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HEALTH-BENEFICIAL COMPOUNDS IN *CORNUS* FRUITS

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SHAIJU KAKKANADAN VAREED

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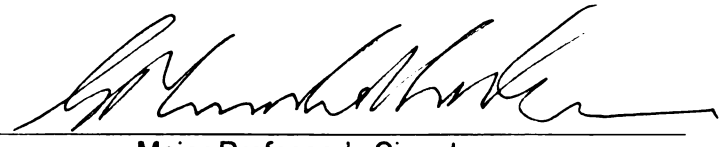
PhD degree in HORTICULTURE



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HEALTH-BENEFICIAL COMPOUNDS IN *CORNUS* FRUITS

By

Shaiju Kakkanadan Vareed

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ABSTRACT

HEALTH-BENEFICIAL COMPOUNDS IN *CORNUS* FRUITS

By

Shaiju Kakkanadan Vareed

The genus *Cornus,* commonly known as dogwood, is widely distributed in eastern Asia, and eastern and western parts of North America. Anecdotal reports indicate that several plants in *Cornus* species are effective for the treatment of various illnesses. Although, *Cornus* plants are well known for its medicinal properties, very little work has been done on the isolation and identification of bioactive compounds. A bioassay-directed investigation of *Cornus kousa*, *Cornus mas*, *Cornus controversa* and *Cornus alternifolia* fruits resulted in the isolation and characterization antioxidant, anti-inflammatory, anti-cancer and anti-diabetic anthocyanins, delphinidin 3-*O*-glucoside (**1**), delphinidin 3-*O*-rutinoside (**2**), delphinidin 3-*O*-galactoside (**3**), cyanidin 3-*O*-galactoside (**4**), pelargonidin 3-*O*-galactoside (**5**) and cyanidin 3-*O*-glucoside (**6**). Acid hydrolysis of the anthocyanin- enriched fruit extracts resulted in the isolation of anthocyanidins, delphinidin (**7**), cyanidin (**8**), pelargonidin (**9**), petunidin (**10**) and malvidin (**11**). The anthocyanins in *Cornus* fruits extracts were quantified by HPLC. The amount of anthocyanins **1**, **2** and **6** in *C. alternifolia, C. controversa* and *C. mas* were determined to be 8-10 times higher than other common fruit sources of anthocyanins.

An investigation of the non-pigmented fraction of *C. kousa* ripened and unripened fruits resulted in the isolation of ursolic acid (**12**), β-sitosterol (**13**), cornin (**14**), kaempherol 3-*O*-rhamnoside (**15**), myricetin 3-*O*-rhamnoside (**16**), and kaempherol 3-*O*-glucoside (**17**), and stenophyllin (**18**). Both ursolic acid (**12**) and β-sitosterol (**13**)

were also isolated from the ripened fruits of *C. kousa*, *C. controversa* and identified in *C. alternifolia*.

Anthocyanins and anthocyanidins were tested for lipid peroxidation, cyclooxygenase (COX-1 and-2) enzymes and tumor cell proliferation inhibitory activities. Anthocyanins **1** and **2** inhibited lipid peroxidation by 71 and 68%, respectively, at 50 μg/ml. Similarly, they inhibited COX-1 enzymes by 39 and 49% and COX-2 enzyme by 54 and 48%, respectively, at 100 μg/mL. In addition, anthocyanins **1** and **2** displayed 50% growth inhibition (IC_{50}) at 21 and 38, 25 and 30, 50 and 76, 60 and 100, and 75 and 100 μg/mL, against HCT-116 (colon), MCF-7 (breast), NCI-H460 (lung), SF-268 (Central Nervous System, CNS), and AGS (stomach), human tumor cell lines, respectively. The most active anthocyanidin malvidin (**11**) inhibited colon, breast, lung, central nervous system and stomach cell growth by 76, 75, 68, 41, and 69%, respectively, at 200 μg/mL. Anthocyanins and anthocyanidins were also studied for their ability to induce insulin secretion by rodent pancreatic β-cells in vitro. The results indicated that anthocyanins **1** and **6** were the most effective insulin secretagogues among the anthocyanins and anthocyanidins tested.

The bioassay guided investigation on *Cornus* fruits indicated that these plants could be cultivated as alternate crop to tart cherries to yield fruits for health beneficial anthocyanins. Ornamental plants in the United States are an untapped and valuable resource for phytochemicals and functional foods. Our results on the health benefits of *Cornus* fruits suggest that *Cornus* plants should be an ideal candidate for the diversification of agricultural crops.

To my parents

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It is a great pleasure and privilege to express my deep sense of gratitude and profound indebtedness towards my advisor Dr. Muraleedharan G Nair for his valuable guidance, direction, constructive criticism and inspiring encouragement to me, without which it would have been impossible to complete the present work.

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KEY TO ABBREVIATIONS

BHA	Butylated hydroxylanisole
BHT	Butylated hydroxytoluene
BuOH	Butanol
$CHCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
CH_3CN	Acetonitrile
cv	Cultivar
DMSO	Dimethyl sulfoxide
D_2O	Deuterium oxide
EIMS	Electron impact ionization mass spectrometry
EtOH	Ethanol
EtOAc	Ethyl acetate
Gal	Galactose
Glc	Glucose
HCl	Hydrochloric acid
HCOOH	Formic acid
HOAc	Acetic acid
HPLC	High performance liquid chromatography
H_2O	Water
H_2SO_4	Sulphuric acid
MeOH	Methanol

MPLC	Medium pressure liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
ODS	Octadecyl silica
PTLC	Preparative thin layer chromatography
Si	Silica
spp.	Species
TBHQ	*tert*-Butylhydroquinone
TFA	Trifluroacetic acid
TLC	Thin layer chromatography
UV	Ultraviolet

INTRODUCTION

The genus *Cornus* (dogwood) belongs to the family Cornaceae that contains about 58 species (Fan and Xiang, 2001). These plants grow mainly in the northern temperate regions of the world. The name "*Cornus*" is the Latin name for Cornelian cherry. The word 'cornu' is for horn (cornu), and refers to the hardiness of its wood (http://plants.usda.gov). *Cornus mas, Cornus officinalis* and *Cornus kousa* bear edible fruits that are consumed in many parts of Europe and Asia (Seeram et al., 2002, Du et al., 1974). All *Cornus* plants produce colorful and attractive flowers and fruits. They are relatively resistant to pest infection compared to many other garden plants and hence are widely grown as ornamental trees in many landscapes.

Although *Cornus* plants are used only for decorative purposes in the United States of America, these plants are used in traditional medicines around the world. Many of the *Cornus* species were reported to have medicinal use and some were used as an ingredient in preservatives and sweets (Bailey, 1977). For example, the extracts of *C. mas* fruits were used for food and cosmetic preparations in Europe (Polinicencu et al., 1980). A decoction from the pulp of *C. mas* fruits was used for the treatment of arthritis, fever and a wide range of other ailments (Millspaugh, 1974). The fruits of *C. officinalis* were used for more than 2000 years in Chinese herbal medicine. *C. officinalis* was used mainly to reduce menstrual bleeding and unusually active secretions including sweating, excessive urine, spermatorrheoa, premature ejaculation and various illnesses associated with liver and kidney (Kim and Kwak., 1998). The fruits of *C. officinalis* were also

reported to possess antibacterial, antifungal, hypotensive, antitumor, astringent and diuretic activities (Kim and Kwak., 1998).

Anthocyanins are the most significant biologically active compounds reported from *Cornus* species fruits. The brilliant red colors of berries, cherries, vegetables and fruits are due to anthocyanins. Recent in vitro and in vivo studies indicated that these compounds possess antioxidant, anti-cancer and anti-inflammatory activities (Seeram et al., 2002, Kang, et al., 2003, Kamei, et al., 1998). Therefore, the consumption of anthocyanin-containing foods as dietary ingredient is considered to be highly beneficial to maintaining health and feeling of wellness.

A detailed literature review of the phytochemicals in *Cornus* spp. revealed that most of the research has been on the isolation of compounds from *C. officinalis*. Very little is known about the chemistry of other *Cornus* plants and the biological activities of the compounds present in them. Moreover, most of the health claims associated with various *Cornus* plants are anecdotal. Based on the previous research on *C. mas* in Dr. Nair's laboratory at Michigan State University, it is my hypothesis that fruits from native *Cornus* species have the potential to yield compounds with anti-carcinogenic, anti-diabetic, anti-inflammatory, and antioxidant activities. In order to test my hypothesis, I have conducted bioassay-directed isolation and characterization of compounds in *Cornus* fruits by using cylooxygenase enzymes, lipid peroxidation and cell proliferation inhibitory and insulin secretion assays. Therefore, the objectives of my research were to conduct bioassay- directed isolation and identification of compounds in *Cornus* fruits using chromatographic and spectral methods, and determine the anticarcinogenic, anti-diabetic, anti-inflammatory, and antioxidant efficacies of purified compounds. By taking

into account of the biological activities associated with *Cornus* plants, my proposed research may lead to planting of *Cornus* trees as an alternate crop for fruit production with bioactive compounds in addition to its current application as an ornamental tree. Also, it is expected that the present work should add to the existing knowledge on the bioactive constituents in *Cornus* fruits.

This dissertation is comprised of a series of chapters detailing the results of this research. **Chapter 1** is a literature review in which the botany, chemical constituents, traditional use, and pharmacological importance of *Cornus* plants are outlined. In **Chapter 2,** the results of characterization and quantification of anthocyanins from *C. kousa, C. florida, C. controversa* and *C. alternifolia* fruits are presented. Detailed investigation of insulin secretion, lipid peroxidation, cyclooxygenase, and cell proliferation inhibitory activities of anthocyanins and anthocyanidins in *Cornus* spp. fruits are presented in **Chapter 3**. All non-pigmented compounds isolated from the unripened and ripened fruits of *C. kousa* are presented in **Chapter 4**. The data in **Chapter 2** has been accepted for publication in *Life Sciences*. The data in **Chapter 3** were published in *Journal of Agricultural and Food Chemistry* and *Life Sciences.* **Chapters 2, 3** and **4** are presented as manuscripts, each with an introduction, material and methods, results and discussion sections. Finally, the conclusions derived from my research on *Cornus* fruits are summarized in **Chapter 5**.

CHAPTER ONE

LITERATURE REVIEW

Introduction

Humankind has benefited from the inherent qualities of over 5000 plants used for drugs, foods, fibers and dyes, which include more than 14 *Cornus* species and their varieties. The genus *Cornus* (dogwood) contains about 58 species, mostly hermaphroditic shrubs or small trees, and is widely grown in North America, Asia, Europe, South America and Africa (Fan and Xiang, 2001). Many *Cornus* spp. with medicinal values are used as one of the components in the preparation of preservatives and sweets (Bailey, 1977). The tree bark of *Cornus alternifolia* and *Cornus florida* has been chewed by many to release analgesic compounds to treat headaches, toothaches, and other pains (Moerman, 1998). The bunchberry dogwoods, *Cornus canadensis*, possess anti-convulsive, anti-fever and analgesic properties in addition to a number of other medicinal uses. Other species such as *Cornus rugosa*, *Cornus racemosa*, *Cornus foemina* and *Cornus amomum* have been used for the treatment of various illnesses associated with kidney, stomach, throat, and lungs, respectively (Moerman, 1998). Many ethnobotanical uses of dogwood species in China have also been reported (www4.ncsu.edu). For example, *Cornus officinalis*, "Zhu Yu" or "Zao Pi" in Chinese medicine, was used as an astringent tonic for impotence, and to treat spermatorrhea, lumbago, vertigo, and night sweats. Fruits of *Cornus oblonga* have been used as a substitute for 'Zao Pi.' Seed oil of several dogwood species including *C. oblonga*,

Cornus alba, *Cornus hemsleyi*, *Cornus walteri*, and *Cornus wilsoniana* have used commercially in various parts of China. The bark from *C. oblonga* and *Cornus capitata* were also used as folk medicines to treat arthritis and injuries. In Japan, several species of *Cornus* including *Cornus controversa* were used to treat the swelling in the body (www4.ncsu.edu). The fruits of several subspecies of *Cornus hongkongensis* are edible and used for wine brewing.

Dogwoods carry attractive flowers and fruits. They are popular ornamental plants in a variety of landscapes (Powell, 1997). The dogwood plants beautifully display a wide range of color in their bracts, foliage and twigs during various seasons of the year. Generally, dogwood plants grow up to a height of 20 - 30 feet and require little maintenance.

The flowering dogwood, *Cornus florida*, is the most popular dogwood in the United States (http://www.ces.ncsu.edu/fletcher/staff/rbir/cornus.html). It is native to the eastern and central United States. *C. florida* flower represents the state flower of North Carolina. Dogwood industry in Tennessee yields more than $30 million annually to the state income from the sale of *C. florida* cultivars (www4.ncsu.edu).

The botany, chemical constituents, and biological activities of *Cornus* plants are outlined in this chapter.

Botany of *Cornus* plants

The *Cornus* species are deciduous shrubs and occasionally grow as trees. All *Cornus* species have opposite leaf system except *Cornus alternifolia*, which have an

alternate leaf system. The fruits of all *Cornus* species are berry-like with white, blue, red or black drupe with one or two seeds.

Dogwood is a member of the genus *Cornus* and it belongs to the family Cornaceae. The family Cornaceae has three genera: *Cornus*, *Aucuba* and *Helwingia.*

Table 1.1 Classification of *Cornus* plant

Kingdom	Plantae
Phylum	Embryophyta
Subkingdom	Tacheobionta
Superdivision	Spermatophyte
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Corneals
Family	Cornaceae
Genus	*Cornus. L*

Bir et al., classified dogwoods according to the season in which they are most attractive. For example, *C. alba* (Tartarian dogwood) and *C. sericea* (Redosier dogwood) are considered winter dogwoods, which are generally grown for their colorful stems (http://www.ces.ncsu.edu/fletcher/staff/rbir/cornus.html). Stem color of *C. alba* and *C.*

sericea change from creamy yellow, orange and shades of red to deep red during winter. The spring dogwood, *C. florida* and *C. kousa* (Oriental dogwood) display attractive and colorful bracts and foliage depending on the cultivar during the spring season.

Among dogwoods, *C. florida, C. kousa*, and *C. nuttallii* (Pacific dogwood) are the three most widely cultivated species in the United States. More than 100 cultivars of *C. florida* have been cultivated in United States (www4.ncsu.edu). Another dogwood, *C. kousa*, native to eastern Asia, is a smaller tree with white or pink bracts and dark green foliage late in the spring season. Popular cultivars of *C. kousa* include 'Greensleeves,' 'Milky Way,' 'Blue Shadow', 'Rosabellla,' 'Rubra,' and 'Rosea'. The fruits of *C. kousa* are red and compound, in contrast to those of *C. florida* and *C. nuttallii*, which are clusters. The fruits from *C. kousa* are edible and sweet, and used in wine making in several Asian countries (Du et al., 1974). *C. nuttallii*, grows mainly in western regions of North America. The numbers of *C. nuttallii* cultivars are much fewer than that of either *C. florida* or *C. kousa*. However, it is still valued as an ornamental dogwood due to its large, colorful bracts. Hybrid varieties of *Cornus* species are common in many parts of United States especially in Michigan (www4.ncsu.edu). The natural hybrids of *C. amomum* x *C. racemosa* (*C.* x *arnoldiana* Rehder) and *C. rugosa* x *C. stolonifera* (*C.* x *slavinii*) are frequently found in Michigan and neighboring states. Wagner reported another hybrid between *C. racemosa* (gray dogwood) and *C. rugosa* (round-leaved dogwood) from Michigan (Wagner, 1990). A number of arboreta or botanical gardens have several examples of natural hybrids of other *Cornus* species.

Chemical Constituents of *Cornus* Species

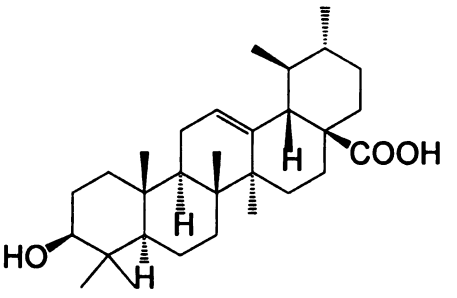
A detailed phytochemical investigation of plants belonging to *Cornus* spp. has resulted in a diverse group of secondary metabolites. Compounds reported from *Cornus* spp. to date are summarized in Table 1.2. The secondary metabolites reported from *Cornus* are divided in to six groups based on structural similarities. These groups include terpenes, steroids, saponins, iridoids, tannins, flavanoids and related compounds.

Terpenes

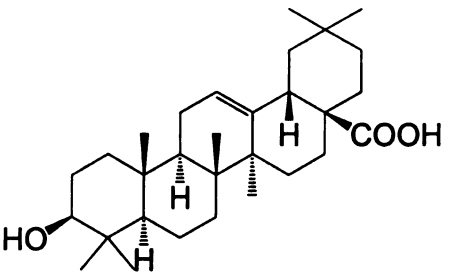
Terpenes are important group of plant secondary metabolites and ubiquitous in several plants. Many of these terpenoids such as menthol, pinene, limonene and camphor impart characteristic odors and flavors unique to a given plant. The building block of a terpenoid is isoprene (CH_2=C-(CH_3)-CH=CH_2). Monoterpene, sesquiterpene, diterpene, triterpene, and tetraterpene are some of the classifications depending on the number of isoprene units in its molecule. The terpenes reported from *Cornus* species are represented in Table 1.2.

Most of the reported terpenes in *Cornus* were isolated from its stems. Yan et al., developed an HPLC method for the determination of ursolic acid, an important constituent of Chinese herb, *C. officinalis*, using Hypersil ODS- column with acetic acid-water-glacial acetic acid mobile system (Yan et al., 2003). This analytical method showed high accuracy and reproducibility and is employed for the quality control of various herbal medicines in China. Another terpenoid compound, betulinic acid, was isolated from the dry roots and bark of *C. macrophylla* (Venketesh and Merchant, 1984). Betulinic acid and ursolic acid were also isolated from the ether fraction of dry roots of

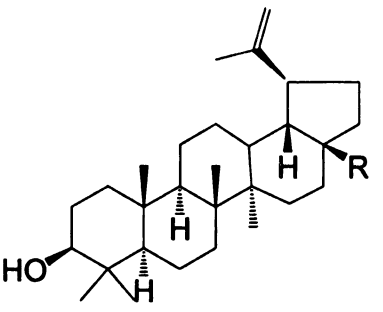
C. excelsa (Dominguez et al., 1981). A pentacyclic trihydroxy triterpenic acid, arjunolic acid, was isolated from the stem of *C. capitata* and exhibited potent insecticidal activity (Bhakuni et al., 2002). This terpenoid was isolated from the hexane extract using silica column and characterized by NMR and mass spectroscopic methods. A detailed phytochemical investigation of the stems of *C. capitata* afforded the terpenoid, 3 β-acetoxy-23-oxo-lup-20 (29)-ene or 3β-acetoxy-24-oxo-lup-20 (29)-ene, in addition to oleanolic and acetyl oleanolic acids (Bhakuni et al., 1988).



