COX	Cyclooxygenase
^{13}C NMR	Carbon nuclear magnetic resonance
DMSO	Dimethyl sulfoxide
d	Doublet
dd	Doublet of doublet
DPA-PA	3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid
DEPT	Distortionless enhancement polarization transfer
EIMS	Electron impact ionization mass spectroscopy
Et_2O	Diethyl ether
EtOH	Ethanol
EtOAc	Ethyl acetate
FABMS	Fast atom bombardment mass spectroscopy
FTIR	Fourier transfer infrared spectroscopy
GC	Gas chromatography
^1H NMR	Proton nuclear magnetic resonance
HMBC	Heteronuclear multiple bond coherence
HMQC	Heteronuclear correlation through multiple quantum coherence
HPLC	High pressure liquid chromatography
IR	Infrared
J	Coupling constant
LC	Lethal concentration Liquid chromatography
m	Multiplet
MeOH	Methanol
MIC	Minimum inhibitory concentration
MOP	3-[N-morpholino] propanesulfonic acid
MPLC	Medium pressure liquid chromatography
MS	Mass spectroscopy
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
NSAID	Non steroidal anti-inflammatory drugs
PDA	Photodiode array
PG	Prostaglandin
PGHS-1	Prostaglandin endoperoxide H synthase-1
PGHS-2	Prostaglandin endoperoxide H synthase-2
ppm	Parts per million
PTLC	Preparative thin layer chromatography
R_f	Reference Value
R_t	Retention time
s	Singlet
TBA	2-thiobrabituric acid

TBHQ	*tert*-butylhydroquinone
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV	Ultraviolet
δ	Chemical shifts

INTRODUCTION

The genus *Amelanchier* (Rosaceae), represented by about 25 species, is found in North America, Europe and Asia where it is better known as serviceberry, shad bush, or Juneberry. A few species are native to North America. The North American species of *Amelanchier* are known by many names such as serviceberry, Saskatoon berry, sarvis, maycherry, Juneberry, Junebush, sugarplum, shadblossom, shadwood, sugar pear, Indian pear, downy shadberry, and grape pear. *Amelanchier bartramiana*, *Amelanchier arborea, Amelanchier laevis, Amelanchier interior, Amelanchier spicata, Amelanchier sanguinea,* and *Amelanchier alnifolia* are grown in Michigan. Most of these *Amelanchier* spp are planted as ornamental landscape plants because of their very early flowering. Horticulturally, these genetically diverse *Amelanchier* spp. are grown in gardens, orchards, and shelterbelts.

The fruits of *A. alnifolia* Nutt, which are commonly called Saskatoon berries, and *A. arborea*, commonly called shadberry, have been a valuable food for the natives and early settlers of the North American prairies. The dried berries were used similar to raisins and prunes, as an ingredient of pemmican and also in the making of juice and jelly. Prior to the cultivation of *Amelanchier* spp., the consumption of its fruit was dependent on the wild source. In the last three decades, however, there has been interest in cultivating these fruit and, in western Canada, several orchards of Saskatoon berries have come into production.

The anthocyanins present in Saskatoon berries are important aesthetically and economically, because they are located in the skin and flesh of the fruit. The stability of these anthocyanins is of significance in the marketability of fresh berries and processed products such as jams, jellies, syrups and juices (Mazza G. 1986). Anthocyanins are also reported to possess strong anti-inflammatory (Seeram et al., 2002; Wang et al, 1999; Vlaskovska et al., 1990) and antioxidant activities (Seeram et al., 2002; Fukumoto et al., 2000; Wang et al, 1999; Costantino et al., 1992; Gabor, 1988).

Besides anthocyanins, *Amelanchier* spp. fruits are reported to contain other polyphenolics such as quercetin, rutin and kaemperol-3-glucoside (Mazza G. 1986, Matsuno et al., 1962). Such plant phenolics are multifunctional and can act as reducing agents, hydrogen-donating antioxidants, and singlet oxygen and free radical quenchers. Flavonoids and other plant phenolics are reported to have multiple biological activities, in addition to their free radical scavenging activities (Kinsella et al., 1993). These types of compounds were reported to have anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, anti-allergenic, antiviral and estrogenic effects (Middleton et al., 1992; 1981; 1984; Robark et al., 1988; Harborne, J. B. 2000).

Studies on *Amelanchier* spp. in the past were mainly focused on phenolic compounds (Sergeeva, et al., 1980), fatty acids and sterols (Zlatanob and Vazvazova, 1999). Fukumoto et al. (2000) detected a higher antioxidant activity in the methanolic extract of Saskatoon berries, which contained mainly anthocyanins, flavonoids and chlorogenic acid during the evaluation of several *Amelanchier* fruits. The antioxidant

activity of the extract was assessed by β-carotene bleaching, DPPH, and HPLC methods (Fukumoto et al., 2000).

Since no research was reported on Michigan grown *Amelanchier* spp., we have conducted a preliminary bioassay evaluation of these *Amelanchier* spp. fruit extracts. The aqueous, methanol and ethyl acetate extracts of *A. canadensis* and hexane, ethyl acetate and methanol extracts of *A. arborea* were evaluated for lipid peroxidation and anti-inflammatory activities in laboratory, the Bioactive Natural Products and Phytoceuticals Laboratory (BNPP) at Michigan State University. These preliminary assays showed that all crude extracts contained bioactive compounds. Based on published reports and preliminary studies carried out on crude extracts of two *Amelanchier* spp. prepared in the laboratory, it is hypothesized that *Amelanchier* spp. fruits contain antioxidant and anti-inflammatory compounds.

The objectives of this study are focused on the isolation, characterization, and determination of bioactivities of anthocyanins from *Amelanchier canadensis*, and quantification of anthocyanins in *A. alnifolia, A. arborea,* and *A. canadensis*. In addition, the study is focused on the bioassay-guided isolation, characterization and determination of bioactivities of the compounds isolated from *A. canadensis*, and *A. arborea.* To accomplish these objectives, various chromatographic techniques, thin layer chromatography (TLC), medium pressure liquid chromatography (MPLC), and high pressure liquid chromatography (HPLC) were used to isolate and purify the compounds. Also, spectroscopic methods such as nuclear magnetic resonance (NMR), ultraviolet

(UV), infrared (IR) spectroscopy, and mass spectrometry were used to characterize the isolated compounds. Antioxidant and anti-inflammatory assays were performed on extracts and compounds.

This dissertation comprises of five chapters. Each chapter, excluding the literature review and summary, is organized in a scientific journal paper format.

CHAPTER 1

LITERATURE REVIEW

This chapter reviews the breadth of literature on the botany, history, phytochemical investigation and biological activities of crude extracts and pure compounds from *Amelanchier* spp. The major classes of compounds reported from *Amelanchier* spp are anthocyanins, flavonoids, polyphenolic acids, sterols and triglycerides. Reports on the bioactivities of *Amelanchiier* fruits extracts are scanty. The only published reports are on antioxidant activity, antimicrobial and antiviral activities. Other miscellaneous articles related to phytochemistry and bioactivities are also presented.

A brief introduction to *Amelanchier spp*

The genus *Amelanchier* (Rosaceae), represented by about 25 species, is found in North America, in Europe and Asia where it is better known as serviceberry, shad bush, or Juneberry. Various species are adapted to every state in the US and province of Canada, but most fruit production occurs in Minnesota, and Canada. The *Amelanchier* spp. is closely related and often difficult to distinguish. Much of the taxonomic confusion is due to the extreme variability in foliage characteristics within any given species; leaf shape and size can differ significantly, depending on the stage of development of the plant and its habitat. The most useful characteristics to distinguish them are associated with the form and structure of the flowers and fruit.

Amelanchier is classified as follows:

Kingdom: *Plantae*, the Plants;

Division: *Magnoliophyta*, the Angiosperms;

Class: *Magnoliopsida*, the Dicotyledons;

Subclass: *Rosidae*, the Roses;

Order: *Rosales*, the Roses;

Family: *Rosaceae*, the Roses;

Genus: *Amelanchier*, the Serviceberries.

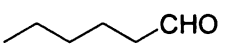
The *Amelanchier* fruits grown in Michigan, used in this study are *A. alnifolia, A. arborea* and *A. canadensis. Amelanchier alnifolia*, commonly called Saskatoon Serviceberry or Alder-leaved Serviceberry is a western North American species. It has a variable form mostly found as a multi-stemmed shrub ranging from 2-3 meters. *A. alnifolia* has been developed for commercial fruit production in the western United States and Canada with several named cultivars available in the nursery trade. Its fruits are approximately 1 cm. in diameter, bluish purple, and ripen in July (Berkheimer et al., 2001).

Amelanchier arborea, Downy Serviceberry, is an eastern North American large shrub or small tree reaching 8-10 meters. *A. arborea* is crossed with *A. laevis* to produce cultivars and used extensively in landscape industry in the United States. *A. arborea* fruits are purplish-black, slightly sweet, and ripen in late June.

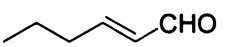
Amelanchier canadensis, Shadblow Serviceberry, is another eastern North American species used in landscape trade. *A. canadensis* is a stoloniferous shrub or small tree reaching 8 meters with similar fruit chararacteristics to *A. arborea*. The fruits of *Amelanchier* spp. are often processed into pies, jellies, jams, syrups and wine. The native Indians used *Amelanchier* berries to make pemmican, a staple food consisting of dried lean meat, fat, and dried berries.

PHYTOCHEMICAL STUDIES

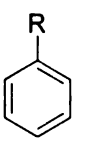
Sensory evaluation and GC/MS showed that the compounds of organoleptic importance to the aroma of the fruit of *A. arborea* were benzaldehyde, phenyl-acetaldehyde, 2-hexenal and hexanal (Parliament, T.H. 2001).



hexanal



2-hexenal

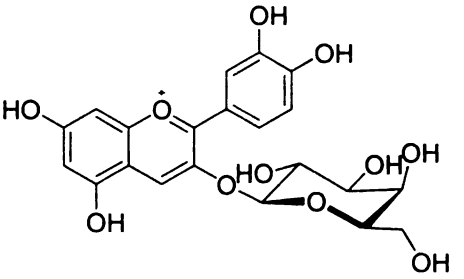


R = CHO; benzaldehyde

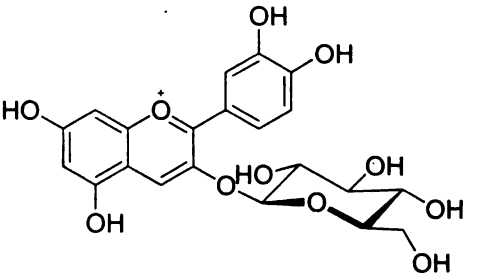
R = CH_2CHO; phenylacetaldehyde

Based on high performance liquid chromatography, paper chromatography and spectral analysis, ten compounds were identified including two major anthocyanins in *A. alnifolia*. The concentration of total anthocyanins in Saskatoon berries was 86-125 mg/100 g fresh berries. The main anthocyanins were cyanidin 3-galactoside (61%) and cyanidin 3-glucoside (21%) of the total anthocyanins reported. The other anthocyanins reported in *A. alnifolia* fruits were cyanidin 3-xyloside, malvidin 3-glucoside (Mazza, G. 1986), cyanidin 3, 5-diglucoside, pelargonidin, pelargonidin 3-glucoside

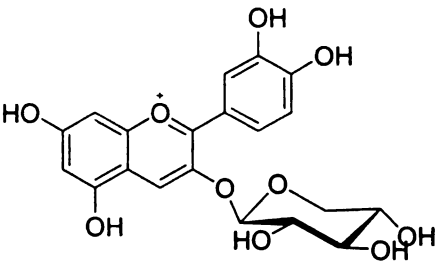
(Vereskovskii et.al., 1982). Flavonoids such as rutin, hyperoside, avicularin and quercetin (Vereskovskii et.al., 1982), chlorogenic, 3 and 5-feruloylquinic acid, and 5-*p*-coumaroylquinic acids (Sergeeva et al., 1980) were also reported in the fruits of *A. alnifolia*. The major aliphatic compounds of the epicuticular wax of Saskatoon berry were reported as alkanes of chain lengths of C23-C29, long chain esters (C36-C42), primary alcohols of C20-C30, and a secondary alcohol with a chain length of C29. The esters were composed of primary alcohols of chain length of C20-C28 and saturated fatty acids with C16-C24. However, the most abundant components in the wax were nonacosan-10-ol and nonacosane (Knowles et al., 1996).



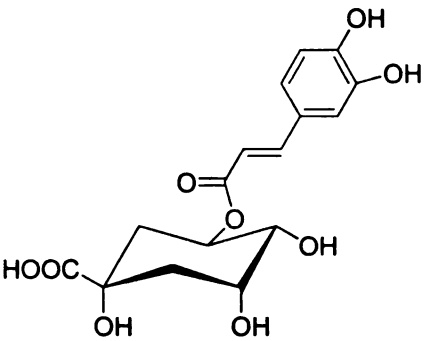
cyanidin 3-galactoside



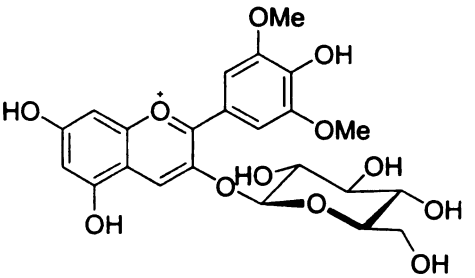
cyanidin 3-glucoside



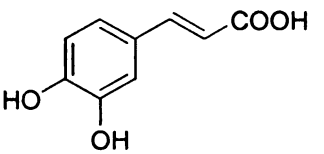
cyanidin-3-xyloside



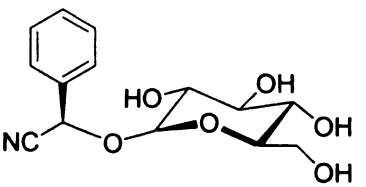
chlorogenic acid



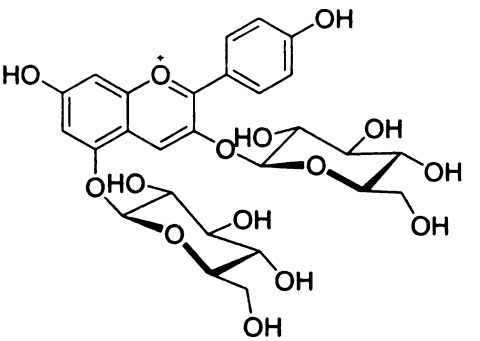
malvidin-3-glucoside



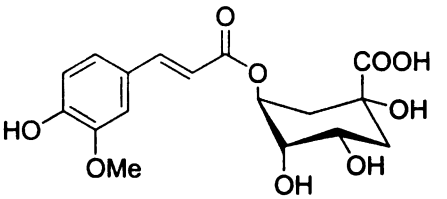
caffeic acid



prunasin

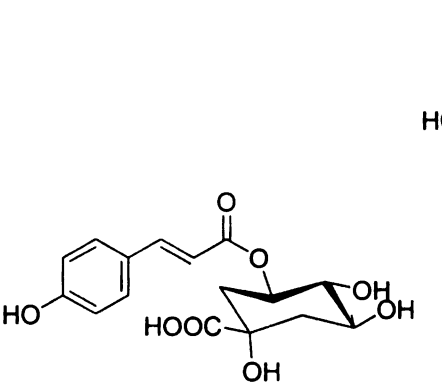


pelargonidin 3,5-diglucoside

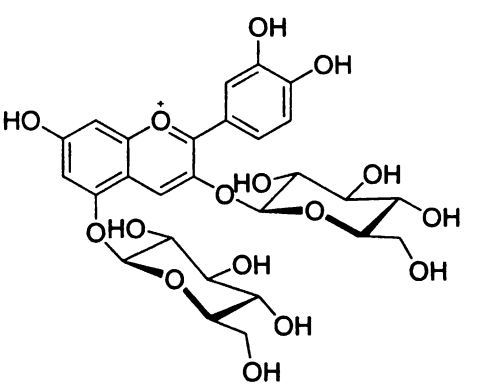


Feruloylquinic acid

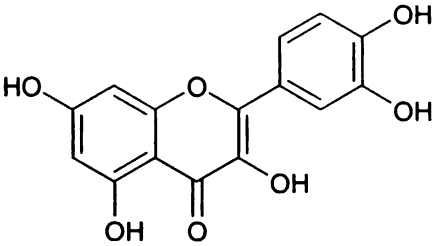
Prunasin was isolated and identified as the cyanogenic glycoside in leaves and twigs of *A. alnifolia* (Majak et al., 1978). The compounds responsible for the aroma of seven cultivars of Saskatoon berries were determined by gas chromatography-mass spectrometry, and colorimetry. The result indicated that benzaldehyde content was 26-168 mg/kg of fresh berries and accounted for 76-96% of the essence (Mazza et al., 1980, 1985). The predominant acid present in fully ripened Saskatoon berry was reported to be malic acid, while the major sugars were fructose and glucose (Wolfe et al., 1971).



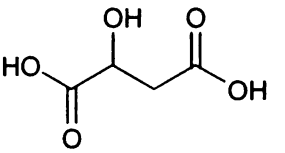
5-*p*-coumaroylquinic acid



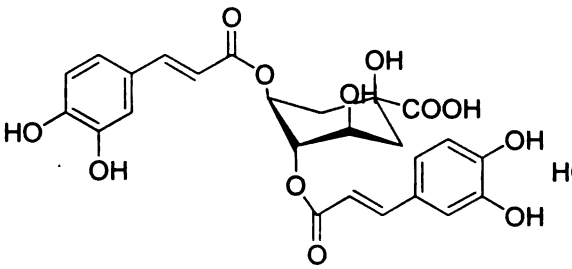
cyanidin 3,5-diglucoside



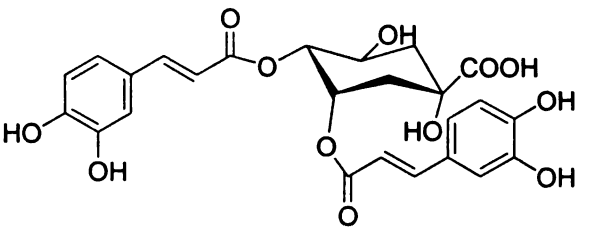
quercetin



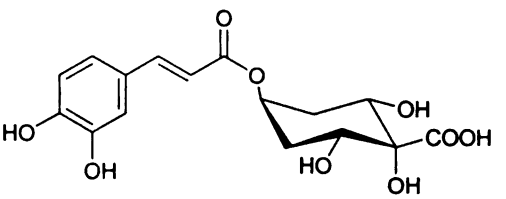
malic acid



isochlorogenic acid b



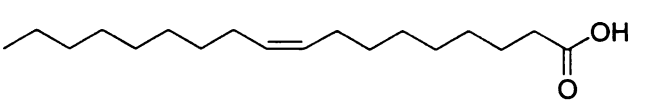
isochlorogenic acid c



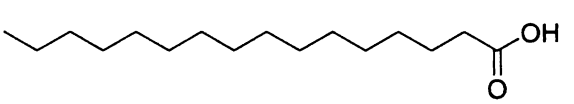
neochlorogenic acid

Panther and Wolfe reported that Saskatoon berries had a negligible ascorbic acid content. Also, ascorbic acid added to Saskatoon juice was rapidly degraded (Panther et al., 1972).

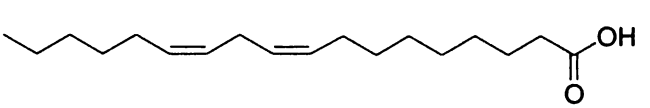
The earlier phytochemical investigation of the seeds of *A. canadensis* by GC/MS showed that it contained 7.2% of oil by weight. Oleic, linoleic and palmitic acids were the main fatty acid constituent in the triacylglycerides isolated. The content of phospholipids in the total triglyceride isolated was 2.8 % and the constituents were phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidic acids. Also, the sterol content was 0.9 % in the oil. β-sitosterol was the main component in addition to trace quantities of campesterol, cholesterol, stigmasterol, brassicasterol, Δ7-stigmasterol, Δ7-avenasterol and Δ5-avenasterol. The tocopherols present in the oil were also identified (Zlatanov et.al., 1999). In addition, caffeic, 3-and 5-feruloylquinic, chlorogenic, isochlorogenic acid c, and neochlorogenic acids were reported in the fruit of *A. canadensis* (Sergeeva et al., 1980).



Oleic acid



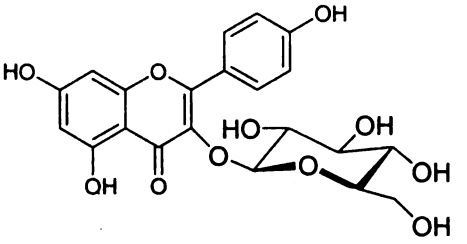
Palmitic acid



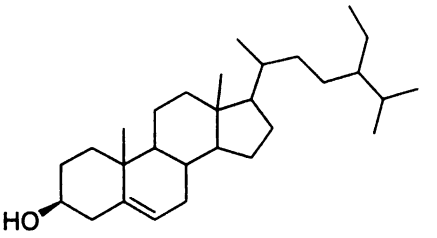
Linoleic acid

The anthocyanins and flavnoids reported from the fruits of *A. spicata* were cyanidin 3,5-diglucoside, cyanidin 3-glucoside, cyanidin 3-galactoside, pelargonidin, pelargonidin 3-glucoside, rutin, quercetin, quercetin 3-α-L-arabinofuranoside (avicularin), and quercetin 3-galactoside (hyperoside) (Vereskovskii et.al., 1982). Similarly, the phenolic acids isolated from the fruits were 3-feruloylquinic, a and b isochlorogenic acids. The compounds isolated from the fruits of *A. laevis* were 5-feruloylquinic, chlorogenic, and isochlorgenic a acids (Sergeeva et al., 1980).

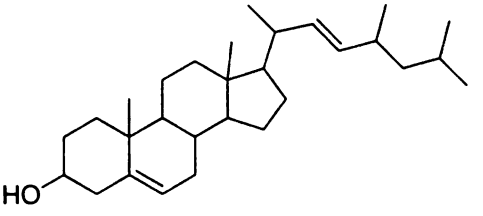
The water-soluble polysaccharides in the fruits of *A. vulgaris* contained galactose, glucose, arabinose, xylose, and rhamnose (Martynov, E. G.1981). Flower petals of *A. asiatica* contained astragalin (I) (Matsuno et al., 1962), and seed oil contained β-sitosterol and fatty acids of chain lengths of C16:0, C18:1, and C18:2 (Mitsuhashi et al., 1977). The major sugars isolated from the leaves of *A. ovalis* were D-galacturonic acid, D-galactose, D-glucose, L-arabinose, D-xylose, and L- rhamnose (Martynov et al., 1985). The anthocyanins and flavonoids reported from the fruits of *A. oligocarpa* and *A. sanguinea* were cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, pelargonidin 3-glucoside, cyanidin 3-glucoside, cyanidin 3-galactoside, rutin, quercetin, hyperoside, and avicularin (Vereskovskii et.al., 1982). Caffeic acid and chlorogenic acids were reported from the fruits of *A. oligocarpa* while *A. sanguinea* contained ferulic, 3-feruloylquinic, *p*-coumaric, and 3-*p*-coumaroylquinic, caffeic and chlorogenic acids (Sergeeva et al., 1979).



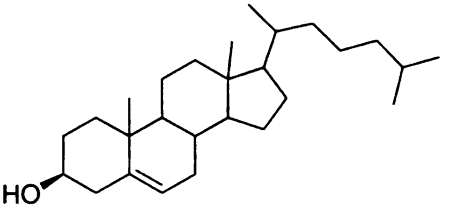
Astragalin (I)



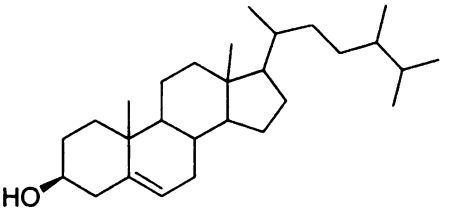
β-sitosterol



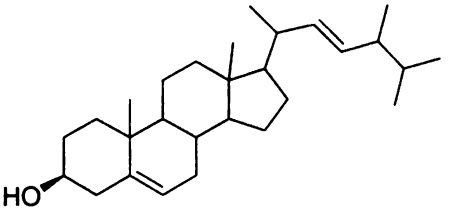
Stigmasterol



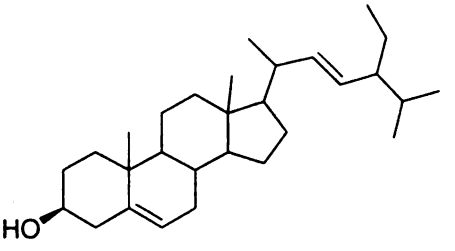
Cholesterol



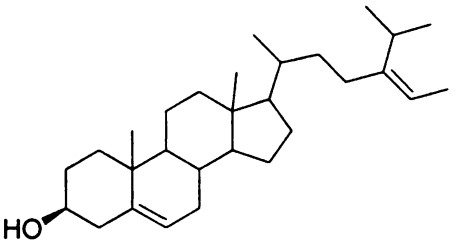
Campesterol

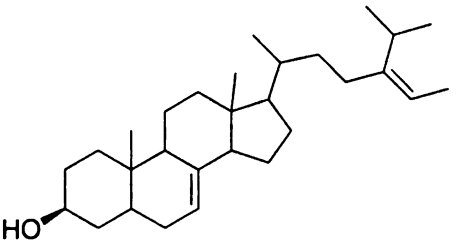


Brassicasterol



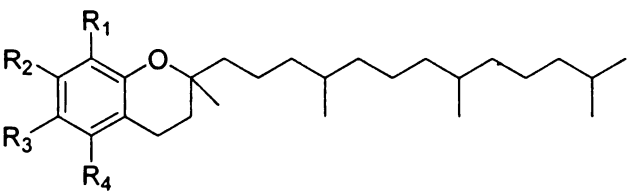
Δ7-Stigmasterol



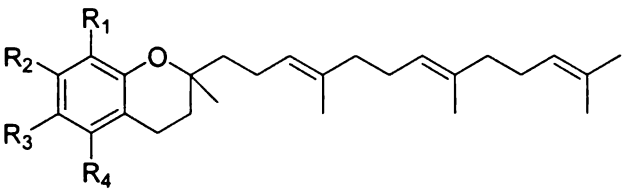


Δ5-Avenasterol

Δ7-Avenasterol



	R1	R2	R3	R4
α-Tocopherol	Me	OH	Me	Me
β-Tocopherol	Me	H	OH	Me
δ-Tocopherol	Me	H	OH	H



	R1	R2	R3	R4
α-Tocotrienol	Me	Me	OH	Me
β-Tocotrienol	Me	H	OH	Me

Biological activity of compounds isolated from *Amelanchier* species:

It is well known that diets rich in fruits and vegetables are correlated with decreasing risk of cardiovascular diseases and cancer (Heim et al., 2002; Proteggente et al., 2002; Block, 1992; 1994). These protective effects have been attributed, in large part, to the antioxidants, vitamin C and beta-carotene, but also to the minor constituents including carotenoids and phenolics such as flavonoids and phenyl propanoids (Heim et al., 2002; Proteggente et al., 2002; Block, 1992; 1994).

Most *Amelanchier* fruits contain anthocyanins, flavonoids and other phenolic acids which are important to human health. The flavonoids have long been considered to possess antiallergenic, anti-inflammatory, antiviral and anticarcinogenic activities. There are various reports of antioxidant activity of anthocyanins and anthocyanidins (Kähkönen et al., 2003; Matsumoto et al., 2002; Seeram et al., 2002; Fukumoto et al., 2000; Wang et al., 1999; Cao et al, 1997; Gabon, 1986).

Kähkönen et al (2003) evaluated the antioxidant activity of the six anthocyanidins, pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, and their glycosidic forms in three lipid-containing models [human low–density lipoprotein (LDL) and bulk oil and emulsified methyl linoleate] in addition to the radical scavenging activity of the compounds against the DPPH (2,2-diphenyl-1-picrylhydrazyl radical. Kähkönen et al (2003) observed that most anthocyanins studied and their aglycones acted as strong antioxidants in the emulsion and LDL, however, weak antioxidant activity or even prooxidant activity was observed in bulk methyl linoleate. They did not observe any correlation between glycosidation and antioxidant activity.

Matsumoto et al. (2002) measured the antioxidant activity of nine anthocyanins, glucosides of delphinidin, cyanidin, pelargonidin, malvidin, and peonidin; galactosides of cyanidin and malvidin, and rutinosides of delphinidin and cyanidin, at pH 7.0 using a chemiluminescence (CL) emission system with hydrogen peroxide–acetaldehyde system as free radical initiator. It was observed that the antioxidant activity was affected by the pH and glycosilation.

Fukumoto et al. (2000) determined antioxidant and prooxidant activities of several phenolic compounds including some anthocyanidins/anthocyanins by β-carotene bleaching method and by free radical DPPH method. Malonaldehyde production in a linoleic acid emulsion system was assayed by an HPLC method to determine antioxidant and prooxidant activities initiated by a metal catalyst (Cu^{2+}). They observed that most

phenolic compounds studied showed prooxidant activity at low concentrations unlike synthetic antioxidants BHA and BHT. Similarly, they observed that antioxidant activity was generally increased with increasing numbers of hydroxyl groups but decreased with glycosylation.

Wang et al.(1999) also observed the same trend of decreasing antioxidant activity with increasing numbers of glycosylation when antioxidant activities were determined by analysis of model lipid oxidation using fluorescence spectroscopy. Lipid peroxidation was initiated by the addition of $FeCl_2$. Gabon (1986) verified the antioxidant effects of anthocyanins based on a number of external factors such as pH, the ratio of L-ascorbic acid and oxygen, reaction time and the nature of organic acids present. Some of the other reported bioactivities performed on crude extracts of *Amelanchier* species were antiviral, antimicrobial and antioxidant.

Antioxidant Activity

The high antioxidant activity was observed in the methanolic extract of Saskatoon berries which contained mainly anthocyanins and chlorogenic acid. The antioxidant activity of the extract was assessed by β-carotene bleaching, DPPH and by HPLC methods (Fukumoto et al., 2000). In the HPLC method, antioxidant activity was characterized by a decrease in the quantity of malonaldehyde detected. Higher antioxidant activity was indicated by the lowest concentration of malonaldehyde. In Saskatoon berry, the concentration of total phenolics was 230 to 460 μM. The chlorogenic acid was used as a marker to determine the antioxidant activity of the extract.

BHA, cyanidin and cyanidin-3-glucoside showed similar antioxidant activity at 200-300 μM.

Nitric Oxide Inhibition

Flavonoids including anthocyanins have been reported to lower oxidative stress and possess beneficial effects on cardiovascular and chronic inflammatory diseases associated with nitric oxide (NO). Common phenolic compounds, including phenolic acids, flavonols, isoflavones, and anthocyanins were investigated for their effects on NO production in LPS/IFN-γ-activated RAW 264.7 macrophages. The anthocyanidins/ anthocyanins pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin-3-glucoside, and malvidin 3,5-diglucosides showed strong inhibitory effects on NO production at the range of 16- 500 μM range. But, methanol extracts of Saskatoon berries at 500 μg/ml had no effect on nitric oxide production in LPS/IFN-γ-activated raw 264.7 macrophages (Wang et al., 2002).

Amelanchier spp. has the potential to be introduced as a commercial fruit in Michigan. The phytochemical studies conducted on *Amelanchier spp.* indicate that anthocyanins are the major component in the fruit. Very little is known about the biological activities of the compounds present in the fruit. However, preliminary result on the juice, methanolic and ethyl acetate fractions of the crude extract of *A. canadensis* and hexane, ethyl acetate and methanol extracts of *A. arborea* showed antioxidant and anti-inflammatory activities. This confirmed the presence of several bioactive compound(s) in

the fruit. Therefore, it is rationalized that *Amelanchier* spp. have the potential to yield value-added bioactive compounds.

The objectives of this study are therefore (1) to isolate, characterize and determine antioxidant and anti-inflammatory activities of anthocyanins from *Amelanchier canadensis*, (2) quantify anthocyanins present in three *Amelanchier* spp. grown in Michigan and (3) isolate, characterize and determine antioxidant and anti-inflammatory activities of compounds in *A. canadensis*, and *A. arborea.* Antioxidant and Anti-inflammatory assays were used to direct the isolation of compounds from *Amelanchier* fruits. Isolation, characterization and determination of bioactivities (antioxidant and anti-inflammatory activities) of anthocyanins in *Amelanchier alnifolia*, *Amelanchier arborea* and *Amelanchier canadensis* are presented in **Chapter 2**. Isolation of bioactive compounds and determination of bioactivities (antioxidant and anti-inflammatory activities) of isolated compounds from *A. canadensis*, and *A. arborea* are presented in **Chapters 3** and **4**, respectively. Summary and conclusion are presented in **Chapter 5**. The proposed research is expected to add additional phytochemical data on *Amelanchier* spp. It is also expected that the results from this study could substantiate the use of *Amelanchier* berries as phytoceutical or nutraceutical supplements as well as their use in pies, jellies, jams, or wine. Also, the use of *Amelanchier* fruit should expand the horticultural market for *Amelanchier* spp. and may contribute to the economy of the tree growers in Michigan and the overall economy of the State of Michigan.

CHAPTER 2

BIOACTIVE ANTHOCYANINS IN THE FRUITS OF MICHIGAN GROWN *AMELANCHIER* SPP.

Abstract: Fruits of *Amelanchier alnifolia*, *Amelanchier arborea* and *Amelanchier canadensis* (Rosaceae) are consumed in North America. However, not much research was done on the fruits of *Amelanchier* spp. In this study, we have determined the levels of bioactive anthocyanins in the fruits of *Amenlanchier* species. HPLC analyses of fresh fruits indicated that all three species contained cyanidin 3-galactoside (**1**), but cyanidin 3-glucoside (**2**) was present only in *A. alnifolia* and *A. canadensis*. The anthocyanins were confirmed by LC-ESI/MS and NMR studies. The concentrations of anthocyanins **1** and **2** in 100 g of fresh fruits of *A. alnifolia*, *A. arborea,* and *A. canadensis* were 155 and 54; 390 and 0; 165 and 48 mg, respectively. Anthocyanin mixtures from *A. alnifolia, A. arborea,* and *A. canadensis* at 100 ppm inhibited cyclooxygenase (COX) -1 and -2 enzymes at 66 and 67%; 60 and 72% and 51 and 76%, respectively. The positive controls used in the COX assays were Aspirin, Celebrex and Vioxx at 180, 1.67 and 1.67 ppm, respectively, and showed 74 and 69%; 5 and 82%; 0 and 85% of COX-1 and COX-2 inhibition, respectively. Anthocyanins **1** and **2** and cyanidin (**3**) inhibited COX-1 enzyme by 50.5, 45.62 and 96.36 %, respectively, at 100 ppm whereas the COX-2 inhibition was the highest for cyanidin at 75%. In the lipid peroxidation inhibitory assay, anthocyanin mixtures at 10 ppm from *A. alnifolia*, *A. arborea,* and *A.*

canadensis showed activities of 72, 73, and 68 %, respectively, compared to 87 % for commercial antioxidant butylated hydroxytoluene (BHT) at 2.2 ppm. Compounds **1-3** at 10 ppm inhibited lipid peroxidation by 70, 75 and 78%, respectively.

INTRODUCTION

Anthocyanins are glycosides of anthocyanidins associated with attractive, colorful, and flavorful fruits. Recently, there has been a resurgence of interest in anthocyanins because of their potential health benefits as antioxidants and anti-inflammatory agents (Seeram et al., 2002). Anthocyanins have gained increased interest as compounds for coloring food, and as potent agents against oxidative stress. There is increasing epidemiological evidence that fruits and vegetable based diets are likely to improve the antioxidant status of human beings. A number of investigators have shown a high correlation between total anthocyanins, total phenolics, and the in vitro antioxidant activities of fruits and vegetable extracts (Stintzing et al., 2002). The beneficial effects of anthocyanins have resulted in the phytoceutical and botanical supplement industries investigating various fruits that have a high content of anthocyanins for purposes of formulating new commercial products. Some of the common sources of anthocyanins are bilberries, elderberries, chokeberries, and tart cherries. As an alternative source of anthocyanins, we have turned our attention to fruits of *Amelanchier* species.

The genus *Amelanchier* (Rosaceae) is represented by about 25 species, are found in North America, in parts of Europe and Asia. *Amelanchier alnifolia*, commonly called Saskatoon Serviceberry or Alder-leaved Serviceberry is a western North American species. It has a variable form mostly found as a multi-stemmed shrub ranging from 2-3 meters. *A. alnifolia* has been developed for commercial fruit production in the western

United States and Canada with several named cultivars available in the nursery trade. Its fruits are approximately 1 cm. in diameter, bluish purple, and ripen in July (Berkheimer et al., 2001). *Amelanchier arborea,* Downy Serviceberry, is an eastern North American large shrub or small tree reaching 8-10 meters. *A. arborea* is crossed with *A. laevis* to produce cultivars and is used extensively in the landscape industry in the United States. *A. arborea* fruits are purplish-black, slightly sweet, and ripen in late June. *A. canadensis,* Shadblow Serviceberry, is another eastern North American species used in landscape trade. *A. canadensis* is a stoloniferous shrub or small tree reaching 8 meters with similar fruit chararacteristics to *A. arborea.* The fruits of *Amelanchier* spp. are often processed into jellies, jams, syrups and wine. The native people and early settlers of the North American prairies used Saskatoon berry as a major food source but until recently it could be picked only in the wild. In the last two decades, however, there has been growing interest in industrial cultivation and utilization of this fruit in Canada and Michigan, USA. It is reported that berries of *A. alnifolia* contain cyanidin 3- galactoside and cyanidin 3- glucoside as major anthocyanins in addition to two other minor anthocyanins. However, the identification was based only by paper chromatography, spectrophotometric, and HPLC-DAD analyses (Mazza, 1986; Green and Mazza, 1986; Rogiers and Knowles, 1997; Sergeeva, et al., 1980). The literature survey shows that there are no reports on anthocyanins in *A. arborea* and *A. canadensis.* Also, phytochemical studies of *Amelanchier* spp., especially *A. arborea* (Parliament, 2001) and *A. canadensis* (Zlatanob and Vazvazova, 1990), were not carried out.

The objectives of this study were to isolate, characterize and quantify anthocyanins in three *Amelanchier* species grown in Michigan and determine their biological activities. This is the first report of the characterization of anthocyanins in *A. canadensis* and *A. arborea.*

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR 500 MHz spectrometer. ^{13}C NMR spectra was recorded at 125 MHz. Chemical shifts were recorded in CD_3OD/DCl, and the values are in δ (parts per million) relative to $CD_3OD/$ at 3.31 ppm for ^{1}H NMR and at 49.15 ppm for ^{13}C NMR. Coupling constants, J, are in hertz. All solvents were of ACS grade and were purchased from Spectrum Chemical Company.

Fruits. Fully ripened fruits of *Amelanchier* spp were collected in the middle of July 2001, in Eaton Rapids, Michigan. The locations of the trees are recorded in the Department of Horticulture Database at Michigan State University. The fruits were stored in plastic zip lock bags at –20° C till extraction and analyses.

Extraction of Anthocyanins for HPLC Analysis. Fresh fruits (10 g) were homogenized separately with methanol containing 1% HCl (150 mL) for 5 min in a Kinematica CH-6010 (Roxdale, ON, Canada) homogenizer and centrifuged (Model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10000 rpm for 20 min at 4° C. The extraction

process was repeated three times to make sure anthocyanins were completely extracted. The supernatant from each fruit sample was evaporated using a Buchi rotavapor at 35°C under vacuum to remove methanol, the residue was dissolved in water quantitatively to 20 mL and stored at –20° C till analyses.

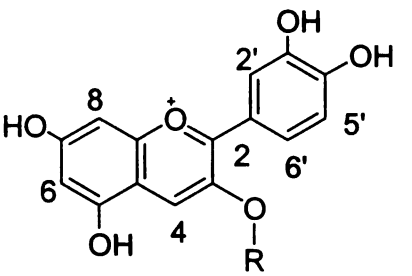
HPLC Quantification of Anthocyanins. All samples (50 μL injection volume) were filtered (0.22μm), and analyzed on an Xterra RP-18 column (250 x 4.6 mm, 5μm, Waters Corp. Milford, MA) with Novapak C-18 guard column at 35°C. The mobile phase was 0.5% aqueous H_3PO_4/ CH_3CN (9:1 v/v) used under isocratic conditions at a flow rate of 0.75 mL/min. Anthocyanins were detected at 520 nm using a PDA detector (Waters Corp., Milford, MA). Quantification of anthocyanins was carried out using Empower Pro (Waters Corp., Milford, MA) software. The solutions of anthocyanins **1** and **2** were prepared by dissolving 13 mg of **1** and 4 mg of **2**, separately, in 2 mL of distilled water. Aliquots of these solutions (1mL and 307μL, respectively, for **1** and **2**) were diluted separately to 2 mL each using 0.5% aqueous H_3PO_4/CH_3CN (9:1 v/v) to prepare a stock solutions containing 1 mg/mL and stored at –20° C till analyses. The stock solutions were diluted with 0.5% aqueous H_3PO_4/CH_3CN (9:1 v/v) to yield solutions containing 0.5, 0.25, 0.125, 0.05625, 0.007825, and 0.0039125 mg/mL, respectively. Each sample was injected in triplicate, and calibration curves were prepared by plotting the mean peak areas against concentration for each standard. The samples were injected in triplicate, and the mean areas of anthocyanins peaks were used to determine the quantities of anthocyanins **1** and **2** present in *Amelanchier* fruits.