Ursolic acid

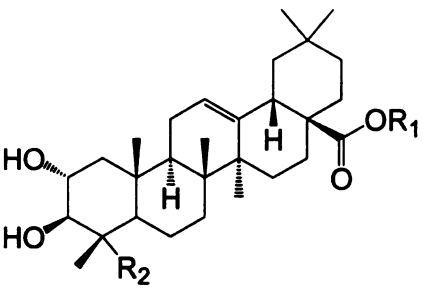


Oleanolic acid



R=CH_2OH Betulin

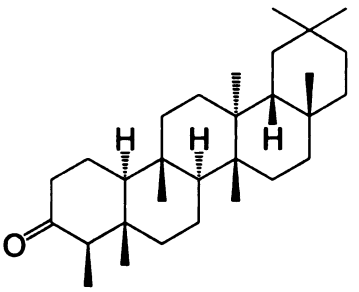
R=COOH Betulinic acid



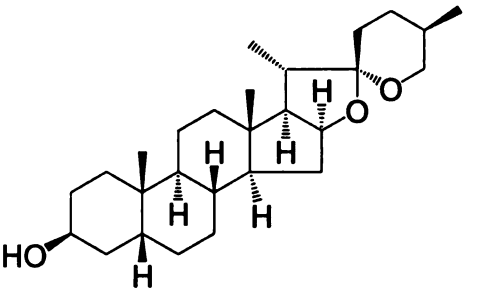
R_1=H, R_2=CH_2OH Arjunolic acid

R_1=H, R_2=CH_3 Maslinic acid

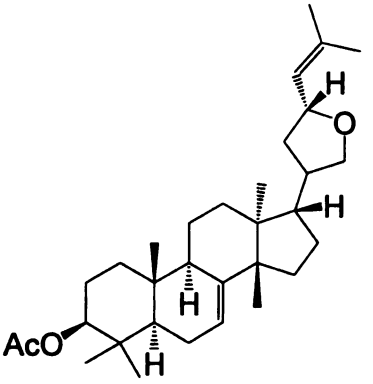
Figure 1.1 Terpenes found in *Cornus* species

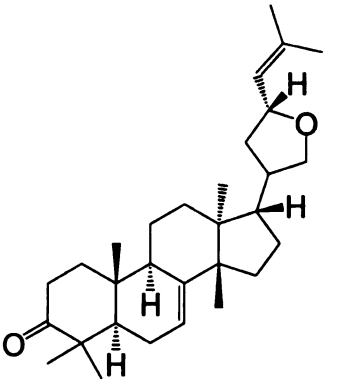


fridelin

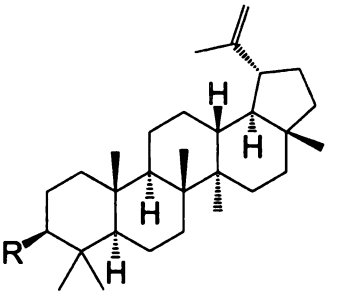


smilagenin





3 β Acetoxy-21, 23-Epoxytirucalla-7, 24-diene 21, 23-Epoxytirucalla-7, 24-diene-3-one



R=OH, 3 β Hydroxy-23-oxo-lup-20 (29)-ene (Lupeol)

R=OAc, 3 β Acetoxy-23-oxo-lup-20 (29)-ene

Figure 1.1. (cont'd). Terpenes found in *Cornus* species

Table 1.2 Chemical constituents reported from *Cornus* species

Compound	Source	Plant parts	References
TERPENES			
ursolic acid	*C. officinalis*	fruits	Yan et al., 2003
	C. excelsa	roots	Dominguez et al., 1981
	C. florida	flower, bracts	Sando et al., 1936
	C. mas	ariel parts	Zorina et al., 1966
olenolic acid	*C. capitata*	stem	Bhakuni et al., 1988
acetyl oleanolic acid	*C. capitata*	stem	Bhakuni et al., 1988
betulin	*C. officinalis*	fruits	Endo and Taguchi, 1973
	C. capitata	stem	Yamahara et al., 1981
betulinic acid	*C. capitata*	stem	Yamahara et al., 198127
	C. excelsa	roots	Dominguez et al, 1981
epibetulinic acid	*C. florida*	bark	Sando et al., 1936
	C. capitata	stem	Yamahara et al., 1981

Table 1.2 (cont'd)

Compound	Source	Plant parts	References
arjunolic acid	*C. capitata*	stem	Bhukani et al., 2002
maslinic acid	*C. capitata*	stem	Yamahara et al., 1981
friedelin	*C. capitata*	stem	Yamahara et al., 1981
3 β acetoxy-21, 23- epoxytirucalla-7, 24-diene	*C. capitata*	stem, bark	Bhukani et al., 1987
3 β acetoxy-23-oxo-lup-20 (29)-ene	*C. capitata*	stem	Bhukani et al., 1988
3 β acetoxy-23-oxo-lup-20 (29)-ene	*C. capitata*	stem	Bhukani et al., 1988
cornusol	*C. controversa*	flowers	Kurihara et al., 1978
smilagenin	*C. capitata*	stem	Yamahara et al., 1981
lupeol	*C. capitata*	stem	Yamahara et al., 1981
SAPONINS			
filferin	*C. excelsa*	roots	Dominguez et al., 1981
arjunglucoside	*C. controversa*	stem	Jang et al., 1998

Table 1.2 (cont'd)

Compounds	Source	Plant parts	References
sarsapogenin *O*-*β*-D-glucosyl-*O*-*β*-D- galactoside	*C. florida*	bark	Hostettmann et al., 1978
sarsapogenin *O*-*β*-D-xylosyl-*O*-*β*-D-galactoside	*C. florida*	bark	Hostettmann et al., 1978
IRIDOIDS			
morroniside	*C. officinalis*	fruits	Zhao and Xue, 1992
loganin	*C. officinalis*	fruits	Li et al., 1999
cornin	*C. nuttalli*	leaves	Jenson et al., 1973
	C. florida	flowers	Jenson et al., 1973
	C. capitata	leaves	Tanaka et al., 2001
	C. kousa	leaves	Jenson et al., 1973
dihydrocornin	*C. nuttalli*	leaves	Jenson et al., 1973
hastatoside	*C. florida*	flowers	Sando et al., 1936
6α-dihydrocornic acid.	*C. nuttalli*	leaves	Stermitz and Krull., 1998
	C. capitata	roots	Tanaka et al., 2001

Table 1.2 (cont'd)

Compound	Source	Plant Parts	References
6β-dihydrocornic acid.	*C. capitata*	roots	Tanaka et al., 2001
monotropein	*C. candensis*	fruits	Stermitz et al., 1998
scandoside	*C. candensis*	fruits	Stermitz et al., 1998
scandoside methyl ester	*C. candensis*	fruits	Stermitz et al., 1998
galioside	*C. candensis*	fruits	Stermitz et al., 1998
sweroside	*C. officinalis*	fruits	Xu et al., 1995
7-dehydrologanin	*C. officinalis*	fruits	Li, 1999
cornuside	*C. officinalis*	fruits	Hatano., 1989
STEROLS			
β-sitosterol	*C. officinalis*	fruits	Zhao et al., 1992
	C. capitata	stem	Bhakuni et al., 1986
	C. sanguniea	fruits	Viano and Gaydou., 1984
	C. florida	seed	Robertson and Soliman., 1939

Table 1.2 (cont'd)

Compound	Source	Plant Parts	References
stigmastanone	*C. capitata*	stem	Bhakuni et al., 1986
TANNINS			
β-glucogallin	*C. kousa*	root culture	Ishimuaru et al., 1993
isoterchibin	*C. officinalis*	fruits	Hatano et al., 1990
tellimagrandin II	*C. officinalis*	fruits	Hatano et al., 1989
gemin D	*C. officinalis*	fruits	Hatano et al., 1989
	C. controversa	leaf	Lee at al., 1995
tellimagrandin I	*C. officinalis*	fruits	Hatano et al., 1989
1, 2, 6, tri-*O*-galloyl -β-D-glucose	*C. officinalis*	fruits	Hatano et al., 1989
	C. controversa	leaf	Nakaoki and Morita., 1958
1, 2, 4, 6- Tetra-*O*-galloyl -β-D-glucose	*C. officinalis*	fruits	Hatano et al., 1989
1-*O*-galloyl-β-D-glucose	*C. officinalis*	fruits	Hatano et al., 1989
	C. controversa	leaf	Nakaoki and Morita., 1958

Table 1.2 (cont'd)

Compound	Source	Plant parts	References
1, 2, 3 tri-*O*-galloyl-β-D-glucose	*C. officinalis*	fruits	Hatano et al., 1989
	C. controversa	leaf	Nakaoki and Morita., 1958
1, 6-Di-*O*-galloyl-β-D-glucose	*C. officinalis*	fruits	Hatano et al., 1989
	C. controversa	leaf	Nakaoki and Morita., 1958
	C. capitata	callus	Tanaka et al., 1997
1, 2, 4, 6- penta-*O*-galloyl -β-D-glucose	*C. capitata*	callus and root culture	Tanaka, 1997
FLAVANOIDS			
quercetin	*C. controversa*	stem, leaf	Nakaoki and Morita., 1958
	C. walterli	bark	Choi et al., 1998
	C. florida	stem, flowers, bracts	Mudry and Schilling., 1983 Sando et al., 1936

Table 1.2 (cont'd)

Compound	Source	Plant parts	References
isoquercetin	*C. controversa*	stem, leaf	Nakaoki and Morita., 1958
	C. walterli	bark	Choi et al., 1998
	C. mas	stem	Egger and Keil., 1969
quercetin glucoside	*C. canadensis*	flowers	Bain and Denford., 1979
quercetin galactoside	*C. florida*	leaves	Mudry and Schilling., 1983
	C. canadensis	flowers	Bain and Denford., 1979
	C. mas	flowers	Egger and Keil., 1969
quercetin sophoroside	*C. canadensis*	flowers	Bain and Denford., 1979
quercetin gentiobioside	*C. canadensis*	flowers	Bain and Denford., 1979
querceturone	*C. mas*	flowers	Egger and keil., 1969
quercetin 3-*O*- glucosyl rhamnoside	*C. mas*	flowers	Egger and keil., 1969
kaempferol	*C. florida*	flowers,bracts	Mudry and Schilling., 1983
kaempferol glucoside	*C. canadensis*	flowers	Bain and Denford., 1979

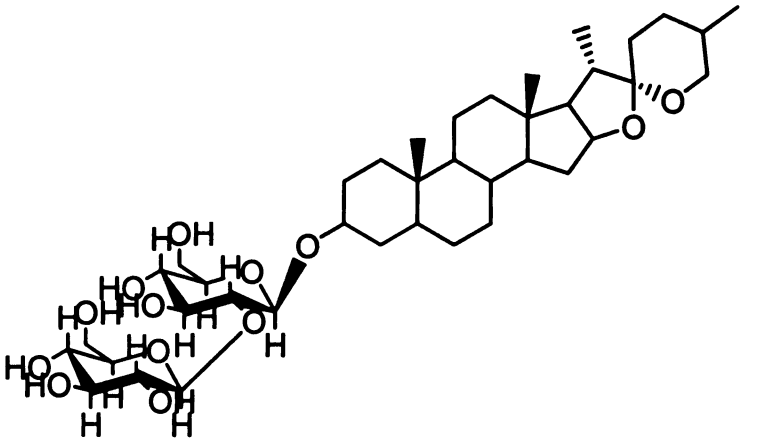
Table 1.2 (cont'd)

Compounds	Source	Plant parts	References
kaempferol galactoside	*C. florida*	leaves	Mudry and Schilling., 1983
kaempferol arabinoside	*C. canadensis*	leaves	Bain and Denford., 1979
kaempferol glucosyl rhamnoside	*C. mas*	flowers	Egger and Keil., 1969
hyperoside	*C. controversa*	leaf	Lee et al., 1995
	C. walterli	stem	Choi et al., 1998
rutin	*C. controversa*	stem, leaf	Nakaoki and Morita., 1958
	C. walterli	stem	Choi et al., 1998
	C. mas	flower	Egger and Keil., 1969
	C. sanguniea	flower	Delaveau and Paris 1961
(+)-catechin	*C. walterli*	stem	Choi et al., 1998
	C. kousa	root cultures	Ishimuaru et al., 1993
procyanidin-3	*C. kousa*	root culture	Ishimuaru et al., 1993

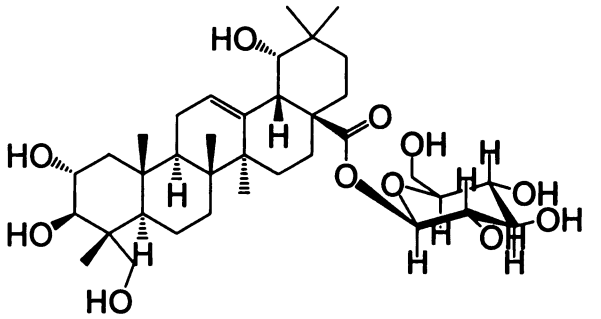
Saponins

Saponins are glycosides of sapogenins. The sapogenin can be a steroid (C-29) or a triterpene (C-30) and the sugar can be glucose, galactose, pentose, or methylpentose. They are considered as natural surfactants, or detergents. Some saponins are known to reduce the feed intake and growth rate of nonruminant animals while others are not very harmful. For example, the saponins found in oats and spinach are implicated to accelerate the body's ability to absorb calcium and silicon, thus assisting in digestion. On the other hand, certain pasture weeds contain substantial quantities of dangerous saponins that are toxic to several animal species. Saponins bind with cholesterol so it cannot be reabsorbed into the system and is excreted from the body. Saponins are being widely researched for cancer prevention and cholesterol control. The saponins reported from *Cornus* species are represented in Table 1.2.

Hostettmann, et al., reported two molluscicidal saponins from *C. florida* (Hostettmann, 1978). These compounds were isolated from the methanol extract of the bark of the plant and characterized as sarsapogenin O-β-D-xylopyranosyl-(1→2)- β-D-galactopyranoside and sarsapogenin O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside by spectroscopic and chemical methods. Another saponin, arjunglucoside was isolated from *C. controversa* (Jang et al., 1998). Sequential extraction of dry roots of *C. excelsa* with ether and methanol afforded a serious of phytochemicals including sarsaponin-*O*-galactosylxyloside (filiferin) (Domoinguez, 1981). Saponins from various *Cornus* spp. are represented in Fig. 1.2



sarsaponin-*O*-galactosylxyloside (filiferin)



arjunglucoside

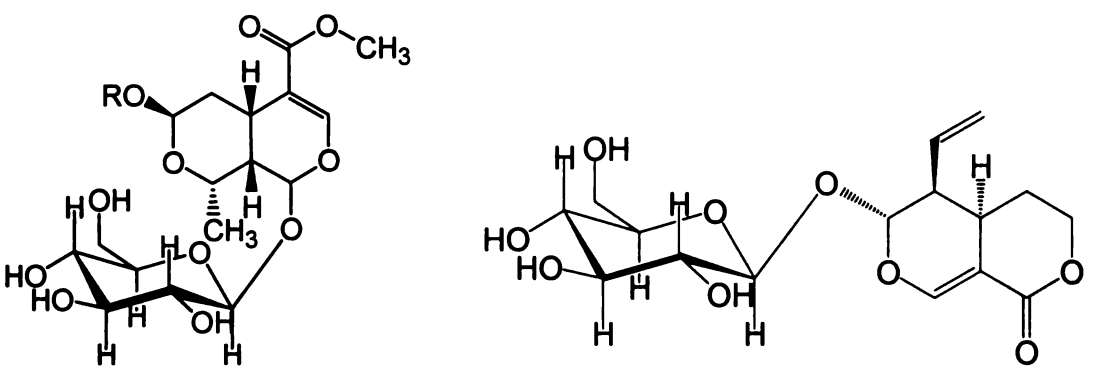
Figure 1.2 Saponins found in *Cornus* species

Iridoids and Iridoid glycosides

Iridoids are cyclopetanoid monoterpene secondary metabolites of plants. Based on chemical structures they are classified into iridoid glycoside, nonglycosidic (aglycones) iridoids, secoiridoids, and bisoridoids. A detailed literature search revealed that plants in the genus *Cornus* are also potential sources of iridoids and its glucosides.

Many iridoids reported from *Cornus* spp. are specifically from *C. capitata*. The iridoids detected in *Cornus* spp. are shown in Table 1.2.

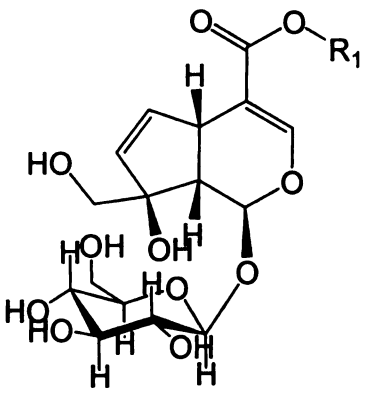
Jenson et al., characterized two glycosides, cornin and phlorin, from the leaves and twigs of *C. capitata* (Jenson et al., 1973). Cornin was reported from *C. kousa* as well (Jenson at al., 1973). The chemical structures of these iridoids were determined by NMR and mass spectroscopic methods. The iridoid glycoside, dehydromorroniaglycone, along with several other iridoids, loganin, 7-dehydrologanin, 7-*O*-methylmorronisde, was reported from *C. officinalis* (Li et al., 1999). Zhang et al, developed an HPLC method by using Kromasil column with 0.05 M NaH_2PO_4:acetonitrile (6.4:1) isocratic mobile system, for the simultaneous determination of morroniside and loganin in *C. officinalis* (Zhang et al., 1999). The iridoids found in *Cornus* spp. are represented in Fig 1.3.



R=H Morroniside Sweroside

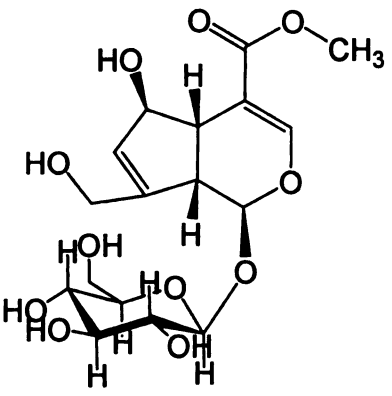
R=CH_3 7-*O*-methyl morroniside

Figure 1.3 Iridoids found in *Cornus* species

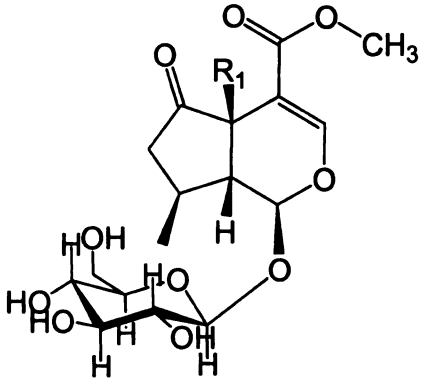


R_1=OH Monotropein

R_1=CH_3 Galioside

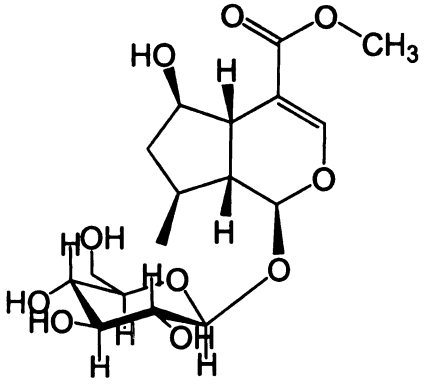


Scandoside methyl ester

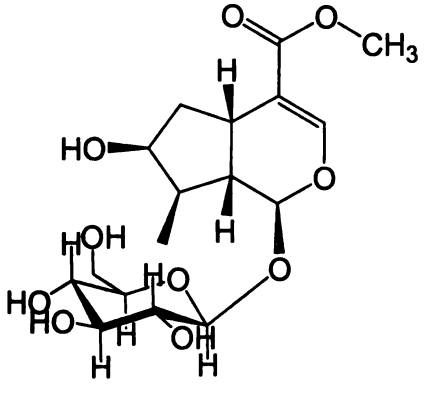


R_1=H Cornin

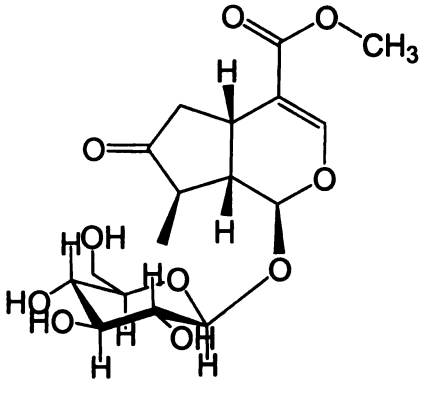
R_1=OH Hastatoside



Dihydrocornin



Loganin



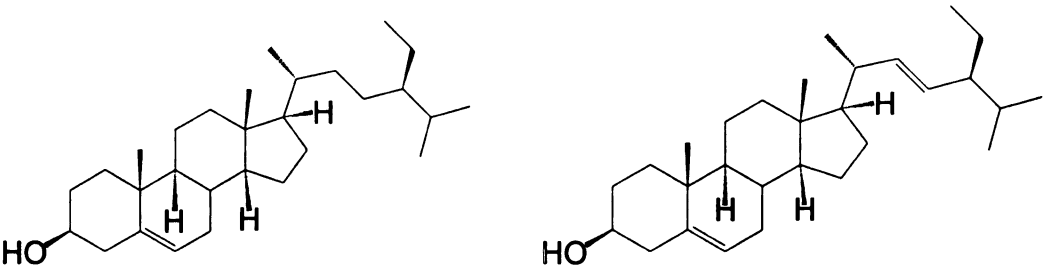
7-Dehydrologanin

Figure 1.3 (cont'd). Iridoids found in *Cornus* species

Sterols

Phytosterols produce a wide spectrum of biological activities in plants and animals. In the natural state, they are bound to the fibers of the plant and for this reason, they are difficult to desorb from the fibers. Seeds are the richest source of the sterols and sterolins. Many animal and human studies showed that phytosterols reduce serum or plasma cholesterol and low density lipoprotein (LDL) cholesterol levels (Ling and Jones, 1995). The major sterols reported from *Cornus* spp. are shown in Table 1.2.

β-sitosterol and stigmasterol are the two major sterols reported from *Cornus* spp. Among these sterols, β-sitosterol is very common and present in many *Cornus* spp., including *C. officinalis, C. capitata, C. sanguniea* and *C. florida* (Xu et al., 1995, Robertson et al., 1939, Yamahara et al., 1981).



β-sitosterol stigmasterol

Figure 1.4 Sterols found in *Cornus* species

Tannins

Tannins are naturally occurring plant polyphenols. They are classified into hydrolysable and condensed tannins (proanthocyanidins). In hydrolyzable tannins, gallic acid or ellagic acid is bonded directly to sugar units and is susceptible to hydrolysis. On the other hand, condensed tannins are oligomers or polymers of flavonoid units and are

not susceptible to hydrolysis. Condensed tannins are more widely distributed than hydrolysable tannins. The tannins found in various *Cornus* plants are represented in Table 1.2.

Many condensed tannins have been isolated from *Cornus* spp. *C. capitata*, an ornamental plant, decorating the mountainsides of Himalayas, is considered as a source of tannins (Bhakuni et al., 2002). Another species, *C. controversa, is* reported to be a good source of gallotannins (Lee et al., 1995, Nakaoki and Morita, 1958). A series of substituted gallotannins including gallic acid, 1-*O*-galloyl-β-D-glucose, 1,6-di-*O*-galloyl-β-D-glucose, 1,2,3-tri-*O*-galloyl-β-D-glucose, 1,2,6-tri-*O*-galloyl-β-D-glucose, 3,4,6-tri-*O*-galloyl-β-D-glucose, eugeniin, and gemin D were isolated from the aqueous extract of the leaves of *C. controversa*. The tannins found in *C. controversa* are represented in Table 1.3

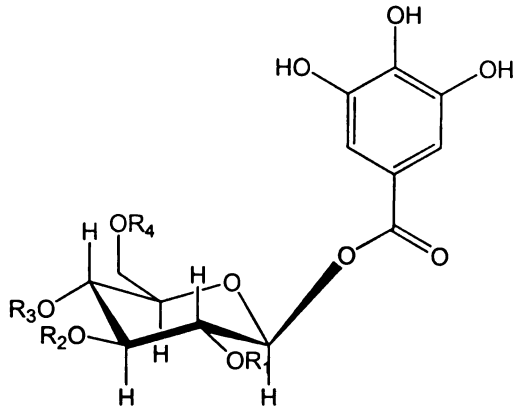


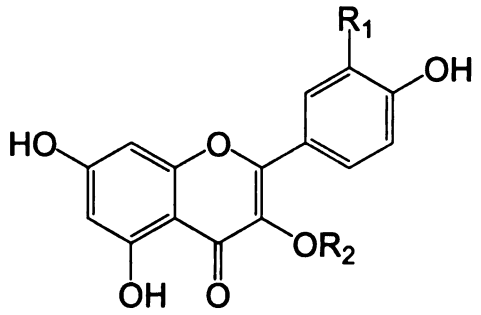
Table 1.3. Common tannins in *Cornus* species

R_1	R_2	R_3	R_4	Tannin
H	H	H	H	*β*-glucogallin
H	H	H	gallic acid	1, 6-di-*O*-galloyl-*β*-D-glucose
gallic acid	gallic acid	H	H	1, 2, 3 tri-*O*-galloyl-*β*-D-glucose
H	H	gallic acid	gallic acid	1, 4, 6 tri-*O*-galloyl-*β*-D-glucose
gallic acid	H	H	gallic acid	1, 2, 6 tri-*O*-galloyl-*β*-D-glucose
gallic acid	H	gallic acid	gallic acid	1, 2, 4, 6 tri-*O*-galloyl-*β*-D-glucose

Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in plants. They are grouped into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanins and chalcones. More than 4,000 flavonoids have been identified, many of which occur in fruits, vegetables, tea, coffee, and also contain in beverages such as beer, wine and fruit drinks. The flavonoids have aroused considerable interest recently because of their beneficial effects to human health. They are known to possess antiviral, anti-allergic, anti-platelet, anti-inflammatory, anti-tumor and antioxidant activities (Harbone and Williams, 2001, Conklin, 2000, Kim et al., 2004)

The flavonoids, kaempferol 3-*O*-galactoside and quercetin 3-*O*-galactoside, have been reported from the bracts and leaves of *C. florida* (Mudry and Schilling, 1983). It is known that bracts have higher amount of flavonoids compared to leaves, which indicated that the role of flavonoids in these plants as pollinator attractants. Quercetin 3-*O*-galactoside was reported from the bark of red-osier dogwood, *C. stolonifera* (Stermitz and Krull, 1998). Grigorescu et al., identified quercetin 3-*O*-rhamnosyl glucoside and kaempferol 3-*O*- rhamnosyl glucoside from the flowers of *C. mas* from Rumania (Grigorescu, 1972). Common flavonoids isolated from *Cornus* species represented in Fig.1.5

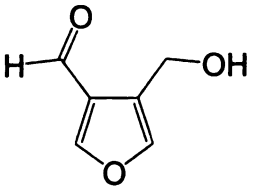


R_1=H	R_2=gal	Kaempferol 3-*O*- galactoside
R_1=H	R_2=glu	Kaempferol 3-*O*- glucoside
R_1=H	R_2= ara	Kaempferol 3-*O*- arabinoside
R_1=H	R_2=glu→rha	Kaempferol 3-*O*- glucosyl rhamnoside
R_1=H	R_2=H	Kaempferol
R_1=OH	R_2=gal	Quercetin 3-*O*-galactoside
R_1=OH	R_2=glu	Quercetin 3-*O*-glucoside
R_1=OH	R_2= ara	Quercetin 3-*O*-arabinoside
R_1=OH	R_2=glu→rha	Quercetin 3-*O*- glucosyl rhamnoside
R_1=OH	R_2=glu	Quercetin

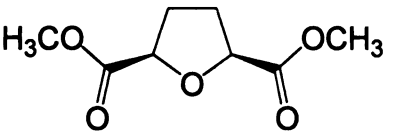
Figure 1.5 Flavanoids found in *Cornus* species

Kim et al., reported a new furan derivative, Di-methyl-tetrahydrofuran-2, 5 dicarboxylate along with a known compound, 5-hydroxy methyl furfural from the fruits

of *C. officinalis* (Kim and Kwak, 1998). Furan derivatives isolated from *C. officinalis* are given below (Fig 1.6).



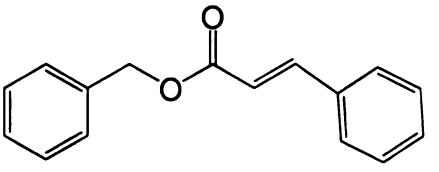
5-hydroxy methyl furfural



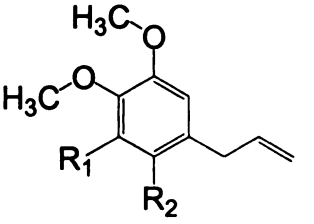
Di-methyl-tetrahydrofuran-2,5dicarboxylate

Figure 1.6 Furan derivatives found in *C. officinalis*

Several volatile flavor compounds have been isolated from the fruits of *C. officinalis* by gas chromatographic method (Mayazawa and Kameoka, 1989). The major volatile compounds reported from *C. officinalis* were benzyl cinnamate, isobutyl alcohol, isoamyl alcohol, furfural, phenethyl alcohol, methyl eugenol, isoasarone and elemicin.

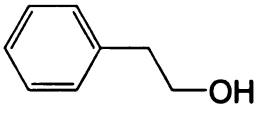


Benzyl cinnamate



$R_1 = R_2 = H$ Methyl eugenol

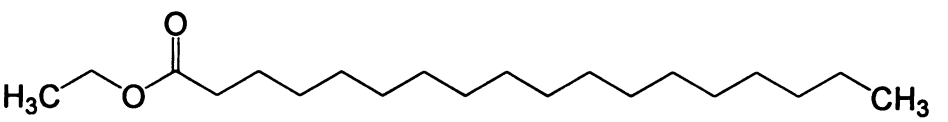
$R_1 = OCH_3$, $R_2 = H$ Elemicin



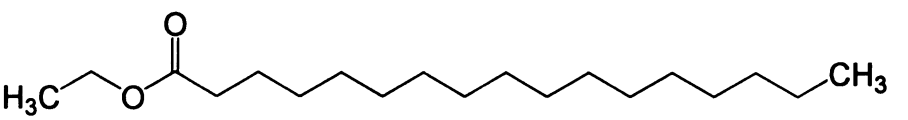
Phenethyl alcohol

Figure 1.7 Representative Volatile compounds found in *Cornus* species

Two lipid-soluble compounds, ethyl octadecanoate and ethyl heptadecanoate were reported from *Cornus cervi* (Long et al., 1991) (Fig. 1.8). These compounds were isolated from *C. cervi* by chromatographic methods and characterized by GC-MS, IR, and NMR spectroscopic methods.



Ethyl octadecanoate

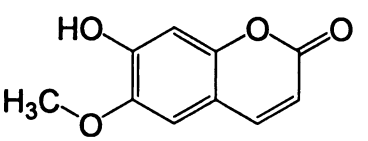


Ethyl heptadecanoate

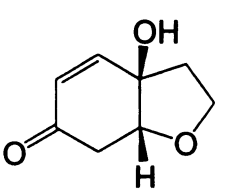
Figure 1.8 Aliphatic esters found in *Cornus* species

Dominguez, et al., reported the isolation of 7-hydroxycadelene from *C. excelsa* roots (Dominguez et al., 1981). It was isolated from the methanolic extract by using normal phase chromatography and characterized by NMR spectroscopy.

Scopoletin and a cytotoxic constituent halleridone were isolated from the Korean and Japanese dogwood, *C. controversa* (Nishino et al., 1988) (Fig 1.9). These compounds were isolated from the methanolic extract of leaves, purified by silica gel column chromatography, and characterized by NMR and mass spectroscopic methods.



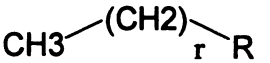
Scopoletin



Helleridone

Figure 1.9 Cytotoxic compounds found in *Cornus* species

An anti-inflammatory agent, n-hentriacontane, a plant growth promoter, triacontanol, triacontanoic acid, tetracosanoic acid, pholoroglucinol and gallic acid were isolated from the stem of *C. capitata* and characterized by chemical, NMR and mass spectroscopic methods (Yamahara et al., 1981 and Bhakuni et al., 2002).



R=CH_3, r = 29 *n*-Hentriacontane

R=OH, r = 29 *n*-Triacontanol

R=COOH, r = 28 *n*-Triacontanoic acid

R=COOH, r = 22 *n*-Tetraconsanoic acid

Figure 1.10 Aliphatic compounds found in *Cornus* species

Gas chromatography has been used to analyze various long chain fatty acids, alcohols, aldehydes and alkenes from flowers and bracts of *C. florida* and *C. sanguinea* (Delaveau and Paris, 1961). Most of these compounds were unbranched lipophilic constituents such as lauric, palmitic, stearic, oleic, linoleic and linolenic acids.

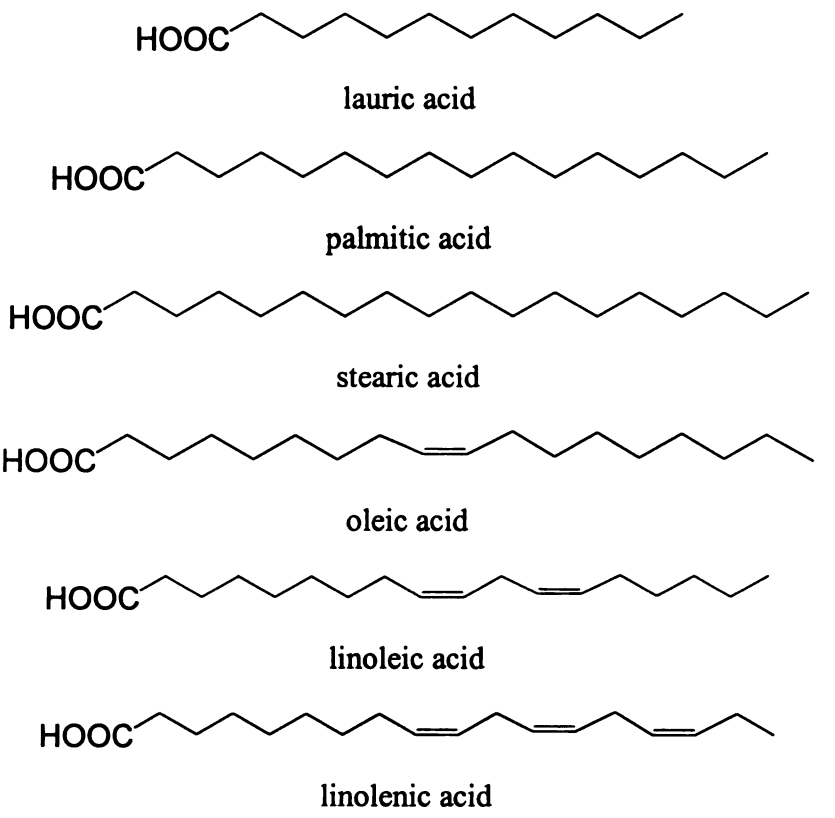


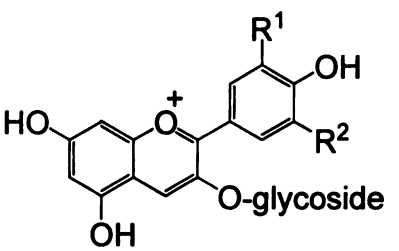
Figure 1.11 Fatty acids found in *Cornus* species

Inositol and scyllitol were reported from the alcoholic extracts of flowers and bracts of *C. florida* (Sando, 1926). Inositol is involved in the glucuronic acid and pentose phosphate pathways that are responsible for the production of various compounds including glucose and glucuronolactone.

Anthocyanins

Anthocyanins constitute one of the important groups of biologically active natural pigments. They are water soluble compounds responsible for the colors of many fruits and vegetables. Even though they are common in fruits and flowers, they may also

occur in roots, stems, leaves and bracts. Aglycons of anthocyanins are known as anthocyanidins. Delphinidin, cyanidin, pelargonidin, petunidin, peonidin and malvidin are the common anthocyanidins which occur in nature. The sugars that are present include glucose, galactose, rhamnose, and arabinose (Mazza and Miniati, 2000). The sugars provide additional sites for modification as they may be acylated with acids such as *p*-coumaric, caffeic, ferulic, sinapic, acetic, malonic or *p*-hydroxybenzoic acid. Because of the diversity of glycosylation and acylation, there are at least 300 naturally occurring anthocyanins. The color and stability of anthocyanins in solution depends on pH. Anthocyanins found in *Cornus* spp. are represented in Table 1.4.



	R^1	R^2
Delphinidin	OH	OH
Cyanidin	OH	H
Pelargonidin	H	H
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	OH

Figure 1.12 Anthocyanins found in *Cornus* species.