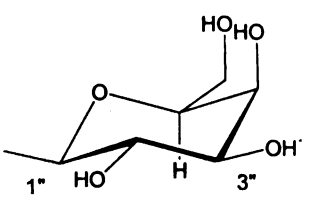
Anthocyanins 1 and 2 from *A. canadensis*. Anthocyanins were isolated from *A. canadensis* berries according to the previously published method (Seeram et al., 2002). Briefly, fresh berries (500 g) were blended with water (1L) in a Waring Blender for 5 min. The slurry was centrifuged (10000 rpm at 4°C) for 20 min. The resulting residue was extracted with acidic methanol (1% conc. HCl in MeOH, 3x1500 mL) for 48 h and the combined methanol extract was evaporated at 35°C to give a red powder (20 g). An aliquot of this crude extract (14 g) was dissolved in water and fractionated by XAD-16 (100 g) resin. XAD-16 column was prepared according to previously published procedure (Wang et al., 1997). The resin with adsorbed anthocyanins was washed with water (8L) till the washing was neutral. The adsorbed anthocyanins were then eluted with acidic MeOH (1% HCl in MeOH, 500 mL) and evaporated at 35°C to yield a red powder (1.4 g). This anthocyanin mixture (1.4 g) was then dissolved in water (2 mL) and fractionated by medium pressure liquid chromatography (MPLC) using a C-18 column (350x 40 mm). The column was eluted with MeOH containing 0.01 % of HCl/H_2O solvent system, under gradient conditions, starting with 30 % MeOH to 100 % MeOH. The fractions collected were I (79 mg, 50mL) and II (71 mg, 350 mL), eluted with 30% acidic methanol in water; fractions III (25mg, 100mL), IV (50 mg, 100mL), and V (32mg, 60mL), eluted with 40% acidic methanol in water and fractions VI (38 mg, 100 mL), VII (37 mg, 100 mL), VIII (64 mg, 150 mL), IX (65 mg, 50 mL), X (83 mg, 120 mL), XI (38 mg, 150 mL), XII (47 mg, 100 mL), and XIII (57 mg, 150 mL), eluted with 100% methanol. The HPLC analysis of these fractions showed that fractions III (25 mg) and IV (50 mg) contained anthocyanins **1** and **2**. Fractions III and IV were dissolved in 3.5 and 6.5 mL of acidic MeOH, respectively, and purified further by preparative HPLC.

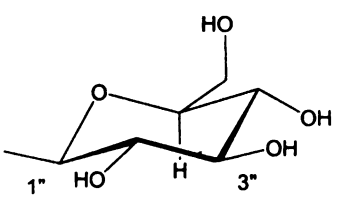
For HPLC purification, fractions III and IV (1 mL injection volume) were filtered (0.22μm) and purified by using an Xterra RP-18 column (250 x 19 mm, 5μm, Waters Corp.) at 35°C. The mobile phase was 4 % aqueous H_3PO_4/ CH_3CN (9:1 v/v) used under isocratic conditions at the flow rate of 4 mL/min. Anthocyanins were detected at 520 nm using a PDA detector (Waters Corp., Milford, MA). Anthocyanins **1** and **2** were eluted at 62 and 78 min respectively. The resulting solutions were passed through XAD-16 to remove phosphoric acid present in the mobile phase. The anthocyanins were then eluted with acidic methanol (1% conc. HCl), evaporated to remove methanol and the residue lyophilized to yield **1** (18 mg) and **2** (7.5 mg), respectively.

Compound **1**: ^{1}H NMR(CD_3OD/DCl): δ 8.98 (1H, s, H-4), 8.23 (1H, dd, J = 8.5, 2.0 Hz, H-6'), 8.04 (1H, d, J = 2.0 Hz, H-2'), 7.00 (1H, d, J = 8.5 Hz, H-5'), 6.90 (1H, d, J = 1.5 Hz, H-8), 6.65 (1H, d, J = 2.0 Hz, H-6), 5.29 (1H, d, J = 7.5 Hz, H-1"), 3.98 (1H, dd, J = 12.5, 3.5 Hz, H-6a"), 3.80 (1H, dd, J = 9.3, 9.0 Hz, H-3"), 3.71 (1H, dd, J = 9.0, 3.5 Hz, H-2"), 3.64 (1H, m, H-5"), 3.62 (1H, dd, J = 12.5, 1.5 Hz, H-6b"), 3.34 (1H, dd, J= 9.6, 9.0 Hz, H-4"); ^{13}C NMR (CD_3OD/DCl): δ 170.37 (C-7), 168.87 (C-2), 159.14 (C-5), 157.57 (C-9), 155.69 (C-4'), 147.28 (C-3'), 145.61 (C-3), 136.85 (C-4), 128.25 (C-6'), 121.18 (C-1'), 118.44 (C-2'), 117.47 (C-5'), 113.31 (C-10), 104.31 (C-6), 103.43 (C-1"), 95.20 (C-8), 77.68 (C-5"), 74.87 (C-3"), 72.07 (C-2"), 70.05 (C-4"), 62.74 (C-6"). ^{1}H NMR and ^{13}C NMR spectra of anthocyanin **1** confirmed it as Cyanidin 3-O-β-galactopyranoside and were consistent with the literature data (Seeram et al., 2002). The

LC-ES/MS gave the following major peaks at *m/z* (% intensity): 449 (M^+, 100), 287 (20, aglycone).







1 R = Galactose **2** R = Glucose **3** R= H

Figure 2.1. Anthocyanins (**1** and **2**) and cyanidin (**3**) present in *Amelanchier* spp.

Compound **2**: ^{1}H NMR (CD_3OD/DCl): δ 9.02 (1H, s, H-4), 8.26 (1H, dd, J = 8.5, 2.0 Hz, H-6'), 8.06 (1H, d, J = 2.0 Hz, H-2'), 7.03 (1H, d, J = 8.5 Hz, H-5'), 6.93 (1H, d, J= 1.5 Hz, H-8), 6.68 (1H, d, J = 1.0 Hz, H-6), 5.29 (1H, d, J= 7.5 Hz, H-1"), 3.89 (1H, dd, J = 10.9, 7.0, Hz, H-6a"), 3.77 (1H, d, J = 9.3, 9.0 Hz, H-3'), 3.71 (1H, dd, J= 9.0, 7.6 Hz, H-2"), 3.64 (1H, m, H-5"), 3.62 (1H, dd, J= 10.9, 1.5 Hz, H-6b"), 3.34 (1H, dd, J = 9.6, 9.0 Hz, H-4'). ^{1}H NMR spectra of anthocyanin **2** confirmed it as Cyanidin 3-O-β-

glucopyranoside and were consistent with the literature data (Wang et al., 1997a). The LC-ES/MS gave the major peaks at *m/z* (% intensity) at 449 (M^+, 100) and 287 (20, aglycone).

Anthocyanins from *Amelanchier* spp. for Bioassays. Fresh fruits of *A. alnifoia* (26.2 g), *A. arborea* (25.3 g) and *A. canadensis* (25.5 g) were homogenized separately with 1% HCl in methanol (3x150 mL) for 5 min using a Kinematica CH-6010 (Roxdale, ON, Canada) homogenizer and centrifuged (Model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10000 rpm for 20 min at 4° C. The extraction process was done three times to make sure that anthocyanins were completely extracted. The supernatant from each fruit sample was evaporated at 35°C under vacuum to give crude anthocyanin mixture. This crude anthocyanin mixture was further purified by XAD-16 (100 g) as described earlier. The yields of pure anthocyanin mixtures were 337, 414, and 314 mg, respectively, for *A. alnifoia*, *A. arborea* and *A. canadensis*.

LC-ESI/MS Analyses. HPLC-ESI/MS analyses were carried out on a MicroMass Quattro II LC-MS/MS system (MicroMass, Division of Waters Corp., Beverly, MA), equipped with a Waters 2690 HPLC pump and a Waters 996 PDA detector (Waters Corp., Milford, MA). Mass-Lynx v.3.4 software (MicroMass) was used for Data handling. The conditions were as follows: column, HP ODS Hypersil HPLC column, 125 mm x 4.0 i.d., 5μm (Agilent Technologies, Wilmington, DE); solvent A, 0.1% TFA/H_2O (v/v); solvent B, 50.4% H_2O/48.5% CH_3CN/1.0% CH_3COOH/1.0%TFA (v/v/v/v); gradient, % B initial (20%); 26 min (60%), 30 min (20%), 35 min (20%); run

time, 35 min; flow rate, 0.80 mL/min; injection volume10 μL (post column split 10:1); column temperature, 30°C; PDA range, 200-799 nm, 500 nm as detection wavelength. MS parameters were as follows: ionization mode, ES+, scan range, 200 – 1000 amu; scan rate, 1 scan/s; cone voltage, 20 eV. Peak identities were obtained by matching their molecular ions (M^+) obtained by ES/MS with the expected theoretical molecular weight from literature data (Chandra, et al., 2001). References standards of commercially available anthocyanins were used (independently and co-injected with test samples) for substantiating identities.

Cyclooxygenase (COX) Enzyme Inhibitory Assay. COX-1 enzyme inhibitory assay was conducted with an enzyme preparation from ram seminal vesicles. The COX-2 enzyme activity was determined by using the enzyme preparation from HPGHS-2 cloned insect cell lysate. COX assays were measured at 37°C and at pH 7.0 according to the published procedure (Wang et al., 1999). Positive controls used in this assay were Aspirin, Vioxx and Celebrex and were at 180, 1.67 and 1.67 ppm, respectively.

Lipid Peroxidation Inhibitory Assay. Antioxidant activities were determined by analysis of model lipid oxidation using fluorescence spectroscopy according to previously published procedure (Wang et al., 1999). Lipid peroxidation was initiated by the addition of $FeCl_2$. BHT was used as positive controls at 2.2 ppm. Compounds **1-3** were tested at 10 ppm. Fluorescence was measured at 384 nm and monitored at 0, 1, 3, and every 3 min up to 21 min using a Turner model 450 digital fluorimeter. The decrease of relative fluorescence intensity with time indicated the rate of peroxidation and data are

reported for 21 min after the initiation of peroxidation. Relative fluorescence (*Ft/Fo*) was calculated by dividing the fluorescence value at a given time (*Ft*) by that time at t = 0 min. (*Fo*).

RESULTS AND DISCUSSION

HPLC separations of anthocyanins in *Amelanchier* species studied were carried out under isocratic conditions utilizing 4% aqueous H_3PO_4/CH_3CN as the mobile phase. The HPLC profiles of anthocyanins in *A. alnifolia, A. arborea* and *A. canadensis* were similar (Figures 2.2a-c) except that the second peak (retention time, 16.8 min) for anthocyanin **2** (Figure 2.1) was not present in *A. arborea* (Figure 2.2b). The third peak (retention time 19.9 min) was present in all three species and the concentration was less than 1%. We did not isolate this since it was confirmed as the aglycone, cyanidin, by comparison with an authentic sample of cyanidin (Figure 2.2a-c). The anthocyanins present in the fruits of these three *Amelanchier* species were different in concentration and hence were quantified by HPLC.

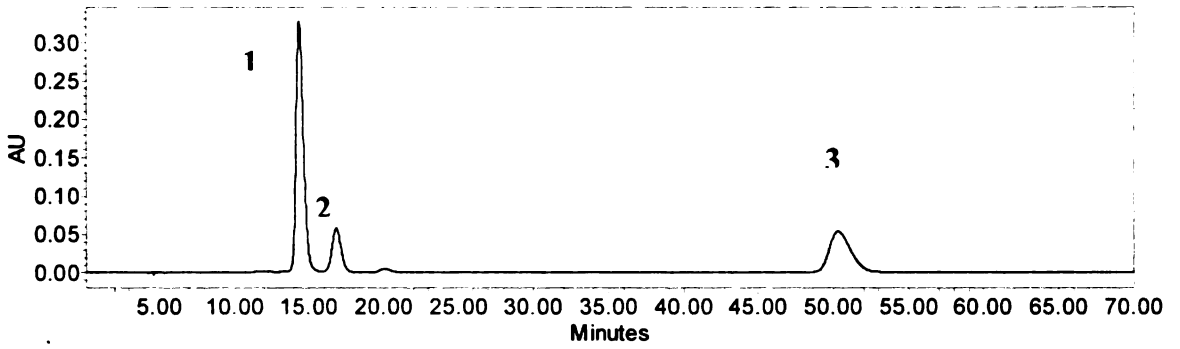


Figure 2.2a

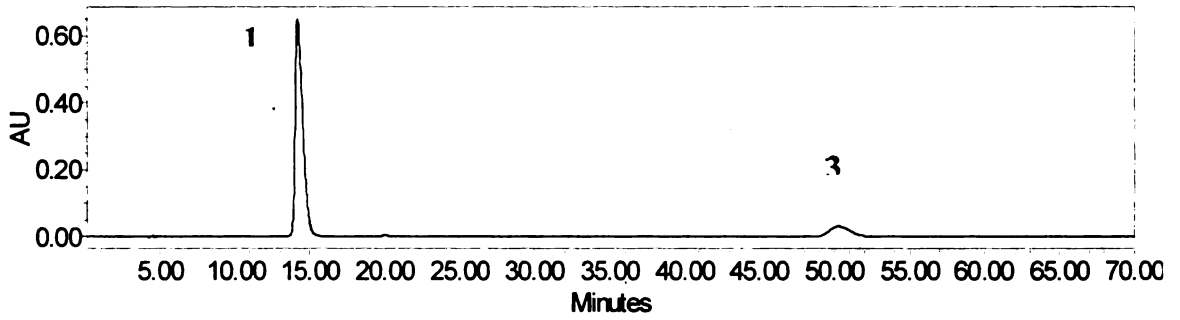


Figure 2.2b

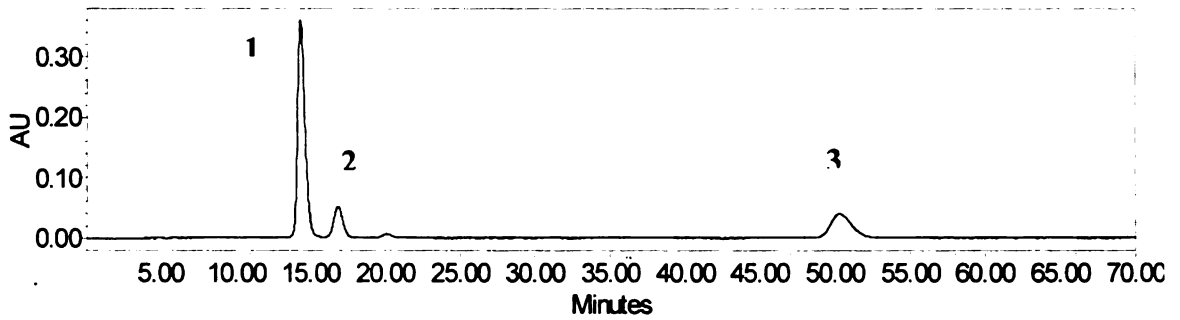


Figure 2.2c

Figure 2.2. HPLC analyses of fresh berries of *A. alnifolia*, *A. arborea* and *A. canadensis*. 2.2**a**. cyanidin 3-galactoside (peak 1, R_t=14.5), cyanidin 3-glucoside (peak 2, R_t=16.8) and cyanidin (peak 3, R_t=50.5) in *A. alnifolia*, **2.2b**. cyanidin 3-galactoside (peak 1, R_t=14.5) and cyanidin (peak 3, R_t=50.5) in *A. arborea* and **2.2c**. cyanidin 3-galactoside (peak 1, R_t=14.5), cyanidin 3-glucoside (peak 2, R_t=16.8) and cyanidin (peak 3, R_t=50.5) in *A. canadensis*.

Anthocyanins **1** and **2** (Figure 2.1) were isolated and purified from *A. canadensis* according to the published report (Seeram et al., 2002). Anthocyanin **1** was identified as cyanidin 3- O-β-galactopyranoside based on its retention time, UV- absorbance from HPLC-PDA detector and NMR data. Figure 2.2a shows the HPLC chromatogram of cyanidin 3-O-β-galactopyranoside at 14.4 min and its aglycone at 50.2 min. The NMR data of **1** was in agreement with the literature values for cyanidin 3-O-β-galactopyranoside (Seeram et al., 2002). The LC-ESI/MS spectral data (Figure 2.3a, peak 1) confirmed its molecular ion at *m/z* 449 (Figure 2.3b). Similarly, the structure of anthocyanin **2** was established as cyanidin 3- O-β-glucopyranoside based on ^{1}H NMR and HPLC analyses. The HPLC retention time of cyanidin 3- O-β-glucopyranoside was 16.8 min (Figure 2.2c). The LC-ESI/MS spectrum of **2** (peak 2, Figure 2.3a) gave a molecular ion at *m/z* 449 and it corresponded to a cyanidin hexose (Figure 2.3d). The peak at *m/z* 287 indicated the presence of a cyanidin aglycone in all *Amelanchier* fruit samples (Figure 2.3c) and accounted as hydrolysis products of **1** and **2** due to glycosidase enzyme activity.

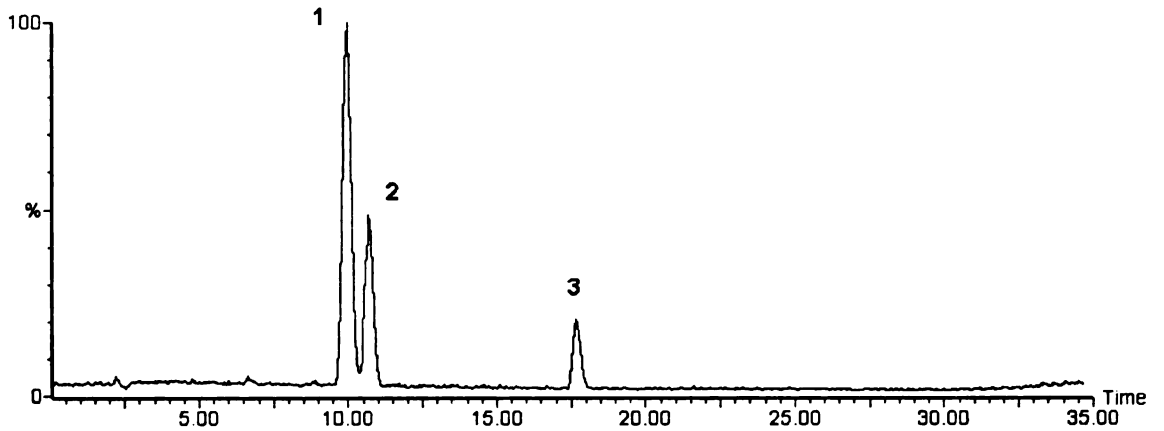


Figure 2.3a

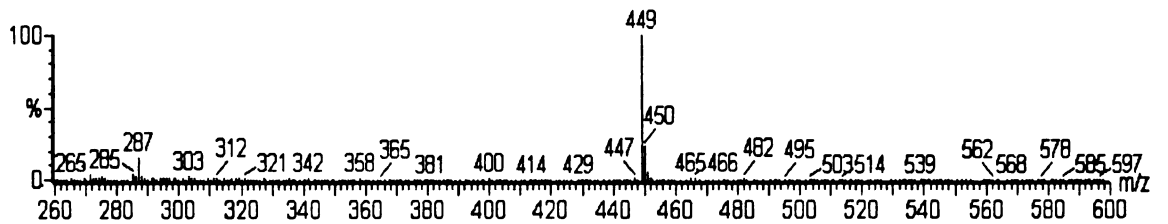


Figure 2.3b

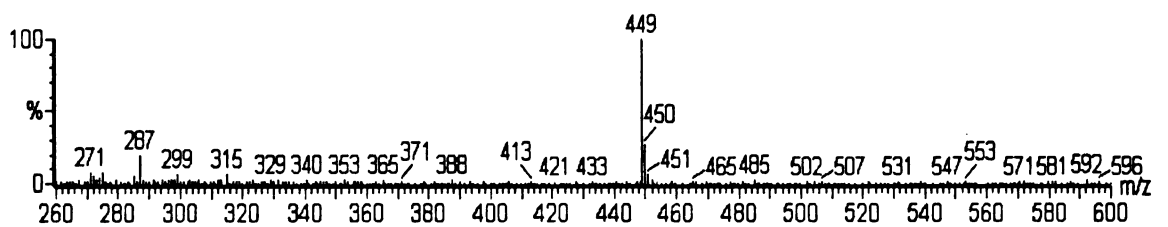


Figure 2.3c

Figure 2.3 3a. LC-ESI/MS total ion chromatogram (TIC) obtained for partially purified acidic methanol extract of *A. canadensis* fruits by XAD-16 and C18 MPLC. Peaks 1, 2 and 3 represent cyanidin galactoside, cyanidin glucoside and cyanidin, respectively. **3b-c**. Molecular ions obtained for peaks 1 and 2 respectively, and confirm these peaks as cyanidin galactoside, cyanidin glucoside respectively.

Quantitative analysis of anthocyanins in *A. alnifolia, A. arborea* and *A. canadensis* fruits was carried out by using calibration curves generated from standard solutions prepared from pure anthocyanins. The standards used for the calibration curve were pure compounds **1** and **2** isolated from the methanol fraction of *A. candensis*. The calibration curves for **1** and **2** were generated separately by plotting mean areas of HPLC peaks against concentrations ranging from 0.0039125 to 0.5 mg/mL (correlation coefficient, $R^2 = 0.99$) (Figure 2.4). The concentrations of anthocyanins **1** and **2** in 100 g of fresh berries of *A. alnifolia, A. arborea* and *A. canadensis* were found to be 155 and 54; 390 and 0 mg and 165 and 48, respectively.