Table 1.4 Anthocyanins reported from *Cornus* species

Anthocyanins	*Cornus* Plant	References
Delphinidin 3-*O*-galactoside	*C. mas, C. officinalis, C. florida, C. alba*	Seeram et al., 2002, Du et al., 1974, Du et al., 1974
Delphinidin 3-*O*- glucoside	*C. controversa, C. alternifolia, C. kousa, C. florida,*	Du et al., 1975, Du et al., 1974, Du et al., 1973
Delphinidin 3-*O*- rutinoside	*C. alternifolia*	Du et al., 1975
Cyanidin 3-*O*-galactoside	*C. mas, C. officinalis, C. controversa, C. alternifolia, C. florida, C. canadensis, C. suecica, C. alba*	Seeram et al., 2002, Du et al., 1975, Du et al., 1974
Cyanidin 3-*O*-glucoside	*C. kousa, C. florida,*	Du et al., 1974
Cyanidin 3-*O*-arabinoside	*C. canadensis, C. suecica*	Du et al., 1975
Cyanidin 3-*O*-rutinoside	*C. florida, C. alba*	Du et al., 1974, Du et al., 1975
Cyanidin 3-*O*- robinobioside	*C. candensis*	Du et al., 1974
	C. mas, C. canadensis	Du et al., 1973, Du et al., 1974

Table 1.4 (cont'd)

Anthocyanins	*Cornus* Plant	References
Cyanidin3-*O*-glucosyl rhamnoside	*C. mas*	Du et al., 1973
Pelargonidin 3-*O*-galactoside	*C. mas, C. officinalis,*	Seeram et al., 2002
	C. controversa, C. canadensis	Du et al., 1974
Pelargonidin 3-*O*- glucoside	*C. canadensis, C. kousa*	Du et al., 1974
Pelargonidin 3-*O*-robionobioside	*C. mas, C. canadensis*	Du et al., 1973, Du et al., 1974
Pelargonidin 3-*O*-rutinoside	*C. canadensis*	Du et al., 1974
Pelargonidin 3-*O*-sophoroside	*C. canadensis*	Du et al., 1974
Pelargonidin 3-*O*-galactosyl rhamnoside	*C. mas*	Du et al., 1973
Petunidin 3-*O*-galactoside	*C. florida*	Du et al., 1974
Petunidin 3-*O*-rutinoside	*C. alternifolia*	Du et al., 1975
Peonidin 3-*O*-glucoside	*C. florida*	Du et al., 1974
Malvidin 3-*O*- galactoside	*C. florida*	Du et al., 1974

Biological activities of *Cornus* species

The genus *Cornus* is a rich source of biologically active compounds. Several species in this genus have been used in traditional medicines in many Asian countries. For example, *C. officinalis* and *C. controversa* have been used in Chinese and Korean traditional medicines. Numerous bioactive anthocyanins, flavanoids, terpenes, saponins, iridoids and tannins have been isolated from several of *Cornus* species. The fermented *C. mas* fruits have been used as a beverage in Turkey (Millspaugh, 1974) and the extracts of *C. mas* fruits are used in European cosmetics. The pulp of *C. mas* was processed for the treatment of arthritis, fever and a wide range of other ailments. Also, it was used for the treatment of senility, lumbago, diabetes, cystitis and tinnitus. Limonene and kaurene were the important odorants found in *C. mas* (Seeram et al, 2002). An important Chinese herb, *C. officinalis,* was used to reduce active secretions including copious sweating, excessive urine, spermatorrheoa and premature ejaculation, etc. It was also used for the treatment of various illness associated with kidney and liver. The fruits were reported to possess antibacterial, antifungal, hypotensive, antitumor, astringent and diuretic activities (Post et al., 1995).

Anthocyanins are one of the major biologically active compounds found in all *Cornus* species. As in the case of cherries and berries, fruits of *Cornus* species contain substantial quantities of anthocyanins. Anthocyanins have several beneficial roles in plants and animals. They protect the plants from the harmful effect of UV radiation (Burger and Edward., 1996). They enhances fertilization and seed dispersal through birds and other organisms. Anthocyanins were reported to possess a wide range of biological activities including antioxidant, anti-inflammatory, anti-cancer and anti-

diabetic activities (Wang et al., 1998; Kamei et al., 1995). Therefore, the use of anthocyanin containing foods as part of diet may be beneficial to human health. This has resulted in the phytoceutical and botanical supplement industries investigating fruits that have a high content of anthocyanin for purposes of formulating new commercial products. Anthocyanins are important ingredients in several herbal folk medicines which have been used in 12^{th} century to induce menstruation (Nahaishi, 2000).

Antioxidant Activity

Galloylglucosides from the roots of *C. capitata* were analyzed for antioxidant and radical scavenging activities (Tanaka et al., 2000). The antioxidant activity of galloylglucosides was higher than that of tannic acid, used as a common medicine. Among various extracts used for study, the methanolic extract of *C. capitata* root showed highest anti-oxidant activity. These results suggested the existence of polyphenols or secondary metabolites with strong antioxidant activity in the extracts of *Cornus* species. The radical scavenging activity of the root extract of *C. capitata* was found to increase with the polyphenols concentration. This revealed that polyphenols including galloylglucose was responsible for the radical scavenging activity in *C. capitata*. The phytochemical investigation of polyphenol content of eight *Cornus* species indicated that mono-galloylglucose, *β*-glucogallin, was the major polyphenol. Similarly *C. capitata* leaves contained large amounts (1.46% as dry weight) of hydrolysable tannin 1, 2, 3, 4, 6-penta-*O*-galloyl-*β*-D-glucose. The level of this tannin was 2-10 times higher in *C. capitata* than in other *Cornus* species (Tanaka et al., 1998). The methanolic extracts of 100 medicinal plants were screened for antioxidant activity using Fenton's reagent/ethyl

linoleate system as well as for free radical scavenging activity using the 1,1,-diphenyl-2-picryl hydrazyl (DPPH) free radical generating system and showed that *C. officinalis* fruits extract has highest activity (Kim et al, 1997). The methanolic extracts of the peel and seeds of *C. officinalis* were also analyzed by oven test methods in soybean oil and lard, for antioxidant properties. Antioxidant activities of both of these extracts were comparable to common synthetic antioxidants, BHA, BHT and TBHQ. Further studies of the methanol extract indicated that gallic acid and methyl gallate were responsible for the antioxidant activity (Sahng et al., 1990).

Antimicrobial Activity

The methanolic extracts of *C. officinalis* fruits inhibited the growth of *Bacillus dysentriae, Staphalococci aureus* and *Escherichia coli* (Mau et al., 2001). An extract mixture, prepared by mixing equal volumes of methanolic extracts of three Chinese herbs, *C. officinalis*, *Cinnamommum cassia* and Chinese chive, showed good antimicrobial activity against common food borne microorganisms. It inhibited the growth of *E. coli* and showed excellent stability to heat, pH and storage conditions (Mau et al., 2001). Among 21 methanolic extracts of Korean medicinal plants studied for antimicrobial activity, the extract from *C. officinalis* was the most effective. The compound from the active fraction identified was ursolic acid (Kim et al., 1996). The extracts from the fruits of *C. drummondii* also showed good antimicrobial activity when tested on *E. coli*, *S. aureus* and *B. psychrophilus* (Post et al., 1995).

Antidiabetic Activity

C. officinalis is an important medicinal plant used for the antidiabetic preparations in China. Twelve Chinese herbal drugs used for the treatment of type II diabetics contained the extracts of *C. officinalis* (Li et al., 1999). Also, the methanolic extract enhanced the proliferations of islets and increased post prandial secretions of insulin (Quian et al., 2001). The ether extract of *C. officinalis* also showed antidiabetic activity against experimental rats with type-1 diabetes induced by streptozotocin. Fractionation of the ether extract revealed that the activity was due to ursolic acid (Kim and Oh, 1999).

Anticancer activity

Anthocyanins in fruits and vegetables exhibited a wide spectrum of anticancer activity. Tart cherry anthocyanins inhibited the growth of human colon cancer cell lines HT-29 in vitro (Kang, et al., 2003). The anthocyanin fraction from red wine have been reported to suppress the growth of HCT-115 cells, which were derived from human colon cancer or AGS cells from human gastric cancer (Kamei, et al., 1998).

Many *Cornus* seeds have substantial amount of oil and are used commercially in China. *Cornus* oil could be a good vegetable oil for patients with coronary heart diseases. It attenuated aortic atherosclerotic lesions in rabbits and reduced cholesterol accumulations in the intima of aorta (Huangfu et al., 1984). The triterpene, arjunolic acid, isolated from the stem of *C. capitata* was tested for insect growth and as antifeedant by using 4 th instar larvae of *Spliarctia oblique.* The effective concentration (EC_{50})

observed to inhibit 50% feeding and growth inhibition was 618 and 667 ppm, respectively (Bhakuni et al., 2002).

Even though several compounds and their biological activities were reported, many more is hidden among various *Cornus* spp., especially *C. kousa*, *C. controversa*, and *C. alternifolia*. It is my objective to investigate the therapeutic potential of the compounds from Michigan grown *Cornus* fruits. I am also looking for the potentials of *Cornus* plants as an alternative crop in Michigan so that it could generate additional income to *Cornus* growers and improve the economy of the State of Michigan. My studies will be focused on the phytochemicals in ripened and unripened *Cornus* fruits for bioactive compounds. Therefore, the present study deals with the isolation, structure elucidation and biological activities of phytochemicals in *C. kousa*, *C. controversa* and *C. alternifolia* fruits.

CHAPTER TWO

ANTHOCYANINS IN *CORNUS ALTERNIFOLIA, CORNUS CONTROVERSA, CORNUS KOUSA AND CORNUS FLORIDA* FRUITS*

Abstract

The anthocyanins in *Cornus alternifolia, Cornus controversa, Cornus kousa* and *Cornus florida* were quantified by HPLC and characterized by spectroscopic methods. The analyses of *C. alternifolia* and *C. controversa* revealed that both contained delphinidin 3-*O*-glucoside (**1**), delphinidin 3-*O*-rutinoside (**2**) and cyanidin 3-*O*-glucoside (**6**), respectively. Similarly, *C. kousa* and *C. florida* showed identical anthocyanin profiles with major anthocyanins as cyanidin 3-*O*-galactoside (**4**) and cyanidin 3-*O*-glucoside (**6**), respectively. The amount of anthocyanins **1**, **2** and **6** in *C. alternifolia* and *C. controversa* were 8.21, 8.44 and 0.02 mg; and 7.74, 5.92, and 0.02 mg/g of fresh fruits, respectively. The anthocyanins **4** and **6** in *C. kousa* and *C. florida* were 0.02 and 0.16 mg; and 0.62 and 0.03 mg/g fresh fruits, respectively. This is the first report of the quantification of anthocyanins in *C. alternifolia, C. kousa* and *C. florida* in addition to the anthocyanins not previously quantified in *C. controversa*.

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Introduction

The genus *Cornus* (dogwood) belongs to the family Cornaceae, which consists of about 58 species (Fan and Xiang, 2001). The *Cornus* spp. is widely distributed in the northern hemisphere, eastern Asia, eastern and northern part of the United States. The dogwood plants in general are characterized by brilliant, colorful and attractive flowers and fruits and hence are widely grown as ornamental plants throughout the United States. There are several reports of its use in traditional medicine and as a food preservative (Jianrong and Daozong, 2003; Hwang and Yeon, 2002). For example, *C. officinalis*, a widely grown *Cornus* spp, has been used in Chinese herbal medicine and known for its tonic, analgesic and diuretic activities (Kean and Hwan, 1998). Fruits from several *Cornus* spp. have used for improved liver and kidney functions (Yongwen et al., 1999). It is also reported to have anti-bacterial, antihistamine, anti-allergic, anti-microbial and anti-malarial activities (Zanyin et al., 1949; Mau et al., 2001). In Europe, *C. mas* or Cornelian cherry fruits were reported to have food and cosmetic applications (Seeram et al., 2002). Similarly, fruits from *C. controversa* have been used as an astringent and as a tonic in Korea and China (Jang et al., 1998).

The only *Cornus* spp. with alternate leaves is *C. alternifolia* and it is native to eastern United States. It produces attractive, dark-blue and berry-like fruits. Another common landscape tree, *C. kousa*, is a small deciduous tree also found in China, Japan and Korea. It bears sweet and edible fruits and is used for the production of wine in many parts of China and Korea (Du et al., 1974). The plant *C. kousa* is highly resistant to diseases and pests and hence it is widely used as a landscape plant. However, C. *florida,* commonly known as flowering dogwood, is the most popular dogwood in the United

States. It is recognized as the state flower of North Carolina and Virginia, the state tree of Missouri and Virginia and the state memorial tree in New Jersey.

The chemical investigation *C. controversa* has resulted in the isolation of several compounds including flavonoids, terpenoids and tannins (Dongho et al., 2000; Nakaoki and Morita, 1958). Fruits from several *Cornus* spp. have been studied for anthocyanin content. The anthocyanins impart bright colors to several fruits and vegetables and possess antioxidant, anti-inflammatory, anticancer and anti-diabetic activities (Wang et al., 1999; Kamei et al., 1995; Jayaprakasam et al., 2005; Chandra et al., 1992). Therefore, the food industry is interested in fruits and vegetables with high content of bioactive anthocyanins to manufacture supplements with preventative and therapeutic uses. Although *C. officinalis* and *C. mas* were studied by us in the past for their anthocyanin content, very little is known about the phytochemicals in the fruits of other *Cornus* spp (Seeram et al., 2002). In this manuscript, we have characterized and quantified the anthocyanins in native *C. alternifolia*, *C. controversa, C. florida* and *C. kousa* fruits. In addition to the anthocyanins not previously quantified in *C. controversa,* this is the first report of the quantification of anthocyanins in the fruits of *C. alternifolia, C. florida* and *C. kousa* species.

Material and Methods

All solvents were of ACS reagent grade. ^{1}H- and ^{13}C NMR spectra were recorded on Varian 500 and 125 MHz spectrometers using CD_3OD/DCl solution. Chemical shifts are given in parts per million relative to CD_3OD at 3.31ppm for ^{1}H NMR.

Plant Material. Fruits of *C. mas*, *C. officinalis*, *C. controversa*, *C. alternifolia*, *C. kousa*, *and C. florida* were collected from Michigan State University campus in August-September, 2004. The locations of the trees are recorded in the Michigan State University Herbarium Plant Database. The fruits were collected and analyzed on the same day to prevent the degradation of anthocyanins due to storage.

Extraction of *Cornus* fruits for anthocyanin quantification. Fresh fruits of *C. mas*, *C. officinalis*, and *C. florida* (25 g each), *C. alternifolia* and *C. controversa,* (10 g each) and *C. kousa* (100 g) were weighed and homogenized separately with methanol (1% HCl, 20 mL) for 3 min using a Kinematica CH-6010 (Roxdale, ON, Canada) homogenizer and centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10000g for 20 min at 4°C. The residue was further extracted with acidic methanol (3 x 15 mL) and the extracts were collected by centrifugation. The combined supernatants were made up to 100 mL (*C. kousa* 200 mL) with acidic methanol.

Quantification of anthocyanins. The fruits from *Cornus* spp. were extracted immediately after the collection. The anthocyanins were quantified by using Waters 2010 HPLC system (Waters Corp.) equipped with Empower Software, Shodex Degasser, Autosampler (Waters 717), Photodiode Array Detector (Waters 996), according to the method published from our laboratory (Yunjun et al., 2005). The separation was performed on a Capcell Pak (Dichrome, Santa Clara, CA) C18 column (150 x 4.6mm i.d.; 5 μm particle size) maintained at 25°C. The mobile phase used under gradient conditions consisted of 0.1% trifluroacetic acid /water (v/v; A); 50.4% water/48.5% acetonitile/1.0% acetic acid/0.1% trifluroacetic acid (v/v/v/v; B). The conditions were 20 % of A to 60% B in 26 min and then to 20 % B in 4 min and maintained for another 10

min to a total of 40 min run time. The flow rate was 1 ml/min. The injection volume for all samples was 50 μL and detection of anthocyanins was performed at 520 nm.

Pure anthocyanins **1, 2, 3, 4, 5,** and **6** were weighed (2 mg each) and dissolved in acidic methanol (1% HCl, 2 mL) separately. The stock solutions were diluted with acidic methanol (1% HCl) to yield 0.50, 0.25, 0.13, 0.063, 0.031, 0.016, 0.0078, 0.0039, and 0.00095 mg/mL concentrations, respectively. The standard solutions of each anthocyanin were analyzed in triplicate. Calibration curves were obtained by plotting the mean peak areas of triplicate injections of each standard against concentrations. The *Cornus* fruit extracts were also analyzed in triplicate at two different concentrations and mean peak areas were used for the quantification of anthocyanins **1-6** in these extracts.

Isolation and characterization of anthocyanins 1-6: The anthocyanins **1-6** were isolated according to the method previously published from our laboratory (Seeram et al, 2002). In summary, the fruits from *C. controversa* plants (1.45 kg) were blended with acidic methanol (1% HCl, 2 x 500 mL) for 2 min at room temperature and centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10000g for 20 min at 4°C. The residue was extracted further with acidic methanol (2 x 600 mL). The combined supernatants were evaporated to dryness at reduced pressure (35 °C) to yield a reddish gummy extract (111 g). A portion of this extract (43 g) was dissolved in water (250 mL) and fractionated by an XAD-2 column (500g, amberlite resin, mesh size 20-50; Sigma Chemical Co., St. Louis, MO). The resin with adsorbed anthocyanins was then washed with water (6 x 10 L). The water fraction was discarded. The adsorbed anthocyanin was eluted with methanol (2 x 2 L) and concentrated. The resulting aqueous concentrate was lyophilized to yield an amorphous red powder (10.3 g). This anthocyanin powder (1 g)

was dissolved in water:methanol (1:1, 1% HCl, 3 mL) and purified further by C-18 MPLC column (350 x 40 mm) using water:methanol (1% HCl) as the mobile phase under gradient conditions, starting with 77.5% of water. Fractions I (125 mL), II (150 mL), III (150 mL) and 1V (200 mL) were collected when water:methanol gradient was at 72:28 (v/v). The HPLC analysis of each fraction revealed that fractions I and II contained pure anthocyanins **1** (20 mg) and **2** (30 mg), respectively.

Anthocyanin **1:** ^{1}H NMR (CD_3OD/DCl) δ 3.46 (1H, *t*, *J* = 9.3 Hz, H-4″), 3.55 (2H, *m*, H-3″ and H-5″), 3.70 (1H, *dd*, *J* = 9.2, 7.8 Hz, H-2″), 3.73 (1H, *dd*, *J* = 12.1, 5.7 Hz, H-6′B), 3.91 (1H, *dd*, *J* = 12.1, 2.2 Hz, H-6″A), 5.30 (1H, *d*, *J* = 7.5 Hz, H-1″), 6.66 (1H, *d*, *J* = 2.0, H-6), 6.89 (1H, br *s*, H-8), 7.75 (2H, *s*, H-2′ and H-6), 8.94 (1H, *s*, H-4). This ^{1}H NMR spectral data was identical to the spectral data of delphinidin 3-*O*-glucoside (Mas et al., 2000).

Anthocyanin **2**

^{1}H NMR (CD_3OD/DCl) δ 1.14 d (1H, d, J = 6.0, H-6‴), 3.32 (1H, *t*, *J* = 9.5, H-4‴), 3.43 (1H, *t*, *J* = 9.5, H-4″), 3.54 (1H, m, H-5‴), 3.56 (1H, *t*, *J* = 9.5, H-3″), 3.59 (1H, *dd*, *J* = 1.5, 11.5, H-6″), 3.64 (1H, dd, J = 3.3, 9.5, H-3‴), 3.68 (1H, *t*, *J* = 9.5, H-2″), 3.72 (1H, *m*, H-5′), 3.80 (1H, *m*, H-2‴), 4.06 (1H, *dd*, *J* = 1.5, 11.3, H-6″), 4.64 (1H, *d*, *J* = 1.5 , H-1‴), 5.32 (1H, *d*, *J* = 7.8, H-1″), 6.71 (1H, *d*, *J* = 2.0, H-6), 6.91 (1H, *d*, *J* = 1.5, H-8), 7.76 (2H, *br s*, H-2′ and H-6′), 8.85 (1H, *br s*, H-4). This ^{1}H NMR spectral data was identical to the spectral data of delphinidin 3-*O*- rutinoside (Norbek and Kondo, 1998).

Anthocyanins **3-5** and **6** were isolated from *C. mas* and *C. kousa*, respectively, by the same protocol as mentioned above. The identities of these anthocyanins were confirmed by co-injection with authentic samples (Seeram et al, 2002).

Results

The *Cornus* plants, known as dogwood, bear attractive flowers and fruits. It produces flowers during May-June and fruits during August-September. The fruits of *C. mas* and *C. officinalis* are morphologically similar. The average weight of ten fruits from *C. mas* and *C. officinalis* were 2.03 and 2.06 g, respectively. The smallest fruits among *Cornus* spp. is from *C. controversa* whereas the largest fruit is from *C. kousa*. The average weight of *C. controversa, C. alternifolia, C. kousa* and *C. florida* per fruit was 0.07, 0.30, 15.56, and 0.54 g, respectively.

The fruits of *C. alternifolia* and *C. controversa* showed similar anthocyanin profiles (Fig. 2C, 2D). The major anthocyanins identified from them were delphinidin 3-*O*-glucoside (**1**), delphinidin 3-*O*-rutinoside (**2**) and cyanidin 3-*O*-glucoside (**6**), respectively. The relative amounts of anthocyanins in *C. alternifolia* were delphinidin 3-*O*-rutinoside > delphinidin 3-*O*-glucoside > cyanidin 3-*O*-glucoside. Both *C. alternifolia* and *C. controversa* contained negligible amount of anthocyanin **6** when compared to anthocyanins **1** and **2** present in them. The fruits of *C. controversa* gave higher amount of delphinidin 3-*O*-glucoside than delphinidin 3-*O*-rutinoside when compared to the fruits from *C. alternifolia*. The relative amounts of anthocyanins **1** and **2** in *C. controversa* were delphinidin 3-*O*-glucoside > delphinidin 3-*O*-rutinoside > cyanidin 3-*O*-glucoside. However, fruits from both of these *Cornus* spp. are rich sources of anthocyanins **1** and **2**.

The anthocyanin profiles of *C. kousa* and *C. florida* were also similar (Fig. 2E, 2F). The anthocyanins identified from these species were delphinidin 3-*O*-glucoside (**1**) cyanidin 3-*O*-glucoside (**6**) and cyanidin 3-*O*-galactoside (**4**), respectively. The

quantities of delphinidin 3-*O*-glucoside in *C. kousa* and *C. florida* were very small compared to cyanidin 3-*O*-glucoside and cyanidin 3-*O*-galactoside. Also, cyanidin 3-*O*-glucoside (**6**) was very high compared to cyanidin 3-*O*-galactoside (**4**) in *C.kousa*. In *C. florida*, on the other hand, cyanidin 3-*O*-galactoside was very high compared to cyanidin 3-*O*-glucoside. The major anthocyanins in *C. mas* and *C. officinalis* were delphinidin 3-*O*-galactoside (**3**), cyanidin 3-*O*-galactoside (**4**) and pelargonidin 3-*O*-galactoside (**5**), respectively (Fig. 2A, 2B). The concentration of anthocyanins was in the order of cyanidin 3-*O*- galactoside > pelargonidin 3-*O*-galactoside > delphinidin 3-*O*-galactoside.

The standard solutions of each anthocyanin were analyzed by HPLC in triplicate. The retention values of anthocyanins **1-6** were 13.50, 14.45, 12.56, 14.79, 16.57, and 15.61 min, respectively. A calibration curve was obtained after plotting the mean peak area of each anthocyanin against respective concentrations. Calibration curves for anthocyanins **1-6** were linear with corresponding correlation coefficients of 0.99, 1.0, 0.99, 1.0, 0.99, and 0.99, respectively. The concentration of anthocyanins in each *Cornus* fruits were then determined from their corresponding calibration curves resulting from the mean peak area of triplicate injections of each *Cornus* fruit extracts.

The contents of anthocyanins **1, 2** and **6** in *C. alternifolia* and *C. controversa* were 8.21, 8.44 and 0.02 mg; and 7.75, 5.92, and 0.02 mg, respectively, per g of fresh fruits (Table 1). Similarly, the concentration of anthocyanins **4** and **6** in *C. kousa* and *C. florida* fruits were 0.02 and 0.16; and 0.62 and 0.03, mg/g, respectively (Table 1). The amount of anthocyanins **3-5** in *C. mas* and *C. officinalis* were 0.47, 1.66 and 1.62; and 0.15, 0.21, and 0.78 mg/g of fruits, respectively (Table 1).

Discussion

The isolation of anthocyanins **1-6** from *C. controversa*, *C. mas* and *C. kousa* was according to the previously published method (Seeram et al., 2002). Based on the quantification studies, *C. alternifolia* fruits showed highest and *C. kousa* fruits showed lowest amount of anthocyanins among the *Cornus* fruits studied so far. Anthocyanins **1** and **2** were not reported previously from *Cornus* spp. Therefore, only these anthocyanins, isolated from *C. controversa*, were further characterized by ^{1}H-NMR in this study. As indicated earlier, the anthocyanin profiles of both *C. controversa* and *C. alternifolia* were identical. The ^{1}H-NMR data of anthocyanins **1** and **2** were in agreement with the literature data of delphinidin 3-*O*-glucoside and delphinidin 3-*O*-rutinoside, respectively (Mas et al., 2000; Norbek and Kondo, 1998).

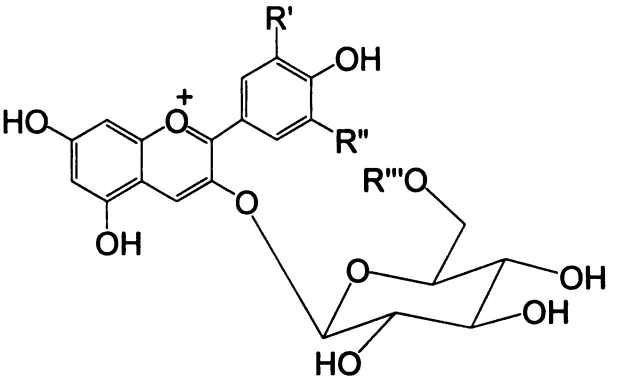
This is the first report of the quantification of anthocyanins in the fruits of *C. alternifolia, C. kousa* and *C. florida.* Both *C. alternifolia* and *C. controversa* fruits are excellent sources of bioactive anthocyanins based on the unusually high concentration of anthocyanins (total anthocyanins in *C. alterniflolia* and *C. controversa* were 16.67, and 13.68, g/kg of fresh fruits, respectively), detected in them. Also, the higher levels of delphinidin 3-*O*-glucoside and delphinidin 3-*O*-rutinoside could be used as taxonomical markers for the identification *C. alternifolia* and *C. controversa* fruits. The anthocyanin content in these fruits was several times higher than other commonly consumed fruits and vegetables. For example, total anthocyanins present in 'Montmorency', which is the major commercial tart cherry cultivated in the United States, and 'Balaton®' tart cherries are 0.24, and 0.08, g/kg of fresh fruits, respectively (Wang et al., 1997). Because anthocyanins are well known for its beneficial activities and non-toxic in nature, it is

possible that these plants could be cultivated as alternate crops to yield fruits for health beneficial anthocyanins.

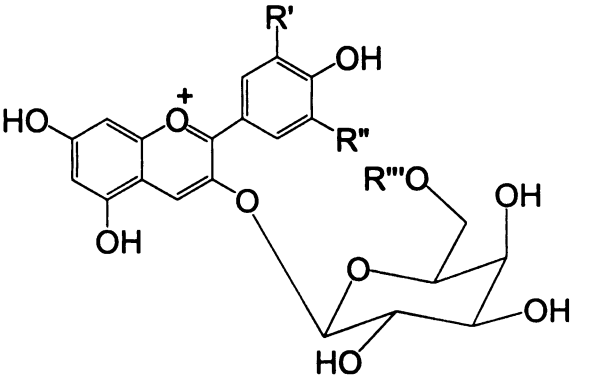
Table 2.1. The concentration of delphinidin 3-*O*-glucoside (**1**), delphinidin 3-*O*-rutinoside (**2**), delphinidin 3-*O*-galactoside (**3**), cyanidin 3-*O*- galactoside (**4**), pelargonidin 3-*O*-galactoside (**5**) and cyanidin 3-*O*-glucoside (**6**) in the fruits of *Cornus* spp.

Cornus fruits	Anthocyanins (mg/g of fresh fruits)					
	1	**2**	**3**	**4**	**5**	**6**
C. alternifolia	8.21	8.44	ND	ND	ND	0.02
C. controversa	7.75	5.92	ND	ND	ND	0.02
C. kousa	ND	ND	ND	0.02	ND	0.16
C. florida	ND	ND	ND	0.62	ND	0.03
C. mas	ND	ND	0.47	1.66	1.62	ND
C. officinalis	ND	ND	0.15	0.21	0.78	ND

ND = Not detected



Anthocyanin	R′	R″	R‴
1	OH	OH	H
2	OH	OH	Rha
6	OH	H	H



Anthocyanin	R′	R″	R‴
3	OH	OH	H
4	OH	H	H
5	H	H	H

Figure 2.1. Anthocyanins characterized from fruits of various *Cornus* spp.

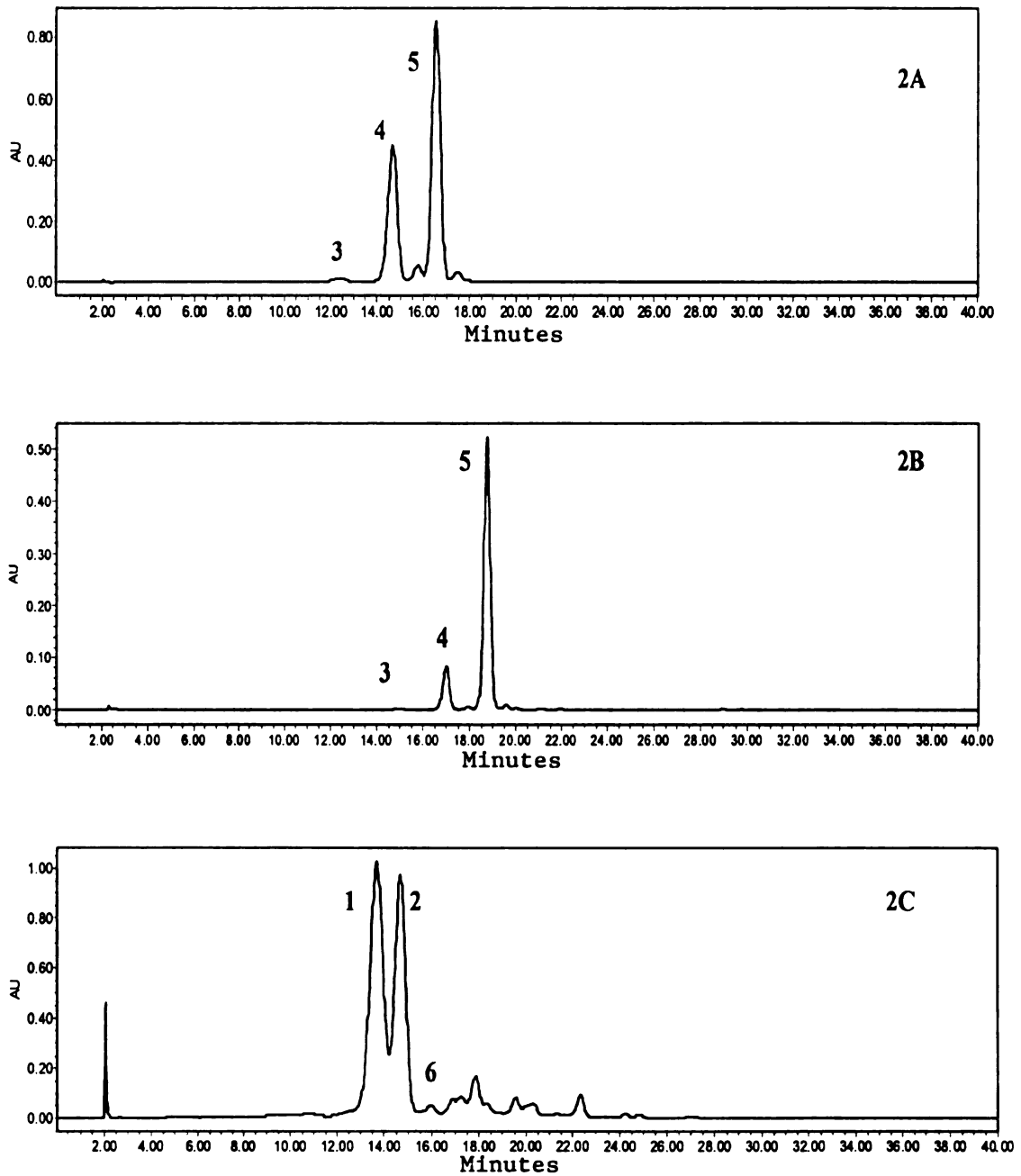


Figure 2.2 HPLC profiles of *Cornus* spp. fruits. (2A) *C. mas*; (2B) *C. officinalis*; (2C) *C. alternifolia*: Anthocyanins **1**. delphinidin 3-*O*-glucoside, **2**. delphinidin 3-*O*-rutinoside, **3**. delphinidin 3-*O*-galactoside, **4**. cyanidin 3-*O*- galactoside, **5**. pelargonidin 3-*O*-galactoside, **6**. cyanidin 3-*O*-glucoside.

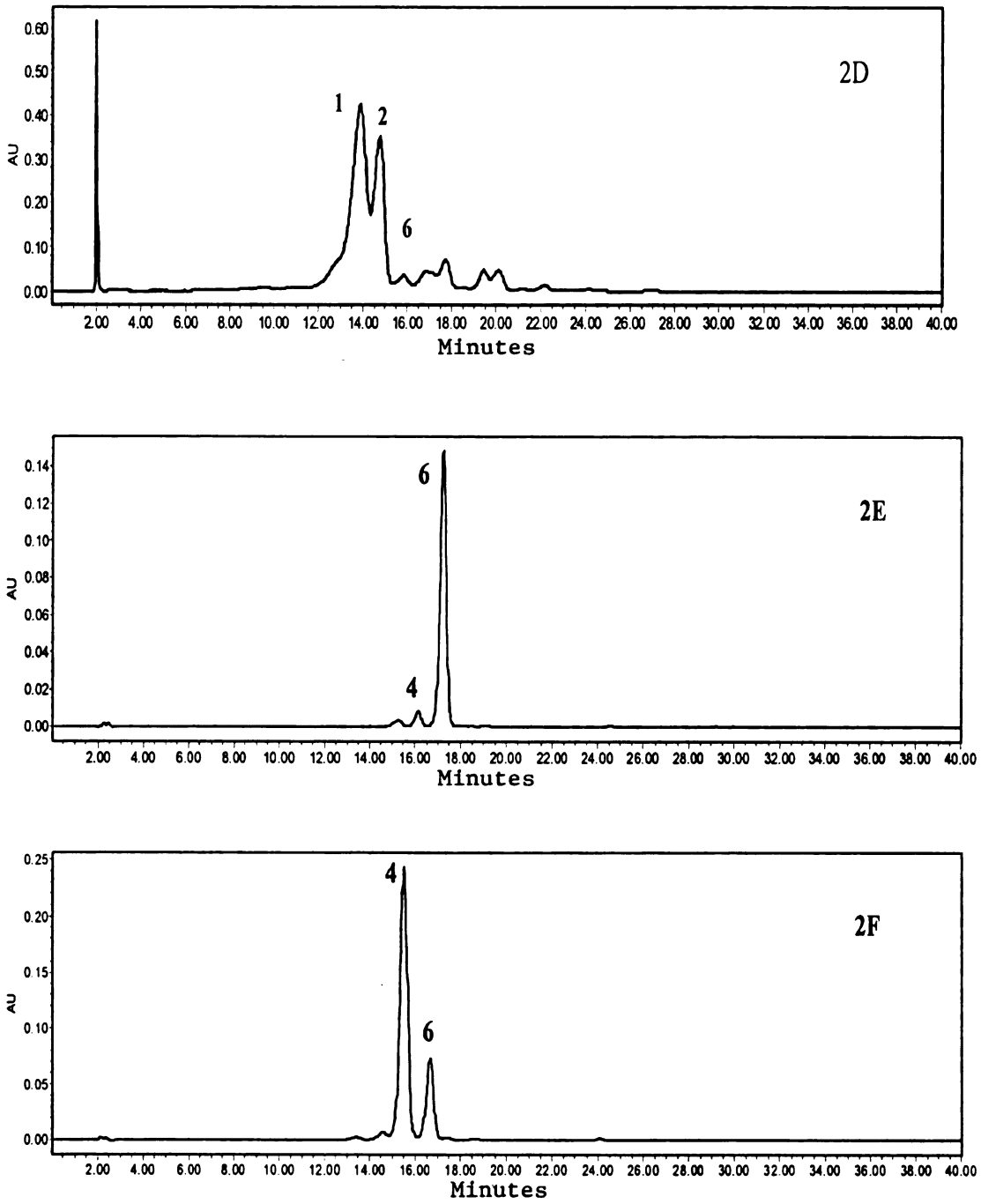


Figure 2.2 (Cont'd). HPLC profiles of *Cornus* spp. fruits. *C. alternifolia*; (2D) *C. controversa*; (2E) *C. kousa,* and (2F) *C. florida*: Anthocyanins **1**. delphinidin 3-*O*-glucoside, **2**. delphinidin 3-*O*-rutinoside, **3**. delphinidin 3-*O*-galactoside, **4**. cyanidin 3-*O*-galactoside, **5**. pelargonidin 3-*O*-galactoside, **6**. cyanidin 3-*O*-glucoside

CHAPTER THREE

ANTHOCYANINS AND ANTHOCYANIDINS IN *CORNUS ALTERNIFOLIA, CORNUS CONTROVERSA, CORNUS KOUSA, CORNUS FLORIDA, CORNUS MAS* AND *CORNUS OFFICINALIS* FRUITS WITH HEALTH BENEFITS*

Abstract

Anthocyanins are natural pigments widely distributed in plant kingdom. They are responsible for a variety of colors in fruits, flowers and vegetables. Anthocyanidins are aglycones of anthocyanins. Both anthocyanins and anthocyanidins are reported as powerful antioxidant and anti-inflammatory agents. Delphinidin 3-*O*- glucoside (**1**) and Delphinidin 3-*O*- rutinoside (**2**) were not studied earlier for their inhibition of lipid peroxidation and cyclooxygenase enzymes (COX-1 and COX-2) activities. At 50 μg/mL, anthocyanins **1** and **2** inhibited lipid peroxidation by 71 and 68%, respectively. Similarly, they inhibited COX-1 enzymes by 39 and 49% and COX-2 enzyme by 54 and 48%, respectively, at 100 μg/mL. Anthocyanins and anthocyanidins were tested for cell proliferation inhibitory activity against human cancer cell lines, AGS (stomach), HCT-

* Results in this Chapter have been

1. Accepted for publication in *Life Sciences* 2005 (Shaiju K Vareed, Muntha K Reddy, Robert E Schtzki, Muraleedharan G Nair. Anthocyanins in *Cornus alternifolia*, *Cornus controversa*, *Cornus kousa* and *Cornus florida* fruits with health benefits).