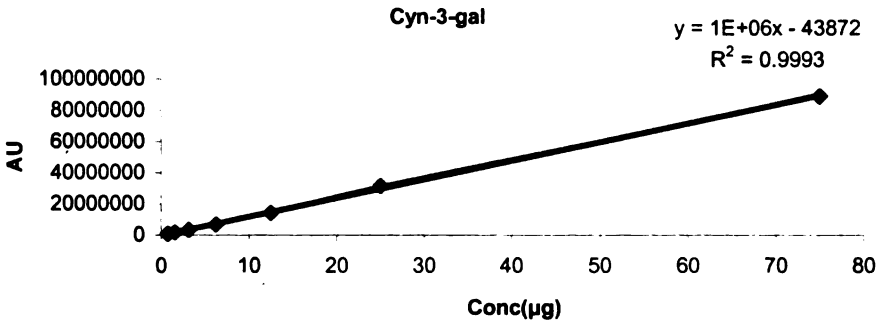


Figure 2.4a. Calibration curve for cyanidin-3-galactoside (**1**)

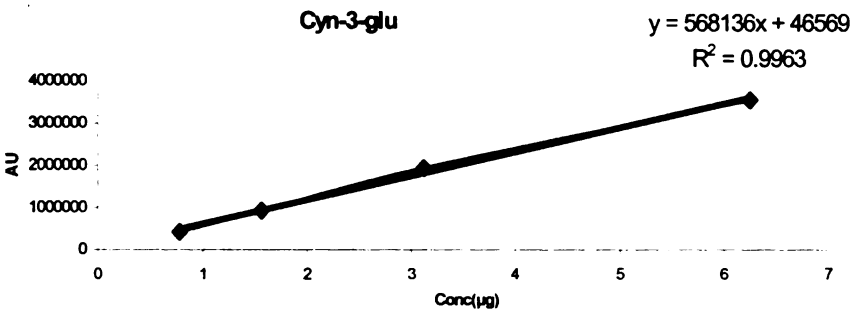


Figure 2.4b. Calibration curve for cyanidin-3-glucoside (**2**)

Our report on cyanidin 3-O-β-galactopyranoside (**1**) and cyanidin 3-O-β-glucopyranoside (**2**) in the Michigan grown *Amelanchier* fruits, based on HPLC, NMR and LC-MS results, are similar to the anthocyanin profiles in Canadian *Amelanchier* fruits (Mazza et al., 1986). However, the concentration of anthocyanins present in *A. alnifolia* in Michigan species was much higher than in the Canadian *Amelanchier* fruits. It was reported (Mazza et al., 1986) that the anthocyanins present in Saskatoon berries

were 86-125 mg/100 g of fresh fruits with a ratio of cyanidin 3-galactoside to cyanidin 3-glucoside as 61:21 based on the area under HPLC peaks detected at 280 nm. Our results indicate that anthocyanins **1** and **2** accounted for about 99 % of total anthocyanins in *A. alnifolia* and the ratio was found to be the same. The concentration of total anthocyanins in *A. alnifolia* was 209 mg/100g fresh fruits. This variation may be due to changes in environmental and nutritional factors under which the plants were grown. *A. arborea* fruits contained mainly anthocyanin **1** and it was about twice the amount detected in *A. alnifolia* and *A. canadensis*.

The anthocyanins, cyanidin 3-glucoside and cyanidin 3-galactoside, were shown to be good antioxidants based on ORAC assay (Wang et al., 1997b) and lipid peroxidation assay (Wang et al., 1999; Seeram et al., 2002). Therefore, the anthocyanin mixtures obtained from *Amelanchier* species were tested for lipid peroxidation inhibitory activities. Anthocyanin mixture containing **1** and **2** was tested for antioxidant activity at 10 ppm using an iron-catalyzed liposomal model system using fluorescence spectroscopy to monitor the inhibition of lipid peroxidation (Wang et al., 1999). The liposome used in this assay mimics the living cell membrane. At test concentrations, antioxidant activities observed were 72, 73, and 68 % for *A. alnifolia*, *A. arborea,* and *A. canadensis*, respectively (Figure 3). Pure anthocyanins **1** and **2** and cyanidin **3**, at 10 ppm, inhibited lipid peroxidation at 70, 75 and 78%, respectively (Figure 2.5). The commercial antioxidant BHT gave 87% inhibition at 2.2 ppm, respectively.

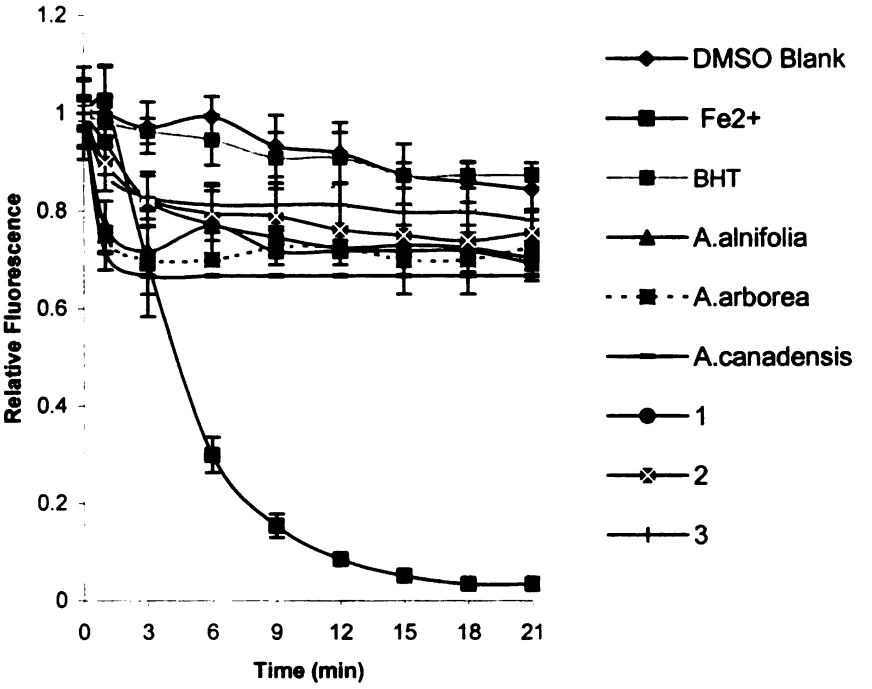


Figure 2.5. Antioxidant activities of anthocyanin mixtures from *Amelanchier* spp. and anthocyanins **1** and **2** and cyanidin (**3**) isolated from *A. canadensis* assayed in a liposomal model system. Samples were tested at 10 ppm. Commercial antioxidant BHT was assayed at 2.20 ppm. Data represent the average ± 1 standard deviation.

Cyanidin glycosides from tart cherries were reported to be inhibitors of COX enzymes (Wang et al., 1999; Seeram et al., 2002). Therefore, the extracts and pure compounds from *Amelanchier* fruits were investigated for COX-1 and COX-2 inhibitory activities. This assay is based on the ability of COX enzymes to convert arachidonic acid to prostaglandins, which evoke the physiological response of inflammation. In COX-1 and COX–2 enzyme inhibitory assays, anthocyanin mixture of **1** and **2** from *A. alnifolia,*

A. arborea, and *A. canadensis* at 100 ppm showed 66 and 67%; 60 and 72%, 51 and 76% of inhibition, respectively. Aspirin (180 ppm), Vioxx (1.67 ppm) and Celebrex (1.67 ppm) inhibited COX-1 and COX-2 enzymes by 74 and 69; 0 and 85 and 5 and 82 %, respectively. Pure anthocyanins **1** and **2** and cyanidin (**3**) inhibited COX-1 enzyme by 50.5, 45.62 and 96.36 %, respectively, at 100 ppm where as the COX-2 inhibition was the highest for cyanidin at 75% (Figure 2.6).

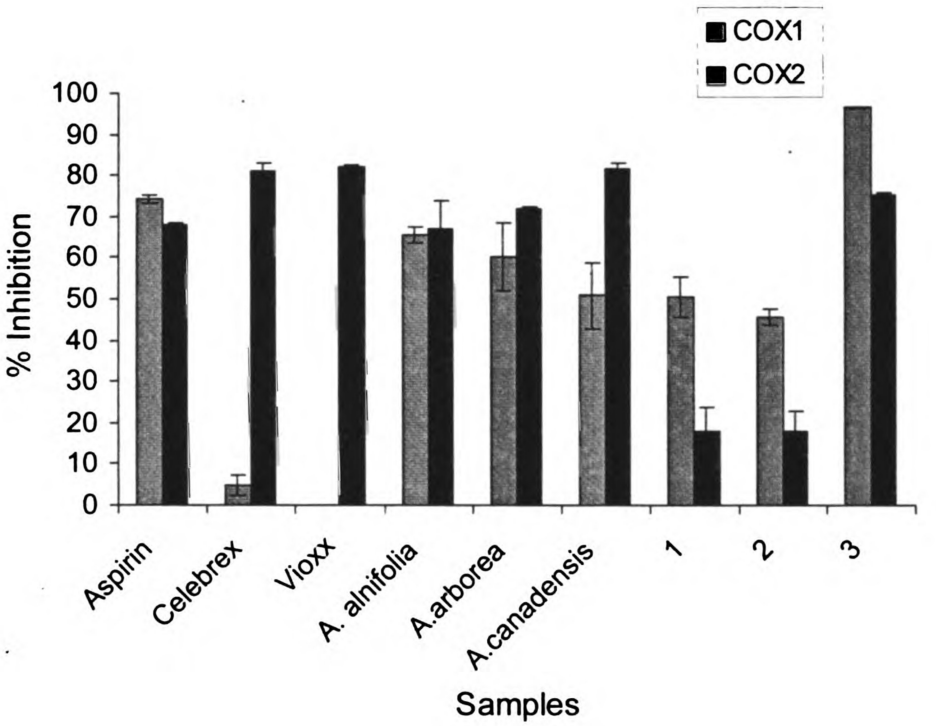


Figure 2.6. Cyclooxygenase inhibitory activities of anthocyanin mixtures from *Amelanchier* spp. and anthocyanins **1** and **2** and cyanidin (**3**) isolated from *A. canadensis*. Samples were tested at 100 ppm. Aspirin, Celebrex, and Vioxx were tested at 180, 1.67, 1.67 ppm, respectively. Data represent the average ± 1 standard deviation.

The concentration of anthocyanins in *A. arborea* (390 mg/ 100 g) was comparable to the anthocyanin content in blueberry and black raspberry (Wang et al., 1997b). The anthocyanins in *Amelanchier* spp. showed good antioxidant and cyclooxygenase enzyme inhibitory activities. The high concentration of anthocyanins, especially cyanidin 3-galactoside, in all three *Amelanchier* species studied suggests that *Amelanchier* fruits may be useful to incorporate in the diet. These fruits may also be considered as an alternate source of anthocyanins to formulate supplements containing bioactive anthocyanins similar to the bioactive tart cherry anthocyanins.

CHAPTER 3

BIOACTIVE COMPOUNDS FROM *AMELANCHIER CANADENSIS*

Abstract. *Amelanchier canadensis* (L.) Medic. is one of seven *Amelanchier* species grown in Michigan. Although triglycerides, sterols and phenolic acids were reported from the species, there is no report of biological activity of the compounds isolated from *Amelanchier* fruits. We have isolated several compounds from the fresh fruits of *A. canadensis* and determined their bioactivity using several in vitro assays. Two triglycerides, 1, 3 - dilinolein 2- olein (**4**) and 1, 3 - diolein 2- linolein (**5**), and and β-sitosterol (**6**) were purified from ethyl acetate extracts of fresh *A. canadensis* fruits. In addition, 5-hydroxymethyl-2-furfural (HMF) (**7**), 5-(Sorbitoloxymethyl)-furan-2-carbaldehyde (**8**), 5-(Mannitoloxymethyl)-furan-2-carbaldehyde (**9**), 5-(β-D-Glucopyranosyloxymethyl)-2-furancarboxaldehyde (**10**), and 5-(α-Glucopyranosyloxymethyl)-2-furancarboxaldehyde (**11**) were isolated from the methanolic extract of *A. canadensis*. When assayed, compounds **4**, **5** and **6** at 100 ppm showed 3.3, 8.7, and 11 % lipid peroxidation inhibitory activity. Respectively. Similarly, compounds **8** and **9**, **10** and **11** at 100 ppm and compound **7** at 10 ppm showed 83, 26 and 8.6 % of lipid peroxidation inhibitory activity, respectively. The commercial antioxidant butylated hydroxytoluene (BHT) at 2.2, ppm gave 87 % lipid peroxidation inhibitory activity. Compounds **4**, **8** and **9**, **10** and **11** at 100 ppm, and compound **7** at 10 ppm inhibited cyclooxygenase (COX) -1 and -2 enzymes at 39 and 0 %; 68 and 49 %; 63 and 45 %; and 5 and 13 %, respectively. The positive controls used in the COX assays were

Aspirin, Celebrex and Vioxx at 180, 1.67 and 1.67 ppm, and showed 74 and 69 %; 5 and 82 %; 0 and 85 % of COX-1 and COX-2 inhibition, respectively. Compounds **7**, **8**, **10** and **11** were isolated for the first time from *A. canadensis* fruits.

INTRODUCTION

Amelanchier canadensis (Rosaceae), commonly called Shadblow Serviceberry, is eastern Northern American species used in landscape trade. *A. canadensis* is a stoloniferous shrub or small tree reaching 8 meters. The fruits, purplish-black, slightly sweet, and ripen in late June, are consumed in Northern America. Earlier phytochemical studies by GC/MS showed that the seed contained 7.2% oil. The major acids present in the oil were oleic, linoleic, and palmitic acid. Similarly, the sterol amount was about 0.9%. The major sterol present in the seed was β-sitosterol in addition to campesterol, stigmasterol, brassicasterol, cholesterol, Δ7-stigmasterol, Δ5-avenasterol and Δ7-avenasterol. The content of phospholipids in the oils, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidic acids was 2.8% respectively. The tocopherols present in *A. canadensis* seeds were identified (Zlatanov et al., 1999). In another study, 3-feruloylquinic, 5-feruloylquinic, chlorogenic, neochlorogenic, isochlorogenic c, and caffeic acids were identified from the ethanolic extracts of *A. canadensis* fruits. The total phenolic acid content was determined as 222.2 mg/100 g fresh fruit (Sergeeva et al., 1980).

Past research on *A. canadensis* was focused on the compounds present in the fruits. There is no report of biological activities on the crude extracts or compounds isolated from the species. The work in our laboratory, in part, involves the preliminary screening of many plant and microbial extracts in order to determine the presence of any biologically active compounds. Here, we report, for the first time, the isolation, structure

determination, and lipid peroxidation and COX inhibitory activities of the compounds from *A. canadensis*.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on Varian INOVA (300 MHz) and VRX (500 MHz) instruments. ^{13}C NMR spectra were recorded at 75 and 125 MHz, respectively. ^{1}H NMR chemical shifts are reported in ppm relative to $CDCl_3$ at 7.24, CD_3OD at 3.31, DMSO-d_6 at 2.54, and D_2O at 4.80. Coupling constants, J, are in hertz. ^{13}C NMR chemical shifts are reported in ppm relative to $CDCl_3$ at 77.0, CD_3OD at 49.0, DMSO-d_6 at 39.50. Standard pulse sequences were employed for all 1D (^{1}H, ^{13}C, DEPT) and 2D (HMBC and HMQC) NMR experiments. Mass spectra were recorded at the Michigan State University Mass Spectrometry Facility using a JOEL HX-110 double focusing mass spectrometer (Peabody, MA) operating in the positive ion mode for FABMS experiments.

A Recycling Preparative Liquid Chromatograph model LC-20, equipped with a model AS-20 fraction collector (both Japan Analytical Industry Co., Tokyo) and a JAIGEL-ODS column (A-343-10, 250 x 20 mm, 10 μm, Dychrom, Santa Clara, CA) was used for purification of extracts. Compounds were detected by UV and refractive index detectors attached to a model D-2500 chromato-integrator (Hitachi, Tokyo). Merck silica gel 60 with a particle size 35-70 μm and C18 with a particle size of 60 μm (Dychrom, Santa Clara, CA) were used for preparative medium-pressure liquid chromatography (MPLC). For preparative TLC separation, 250, 500, 1000 μm silica gel plates (Analtech,

Inc., Newark, DE) were used. Gas chromatographic-mass spectrometric (GC-MS) analysis was carried out using an HP 6890 system equipped with an electron capture detector operating at 250 ^{0}C, an HP-5MS (30 m x 250 μm x 0.25 μm) column and a 7673 model injector operating at 250 ^{0} C in the splitless mode; the injection volume was 1.0 μL. Helium was used as carrier gas at 0.8 mL/min. The initial temperature was started at 50 ^{0}C, held for 2 min, elevated to 250 ^{0}C at 10 ^{0}C/min and held until the end of analysis. The quadrupole mass filter was set to scan from 40 to 550 *m/z* units. The samples were dissolved in hexane to yield a solution of 1 mg/mL.

The solvents used were of ACS reagent grade and purchased from Spectrum Chemical Co. (Gardena, CA). Butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), acetic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Celebrex and Vioxx were physician's professional samples supplied by Dr. S. Gupta, Sparrow Hospital, Michigan. 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Alabastaer, AL), 3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid from Molecular Probes (Eugene, OR). COX-1 and COX-2 enzymes were prepared in the Bioactive Natural Products and Phytoceuticals Laboratory (BNPP) from ram seminal vesicles and prostaglandin endoperoxide H synthase-2 (PGHS-2) cloned insect cell lysate, respectively.

Fruits. Fully ripened fruits of *A. canadensis* were collected in mid July, 2001 in Eaton Rapids, MI. The locations of trees were recorded in the Data Base maintained by the

Department of Horticulture at Michigan State University. The fruits were stored in plastic zip lock bags at –20° C prior to analyses.

Extraction. Fresh fruits of *A. canadensis* (750 g) were blended with water (2x750 mL) in a Waring Blender (5 min) at high speed and centrifuged at 10000 *g* at 4^0C for 20 min. The residue was extracted with acidic methanol (1% HCl) (3x1000 mL) for 36 h and ethyl acetate (3x1000 mL) for 36 h, respectively. The yields of aqueous, methanol and ethyl acetate extracts, after removal of the solvents, were 55, 90, and 6 g, respectively. An aliquot of the ethyl acetate extract (4.8 g) was fractionated into $CHCl_3$ soluble (3 g) and insoluble fractions (1.8 g) by stirring with 30 mL of $CHCl_3$. The chloroform soluble fraction (1.9 g) was subjected to silica gel MPLC column (350 x 40 mm) and eluted with 100 % hexane, followed by gradients of acetone/hexane, chloroform, chloroform/methanol, and methanol. A total of sixteen 200 mL fractions were collected. Based on TLC profiles, the fractions were combined to yield fractions I (160 mg, hexane, 300 mL, hexane/acetone/20/80, 500 mL), II (1.05 g, hexane/acetone/20/80, 150 mL), III (32 mg, hexane/acetone/40/60, 250 mL), IV (74 mg, hexane/acetone/40/60, 150 mL, $CHCl_3$ 225 mL, MeOH/ $CHCl_3$ / 25/75 500 mL), V (89 mg, MeOH/ $CHCl_3$/25/75 300 mL, MeOH 200 mL), and VI (367 mg, MeOH 260 mL).

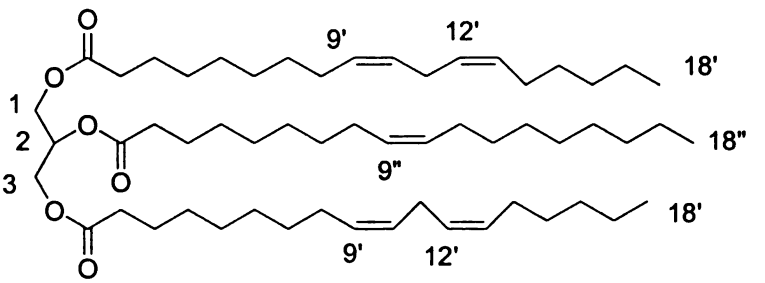
The fraction II (1.012g) was further fractionated by MPLC silica gel column (350 x 40 mm) and eluted with hexane, hexane/acetone and methanol. Aliquots of 15 mL fractions (180) were collected in 20 mL test tubes and altogether 180 fractions were used. Based on TLC, these fractions were combined to yield fractions II A (805 mg,

acetone / hexane/4/96, 300 mL), II B (20 mg, acetone/hexane/8/92 ,225 mL), IIC (18 mg, acetone/hexane/8/92 mL 150 mL), IID (9.4 mg, acetone/hexane/4/96, 75 mL), IIE (100 mg, acetone/hexane/8/92 150mL, acetone/hexane/16/84 75 mL), and IIF (54 mg, acetone/hexane/16/84, 600 mL).

Isolation of compounds 4, 5 and 6: Fraction IID, was pure by TLC analysis and gave compound **5**. An aliquot of fraction IIA (230 mg) was subjected to repeated silica gel preparative TLC using acetone/hexane (5/95) as the mobile phase followed by developing with (acetic acid / diethylether / hexane) (1/20/80) yielded compound **4** (40 mg). Repeated preparative silica gel TLC of II E (100 mg) using acetone / hexane (20/80) as the mobile phase gave compound **6** (37 mg).