1. Published in *Journal of Agriculture and Food Chemistry* (Jayaprakasam Bolleddula, Shaiju K Vareed, Lawrence K Olson, Muraleedharan G Nair. Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. 2005, 53, 28-31).

2. Published in *Life Sciences* (Zhang Yunjun, Shaiju K Vareed, Muraleedharan G Nair. Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. 2005, 76, 1465-72).

116 (colon), MCF-7 (breast), NCI-H460 (lung), and SF-268 (Central Nervous System, CNS) at 12.5 - 200 μg/mL concentrations. Anthocyanin **1** displayed 50% growth inhibition (IC_{50}) at 21, 25, 50, 60, and 75 μg/mL, against colon, breast, lung, central nervous system, and stomach, human tumor cell lines, respectively. Similarly, IC_{50} values for anthocyanin **2** were 38, 30, 76, 100, and 100 μg/mL against colon, breast, lung, central nervous system, and stomach, respectively. Anthocyanins, delphinidin 3-*O*-galactoside (**3**), cyanidin 3-*O*- galactoside (**4**), pelargonidin 3-*O*-galactoside (**5**) and cyanidin 3-*O*-glucoside (**6**) did not show activity at a concentration lower than 200 μg/mL. The anthocyanidin malvidin (**11**) inhibited stomach, colon, lung, breast, and central nervous system cell growth by 69, 75.7, 67.7, 74.7 and 40.5%, respectively, at 200 μg/mL. Similarly, pelargonidin (**9**) inhibited stomach, colon, lung, breast, and central nervous system cell growth by 64, 63, 62, 63 and 34%, respectively, at 200 μg/mL. At 200 μg/mL, cyanidin (**8**), delphinidin (**7**) and petunidin (**10**) inhibited the breast cancer cell growth by 47, 66 and 53%, respectively. Anthocyanins **1**, **4-6** and anthocyanidins **7-11** were studied for their insulin secretion ability by rodent pancreatic beta cells (INS-1 813/32) in vitro. For insulin secretion studies, the compounds were tested in the presence of 4 and 10 mM glucose concentrations. Our results indicated that cyanidin-3-glucoside (**6**) and delphindin-3-glucoside (**1**) were the most effective insulin secretagogues among the anthocyanins and anthocyanidins tested at 4 and 10 mM glucose concentrations. Pelargonidin-3-galactoside (**5**) is one of the major anthocyanins and its aglycone, pelargonidin, caused a 1.4-fold increase in insulin secretion at 4 mM glucose concentration. Rest of the anthocyanins and anthocyanidins tested in our assay had only marginal effects on insulin at 4 and 10 mM glucose concentrations.

Introduction

Anthocyanins belong to an important class of plant compounds, known as flavanoids, that impart colors to flowers, fruits and vegetables (Mazza and Miniati, 1993). Anthocyanins occur in almost all parts of plants but are more common in fruits and flowers. They are water soluble pigments derived from the flavylium cation (2-phenyl-benzopyrylium). The number and position of hydroxyl and methoxyl groups, sugar moieties with or without aliphatic or aromatic acids bonded to the sugar moiety make anthocyanins different from one another (Mazza and Miniati, 1993). Anthocyanins with major aglycons in plants are delphinidin, cyanidin, pelargonidin, peonidin, petunidin and malvidin. The common sugar moieties bonded to anthocyanidins are glucose, galactose, rhamnose and arabinose. In plants anthocyanin content depends on various factors including light intensity, temperature, nutrient stress and pathogen attack (Beckwith et al., 2004). Intense light and low temperature are the most favorable conditions for anthocyanin production (Beckwith et al., 2004).

Anthocyanins are reported to have wide range of biological activities including antioxidant, anti-inflammatory, anti-cancer and anti-diabetic activities (Kim, et al., 2004, Harbone and Williams.,2000, Conklin, 2000). A recent in vitro study indicated the absorption of anthocyanins in the small intestine of rats (Talavera et al., 2004). The health benefits of anthocyanins, for example, prevention of chronic diseases such as cancer, heart diseases, and aging, may be closely associated with their antioxidant properties (Omenn, 1995). Today, food industries prefer to use these natural colorants over synthetic colors may be due to its health benefits and lower risk of side effects (Chandra et al., 2001).

Heart diseases, cancer and stroke cause numerous deaths in the United States. In 2002, 58% of the total mortality in US was due to these diseases (www.cdc.gov.). Even though good treatments are available now for these deadly diseases, the incidence and the mortality rate are still quite high. Epidemiological studies suggested that incorporation of fruits and vegetables in diet reduces the incidence of cancer. It is also reported that low intake of fruits and vegetables may increase the chance of degenerative diseases by two times. This suggests that phytochemicals in fruits and vegetables have beneficial effects in preventing many of the diseases. Recent studies show that protective action of fruit and vegetables against the chronic and degenerative diseases may be due to the antioxidant activity of compounds present in them (Kenneth, 2000). Many plant phenolics, tocopherols, carotenoids and ascorbic acid were reported to act as good antioxidants (Frankel, 1998). Most of these antioxidants exhibit anticarcinogenic and antimutagenic activities. Oxidative stress in body is strongly associated to the coronary heart diseases. Reactive oxygen species such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), peroxyl radical ($ROO^{\cdot}$), alkoxyl radical ($OR^{\cdot}$) and the hydroxyl radical ($-OH^{\cdot}$) are responsible for the oxygen toxicity in our body. These reactive species are produced from molecular oxygen due to the action of pollutants, drugs, activated leucocytes and normal cellular respiration. Various studies suggest that oxidation has a major role in heart diseases, cancer, diabetes, and Alzheimer's disease (Frankel. 1998).

Cyclooxygenase enzymes, COX-1 and COX-2, are mediators of inflammatory reactions. These enzymes catalyze the conversion of arachidonic acid to PGH_2, the precursor of all prostaglandins and thromboxanes (Smith et al., 1996). COX enzymes

mediate two distinct reactions. Firstly, it converts AA to PGG_2 through cyclooxygenase activity. Secondly it reduces PGG_2 to PGH_2 via the peroxidase activity. PGH_2 is further converted into various prostaglandins (Trifan et al., 1999). COX-1 and COX-2 are similar in catalytic properties but are different in biological activities. COX-1 is expressed constitutively in cells and is regarded as housekeeping enzyme where as COX-2, is induced in response to various growth factors and inflammatory stimuli. Inhibition of COX-1 may cause several side effects including gastric ulceration. Several studies using animal model for colon cancers indicate that expression of COX-2 is responsible for the transformation of colon epithelial cells (Srikant et al., 2003). Recent studies also showed that selective COX-2 inhibitors possess anticancer activities (Shone et al., 2003).

Diabetes mellitus is a metabolic disorder characterized by high levels of glucose in the blood resulting from the defects in insulin production, insulin action, or both (www.cdc.gov.). The major function of insulin is to counter the concerted action of a number of hyperglycemia-generating hormones and to maintain normal blood glucose levels. Diabetic patients have a shortage of insulin or decreased ability to use insulin, which leads to the accumulation of glucose in the blood. Heart disease is the leading cause of diabetes-related deaths. Diabetes can also cause a number of other diseases including stroke, blindness, kidney failure, pregnancy complications, lower-extremity amputations, and deaths related to flu and pneumonia. There are two types of diabetes, type-1 or insulin-dependent diabetes (IDDM) and type-2 or non-insulin-dependent diabetes (NIDDM). Type 1 diabetes results from the destruction of pancreatic β-cells of the body's immune system. Type-1 diabetes may account for 5 to 10% of all diagnosed diabetes and common among children and young adults. Type-2 diabetes is linked to

obesity and physical inactivity and accounts for 90-95% of diabetic cases. Type-2 diabetes is prevalent among people older than 40 (www.cdc.gov.).

One of the significant health benefits implicated to the consumption of anthocyanins is the low risk of coronary heart diseases. Several studies have shown that tart cherry anthocyanins, cyanidin-3-glycosides, exhibited in vitro antioxidant and anti-inflammatory activities (Wang, et al., 1999, Seeram, et al., 2001). The antioxidant property of anthocyanins and anthocyanidins suggested that they played an important role in the prevention of mutagenesis and carcinogenesis (Omenn, 1995). The anthocyanins in purple colored sweet potato and red cabbage suppressed colon carcinogenesis induced by 1, 2-dimethylhydrazine (DMH) and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine in rats (Hagiwara et al., 2002).

Although anthocyanins present in fruits and vegetables are known for their health benefits, the cell proliferation inhibitory activity and insulin secretion activity of pure anthocyanin and anthocyanidins are not reported. This chapter deals with lipid peroxidation, cyclooxygenase, tumor cell proliferation inhibitory and insulin secretion activities of anthocyanins in *Cornus* species and their corresponding anthocyanidins.

Materials and Methods

All solvents were of ACS reagent grade. *tert*-Butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), aspirin, ibuprofen, naproxen, and 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Company Co (St.Louis, MO). Celebrex ™and Vioxx® were provided by Dr. Subash Gupta, Sparrow Hospital, MI. The COX-1

enzyme was prepared from ram seminal vesicles purchased from Oxford Biomedical research, Inc. (Oxford, MI). The COX-2 enzyme was prepared from prostaglandin endoperoxide H synthase-2 (PGHS-2) –cloned insect lysate. Fetal bovine serum (FBS) and Roswell Park Memorial Institute 1640 (RPMI-1640) medium were purchased from Gibco BRL (Grand Island, NY). HEPES, penicillin- streptomycin, glutamine, sodium pyruvate, 2-mercaptoethanol, trypsin-EDTA, BSA (Bovine, Albumin; RIA Grade), Folin-Ciolatues reagent and chemicals used for the preparation of buffers were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Human tumor cell lines SF-268 (Central Nervous System, CNS), NCI H460 (lung), and MCF-7 (breast) were purchased from the National Cancer Institute (NCI, Bethesda, MD). AGS (stomach) and HCT-116 (colon) were purchased from American Type Culture Collection (ATCC, Rockville, MD). INS-1 832/13 cells were obtained from Dr. Christopher Newgard, Duke University, NC. All cell lines were maintained in a humidified chamber at 37 °C with 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (1 unit/100 mL), and streptomycin (1 μg/100 mL) in the Bioactive Natural Products and Phytoceuticals Laboratory (BNPP) at Michigan State University.

Preparation of Anthocyanins 1-6. Described in Chapter 2

Preparation of Anthocyanidins 7-11. Anthocyanidins were prepared by the acid hydrolysis of respective anthocyanins under nitrogen atmosphere. During our insulin secretagogues studies with grape skin, we have found that one of the red grapes, cabernet sauvignon, contained substantial quantities of delphinidin, cyanidin, petunidin and malvidin glycosides (Zhang et al., 2004). Therefore, pure delphinidin (**7**), cyanidin (**8**), petunidin (**10**) and malvidin (**11**) aglycones were prepared by the acid hydrolysis of

purified grape skin anthocyanins. The purified anthocyanins from grape skin (1.0 g) were hydrolyzed with HCl (3 M, 3h at 95 °C). The solution containing anthocyanidins thus obtained was cooled at room temperature and evaporated under reduced pressure (40°C). The residue from the hydrolysis (0.74 g) was then dissolved in MeOH:H_2O (1:1, 2 mL), and purified by medium pressure liquid chromatography (MPLC) using a C_{18} column (350 x 40 mm). The solvent system used was acidic MeOH:H_2O (pH = 3.0) under gradient conditions starting from 45% to 55% MeOH. The fractions collected from MPLC were analyzed by high performance liquid chromatography (HPLC) to ensure purity. Similarly, pure pelargonidin (**9**) aglycone was prepared from the acid hydrolysis of *C. mas* fruit anthocyanins.

HPLC analysis. The purity of anthocyanidins **7-11** was determined by HPLC. (The HPLC method is described in Chapter 2).

Lipid Peroxidation Inhibitory Assay. The anthocyanins **1** and **2** were tested in vitro for their ability to inhibit the oxidation of large unilamellar vesicles (LUVs) (Arora et al., 1998). The assay was conducted in a buffer consisted of HEPES (100 μL), NaCl (200 μL), N_2- sparged water (1.64 mL), test sample (20 μL) in water and LUV (20 μL) suspension. The peroxidation was initiated by the addition of $FeCl_2$ (20 μL, 0.5 mM) solution and was monitored by observing the fluorescence at 0, 1, 3, 6, 9, 12, 15, 18, and 21 min using a Turner model 450 digital fluorometer (Barnstead Thermolyne, Dubuque, IA) at 384 nm. The decrease of relative fluorescence intensity with time indicated the rate of lipid peroxidation. All compounds were tested at 50 μg/mL. The antioxidant standards BHA, BHT and TBHQ were tested at 1.66, 2.2 and 1.8 μg/mL, respectively. Inhibition of lipid peroxidation by anthocyanins **3-6** and anthocyanidins **7-11** were

reported earlier from our laboratory and hence was not studied at this time (Seeram et al., 2002).

Cyclooxygenase Inhibitory Assay. COX activities of anthocyanins **1** and **2** were assessed by monitoring the initial rate of O_2 uptake using a micro oxygen chamber and electrode (Instech Laboratories, Plymouth Meetings, PA) attached to a YSI model 5300 biological oxygen monitor (Yellow springs Instrument, Inc., Yellow Springs, OH) at 37 °C. The assay was conducted according to the previously reported procedure (Wang et al., 1999). The test samples and controls were dissolved in water. Each assay mixture contained Tris buffer (0.6 mL, 0.1 M, pH 7), phenol (1 mM), hemoglobin (85 μg), and water or test samples (10 μL). COX-1 or COX-2 enzyme (10 μL) was added to the chamber and incubated for 3 min. The reaction was initiated by the addition of arachidonic acid (10 μL of a 1 mg/mL solution). Duplicate analysis was performed for each sample and the standard deviation was calculated for n=2. The data were recorded using QuickLog for windows data acquisition and control software (Strawberry Tree, Inc., Sunnyvale, CA). Commercial anti-inflammatory drugs aspirin, ibuprofen, naproxen, and vioxx were tested at 180, 2.52, 2.06, and 1.67 μg/mL, respectively. COX-1 and -2 inhibitory activities of anthocyanins **3-6** anthocyanidins **7-11** were reported earlier from our laboratory and hence were not studied again (Seeram et al., 2002).

Tumor Cell Proliferation Assay (MTT). The assay was performed according to the previously published method (Denizot and Lang, 1986). MCF-7 (beast), SF-268 (CNS), NCI H460 (lung), HCT-116 (colon) and AGS (gastric) human tumor cells were cultured in RPMI-1640 medium containing penicillin-streptomycin (10 units/mL for penicillin and 10 μg/mL for streptomycin) and 10% fetal bovine serum (FBS). The cells were grown in

a humidified incubator (37 °C, 5% CO_2), counted using hemacytometer, and transferred in to 96-well microtiter plates and incubated for 24 h. The samples were dissolved in water/DMSO and further diluted with RPMI medium. After 24 h of incubation, test samples (100 μL) in appropriate dilution were added to each well containing the appropriate tumor cells and further incubated 48 h. After incubation, an aliquot (25 μL) of MTT solution (5 mg MTT dissolved in 1 mL of phosphate-buffered saline solution) was added and the plates were further incubated for 3 h at 37 °C after wrapping it with aluminum foil. The medium was removed from each well and cells treated with DMSO (200 μL). The plates were then shaken and optical density was measured using a microplate reader at 570 nm. Adriamycin, dissolved in 0.1% DMSO and 0.1% DMSO or Water in RPMI 1640 media were used as positive control and solvent control, respectively. The sample was assayed in triplicate, and three independent experiments were carried out to calculate IC_{50} values.

The assay was conducted in triplicate for each sample concentration, positive and solvent controls. Three parallel experiments were performed. The cell viability of samples at each concentration was calculated with respect to solvent control. The cell viability at each concentration was calculated by dividing the optical density of samples with the optical density of solvent control.

Insulin Secretion Studies. INS-1 832/13 cells were maintained in the Bioactive Natural Products and Phytoceutical Laboratory at Michigan State University in 5% CO_2/air at 37 °C. The cells were cultured in RPMI-1640 medium containing 11.1 mM glucose and supplemented with 10% FBS (Fetal Bovine Serum), 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-

mercaptoethanol. Cells were passed weekly after trypsin-EDTA detachment. The cells were counted using a hemacytometer and transferred into 24-well plates at a density of 0.64 x 10^6 cells per well and incubated for 24 h. The cells were then cultured for an additional 24 h in RPMI-1640 containing 4 mM glucose and the supplements described above. Cells were then incubated twice for 30 min in Krebs-Ringer Bicarbonate buffer (KRBB) containing 4 mM glucose and 0.1% BSA. Cells were rapidly washed with KRBB and incubated for 60 min KRBB containing 4 or 10 mM glucose with or without the indicated anthocyanins or anthocyanidins. The medium was then removed for determination of insulin release. The cells were washed twice with PBS and dissolved in 1 M NaOH. Cellular protein concentration was then determined by Lowry assay. Anthocyanins and anthocyanidins were dissolved in DMSO to obtain desired concentrations. Final concentration of DMSO was maintained at 0.1%. The insulin secreted into the medium by the cells was determined by radioimmunoassay and normalized to total cellular protein.