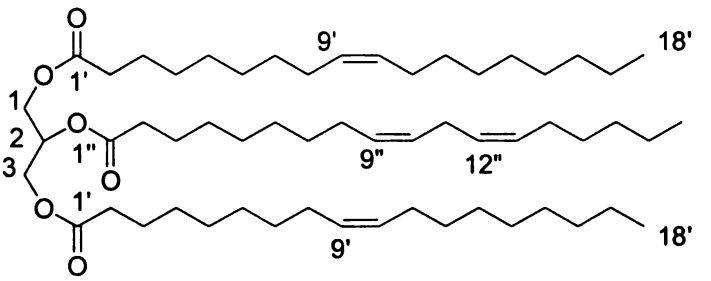
Compound 4. Colorless oil. ^{1}H NMR (500MHz, $CDCl_3$): δ 5.35 (10 H, m, H-(9', 10', 12', 13') x 2 and H-(9", 10")), 5.25 (1H, m, H-2), 4.29 (2H, dd, J = 12.0, 4.5 Hz, H-1b, H-3b), 4.14 (2H, dd, J = 12.0, 6.0 Hz, H-1a, H-3a), 2.77 (4H, m, H-11" x 2), 2.31 (2H, t, J = 7.5 Hz, H-2"), 2.31 (4H, t, J = 7.5 Hz, H-2 x 2'), 2.04 (12H, m, H-(8', 14') x 2 and H-(8", 11")), 1.61 (9H, m, H-3'x 2 and H-3"), 1.25 -1.37 (48H, m, H-(4'-7' and 15'-17') x 2 and H-(4"-7" and 12"- 17")), 0.89 (6H, t, J = 7.0 Hz, H-18'), 0.88 (3H, t, J = 7.0 Hz, H-18"). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.24 (C-1' x 2), 172.86 (C-2"), 127.90-130.23 (C- (9', 10', 12', 13') x 2 and C-(9", 10"), 68.90 (C-2), 62.09 (C-1, 3), 34.19, 34.11, 34.03 (C-2' x 2 and C-2"), 31.92, 31.90, 31.52 (C-3' x 2 and C-3"), 29.05 to 29.76, 22.55-27.96 (C-(4'-8' and 14'-17') x 2 and C-(4"-8", and 11"- 17")), 14.09 (C-18' x 2), 14.04 (C-18"). These

spectral data were identical to the published spectral data of 1,3-dilinoleoyl-2-olein (Ramsewak et al., 2001).



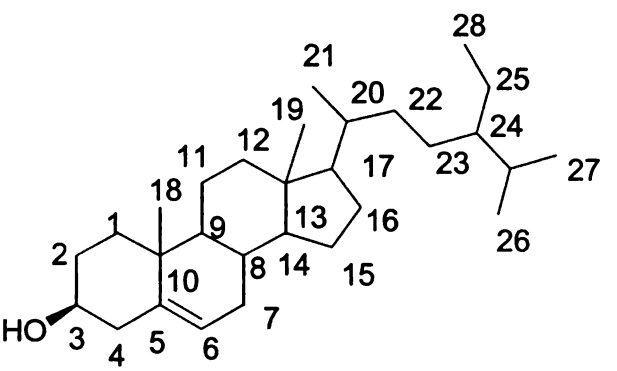
4

Compound 5. Colorless liquid. ^{1}H NMR (300MHz, $CDCl_3$): δ 5.35 (8H, m, H- (9', 10') x 2 and H-(9", 10", 12", 13")), 5.29 (1H, m, H-2), 4.29 (dd, J=12, 4.2 Hz, 2H), 4.14 (dd, J=12, 6 Hz, 2H), 2.77 (2H, m, t, J=7.0 Hz, H-11"), 2.31 (6H, t, J = 7.5 Hz, H-2' x 2 and 2"), 2.03 (12H, m, H-(8', 10') x 2 and H-(8", 14")), 1.61 (6H, m, H-3'x 2 and H-3"), 1.20-1.41 (50 H, m, H-(4'-'7 and 14'-17') x 2 and H-(4"-7" and 12"- 17")), 0.86 (3H, t, J = 8.5 Hz, H-18"), 0.85 (6H, t, J = 7.0 Hz, H-18'). ^{13}CNMR (125 MHz, $CDCl_3$): δ 173.23 (C-1' x 2), 172.80 (C-1"), 127.90 – 130.23 (C-(9',10') x 2 and C-(9", 10", 12", 13")), 68.90 (C-2), 62.08 (C-1 and 3), 34.19, 34.107 (C-2' x 2 and C-2"), 31.92, 31.90 31.52 (C-3' x 2 and C-3"), 22.56 - 29.76 (C-(4', 5', 6', 7', 8', 11', 12', 13', 14', 15', 16', 17') x 2 and C-(4", 5", 6", 7", 8", 11", 14", 15", 16", 17")), 14.09 (C-18'), 14.04 (C-18"). These spectral data were in agreement with the published data and confirmed that it was 1, 3,- diolein 2-linolein (Chandra et al., 1993).



5

Compound 6. Colorless solid. ^{1}H NMR (500MHz, $CDCl_3$): δ 5.35 (1H, m, H-6), 3.53 (1H, m, H-3), 2.28 (1H, d, J=13.0 Hz, 4a-H), 2.03 (1H, d, J=13.0 Hz, 4b-H), 0.80 – 2.00 (27H, m, 1, 2, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 24, 25, 28-H), 0.86 (3H, s, H-19), 0.84 (3H, d, J=7.5 Hz, H-21), 0.82 (3H, t, J = 7.5 Hz, H-29), 0.80 (6H, d, J = 7.5 Hz, H-26, 27), 0.70 (3H, s, H-18). ^{13}C NMR (500MHz, $CDCl_3$): δ 140.84, 121.68, 71.82, 56.86, 56.20, 50.27, 45.99, 42.39, 39.87, 37.33, 36.57, 36.17, 34.07, 31.99, 31.96, 31.76 (2C), 29.33, 26.32, 23.18, 21.16, 19.79, 19.39, 19.10, 18.81, 11.89 (2C). These spectral data of compound **6** were in agreement with the published data and confirmed that it was β-sitosterol (Hung et al., 2001).

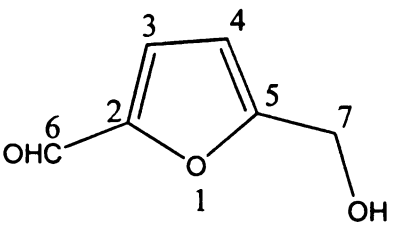


6

Isolation of compounds 7, 8, 9, 10 and 11: The methanol extract (31.4 g) was suspended in water (150 mL) and partitioned with ethyl acetate (4x150 mL) to yield ethyl acetate (1.3 g) and water soluble fractions. The water soluble fraction was cloudy so it was centrifuged. The resulting residue (0.7 g) was soluble in methanol. The aqueous portion afforded a gray residue (26.8 g). An aliquot of (25.4 g) was subjected to C-18 MPLC (450 x 51 mm) and eluted with methanol/water gradient from 2 to 100 % methanol. The fractions were collected as 200 mL aliquots. A total of twenty four fractions were collected. The fractions were combined based on TLC profiles and afforded fractions I (21.79 g, 2% MeOH in water, 1200 mL), II (1.032 g 10% MeOH in water, 650 mL 30% MeOH in water, 300 mL), III (473 mg, 30% MeOH in water, 200 mL), IV (581 mg, 30% MeOH in water, 75 mL), V (422 mg, 30% MeOH in water 175 mL), VI (192 mg, 30% MeOH in water, 825 mL) and VII (400 mg, 50% MeOH in water, 800 mL, MeOH , 900 mL). Based on TLC, fractions I and II contained mainly sugars. Similarly, HPLC analysis showed that fraction VII contained mainly anthocyanins. Therefore, fractions I, II and VII were not purified further. Fraction VI (414 mg) was purified by LC-20 using 30 % methanol in water. The fractions collected were VIA (19.4 mg, R_t = 20 min), VIB (54 mg, R_t = 22 min), VIC (172 mg, R_t = 26 min), VID (123 mg, R_t = 36 min), and VIE (47 mg, R_t = 44 min) fraction VIC (172 mg, R_t = 26 min) was subjected to silica gel prep TLC 4 x (20x20cmx500 μm) and developed with MeOH/$CHCl_3$ (10/90) as the mobile phase to yield compound **7** (22 mg). Similarly, compound **7** (60 mg) was also obtained from fraction V. Fraction IV (260 mg) was purified by repeated silica gel Prep TLC (4 x 20 x 20 cm, 1000 μm)

using MeOH/$CHCl_3$ (20/80) and (10/90) as mobile phases to yield an inseparable mixture of compounds **8** and **9** (32 mg), and compounds **10** and **11** (26 mg).

Compound 7. Viscous liquid; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.54 (1H, s, 6-H), 7.47 (1H, d, J = 3.5Hz, 3-H), 6.59 (1H, dd, J=3.5, 1.0 Hz, 4-H), 4.50 (2H, s, 7-H), 4.05 (1H, brs, 7-OH). ^{13}CNMR (125 MHz, DMSO-d6): δ 177.90 (C-6), 162.15 (C-5), 151.68 (C-2), 124.29 (C-3), 109.59 (C-4), 55.87 (C-7).

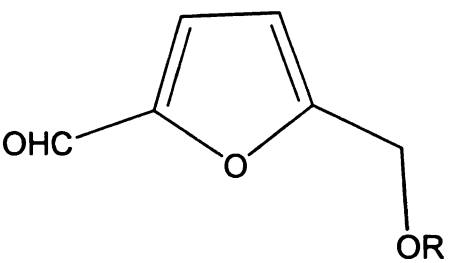


7

Compounds 8. Colorless syrupy liquid; FABMS (relative intensity): $[M^+ + Na]^+$ 313 (20), $[M^+ + H]^+$ 291 (40), $[M^+ - HMF + H]^+$ 183 (10), $[M^+ - sorbitol + H]^+$ 110 (10), $[M^+ - alditol]^+$ 109 (30). ^{1}H NMR (500 MHz, CD_3OD): δ 9.54 (1H, s, 6-H), 7.38 (1H, d, J = 3.5 Hz, 3-H), 6.68 (1H, d, J = 3.5, 4-H), 4.62 (2H, s, H-7), 3.4 - 4.0 (8H, m, 1', 2', 3', 4', 5', 6'-H). ^{13}CNMR (125MHz, CD_3OD): δ 179.55 (C-6), 160.26 (C-5), 154.17 (C-2), 124.33 (C-3), 112.68 (C-4), 75.03 (C-2'), 73.68 (C-1'), 73.49 (C-4'), 73.08 (C-5'), 71.68 (C-3'), 66.15 (C-7), 64.19 (C-6').

Compounds 9. Colorless syrupy liquid; FABMS (relative intensity): $[M^+ + Na]^+$ 313 (20), $[M^+ + H]^+$ 291 (40), $[M^+ - HMF + H]^+$ 183 (10), $[M^+ - mannitol + H]^+$ 110 (10), $[M^+ -$

alditol]$^+$ 109 (30). ^{1}H NMR (500 MHz, CD_3OD): δ 9.54 (2H, s, 6-H), 7.38 (1H, d, J = 3.5 Hz, 3-H), 6.68 (1H, dd, J = 3.5, 1.0 Hz, 4-H), 4.61 (2H, s, H-7), 3.50- 4.01 (8H, m, 1', 2', 3', 4', 5', 6'-H). ^{13}CNMR (125MHz, CD_3OD): δ 179.56 (C-6), 160.12 (C-5), 154.17 (C-2), 124.31 (C-3), 112.62 (C-4), 73.27 (C-2'), 73.27 (C-1'), 73.05 (C-5') 70.99 (C-3'), 70.74 (C-4'), 66.15 (C-7), 64.82 (C-6')

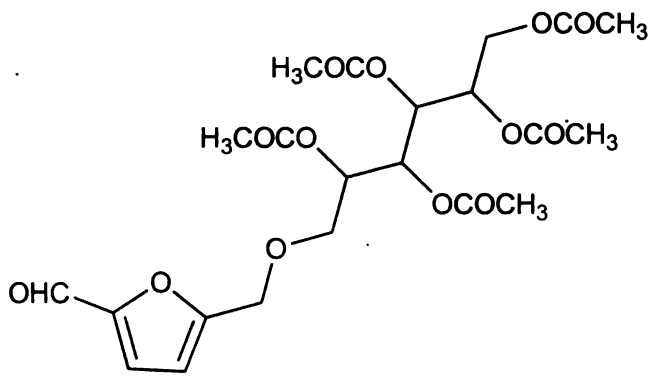


8 R = Sorbitol

9 R = Mannitol

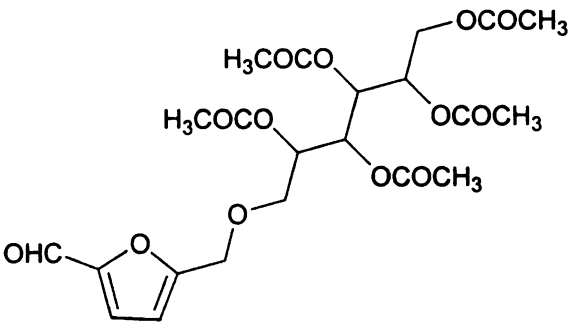
Acetylation of compounds 8 and **9**. An aliquot (5 mg) of the mixture of compounds **8** and **9** was dissolved in pyridine (1 mL) and acetic anhydride (2 mL) was added. The solution was kept at room temperature for 72 h. Deionized water was added to the reaction mixture and the product was extracted with $CHCl_3$ (3x 10 mL). The solvent was evaporated under vacuum and purified by preparative TLC using silica gel plates, developed with hexane/acetone (2:1) as the mobile phase. The UV active band was removed and eluted with chloroform. Removal of the solvent afforded an inseparable mixture of acetylated products **8A** and **9A** (8 mg) as a colorless syrup.

Compound 8A. Colorless liquid. ^{1}H NMR (500 MHz, $CDCl_3$) : δ 9.62 (1H, s, H-6), 7.26 (1H, d, J = 3.5 Hz, H-2), 6.58 (1H, d, J = 3.5, Hz, H-3), 5.50 (1H, dd, J = 6.5, 4.0 Hz, H-3'), 5.43 (1H, dd, J = 11.0, 5.0 Hz, H-4'), 5.28 (1H, m, H-2'), 5.06 (1H, m, H-5'), 4.54 (2H, s, H-7), 4.34 (1H, dd, J = 11.5, 4.5 Hz, H - 6a'), 4.11 (1H, dd, J = 11.5, 6 Hz, H - 6b'), 3.70 (1H, dd, J = 11, 4.5 Hz, H-1a'), 3.56 (1H, dd, J = 10.5, 5Hz, H - 1b'), 2.09 (3H, s), 2.06 (3H, s), 2.04 (3H, s), 2.02 (3H, s), 2.01 (3H, s).



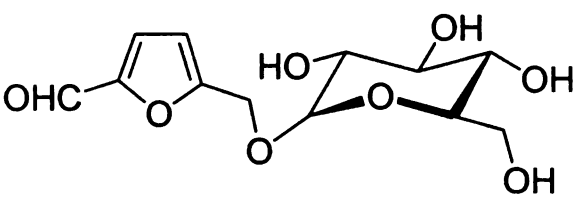
8A

Compound 9A. Colorless liquid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.62 (1H, s, H-6), 7.19 (1H, d, J = 3.5Hz, H-2), 6.54 (1H, d, J = 3.5, Hz, H-3), 5.43 (1H, dd, J = 11.0, 5.0 Hz, H-4'), 5.35 (1H, m, H-3'), 5.06 (1H, m, H-2'), 5.04 (1H, m, H-5'), 4.54 (2H, s, H-7), 4.24 (1H, dd, J = 11.5, 4.5 Hz, H-6a'), 4.01 (1H, dd, J = 11.5, 6 Hz, H-6b'), 3.66 (1H, dd, J = 10.5, 5.5 Hz, H-1a'), 3.56 (1H, dd, J = 10.5, 5Hz, H-1b'), 2.08 (3H, s), 2.05 (3H, s), 2.03 (3H, s), 2.02 (3H, s), 2.00 (3H, s).



9A

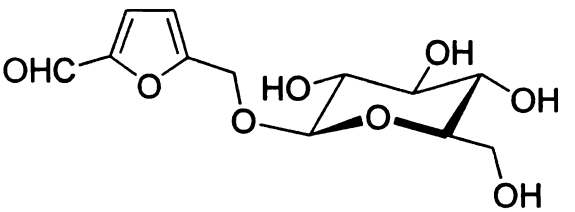
Compound **10.** Light yellow syrupy liquid; FABMS (relative intensity): $[M^+ + H]^+$ 289 (6). ^{1}H NMR (500 MHz, CD_3OD): δ 9.54 (1H, s, 6-H), 7.36 (1H, d, J = 3.5 Hz, 3-H), 6.65 (1H, d, J = 3.5 Hz, 4-H), 5.08 (1H, d, J = 3.5 Hz, 1'-H), 4.61 (2H, s, H-7), 3.1- 4.0 (6H, m, 2', 3', 4', 5', 6'-H) ^{13}CNMR (125 MHz, CD_3OD): δ179.54 (C-6), 160.15 (C-5), 154.17 (C-2), 124.27 (C-3), 112.64 (C-4), 93.98 (C-1'), 76.99, 74.90, 72.07, 71.44, 66.30, 62.78 (C-6').



10

Compound **11.** Light yellow syrupy liquid; FABMS (relative intensity): $[M^+ + H]^+$ 289 (6). ^{1}H NMR (500 MHz, CD_3OD): δ 9.54 (1H, s, 6-H), 7.36 (1H, d, J = 3.5 Hz, 3-H),

6.65 (1H, d, J = 3.5 Hz, 4-H), 4.61 (4H, s, H-7), 4.45 (1H, d, J = 7.0 Hz, H'), 3.1- 4.0 (6H, m, 2', 3', 4', 5', 6'-H) ^{13}CNMR (125 MHz, CD_3OD): δ179.54 (C-6), 160.15 (C-5), 154.17 (C-2), 124.27 (C-3), 112.60 (C-4), 98.24 (C-1'), 78.12, 76.99, 74.90, 72.07, 66.30, 62.78 (C-6').



11

Saponification of compounds 4 and 5 and methylation of the resulting fatty acids. Compounds **4** and **5** (2 mg each) were reacted with 5 % NaOH in methanol (1 mL) separately. Methanolic 6N HCl then was added to acidify the solution and dried under nitrogen. Diazomethane was prepared according to previously published method (Kelm et al., 1997). Briefly, N-nitroso-N-methyl urea was reacted with concentrated KOH solution in ether. The diazomethane formed dissolved in the organic phase. This yellow-colored ether solution containing diazomethane was separated and used to methylate the free fatty acids obtained from the hydrolysis of **4** and **5**. The methylated products dissolved in hexane and were filtered prior to GC-MS analysis.

Cyclooxygenase enzymes inhibitory assay. Cyclooxygenase enzymes inhibitory activities of the compounds isolated from *A. canadensis* were evaluated using COX-1 and

COX-2 enzymes. The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction with arachidonic acid as substrate was measured using a Model 5300 biological oxygen monitor (Yellow Spring Instruments, Inc., Yellow Springs, OH). The reaction mixture consisted of 0.1 M tris, 1.0 mM phenol, 17 μg hemoglobin, the enzyme, 6 μL test sample dissolved in DMSO at 1.5 % (DMSO alone as solvent control). The reaction was performed in a 600 μL micro chamber (Instech Laboratory, Plymouth Meeting, PA) at 37 ^{0} C. After 2 min of incubation of the enzyme and test samples, 10 μL of arachidonic acid (0.25 mg/ 0.5 mL of Tris buffer) was added to initiate the reaction. Data was recorded using Quicklog for Windows (Strawberry Tree Inc., Sunnyvale, CA). Aspirin, Celebrex and Vioxx at 180, 1.67 and 1.67 ppm, respectively, were used as positive controls (Wang et al., 1999).

Lipid Peroxidation assay. Lipid peroxidation assay was performed according to the previously published method (Wang et al., 1999, Arora et al., 1997). Inhibition of lipid peroxidation was measured by fluorescence spectroscopy using large unilamellar vesicles (LUVs) of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine containing a fluorescent probe (3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid) and reported after 21 min as relative fluorescence compared to control. Butylated hydroxytoulene (BHT) at 2.2 ppm was used as positive controls. Briefly, the lipid and probe were dissolved in DMF and evaporated *in vacuo*. The solvent-free mixture was resuspended in 0.15 M NaCl, 0.1 mM EDTA and 0.01 M MOPS (kept over Chelex resin), subjected to ten freeze-thaw cycles using a dry ice-ethanol bath, and extruded 29 times through a 100 nm pore size membrane to form the LUVs. The assay buffer

used consisted of a mixture of 100 μL HEPES buffer, 200 μL 1 M NaCl, 1.64 mL nitrogen-sparged milipore water. An aliquot of 20 μL of test sample in DMSO or DMSO (control) and 20 μL of liposome suspension were mixed with the assay buffer to achieve the desired concentration of the test compounds. Peroxidation was initiated by adding 20μL of $FeCl_2.4H_2O$ (0.5 mM) and the samples were gently vortexed. The fluorescence was monitored using a Turner fluorometer with a narrow band pass 360 nm filter and a sharp cut 415 nm filter at 0, 1, 3, 6, and every three minutes thereafter for 21 min. Analyses of samples and controls were performed in duplicate. Relative fluorescence (*Ft/Fo*) was calculated by dividing the fluorescence value at a given time (*Ft*) by that time at t = 0 min. (*Fo*).

RESULTS AND DISCUSSION

Fresh fruits of *A. canadensis* were sequentially extracted with water, acidic methanol and ethyl acetate to yield 7.3, 5.3, and 0.8 % of crude extracts, respectively. Compounds **4**, **5** and **6** were isolated from chloroform soluble fractions of ethyl acetate extract by successive silica gel MPLC, and preparative TLC. Water soluble portion of the methanol extract afforded compounds **7**, **8**, **9**, **10** and **11**.