Radioimmuno Assay (RIA). The RIA Kit was purchased from LINCO Research Inc. (St Charles, MO), and the assay was conducted according to the manufacturer's directions. Briefly, insulin standards were prepared in the 0.1-10 ng range and added (100 µl) to 12 x 75 mm test tubes. An aliquot of samples (25 µl) from the insulin secretion studies, assay buffer (75 µl) and ^{125}I labeled insulin (100 µl) was then added to each test tube. An aliquot of anti-rat insulin antibody (100 µL) was added and the tubes were incubated at 4°C for 24 h. To the solutions, 1 ml aliquot of the precipitating reagent was added and the tubes were further incubated for 20 min at 4°C to precipitate the

insulin bound to the antibody. The tubes were then centrifuged and the radioactivity was measured using a gamma counter.

Lowry protein Assay. The amount of protein in the assay wells was determined by Lowry method (*19*). The Lowry assay solution was prepared by mixing the Lowry solution, $CuSO_4.5H_2O$ (1%), and sodium tartarate (1%). The protein sample (100 µl) and Lowry assay solution (1 mL) were mixed in a test tube (12 x 75). An aliquot of Folin-Ciolatues reagent (100 µl) was added to these tubes and incubated for 30 min at room temperature. The optical density of resulting solutions was recorded using UV spectrophotometer at 700 nm.

Results

The methanol extracts of *C. alternifolia* (**A**) and *C. controversa* (**B**) inhibited lipid peroxidation by 56 and 53%, respectively, at 250 µg/mL. Inhibition of lipid peroxidation by anthocyanins **1** and **2** were 71 and 68%, respectively, at 50 µg/mL (**Fig. 3.2**). Commercial antioxidants BHA, BHT and TBHQ were used as positive controls in the lipid peroxidation assay at 1.8, 2.2 and 1.66 µg/mL, respectively, to yield about 80-90% inhibition and inhibited the lipid peroxidation by 81, 85 and 84%, respectively.

The anthocyanins **1** and **2** inhibited COX-1 enzyme by 39 and 49%, respectively, at 100 µg/mL. Similarly, they inhibited COX-2 enzyme by 54 and 48%, respectively, at 100 µg/mL (**Fig. 3.3**). The positive controls aspirin (180 µg/mL,) ibuprofen (2.52 µg/mL), naproxen (2.06 µg/mL) and Vioxx® (1.67 µg/mL) inhibited COX-1 and COX-2 enzymes by 61 and 24, 53 and 59, 80 and 96, and 0 and 76%, respectively. The varying concentrations of positive controls were used to obtain the inhibitions between 50-100 %.

Anthocyanins **1-6** and anthocyanidins **7-11** were assayed for their ability to inhibit the proliferation of colon, breast, lung, central nervous system, and stomach human tumor cell lines. These compounds were assayed at 200, 100, 50, 25 and 12.5 μg/mL to obtain their 50% growth inhibitory (IC_{50}) values. The anthocyanin, **1,** exhibited 50% growth inhibition (IC_{50}) at 21, 25, 50, 60 and 75 μg/mL, against colon, breast, lung, central nervous system and stomach, respectively (**Fig. 3.4A**). The IC_{50} values observed for anthocyanin **2** were 38, 30, 76, 100 and 100 μg/mL against colon, breast, lung, central nervous system and stomach, respectively (**Fig. 3.4B**). Anthocyanins **3-5** showed good inhibition of tumor cell proliferation at 200 μg/mL. However, inhibitory activities of anthocyanins **3-5** were marginal below 100 μg/mL. Anthocyanin **3** inhibited the growth of breast and lung tumor cell lines by 34 and 22%, respectively, at 200 μg/mL (**Fig. 3.4C**). Anthocyanin **4** displayed 50% inhibition towards breast cancer cell line at 200 μg/mL (**Fig. 3.4D**). It did not show inhibition to the proliferation of any other cancer cell line. Anthocyanin **5** inhibited breast, central nervous system, lung, and colon cell growth by 40, 20, 51 and 76%, respectively, at 200 ppm (**Fig. 3.4E**).

Anthocyanidins were prepared by the hydrolysis of anthocyanin enriched extract from *Cabernet sauvignon* grape skin under acidic condition. The hydrolyzed mixture was cooled, evaporated under reduced pressure and purified by MPLC afforded delphinidin (**7**), cyanidin (**8**), petunidin (**10**) and malvidin (**11**). Similarly, pure pelargonidin (**9**) aglycone was prepared from the acid hydrolysis of *C. mas* fruit anthocyanins.

The anthocyanidin cyanidin (**8**) inhibited the growth of breast cancer cells by 35 and 47% at 100 and 200 μg/mL (**Fig. 3.5B**), respectively. However, cyanidin did not

inhibit the growth of other cancer cell lines at 100 and 200 μg/mL. Similarly, delphinidin (**7**) inhibited the growth of breast cancer cell lines by 27 and 64% at 100 and 200 μg/mL, respectively, but did not affect the growth of stomach, lung, CNS and colon (**Fig. 3.5A**) cancer cell lines. The anthocyanidin malvidin (**11**) was effective against the cell proliferation on all cell lines tested. It inhibited the cell proliferation of colon, breast, lung, central nervous system, and stomach cancer cells by 75.7, 74.7, 67.7, 40.5, and 69 %, respectively, at 100 μg/mL (**Fig. 3.5E**). At 200 μg/mL, pelargonidin (**9**) inhibited 34, 62, 64, 63, and 65% of colon, breast, lung, central nervous system, and stomach cancer cells growth, respectively (**Fig. 3.5C**). One of the less abundant anthocyanidins found in nature, petunidin (**10**), showed 53 and 24% cell growth inhibitions of breast and stomach cancer cells at 200 μg/mL (**Fig. 3.5D**). Adriamycin (doxorubicin), the positive control, was tested at 0.181, 0.363, 0.725 and 1.45 μg/mL.

Anthocyanins **1**, **4-6**, and anthocyanidins **7-11** were assayed for their ability to stimulate insulin secretion by rodent pancreatic beta cells (INS-1 813/32). Anthocyanins and anthocyanidins were assayed at 4 and 10 mM glucose levels in the cell growth medium at 50 μg/mL initial concentration. The insulin secretion at 4 mM and 10 mM glucose were 27 and 83 ng of insulin/mg of protein, respectively (**Fig. 3.6A**). Anthocyanins delphinidin 3-*O*- glucoside (**1**) and cyanidin 3-*O*-glucoside (**6**) secreted 49.48 and 36.14 ng of insulin/mg of protein, respectively, at 4 mM glucose concentration. At 10 mM glucose level, anthocyanins **1** and **6** showed insulin secretion of 113 and 119 ng of insulin/mg of protein, respectively. The anthocyanins cyanidin-3-*O*-galactoside (**4**) and pelargonidin-3-*O*-galactoside (**5**) did not impact the insulin secretion at 4 mM glucose concentration. However, cyanidin-3-*O*-galactoside showed an increase of 17 ng

of insulin/mg of protein at 10 mM glucose concentration (**Fig. 3.7**). The pelargonidin-3-*O*-galactoside (**5**) was tested only once due to the limited amount of sample. The anthocyanin cyanidin-3-glucoside (**6**) was also evaluated for dose dependent insulin secretion at 5, 10, 50, 100 and 250 μg/mL concentrations. At 4 mM glucose level, the untreated cells and the cells treated with anthocyanin **6** secreted 33 and 46 ng of insulin/mg of protein, respectively. However, there was no significant difference in insulin secretion by compound **6** at 10, 50, 100 and 250 μg/mL concentrations. Anthocyanidins delphinidin (**7**) and cyanidin (**8**) secreted 25 and 29 ng of insulin/mg of protein, respectively, at 4 mM glucose concentration. At 10 mM glucose level, anthocyanidins **7** and **8** showed insulin secretion of 48 and 88 ng of insulin/mg of protein, respectively (**Fig 3.6B**). Pelargonidin (**9**) secreted 49 and 91 ng of insulin/mg of protein at 4 and 10 mM glucose, respectively (**Fig 3.7**). The aglycone petunidin (**10**) increased insulin secretion by 4 ng of insulin/mg protein at 4 mM glucose concentration and no significant increase in insulin level was observed at 10 mM level. However, malvidin (**11**) did not show an increase in insulin secretion with respect to the untreated cells. Anthocyanin **2** and **3** were not tested in this assay due to the limited supply of samples.

Discussion

Anthocyanins are pigments responsible for the orange, red, purple and blue colors of many fruits, vegetables, flowers, leaves, and roots. They are found in nature as polyhydroxylated and or methoxylated heterosides, derived from the flavylium ion or 2-phenylbenzopyrilium. Anthocyanins are biogenetically produced from tetrahydroxychalcone, naringenin, a precursor involved in the pivotal step of flavonoid

biosynthesis (Strack and Wray, 1993). Out of the 21 anthocyanidins reported, only 6 anthocyanidins are commonly found in fruits and vegetables. They are delphinidin, cyanidin, pelargonidin, peonidin, petunidin and malvidin. Anthocyanidins could very well be the immediate metabolite after ingestion of anthocyanins. This is because the β-glucosidase enzyme found in intestinal bacteria can easily hydrolyze respective anthocyanins (glycosides) to anthocyanidins (aglycones) (Miyazawa, et al., 1999). Although anthocyanins are well known for their antioxidant and anti-inflammatory activities, very little is known about their insulin secretion and anticancer activities.

Delphinidin 3-*O*- glucoside (**1**) showed higher inhibitory activity than delphinidin 3-*O*- rutinoside (**2**) in the lipid peroxidation, COX, and cell proliferation inhibitory assays. This is true because the bioactivity of anthocyanins is proportional to the molar amount of respective aglycones present in them. The aglycone, delphinidin, was not active against the cell lines tested except breast (IC_{50} =162.4 μg/mL). Also, delphinidin 3-*O*-galactoside did not show activity towards any cancer cell lines tested even at 200 μg/mL. However, delphinidin 3-*O*- glucoside and delphinidin 3-*O*- rutinoside showed potent growth inhibitory activity towards all tumor cell lines tested. Also, delphinidin 3-*O*- glucoside showed higher growth inhibitory activity than delphinidin 3-*O*- rutinoside in all cell lines studied. This indicated that each anthocyanin exhibits different biological activities with varying potency and the activity is dependent on both the aglycone and the glycoside substitution on its 3-position.

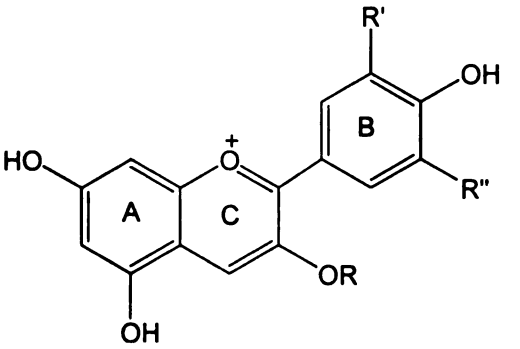
The cell proliferation inhibitory results obtained in this study for cyanidin, delphinidin, pelargonidin, petunidin and malvidin on stomach, colon, lung, breast and CNS cancer cell lines are in agreement with the effects reported for cyanidin on human

colon tumor cell lines HT-29 and HCT-115 (Kang, et al., 2003 and Kamei, et al., 1998). The data from the MTT assay indicated that a free hydroxyl group at 3-position in the flavylium moiety in anthocyanidins contributed to the cell proliferation inhibitory activity against human cancer cell lines studied. Also, the number of hydroxyl and methoxyl groups in B ring of anthocyanidin strongly influenced the growth inhibition of cancer cell lines studied. The highest inhibitory activity was demonstrated by malvidin with hydroxyl groups at 3 and 4' positions and methoxy groups at 3' and 5' positions.

It is well known that dietary antioxidants protect pancreatic β-cells from the damage due to oxidative stress. Various reports also indicated that consumption of fruits and vegetables, especially rich in polyphenols, decreased the incidence of type-2 diabetes (Anderson and Polansky, 2002; Landrault, 2003). The insulin secretion studies in our laboratory with pancreatic β-cells suggested that both anthocyanins and anthocyanidins are insulin secretagogues. Among the anthocyanins studied, delphinidin-3-*O*-glucoside (**1**) showed highest activity at lower glucose concentration. Although cyanidin-3-*O*-glucoside (**6**) was less active than delphinidin-3-*O*-glucoside at lower glucose concentration, it was more active at higher glucose concentration. Among the galactosides, pelargonidin-3-galactoside (**5**) did not induce insulin secretion at 4 and 10 mM glucose concentrations studied where as cyanidin-3-galactoside (**4**) showed significant increase in insulin secretion. The ability of anthocyanins studied to secrete insulin was in the increasing order of delphindin-3-*O*-glucoside > cyanidin-3-*O*-glucoside > pelargonidin-3-*O*-galactoside. This indicated that the number of hydroxyl groups in ring-B of anthocyanins played an important role in their ability to secrete insulin. Among the anthocyanidins tested, pelargonidin was the most active at 4 mM glucose. Other

aglycones did not potentiate significant insulin secretion at 4 or 10 mM glucose concentrations studied.

In summary the in vitro studies with various human tumor cell lines and pancreatic β-cells suggest that both anthocyanins and anthocyanidins in *Cornus* fruits may be effective for the prevention of cancer and type-2 diabetes. However, in vivo studies and clinical evaluation of these compounds must be carried out to validate the in vitro results.



	R	R′	R″
1	Glc	OH	OH
2	Glc→Rha	OH	OH
3	Gal	OH	OH
4	Gal	OH	H
5	Gal	H	H
6	Glc	OH	H
7	H	OH	OH
8	H	OH	H
9	H	H	H
10	H	OCH_3	OH
11	H	OCH_3	OCH_3

Figure 3.1 Structures of anthocyanidins (**1-6**) and anthocyanidins (**7-11**).

1. Delphinidin 3-*O*-glucoside: **2**. Delphinidin 3-*O*- rutinoside; **3**. Delphinidin 3-*O*-galactoside; **4**. Cyanidin-3-*O*-galactoside; **5**. Pelargonidin-3-*O*-galactoside; **6**. Cyanidin-3-*O*-glucoside; 7. Delphinidin; **8**. Cyanidin; **9**. Pelargonidin; **10**. Petunidin and **11**. Malvidin.

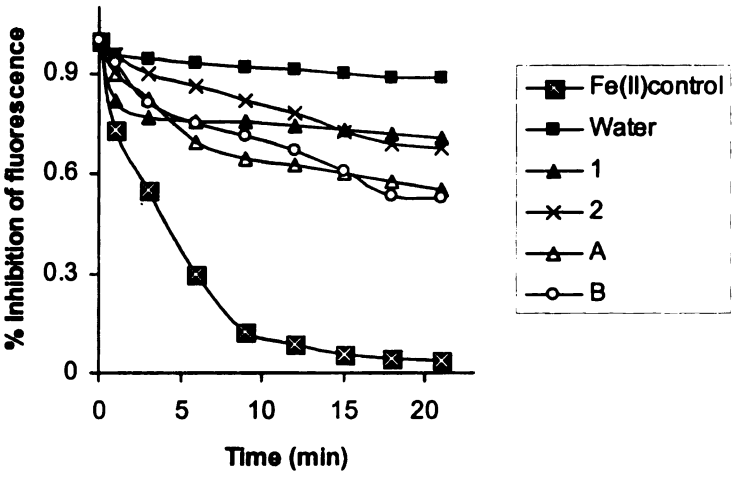


Figure 3.2 Lipid peroxidation inhibitory activities of anthocyanins **1** and **2** and methanol extracts of *C. alternifolia* (**A**) and *C. controversa* (**B**). Synthetic commercial antioxidants BHA, BHT, and TBHQ were tested at 1.66, 2.2 and 1.8 μg/mL, respectively. Oxidation of lipid was initiated by the addition of ferrous ions. The rate of peroxidation was monitored by the measurement of decrease in fluorescence intensity with respect to time. Anthocyanins and extracts were tested at 50 and 250 μg/mL respectively. Antioxidant standards BHA, BHT and TBHQ inhibited lipid peroxidation by 81, 85 and 84%, respectively. Vertical bars represent the standard deviation of each data point (n = 2).

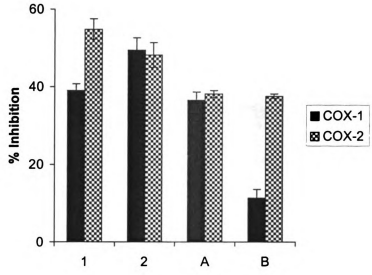


Figure 3.3. COX-1 and COX-2 inhibitory activities of anthocyanins **1** and **2** and methanol extracts of *C. alternifolia* (**A**) and *C. controversa* (**B**). Anthocyanins and extracts were tested at 50 and 250 μg/mL respectively, at pH 7.0. Commercial anti-inflammatory drugs, aspirin (180 μg/mL,) ibuprofen (2.52 μg/mL), naproxen (2.06 μg/mL) and vioxx (1.67 μg/mL) were used as positive controls. Aspirin, ibuprofen, naproxen and vioxx inhibited COX-1 activity by 61, 53, 80 and 0% respectively, and COX-2 activity by 24, 59, 96 and 76%, respectively. Vertical bars represent the standard deviation of each data point (n=2).

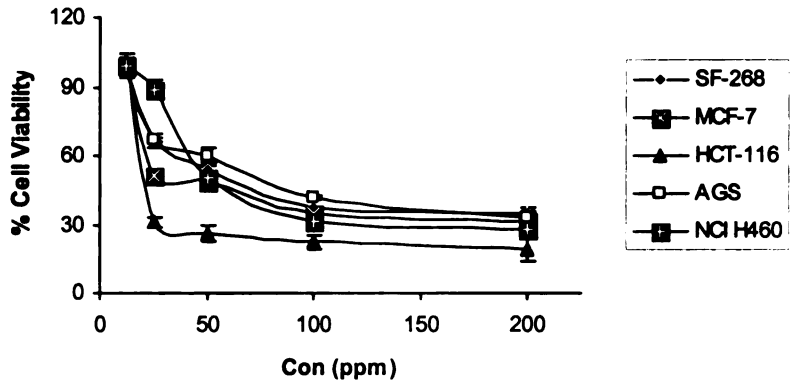


Figure. 3.4A

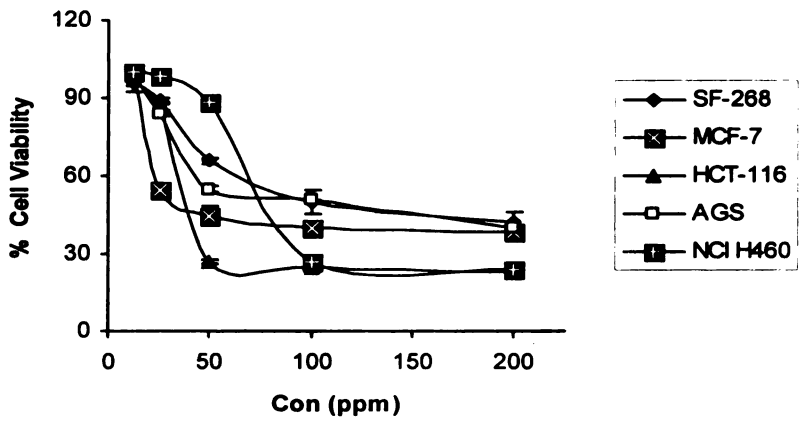


Figure. 3.4B

Figure 3.4. In vitro cell proliferation inhibitory results of anthocyanins **1-6** against human cancer cell lines. Adriamycin was used as positive control. The vertical bars represent ± SD of three individual experiments conducted in triplicate. **3.4A**. Delphinidin 3-*O*-glucoside; **3.4B**. Delphinidin 3-*O*-rutinoside. Anthocyanins were tested at 200, 100, 50, 25, and 6.25 μg/mL, respectively. At 200 μg/mL, all cell lines exposed to anthocyanins displayed growth characteristics identical to the solvent control.