The structure of compound **4** was determined by using ^1H and ^{13}C NMR, and GC/MS spectral experiments. The ^1H NMR signals of **4** showed two doublets at 4.1 and 4.3 ppm, integrated for two protons each, and a one proton multiplet at 5.32 ppm. This indicated the presence of a triglyceride in the molecule. Similarly, ^{13}C- NMR gave

signals at 173.24, 171.86, 68.90, and 62.09 ppm and confirmed that the compound contained an acylated glycerol moiety. The overlapping multiplets at 5.25 ppm, integrated for 10 protons and carbons appeared at 130.19, 129.99, 129.98, 129.95, 129.66, 128.08, 128.06, 127.89 and 127.89 ppm indicated the presence of five carbon double bonds in this molecule. The compound was identified as 1, 3- dilinoleoyl 2-olein.

The GC profile of methylated fatty acids resulting from the hydrolysis of this molecule confirmed the presence of oleic and linoleic acid methyl esters with a ratio of 2:1. The retention times for both methyl esters of oleic acid and linoleic acids were identical to those of authentic samples of oleic and linoleic acids. The GC/MS data supported that compound **4** was 1, 3- dilinoleoyl 2-olein. The spectral data of compound **4** were identical to the literature values of 1, 3- dioleoyl 2-linoolein (Ramsewak et al., 2001).

The ^{1}H NMR of **5** showed doublets integrated for two protons each at 4.29 and 4.14 ppm and a one proton multiplet at 5.29 ppm. This indicated the presence of a triglyceride moiety in the molecule. Similarly, ^{13}C-NMR spectra gave carbon signals at 173.23, 172.80, 68.90 and 62.08 ppm. The overlapping multiplets at 5.29 ppm in its ^{1}H-NMR spectrum and the signals appeared between 127.90-130.23, assigned for eight carbons belonging to the olefinic carbons in the fatty acid moieties of the triglyceride confirmed four double bonds in the molecule.

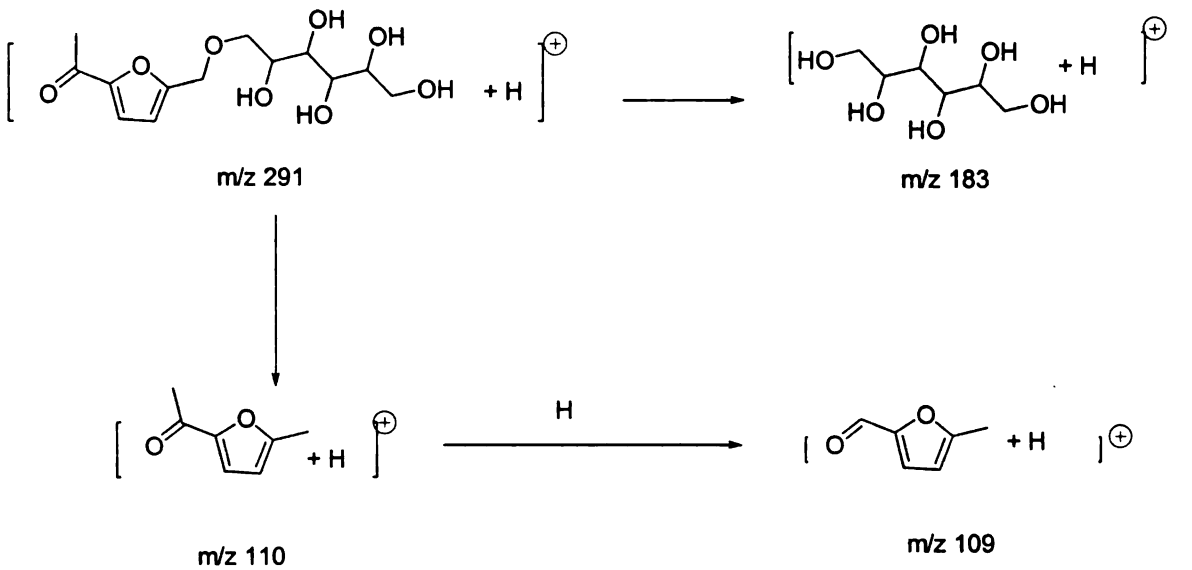
The GC profile of methylated hydrolysis product of this molecule confirmed the presence of oleic and linoleic acids in the triglyceride. The retention time for methyl ester of oleic acid obtained from compound **5** was identical to that of an authentic sample of oleic acid under the same conditions. GC/MS data supported the identity of compound **5** as 1, 3-dioleoyl-2-linolein. The spectral data of compound **5** were identical to the literature values of 1, 3-dioleoyl-2-linolein (Chandra et al., 1993).

^{1}H NMR of compound **6** showed that there were three methyl singlets at 0.86, 0.84 0.70, methyl doublets at 0.82, and 0.80 ppm and multiplets integrated for one proton each at 5.35 and 3.53 ppm. This suggested a sterol moiety in the molecule. ^{13}C-NMR confirmed the identity of compound **6** as β-sitosterol. β-sitosterol is ubiquitous in plants and was previously reported from the seeds of *A. canadensis* by GC analysis (Zlatanov et.al., 1999).

^{1}H NMR of compound **7** showed four set of signals. The singlet at 9.54 ppm, integrated for one proton and did not exchange with D_2O, indicated that it was due to an aldehyde. Two doublets with 3.5 Hz coupling constant at 7.47 and 6.59 ppm showed that the compound contained a furan moiety. There were six carbon signals in its ^{13}C-NMR spectrum, and appeared at 177.90 ,162.15, 151.68, 124.29, 109.59 and 55.87 ppm. Based on ^{1}H and ^{13}C- NMR data, carbon signal, the compound was identified as 5-hydroxymethyl–2- furfural. The spectral data of compound **7** was identical to the literature data of 5-hydroxymethyl-2- furfural (Hearn, 1976).

5-hydroxymethyl-2-furfural was reported as a natural product from several plants (Lee et al., 2002, Chuda et al., 1999, Santi et al., 1999, Kim et al, 1999, Xu et al., 1995, Waruna et al., 1994). This is the first report of this compound from *A. canadensis*. Chuda et al (1999) isolated and characterized 5-hydroxymethyl-2-furfural from *Prunus mume*. It was observed that this compound improved fluidity in human blood in in vitro assay. Santi et al (1999) isolated 5-hydroxymethyl-2-furfural from *Arfeuillea arborescens* as an antibacterial compound. It was reported that 5-hydroxymethyl-2-furfural exhibited antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella debry*, *E.coli* O 157:H7 and *Listeria monocytogenes*. Kim et al. (1999) isolated this compound as a moderate inhibitor of NO production with an IC50 value of 803 μM.

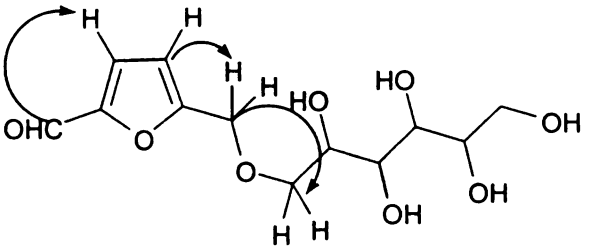
The structure of compounds **8** and **9** were identified based on their ^{1}H- and ^{13}C NMR, DEPT, HMQC, HMBC, mass spectrometry and analysis of acetylated products. Initial analysis of the ^{1}H NMR and ^{13}C NMR spectra of this product indicated a doubling of most proton and carbon signals and suggested that it was a mixture. The positive FABMS indicated a molecular ion $[M+H]^{+}$ at 291 and suggested that the product was a mixture of two structurally related isomers with a molecular formula of $C_{12}H_{18}O_8$. This was further supported by the presence of significant fragment ions at 183 and 109 resulting from the loss of a side chain and a hydrogen atom. All attempts to separate this into pure compounds were not successful. The acetylated product also gave a single band. Therefore, the structure elucidation was performed on the inseparable 1:1 mixture of these two stereoisomers.



The molecular formula of the compounds **8** and **9** were derived as $C_{12}H_{18}O_8$ from its molecular ion at with 291.1081([M+H]$^+$, calc. 291.1080) in its HRFABMS spectrum. ^{1}H NMR of compounds **8** and **9** showed that the signals appeared as a pair. The signal at 9.54 ppm, integrated for 1 proton appeared as a pair and did not exchange with D_2O. This confirmed an aldehyde moiety in the molecule. Two doublets with a coupling constant of 3.5 Hz at 7.47 and 6.59 ppm showed that the compound contained a furan moiety. It is important to note that the ^{1}H-NMR of **8** and **9** were did not show an anomeric proton signal. ^{13}C-NMR spectrum showed that compounds **8** and **9** contained a furanoid moiety. There were six oxygenated carbon signals between 64 and 74 ppm in addition to the furanoid carbons. Carbon signals at 75.03, 73.68, 73.49, 70.08, 71.68 and 64.19 ppm corresponded to 2', 1', 5', 3', 4', and 6' carbons of sorbitol moiety respectively.

Similarly, carbon signals at 73.27, 73.27, 73.05, 70.99, 70.74 and 64.82 ppm were assigned to 2', 1', 5', 3', 4', 6' carbons of mannitol moiety, respectively.

DEPT spectra of **8** and **9** showed that there were three secondary, seven tertiary and two quaternary carbons in both compounds. Because of the absence of anomeric proton and a corresponding carbon signal, the compound contained a furan moiety connected to an open chain poly hydroxyl moiety. Significant HMBC correlations supported the proposed structure. HMBC correlation between C-4 to H-7, C-1' to H-7 confirmed the connectivity between furfural moiety to an open chain poly hydroxyl moiety. Acetylation of compounds **8** and **9** showed that there were a total of ten acetates resulting from both compounds. Chemical shifts of protons bonded to carbons 2', 3', 4', 5' and 6' of polyhydroxyl moiety were shifted to down field in the acetylated product while protons attached to C-1' remained unchanged (Angyal, 1972). Therefore, compounds **8** and **9** were characterized as 5-sorbitol-furan-2-carbaldehyde and 5-mannitol-furan-2-carbaldehyde, respectively.



Significant HMBC correlations observed in **8** and **9**.

^{1}H NMR and ^{13}C-NMR of compound **10** was similar to compound 7 and indicated that it contained a furan moiety as well. In addition, proton doublets at 5.15 ppm with coupling constant 3.5 Hz along with carbon signals at 93.98, 76.99, 74.90, 72.07 and 62.78 ppm indicated that the furfural moiety was connected to an α-glucose moiety. Based on its spectral data, it was identified as 5-hydroxymethyl-2-furfural-α-glucoside. This was also supported by FABMS as indicated by a molecular ion $[M+H]^{+}$ at m/z 289. This compound was synthesized earlier from glucose and 5-hydroxymethyl 2 - furfural at high temperature (Urashima et al, 1988, Lichtenthaler et al., 1993) but this is the first report of it as a natural product as well as from *Amelanchier* spp.

Spectral analysis revealed that compound **11** was similar to compound **10.** The only difference between compounds **10** and **11** was the appearance of a doublet in its ^{1}H-NMR at 4.24 ppm. The doublet at 5.15 ppm was absent in its spectrum. Therefore, proton and carbon signals indicated that hydroxymethyl furfural was connected to a β-glucose moiety. This was also supported by FABMS which showed a molecular ion, $[M+H]^{+}$ at m/z 289. This compound was also reported as a reaction product between glucose and 5-hydroxymethyl 2- furfural formed at high temperature (Urashima et al, 1988). This is the first report of it as a natural product from. The attempts to separate compounds **10** and **11** using various chromatographic techniques were not successful.

Lipid peroxidation inhibitors of compounds **4 - 11** were determined according to previously published procedures (Wang et al., 1999, Arora et al, 1998, Arora et al 1997).

The lipid peroxidation inhibitory activity of these compounds at 100 ppm, except compound **7**, which was tested at 10 ppm; are presented in Figures 3.1 and 3.2, respectively.

The compounds from *A. canadensis* did not exhibit good lipid peroxidation inhibitory activity. For example, compounds **4**, **5**, and **6** at 100 ppm, and **7** at 10 ppm showed only 3.3, 8.7, 11, and 8.6 % of lipid peroxidation inhibitory activity, respectively. However, compounds **8** and **9** at 100 ppm showed 83 % lipid peroxidation inhibitory activity while the acetylated derivative gave 8.3 % inhibition at the same test concentration. Compounds **10** and **11** at 100 ppm showed 26 % inhibitory activity. The commercial antioxidant butylated hydroxytoluene (BHT), at 2.2 ppm gave 87 % lipid peroxidation inhibitory activity.

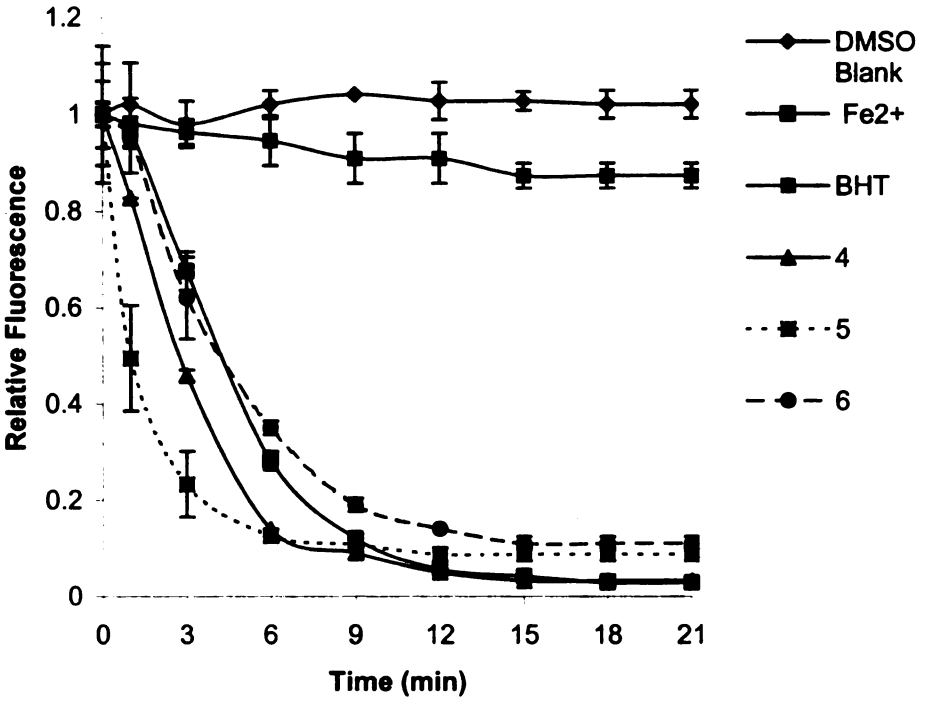


Fig 3.1 Lipid peroxidation inhibitory activity exhibited by compounds **4**, **5** and **6** from *A. canadensis* fruits assayed at 100 ppm. Activity was calculated based on relative fluorescence and compared to standard synthetic antioxidant BHT (2.2 ppm). Data represent the average ± 1 standard deviation.

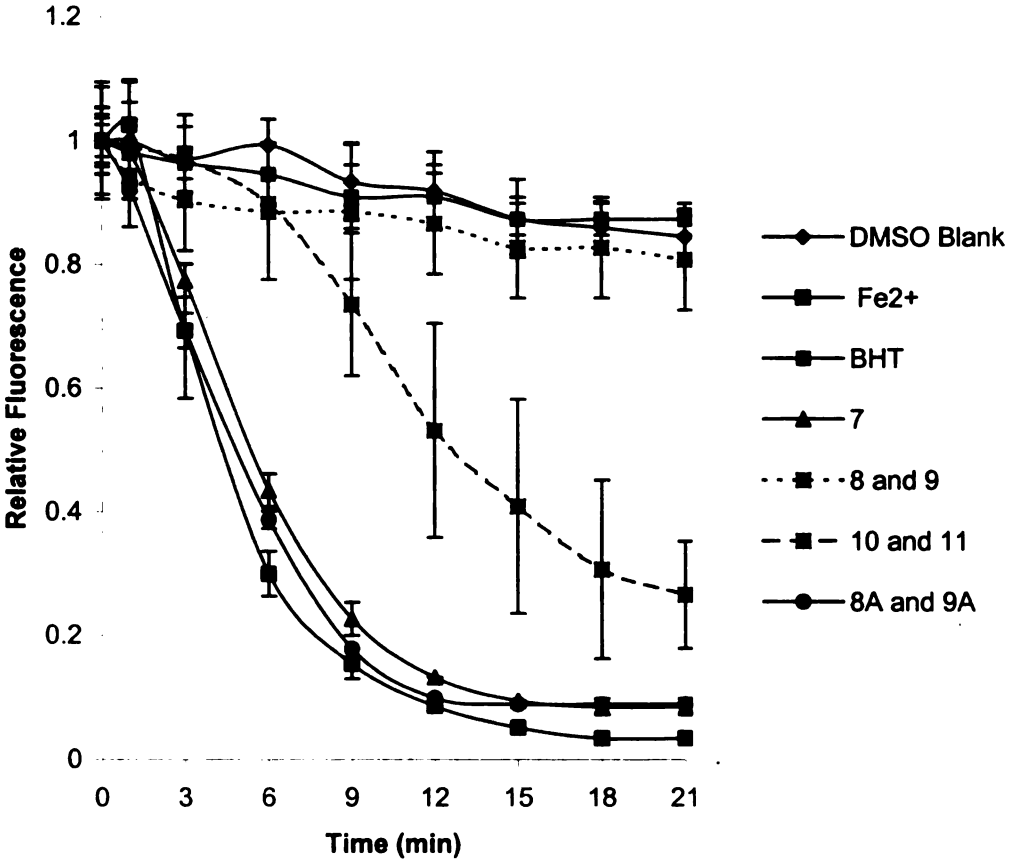


Fig 3.2 Lipid peroxidation inhibitory activity exhibited by compounds **7** at 10 ppm, **8** and **9**, **8A** and **9A**, and **10** and **11** from *A. canadensis* fruits assayed at 100 ppm. Activity was calculated based on relative fluorescence and compared to standard synthetic antioxidant BHT (2.2 ppm). Data represent the average ± 1 standard deviation.

Cyclooxygenase enzymes (COX-1 and COX-2) catalyze the conversion of arachidonic acid to prostaglandins, which are responsible for the onset of inflammation in the body. It has been confirmed that inhibition of COX-1 reduces the production of prostaglandins in the stomach, cause gastric ulceration and other side effects in the body. The inhibition of COX-2 enzyme reduce the inflammation with minimum side effects since COX-2 enzyme is produced mostly in inflamed tissues. Therefore, selective COX-2 inhibitors are better anti-inflammatory products (Smith et al, 2000, Meade et al., 1993). COX-1 and COX-2 enzyme inhibitory activities were conducted for *Amelanchier* according to the previously published procedures (Wang et al., 1999; Laneuville et al, 1994).

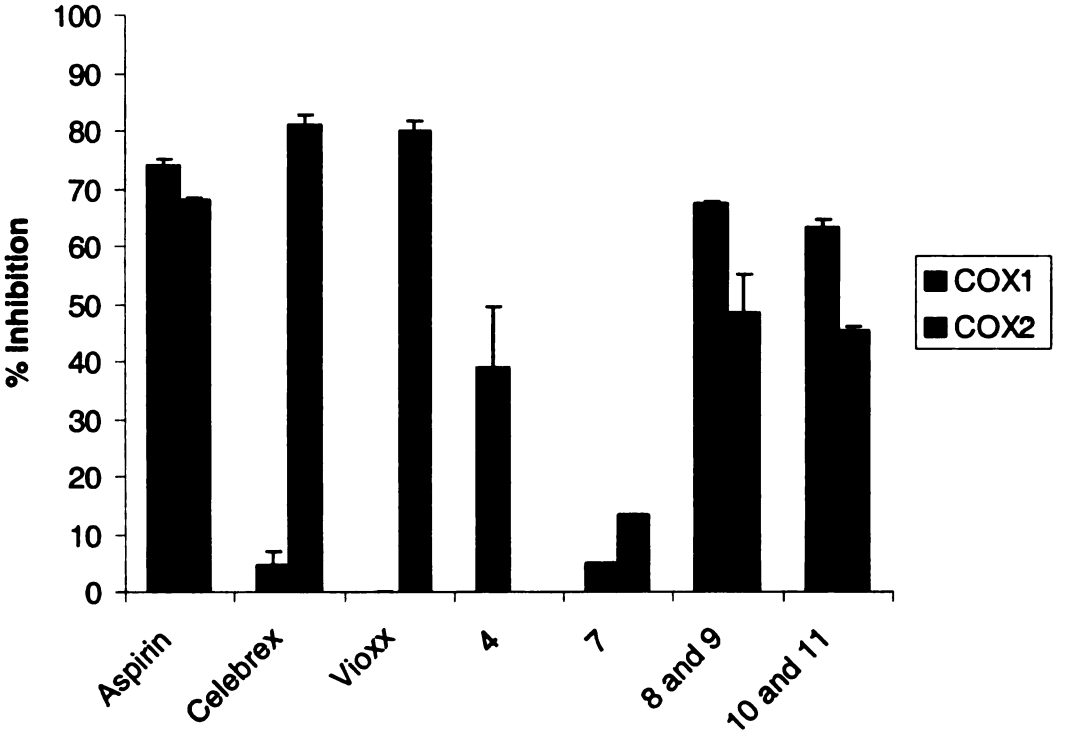


Fig 3.3 COX-1 and COX-2 enzyme inhibitory activities of compounds isolated from *A. canadensis* fruits. Compounds were assayed at 100 ppm and compared to NSAID standards aspirin (180 ppm), Celebrex™ (1.67 ppm) and Vioxx™ (1.67 ppm). Data represent the average ± 1 standard deviation.

Result for COX-1 and COX-2 enzyme inhibitory assays are presented in Figure 3.3. Compound **4** showed 39 % COX-1 inhibition but was not active in COX-2 enzyme assay. This might be due to the hydrolysis of triglyceride into linoleic acid and oleic acid in the assay chamber. The pH of the buffer used in this assay was 7.4 and triglycerides could potentially be hydrolyzed into its corresponding fatty acids. Polyunsaturated fatty acids like linoleic and oleic acids were reported as good COX- enzyme inhibitors (Henry et al., 2002). It is interesting to observe that compound **4** did not show COX-2 enzyme inhibitory activity.

Compounds **5** and **6** did not show COX enzyme inhibitory activities. It has been reported that compound **6**, β-sitosterol, showed analgesic activity (Villaseñor et al., 2002). The acetic acid-induced writhing test showed that β-sitosterol decreased the number of squirms induced by acetic acid by 70 % at a dose of 100 mg/kg mouse which was also confirmed by the hot plate method (Villaseñor et al., 2002). Phytosterols including β-sitosterol have been used to decrease the absorption of cholesterol in the digestive system. Similarly, plant sterols and stanols have been reported to reduce micellar solubilization of cholesterol (Wester 2000).

COX-1 and COX-2 enzyme inhibitory activities compound **7** at 10 ppm were 5 and 13 %, respectively. The mixture of compounds **8** and **9** demonstrated a greater enzyme inhibitory activity of 68 % inhibition in COX-1 assay. Similarly, a mixture of compounds **10** and **11** showed 63 % of COX-1 inhibition. The standards Aspirin, Celebrex, and Vioxx at 180, 1.67 and 1.67 ppm showed 75, 5 and 0% COX-1 inhibition,

respectively. As in the case of COX-1 assay, the mixture of compounds **8** and **9**, and **10** and **11** at 100 ppm demonstrated COX-2 enzyme inhibitory activity of 49 and 45 %, respectively. The standards Aspirin, Celebrex, and Vioxx at 180, 1.67 and 1.67 ppm showed 69, 82 and 85 % of COX-2 inhibition, respectively. The acetylated derivatives of compounds **8** and **9**, **8A** and **9A**, did not exhibit COX enzyme inhibitory activities.

Eight compounds were isolated from ethyl acetate and methanol extracts of *A. canadensis* fruits. Two tryglycerides, 1,3-dilinoleoyl-2-olein (**4**), and 1, 3, - diolein 2-linolein (**5**), and β-sitosterol (**6**) were previously reported from *A. canadensis*. Three other compounds, 5-hydroxymethyl-2- furfural (7), 5-(α-D-Glucopyranosyloxymethyl)-2-furancarboxaldehyde (**10**), 5-(β-D-Glucopyranosyloxymethyl)-2-furancarboxaldehyde (**11**), are reported for the first time from this plant. This is the first report of COX inhibitory activities of compounds **7**, **8**, **9**, **10** and **11**. Compounds **8** and **9** are novel. The findings of these bioactive compounds and anthocyanins in *A. canadensis* substantiated that consumption of *A. canadensis* fruits might be beneficial to health.

CHAPTER 4

CHARACTERIZATION OF

BIOACTIVE COMPOUNDS IN *AMELANCHIER ARBOREA* FRUITS.

Abstract. Bioassay-guided isolation and purification of the ethyl acetate extract of *Amelanchier arborea* (Michx.) Fern. fruits led to the characterization of five compounds, β-sitosterol-3-glucoside (**12**), mixture of oleanolic acid (**13**) and ursolic acid (**14**), kaempferol-3-*O*-α-L-rhamnosylrhamnoside (**15**) and kaempferol-3-*O*-α-L rhamnoside (**16**). The structures of these compounds were established by using ^{1}H and ^{13}C-NMR spectral methods. The mixture of compounds **13** and **14** showed 76% while compounds **15** and **16** showed 85, and 83 % of lipid peroxidation inhibitory activity at 100 ppm. The commercial antioxidant butylated hydroxytoluene (BHT) at 2.2 ppm gave 87 % lipid peroxidation inhibitory activity. Compounds **13** and **14**, and **15** at 100 ppm inhibited cyclooxygenase (COX) -1 and -2 enzymes at 4 and 6%; and 16 and 15 % respectively. The positive controls used in the COX assays were Aspirin, Celebrex and Vioxx at 180, 1.67 and 1.67 ppm, respectively, and showed 74 and 69%; 5 and 82%; 0 and 85% of COX-1 and COX-2 inhibition, respectively. Compounds **12**, **13**, **14**, **15** and **16** were isolated for the first time from *A. arborea* fruits, although compound **12** was not biologically active.

INTRODUCTION

Amelanchier arborea (Michx.) Fern. (Rosaceae), commonly called shadberry bears the edible fruits, which are consumed in the Northern America. The berries are known by many names including serviceberry, Juneberry, downy shadberry and grape pear. *A. arborea*, Downy Serviceberry, is an eastern North American large shrub or small tree reaching 8-10 meters. *A. arborea* is crossed with *A. laevis* to produce cultivars and used extensively in landscape industry in the United States. *A. arborea* fruits are 1/4 to 3/8 inch in diameter, rounded, purplish-black, slightly sweet, and ripen in late June. The *A. arborea* or shadberry was a very important fruit to the early settlers and the Native Americans. Previous research on *A. arborea* fruits was focused on the identification of the compounds of that are important to organoleptic importance. Therefore, phytochemical investigation of *A. arborea* fruits using GC/MS and sensory evaluation showed that the compounds of organoleptic importance to the aroma of the fruit were benzaldehyde, phenyl-acetaldehyde, 2-hexenal and hexanal (Parliament, 2002).

A. arborea fruits have been used in making juice, jelly and pies. There is not enough data available on the phytochemistry of *A. arborea* fruits. Therefore, we have investigated the presence of biologically active compounds in *A. arborea* fruits. In this chapter we report four biologically active compounds and β-sitosterol-3-glucoside isolated from the ethyl acetate extract of the fruits of *A. arborea*.

MATERIALS AND METHODS

General Experimental Procedures. All NMR spectra ^{1}H and ^{13}C were recorded on Varian INOVA (300 MHz) and VRX (500 MHz) instruments. ^{13}C NMR spectra were recorded at 75 and 125 MHz, respectively. Chemical shifts (^{1}H NMR) were recorded in $CDCl_3$ at 7.24, CD_3OD at 3.31, DMSO-d_6 at 2.54 and D_2O at 4.80 ppm. Coupling constants, J, are in hertz. ^{13}C NMR chemical shifts are reported in ppm relative to $CDCl_3$ at 77.0, CD_3OD at 49.0, DMSO-d_6 at 39.50 ppm. HPLC system (Waters Corp., Adena) used was equipped with a Controller, a 717 Autosampler, a 2410 RI detector and a 996 photodiode array detector and an Xterra Prep RP-18 and/or 8 column (250 x 19 mm i.d. 10μm). Data were recorded and processed using Empower Pro (Waters Corp., Milford, MA) soft ware. Merck silica gel 60 with a particle size of 35-70 μm and C18 with a particle size of 60 μm (Dychrom, Santa Clara, CA) were used for preparative medium-pressure liquid chromatography (MPLC). For preparative TLC separation, 250, 500, 1000 μm silica gel plates (Analtech, Inc., Newark, DE) were used.

Solvents used were of ACS reagent grade and purchased from Spectrum Chemical Co. (Gardena, CA). Butylated hydroxytoulene (BHT), dimethyl sulfoxide (DMSO), acetic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Celebrex and Vioxx were physician's professional samples supplied by Dr. S. Gupta. 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Alabastaer, AL), 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid from Molecular Probes (Eugene, OR). COX-1 and COX-2 enzymes were prepared in the Bioactive Natural Products and Phytoceuticals Laboratory (BNPP) from ram seminal

vesicles and prostaglandin endoperoxide H synthase-2 (PGHS-2) cloned insect cell lysate respectively.

Fruits. Fully ripened fruits of *Amelanchier arborea* were collected in mid July, 2001 in Eaton Rapids, MI. The locations of the trees are recorded in the Michigan State University, Department of Horticulture Database. The fruits were stored in plastic zip lock bags at –20° C prior to analyses.

Cyclooxygenase enzymes inhibitory assay. Cyclooxygenase enzymes inhibitory activities of the compounds isolated from *A. arborea* were evaluated using COX-1 and COX-2 enzymes. The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction with arachidonic acid as substrate was measured using a Model 5300 biological oxygen monitor (Yellow Spring Instruments, Inc., Yellow Springs, OH). The reaction mixture was consisted of 0.1 M tris, 1.0 mM phenol, 17 μg hemoglobin, the enzyme and 6 μL test sample dissolved in DMSO at 1.5 % (DMSO alone as solvent control. The reaction was performed in a 600 μL micro chamber (Instech Laboratory, Plymouth Meeting, PA) at 37 ^{0} C. After 2 min of incubation of the enzyme and test samples, 10 μL of arachidonic acid (0.25 mg/ 0.5 mL of Tris buffer) was added to initiate the reaction. Data was recorded using Quicklog for Windows (Strawberry Tree Inc., Sunnyvale, CA). Aspirin, Celebrex and Vioxx at 180, 1.67 and 1.67 ppm, respectively, were used as positive controls (Wang et al., 1999).

Lipid Peroxidation assay. Lipid peroxidation assay was performed according to the previously published method (Wang et al., 1999). Inhibition of lipid peroxidation was measured by fluorescence spectroscopy using large unilamellar vesicles (LUVs) of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine containing a fluorescent probe (3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid) and reported after 21 min as relative fluorescence compared to control. For comparison, butylated hydroxytoulene (BHT) at 2.2 ppm, was used as positive control. Briefly, the lipid and probe were dissolved in DMF and evaporated *in vacuo*. The solvent-free mixture was resuspended in 0.15 M NaCl, 0.1 mM EDTA and 0.01 M MOPS (kept over Chelex resin), subjected to ten freeze-thaw cycles using a dry ice-ethanol bath, and extruded through a 100 nm pore size membrane to form the LUVs. The assay buffer used consisted of a mixture of 100 μL HEPES buffer, 200 μL 1 M NaCl, 1.64 mL nitrogen-sparged milipore water. An aliquot of 20 μL of test sample in DMSO at 2.5 % or DMSO (control) and 20 μL of liposome suspension were mixed with the assay buffer to achieve of 100 or 10 ppm of the compound. Peroxidation was initiated by adding 20μL of $FeCl_2.4H_2O$ (0.5 mM) and the samples were gently vortexed. The fluorescence was monitored using a Turner fluorometer with a narrow band pass 360 nm filter and a sharp cut 415 nm filter at times 0, 1, 3, 6, and every three minutes thereafter for 21 min. Analyses of samples and controls were performed in duplicate. Relative fluorescence (*Ft/Fo*) was calculated by dividing the fluorescence value at a given time (*Ft*) by the value at t = 0 min. (*Fo*).

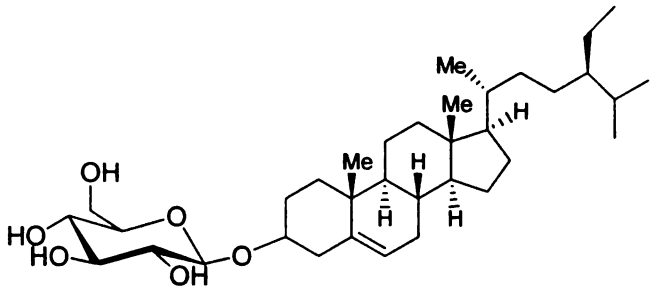
Extraction. The lyophilized fruits (148 g) were blended with hexane (500 mL) in a Waring Blender (5 min) at high speed and extracted with hexane (3x500 mL) for 24 h.

The residue was extracted with ethyl acetate (3x1000 mL) for 36 h and finally with methanol (3x1000 mL) for another 36 h. The yields of hexane, ethyl acetate, and methanol extracts after removal of the solvents were 2, 1.2, and 98 g, respectively. The TLC comparison of hexane extract showed that it contained mainly triglycerides. Therefore, it was not further analyzed. Ethyl acetate extract (1.2 g) was subjected to medium pressure liquid chromatography (MPLC) using a silica gel column (350 x 40 mm). The ethyl acetate extract (1.1 g) was dissolved in chloroform (5 mL) and applied to the column and eluted with chloroform, methanol/ chloroform gradient and methanol, respectively. The flow rate was set to 4 mL/min. The eluent was collected in fraction collector in 15 ml test tubes. Total of 180 fractions were collected. Based on TLC profiles, fractions were combined. Fraction I (186 mg, $CHCl_3$, 100 mL), II (115 mg, $CHCl_3$, 50 mL), III (37 mg, MeOH/$CHCl_3$/1/10, 100 mL), IV (66 mg, MeOH/$CHCl_3$/1/10, 340 mL), V (14.5 mg, MeOH/$CHCl_3$ /1/5, 150 mL), VI (34 mg, MeOH/ $CHCl_3$/1/5, 200 mL), VII (18 mg, MeOH/ $CHCl_3$/1/5, 45 mL), VIII (61 mg, MeOH/ $CHCl_3$/1/5, 200 mL MeOH/$CHCl_3$/1/2, 300 mL), IX (23 mg, MeOH/ $CHCl_3$/1/2, 150 mL), X (88 mg, MeOH/$CHCl_3$/1/1, 120 mL), XI (52 mg, MeOH, 130 mL), and XII (182 mg, MeOH, 350 mL). Fraction I contained mostly triglycerides and was not analyzed further.

Isolation of compounds 12, 13, 14: Precipitation of fraction V (34 mg) of ethyl acetate extract in chloroform and methanol yielded compound **12** (13 mg). Fraction II was subjected to silica gel preparative TLC (1000μm, 20 x 20cm) in acetone/hexane in

the ratio of 15/60 as the mobile phase and developed twice to yield mixtures of compounds, **13** and **14** (10.2 mg).

Compound **12.** White powder. ^{1}H NMR (DMSO-d_6): δ 5.31 (1H, brs, 6-H), 4.82 (3H, brs, 2'-OH, 3'-OH, 4'-OH), 4.38 (1H, brt, 6'-OH), 4.20 (1H, d, J = 8.0 Hz, 1'-H), 3.63 (1H, d, J = 8.5 Hz, 6a'-H), 3.45 (1H, m, 3-H), 3.00-3.40 (4H, m, 3', 4', 5', 6b'-H), 2.88 (1H, dd, J = 8.5 Hz, 8.0 Hz, 2'-H), 2.35 (1H, d, J = 13.0 Hz, 4a-H), 2.15 (1H, d, J = 13.0 Hz, 4b-H), 0.80 – 2.00 (27H, m, 1, 2, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 24, 25, 28-H), 0.94 (3H, s, H-19), 0.93 (3H, d, J=7.5 Hz, H-21), 0.81 (3H, t, J = 7.5 Hz, H-29), 0.80 (6H, d, J = 7.5 Hz, H-26, 27), 0.64 (3H, s, H-18), The spectral data confirmed that compound **12** is β-sitosterol-3-glucoside (Faizi et al., 2001).



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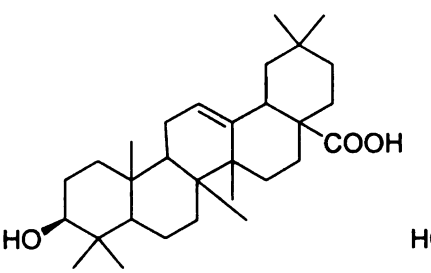
Compounds **13** and **14.** White powder. ^{1}H NMR (DMSO-d_6): δ 5.14 (1H, brs, H-12), 5.11 (1H, brs, H-12), 4.26 (brs, 2H, 3-OH), 2.99 (2H, brs, 3-H), 2.27 (1H, t, J = 6.5Hz), 2.10 (1H, m), 1.79- 1.91(6H, m), 1.37-1.65 (14H, m), 1.13-1.31 (6H, m,), 1.08 (3H, s), 1.03 (3H, s), 0.90 (3H, s), 0.89 (3H, d, J = 8.5 Hz,), 0.89 (3H, s), 0.86 (6H, s), 0.84 (3H, s),

0.81 (3H, d, J = 6.5Hz), 0.74 (3H, s), 0.71 (3H, s), 0.67 (6H, s, H-26). ^{1}H NMR of compound **13** and **14** were found to be in agreement with previously published data (Hung et al., 2001, Seebacher et al., 2003).



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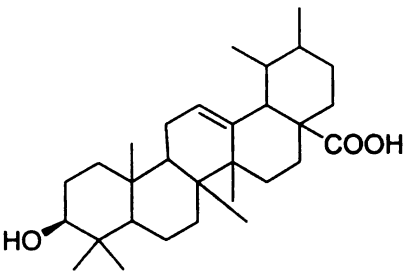

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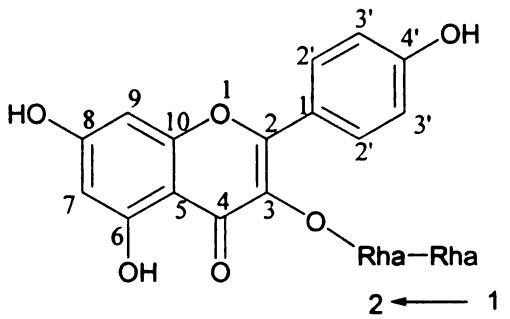
Isolation of compounds 15 and 16: Fractions IX and VIII were dissolved in methanol/water (50/50) (3ml) filtered (0.22μm) and purified by using an Xterra RP-18/8 column (250 x 19 mm, 5μm, Waters Corp.) at 35°C Fraction IX (23 mg) was subjected to repeated preparative HPLC Xterra RP-18 column using 20% CH_3CN in water with the flow rate of 3mL/min yielded compound **15** (rt 44 min, 1.7 mg). Similarly, compound **15** was purified from fraction VIII (61 mg) by repeated preparative HPLC in Xterra RP-8 using 30% CH_3CN in water with flow rate of 3mL/min yielded compound **16** (R_t = 48 min, 1.1 mg).

Compound **15.** Light yellow powder; ^{1}H NMR (DMSO-d_6/D_2O): δ 7.77 (2H, d, J = 9.0 Hz, 2'H), 6.90 (2H, d, J = 9.0 Hz, 3'H), 6.76 (br, 6-H), 6.44 (br, 8-H), δ 5.52 (br, 1"-H), 5.27 (br, 1'"- H), 3.16-3.99 (m, 8H, rhamnosyl), 1.11 (3H, d, J = 6.5Hz, 6"- H), 1.07 (3H,

d, J = 7.0 Hz, 6'''- H). The ^{1}H NMR of compound **15** was found to be in agreement with previously published data (Rao et al., 1999).



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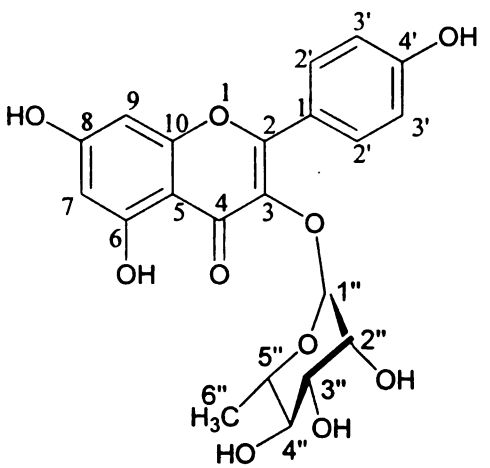
Compound 16. Light yellow powder; ^{1}H NMR (DMSO-d_6/D_2O): δ 8.04 (2H, d, J = 8.5 Hz, 2'-H), 6.87 (2H, d, J = 9.0 Hz, 3'-H), 6.36 (1H, s, 6-H), 6.13 (1H, s, 8-H), 5.30 (1H, d, J = 5.5 Hz, 1"-H), 3.12-3.96 (4H, m, 2", 3", 4", 5"-H), 1.09 (3H, d, J = 6.5Hz, 6"-H). The ^{1}H NMR of compound **16** was found to be in agreement with previously published data (Fossen et al., 1999).



16

RESULTS AND DISCUSSION

Lyophilized fruits of *A. arborea* were sequentially extracted with hexane, ethyl acetate and methanol. Based on TLC, hexane extract contained mainly tryglycerides and were identical to the triglycerides isolated from *A. canadensis.* Similarly, methanol extract contained predominantly sugars and anthocyanins based on TLC and HPLC analysis. More ever, preliminary antioxidant and lipid peroxidation inhibitory assay showed that ethyl acetate extract was the most active extract. Therefore, ethyl acetate extract was studied. further. The ethyl acetate extract was fractionated by silica gel MPLC and purification of the fractions by preparative TLC and HPLC yielded five compounds **12, 13, 14, 15,** and **16**. Precipitation of fraction V from chloroform and methanol yielded compound **12.** Fraction III was subjected to silica gel preparative TLC purification and yielded an inseparable mixture of compounds **13** and **14.** The repeated preparative HPLC of fractions IX and VIII gave compounds **15,** and **16** at R_t = 44 and 48 min respectively.

^{1}H NMR of compound **12** at 5.31, 3.45; two singlets at 0.94 and 0.64 integrated to three protons each; two doublets at 0.93 0.80 ppm and one triplet at 0.807 ppm indicated a sterol moiety in the molecule. The^{1}H NMR signal appeared as a doublet at 4.20 ppm with a coupling constant of 8.0 Hz and integrated for one proton indicated a β-glucosidic moiety in that molecule. The spectral data confirmed that compound **12** was β-sitosterol-3-*O*-β-glucoside (Faizi et al., 2001). TLC comparison of compound **12** gave an identical R_f value with an authentic sample of β-sitosterol-3-*O*-β--glucoside. Therefore,

compound **12** was identified as β-sitosterol-3-*O*-β-glucoside based on its ^{1}H NMR (Faizi et al., 2001) and TLC comparison with an authentic sample

Compounds **13** and **14** were obtained as a mixture. These compounds did not separate under various TLC system used with various solvent systems such as methanol/chloroform, acetone/hexane, ethyl acetate/hexane with varying proportions. ^{1}H-NMR was used to determine the structures of compounds **13** and **14**. The chemical shifts at 5.14 (bt) and 5.11 (bt), along with doublets and singlets at 1.08, 1.03, 0.93, 0.89 (d, J = 8.5 Hz), 0.89, 0.86, 0.84, 0.81 (d, J = 6.5Hz), 0.74, 0.71, 0.67 were indicative of a 1:1 mixture of oleanolic and ursolic acids. The ^{1}H NMR spectrum of compounds **13** and **14** were found to be in agreement with previously published data of ursolic and oleanolic acids (Hung et al., 2001).

Compound **15** was obtained as a light yellow powder and its structure was determined by ^{1}H NMR spectral studies. Two doublets at 7.77 and 6.90 ppm, integrated for two protons each, and two singlets at 6.6.76 and 6.44 ppm indicated that it contained a kaempferol moiety. Other doublets at 5.52 and 5.27 ppm, integrated for one proton each, along with two doublets at 1.11 and 1.07 ppm and integrated for three protons each suggested two rhamnose moieties in the molecule. Therefore, compound **15** was characterized as kaempferol-3-*O*-α-L- rhamnosyl (2 ←1) rhamnoside. The ^{1}H NMR of compound **15** was found to be in agreement with previously published data of kaempferol-3-*O*-α-L- rhamnosyl (2 ←1) rhamnoside (Rao et al., 1999).

Compound **16** was obtained as light yellow powder and its structure was determined using ^{1}H NMR spectral experiment. Two doublets at 8.04 and 6.87 ppm, integrated for two protons each, and two singlets at 6.34 and 6.13 ppm integrated for one proton each suggested that it contained a kaempferol moiety. Also, doublets at 5.30 and 1.09 ppm, integrated for one and three protons, respectively, indicated rhamnose moiety. The ^{1}H NMR of compound **16** was found to be in agreement with previously published data of kaempferol-3- *O*-α-L rhamnoside (Fossen et al., 1999).

Lipid peroxidation inhibitory activity assay was conducted according to previously published procedures (Arora et al, 1998, Arora et al., 1997, Wang et al., 1999). Results of lipid peroxidation inhibitory activities of *A. arborea* compounds are presented in Figures 4.1, 4.2 and 4.3, respectively.

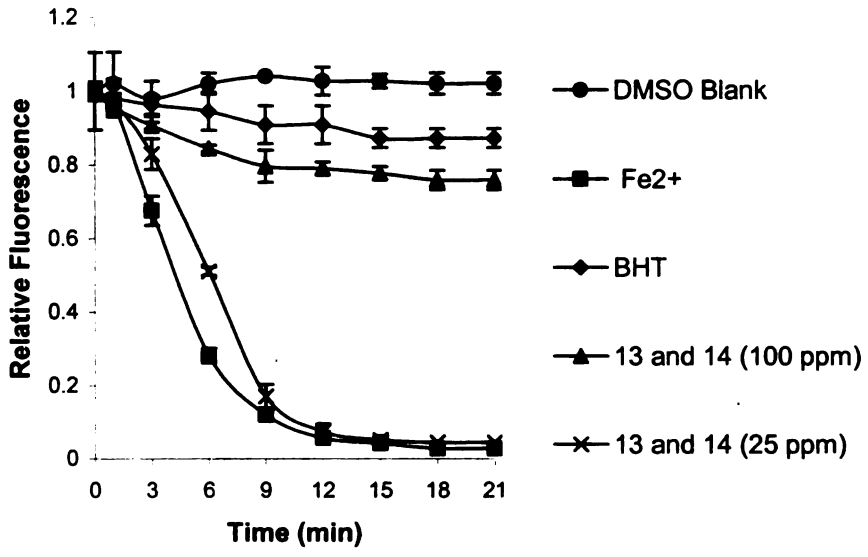


Fig 4.1 Lipid peroxidation inhibitory activity exhibited by compounds **13** and **14** from *A. arborea* assayed at 100 and 25 ppm. Activity was calculated based on relative fluorescence and compared to standard synthetic antioxidant BHT (2.2 ppm). Data represent the average ± 1 standard deviation.

All compounds isolated from *A. arborea* demonstrated lipid peroxidation inhibitory activity. However, compound **12** showed only 6.4% lipid peroxidation inhibitory activity at 100 ppm. Mixture of compounds **13** and **14** showed 76 and 4% of lipid peroxidation inhibitory activity at 100 and 25 ppm, respectively. Compounds **13** and **14** were reported as good antioxidants (Wu et al., 1982; Chen et al., 1992; Balanehru et al., 1992; Balanehru et al., 2001; Hung et al., 2001). Hung et al. (2001) observed 38 and 29 % antioxidant activity for ursolic and oleanolic acid compared to BHA, 91% at 0.2 g/kg when antioxidant activity were determined by thiocyanate method.

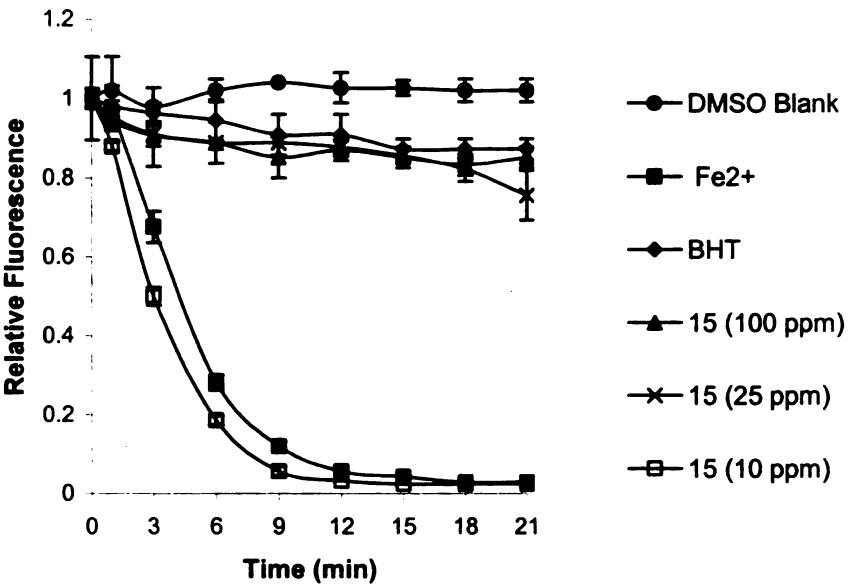


Fig 4.2 Lipid peroxidation inhibitory activity exhibited by compound **15** from *A. arborea* assayed at 100, 25 and 10 ppm. Activity was calculated based on relative fluorescence and compared to standard synthetic antioxidant BHT (2.2 ppm). Data represent the average ± 1 standard deviation.

Compound **15** showed 85, 75 and 24 % of lipid peroxidation inhibitory activity at 100, 25 and 10, ppm, respectively. Compound **16** showed 83, 73 and 6% of lipid peroxidation inhibitory activity at 100, 25 and 10 ppm, respectively. From these results, it was evident that the inhibitory activity of monosachharide and disaccharides of kaempferol were not much different. The flavonoids are well known for their antioxidant activities (Pietta, 2000; Wang et al., 1999; Arora et al., 1998; Cao et al., 1997, Saija et al.,

1994; Bors et al., 1990; Afanas'ev et al., 1989). The antioxidant activity of compound **16** is consistent with previously published report (Braca et al., 2002).

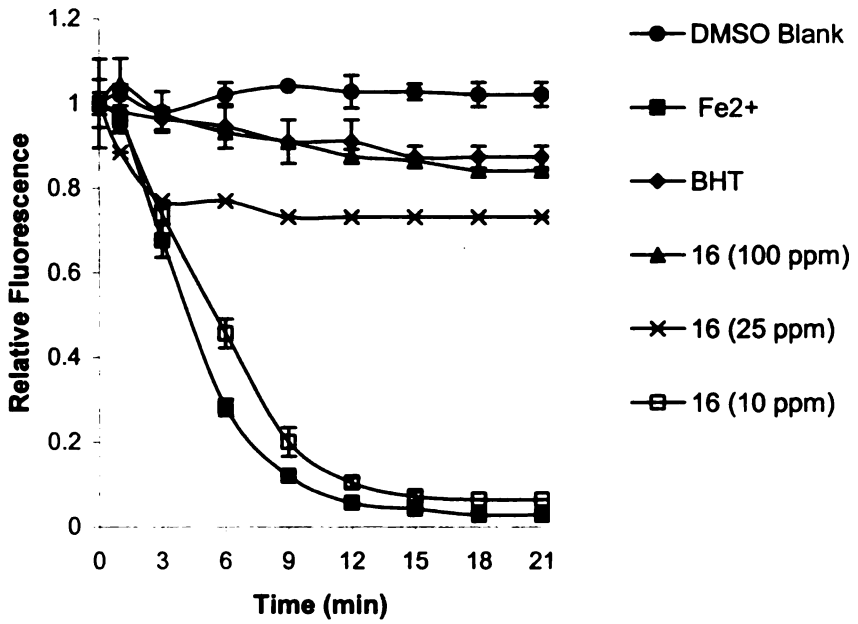


Fig 4.3 Lipid peroxidation inhibitory activity exhibited by compounds **16** from *A. arborea* assayed at 100, 25 and 10 ppm. Activity was calculated based on relative fluorescence and compared to standard synthetic antioxidant BHT (2.2 ppm). Data represent the average ± 1 standard deviation.

COX-1 and COX-2 inhibitory assays of compounds were conducted according to previously published procedures (Wang et al., 1999).

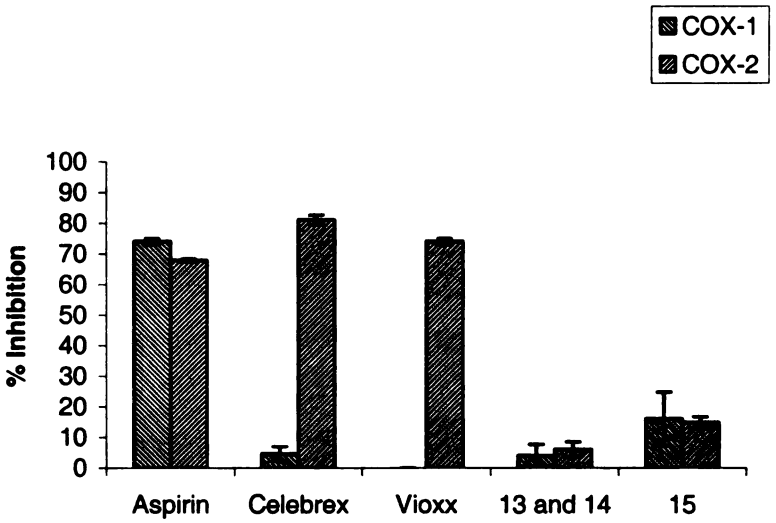


Fig 4.4 COX-1 and COX-2 inhibitory activities of compounds isolated from *A. arborea* fruits. Compounds were assayed at 100 ppm and compared to NSAID standards aspirin (180 ppm), Celebrex™ (1.67 ppm) and Vioxx™ (1.67 ppm). Data represent the average ± 1 standard deviation.

The in vitro COX-1 and COX-2 inhibitory activities of compounds **13** and **14**, and **15** demonstrated weak anti-inflammatory activity as shown in Figure 4.4. The mixture of compounds **13** and **14**, and compound **15** at 100 ppm demonstrated 4 and 16% inhibition of COX-1. Similarly, mixture of compounds **13** and **14**, and compound **15** showed 6 % and 15 % COX-2 inhibition, respectively. The standards aspirin, celebrex, and vioxx at 180, 1.67 and 1.67 ppm showed 74, 5 and 0% COX-1 inhibition and 69, 82 and 85 % COX-2 inhibition. Compounds **12** and **16** did not show any COX inhibitory activities.

Compounds **13** and **14,** a mixture of ursolic acid and oleanolic acid did not show much COX inhibitory activities at test concentration in our studies. Previous investigation showed that ursolic and oleanolic acids were good anti-inflammatory compounds and preferentially inhibited COX-2 activity at higher concentrations (Ringborn et al., 1998). The flavonoid, compound **15,** showed weak COX-1 and COX-2 inhibitory activities, but compound **16** was inactive. This is the first report of COX enzyme inhibitory activity of compounds **15**. Compound **12** did not show any COX inhibitory activities which is in agreement with the previously published result (Villaseñor et al., 2002). It has been reported that compound **12** showed analgesic activity (Villaseñor et al., 2002). The acetic acid-induced writhing test showed that β-sitosterol-3-glucoside **12** decreased the number of squirms induced by acetic acid by 73.0% at a dose of 100 mg/kg mouse.

The compounds from *A. arborea,* β-sitosterol-3-glucoside **12**, mixture of oleanolic acid **13** and ursolic acid **14**, kaempferol-3-*O*-α-L-rhamnosylrhamnoside **15**, and kaempferol-3- *O*-α-L rhamnoside **16**, are reported for the first time from this plant. The results from the lipid peroxidation inhibitory activity assay of the compounds isolated from *A. arborea* indicate that *A. arborea* fruits are a potential source of bioactive compounds. The findings of these compounds and anthocyanins in *A. arborea* substantiated that consumption of *A. arborea* fruits might be beneficial to health.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The genus *Amelanchier* (Rosaceae), represented by about 25 species, is found in North America, Europe and Asia. *Amelanchier* fruits have been a valuable fruits for the native and early settlers of the North Americans. The dried berries were used similar to raisins and prunes, as an ingredient of pemmican and also in the making of juice and jelly. Horticulturally, *Amelanchier* spp. are grown in gardens, orchards, and shelterbelts.

The research prior to our present study was mainly focused on phytochemistry of *Amelanchier* spp. A brief report of this study is presented in **Chapter 1.** The Literature review summarized in **Chapter 1** is also focused on biological activity of *A. alnifolia*. Based on preliminary assays conducted in our laboratory and literature review, we rationalized that the fruits of *A. canadensis, A. alnifolia* and *A. arborea* contain bioactive compounds. Our research resulted in the isolation of compounds with lipid peroxidation and cyclooxygenase inhibitory activity from the fruits of *A. alnifolia*, *A. arborea*, and *A. canadensis.*

Bioassay-directed isolation and purification of anthocyanins/anthocyanidins from methanol extracts of *A. canadensis* fruits and their bioactivities are presented in **Chapter 2**. Three anthocyanins/anthocyanidins, cyanidin-3-*O*-β-galactopyranoside (**1**), cyanidin-3-*O*-β-glucopyranoside (**2**), and cyanidin (**3**), were isolated from the methanol extract of *A. canadensis*. The structures of these compounds were determined by spectral

techniques such as ^{1}H- and ^{13}C- NMR, and MS. In addition, characterization, quantification and lipid peroxidation and cyclooxygenase inhibitory assays were performed on anthocyanins present in *A. alnifolia*, *A. arborea* and *A.* canadensis fruits collected in Michigan during 2000. Fruits of *A. alnifolia*, *A. arborea* and *A. canadensis* were extracted with acidic methanol to isolate anthocyanis. The anthocyanins present in the fruits of these three species were characterized and quantified by HPLC techniques. The level of anthocyanins, **1** and **2** in Michigan grown *A. alnifolia*, *A. arborea* and *A. canadensis* were determined by HPLC method and found 155 and 54, 390 and 0, and 165 and 48 mg in 100 g of fresh fruits, respectively.

Compounds **1**, **2** and **3** were isolated as red powder, and at 10 ppm, demonstrated 70, 75, and 78 % lipid peroxidation inhibitory activity, respectively. Commercial antioxidant such as BHA, BHT, and TBHQ at 180, 2.2, and 1.67 ppm gave 89, 87, and 98 % lipid peroxidation inhibitory activity, respectively. Similarly, compounds **1**, **2**, and **3** showed 50 and 18; 46 and 18; and 96 and 75 % COX-1 and COX-2 inhibitory activity, respectively. Commercial NSAIDs aspirin, celebrex and vioxx at 180, 1.67 and 1.67 ppm showed 74 and 69; 5 and 82; and 0 and 85 % COX-1 and COX-2 inhibitory, respectively.

Bioassay directed isolation, purification, lipid peroxidation and cyclooxygenase inhibitory assays of isolated compounds from fruits of *A. canadensis* are presented in **Chapters 3**. The structures of purified compounds were determined by spectral techniques such as ^{1}H- and ^{13}C- NMR, DEPT, HMQC, HMBC, MS. *A. canadensis* fruits were sequentially extracted with water, acidic methanol and ethyl acetate. The separation

and purification of ethyl acetate extract of *A. canadensis* fruits by various chromatographic techniques yielded three compounds, 1,3-dilinoleoyl 2-olein (**4**), 1,3-diloyl 2-linolein (**5**), and β-sitosterol (**6**). Five compounds, 5-hydroxymethyl-2 furfural (**7**), 5-sorbitol-furan-2-carboxaldehyde (**8**), 5-mannitol-furan-2-carboxaldehyde (**9**), and 5-(D-α-glucopyranosyloxymethyl) furan-2-carboxaldehyde (**10**), 5-(D-β-glucopyranosyloxymethyl) furan-2-carboxaldehyde (**11**), were isolated from water-soluble fraction of methanol extract of *A. canadensis*. Compounds **7**, **10** and **11** were isolated for the first time from *A. canadensis* fruits. Also, compounds **8** and **9** were isolated from *A. canadensis* as an inseparable mixture for the first time. The structures of compounds **8** and **9** were determined by various spectral techniques such as ^{1}H- and ^{13}C-NMR, DEPT, HMQC, HMBC, MS and chemical methods.

Mixture of compounds **8** and **9**, **10** and **11** at 100 ppm showed 83 and 26% lipid peroxidation inhibitory activity, respectively. Compound **4**, mixtures of compounds **8** and **9**, **10** and **11** at 100 ppm gave 39 and 0; 68 and 49; 63 and 45 % of COX-1 and COX-2 inhibitory activity, respectively. Compound **7** at 10 ppm gave 5 and 13 % of COX-1 and COX-2 inhibitory activity, respectively. Compounds **5**, **6**, and acetates of compound **8** and **9** did not exhibit COX inhibitory activities.

Bioassay directed isolation, purification, lipid peroxidation and cyclooxygenase inhibitory assays of isolated compounds from fruits of *A. arborea* are presented in **Chapters 4**. *A. arborea* fruits were sequentially extracted with hexane, ethyl acetate and methanol. The separation and purification of ethyl acetate extract of *A. arborea* fruits by

various chromatographic techniques such as TLC, MPLC, and HPLC yielded five compounds, β-sitosterol-3-glucoside (**12**), mixture of oleanolic acid (**13**) and ursolic acid (**14**), kaempferol -3-*O*- α- L- rhamnopyranosyl (1 ←2) rhamnopyranoside (**15**) and, kaempferol-3-*O*- α- L- rhamnopyranoside (**16**). The structures of these compounds were determined by ^{1}H- NMR. Mixture of compounds **13** and **14**, and compounds **15** and **16** at 100 ppm showed 76, 85 and 83 % lipid peroxidation inhibitory activity, respectively. A dose response study of **13-16** was carried for lipid peroxidation inhibitory activities. Mixture of compounds **13** and **14**, and compound **15** at 100 ppm showed 4 and 6; 16 and 15 % of COX-1 and COX-2 enzyme inhibitory activity, respectively. Compound **12** did not exhibit COX inhibitory activities.

Lipid peroxidation and COX enzyme inhibitory activities of the compounds isolated from *Amelanchier* fruits showed that the fruits contained many bioactive compounds. This research revealed that *Amelanchier* fruits contained appreciable amount of anthocyanins, which are known to possess anticarcinogenic, anti-inflammatory and antioxidant activities. Similarly, the seeds contained appreciable quantities of triglycerides that are composed of mainly unsaturated fatty acids. Compounds 5-hydroxymethyl 2-furfural and its derivatives are present in *A. canadensis* fruits and they showed lipid peroxidation and cyclooxygenase enzyme inhibitory activities. The bioassays results suggest that *Amelanchier* fruits may possess health benefit when included in the diet. However, further research is needed to prove the beneficial effects of these fruits in in vivo studies.

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