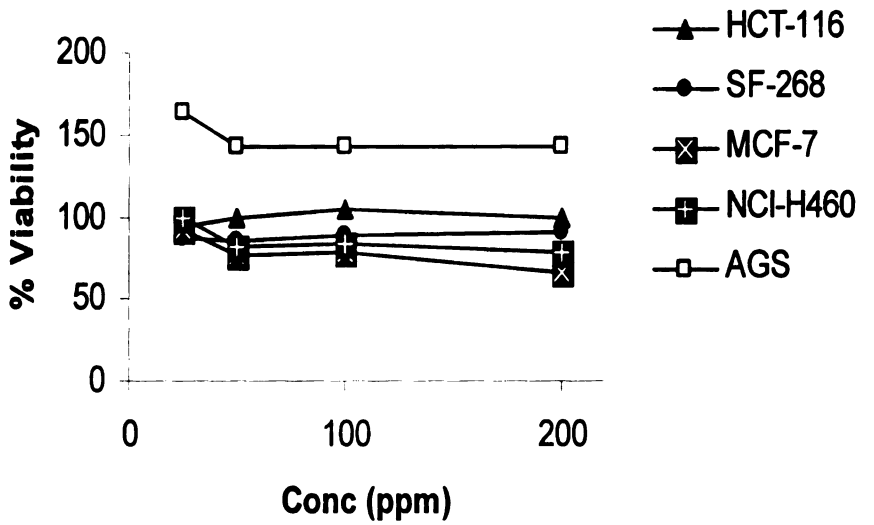


Figure. 3.4C

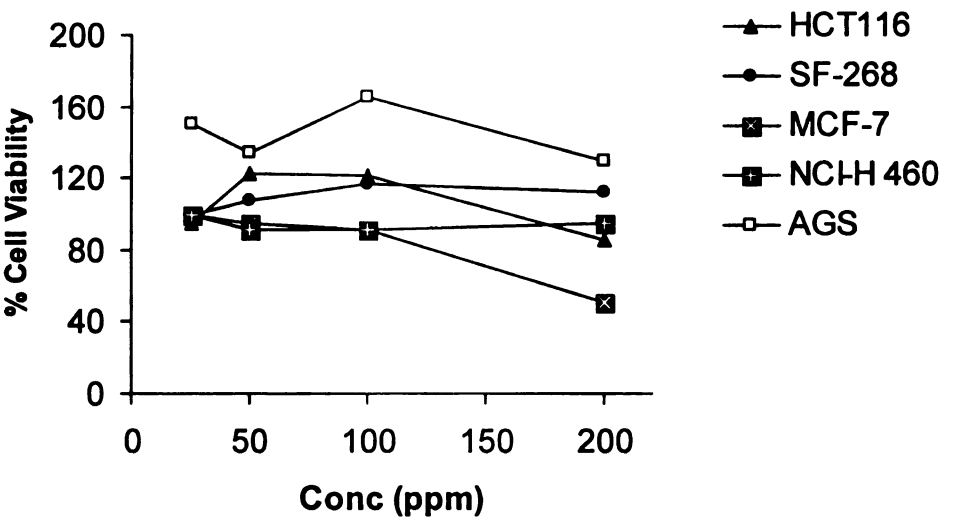


Figure. 3.4D

Figure 3.4 (cont'd). In vitro cell proliferation inhibitory results of anthocyanins against human cancer cell lines. **3.4C**. Delphinidin 3-*O*-galactoside; **3.4D**. Cyanidin 3-*O*-galactoside.

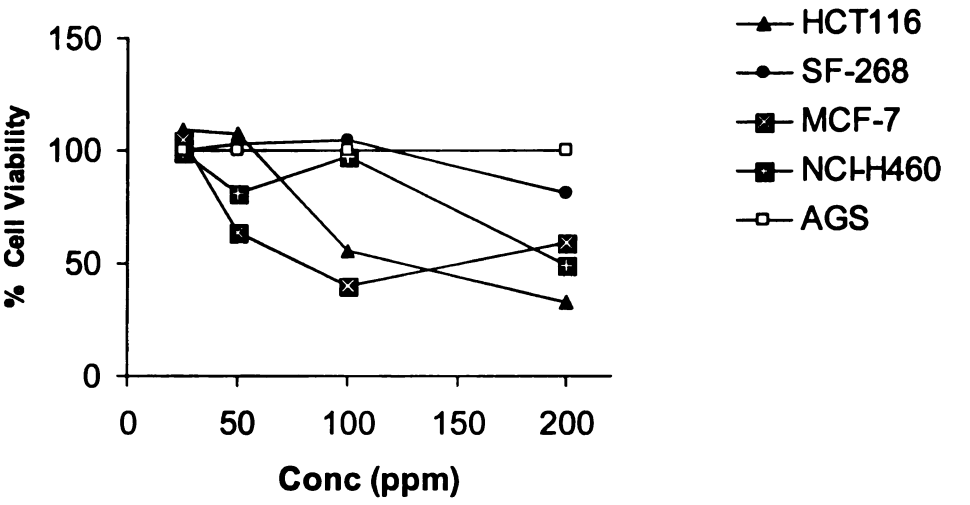


Figure. 3.4E

Figure 3.4 (cont'd). In vitro cell proliferation inhibitory results of anthocyanins against human cancer cell lines. **3.4E**. Pelargonidin 3-*O*-galactoside.

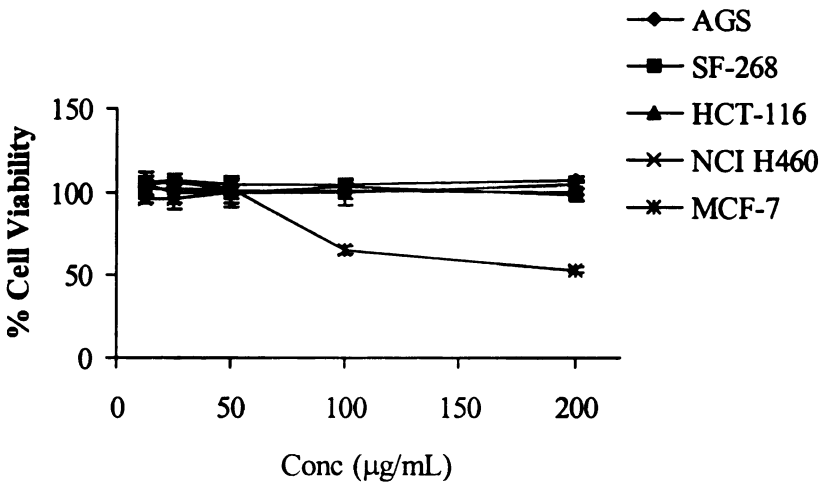


Figure 3.5A

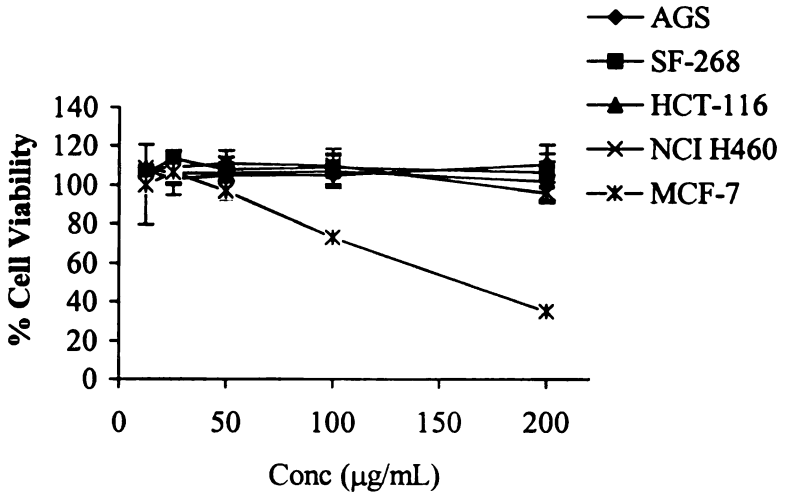


Figure 3.5B

Figure 3.5. In vitro cell proliferation inhibitory results of anthocyanidins **7-11** against human cancer cell lines. DMSO and adriamycin were used as solvent and positive controls, respectively. Anthocyanidins were tested at 200, 100, 50, 25, and 6.25 μg/mL, respectively. The vertical bars represent ± SD of three individual experiments conducted in triplicate. **3.5A**. Delphinidin; **3.5B**. Cyanidin.

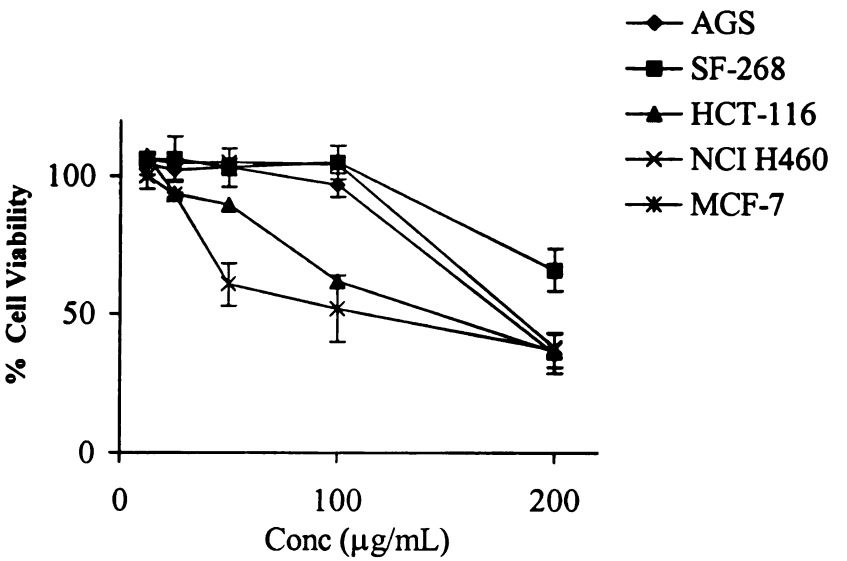


Figure 3.5C

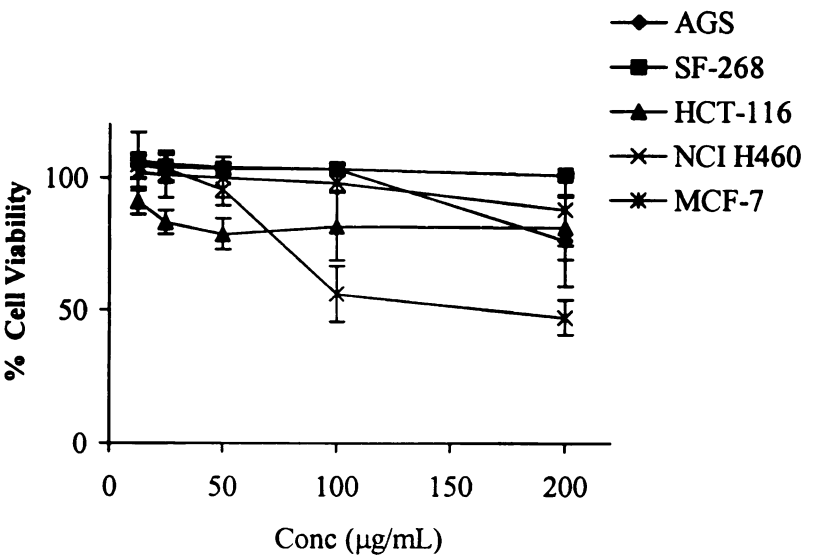


Fig 3.5D

Figure 3.5 (cont'd). In vitro cell proliferation inhibitory results of anthocyanidins against human cancer cell lines. **3.5C**. Pelargonidin; **3.5D**. Petunidin.

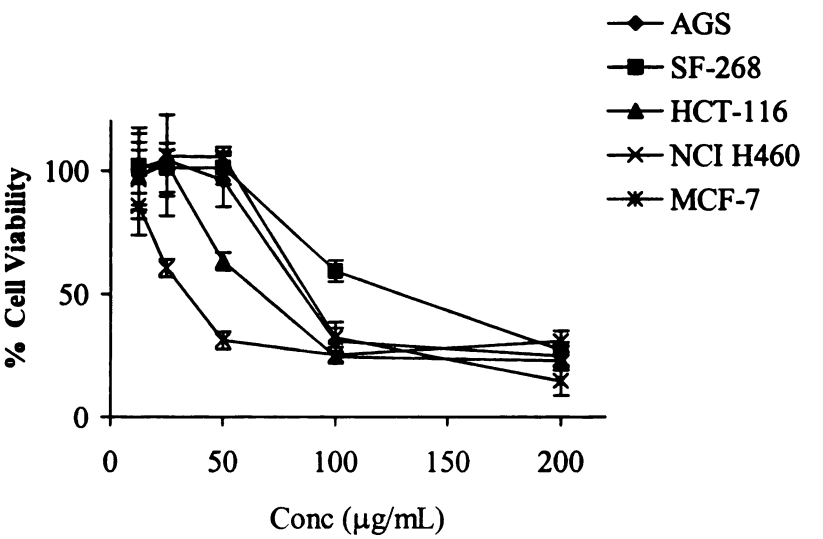


Fig 3.5E

Figure 3.5 (cont'd). In vitro cell proliferation inhibitory results of anthocyanidins against human cancer cell lines. **3.5E**. Malvidin.

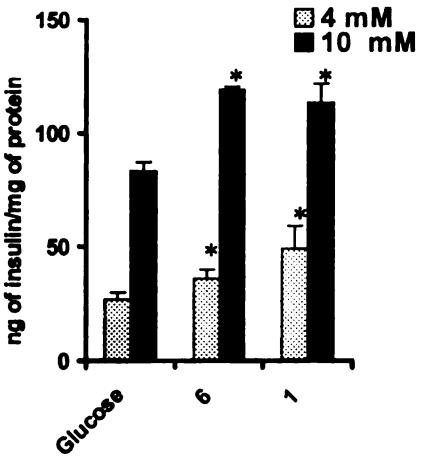


Fig 3.6A

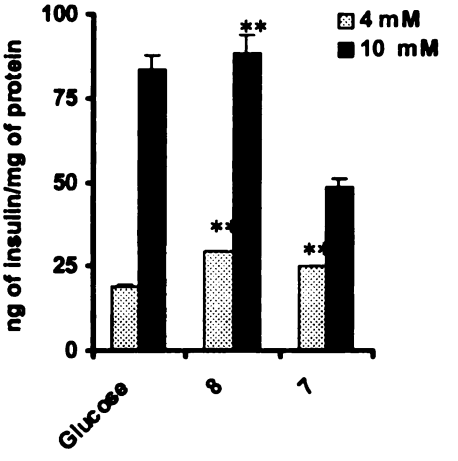


Fig 3.6B

Figure 3.6 (A) The amount of insulin secreted per milligram of protein by compounds **1** and **6** and **(B)** by compounds **7** and **8** in the presence of 4 and 10 mM glucose. The final DMSO concentration in the assay wells was 0.1%. The results represented are the average of three or five independent experiments and each sample was assayed in duplicate. Insulin secretion by compounds **1, 6, 7** and **8** were significant at * (95% or $p \leq 0.05$) or ** (99% or $p \leq 0.01$) as determined by LSD using the t-test

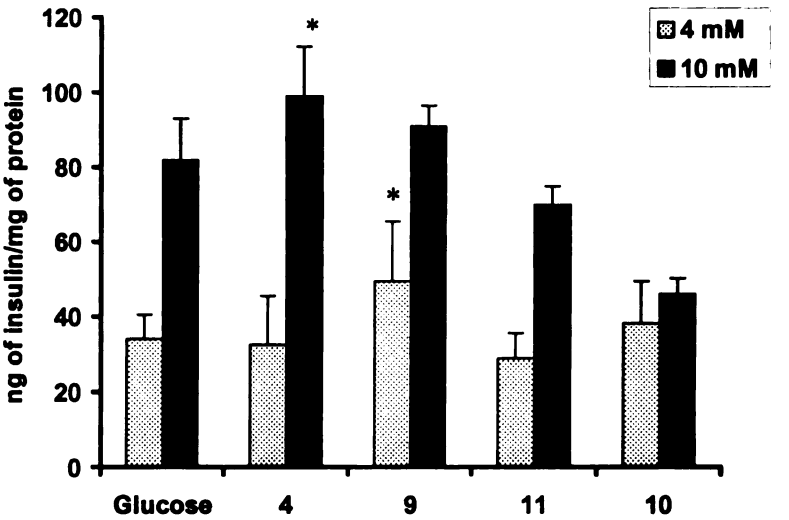


Figure 3.7 The insulin secreted by compounds **4**, **9-11** at 4 and 10 mM glucose concentrations. The amount of insulin secreted was normalized to milligram protein. The final DMSO concentration in the assay wells was 0.1%. The results represented are the average of three independent experiments and each sample was assayed in duplicate. Insulin secretion by compounds **4**, **9-11** was significant at * (95% or $p \leq 0.05$) as determined by LSD using the t-test.

CHAPTER FOUR

FRUIT MATURITY, FLAVONOID AND ANTHOCYANIN PRODUCTION IN *CORNUS* FRUITS

Abstract

The genus *Cornus* is well known for its medicinal properties. Several *Cornus* species are used as traditional medicines in Asian countries including China, India, Korea and Japan. *Cornus kousa*, a widely grown plant among *Cornus* species, has not been investigated for its bioactive constituents. Bioassay-guided isolation and characterization of ripened red fruits of *C. kousa* afforded ursolic acid (**12**) and β-sitosterol (**13**) in addition to cyanidin-3-*O*-glucoside. The matured green fruits of *C. kousa* afforded cornin (**14**), kaempherol 3-*O*-rhamnoside (**15**), myricetin 3-*O*-rhamnoside (**16**), kaempherol 3-*O*-glucoside (**17**) and stenophyllin (**18**) in addition to compounds **12** and **13**. The green fruits were devoid of anthocyanins. These compounds are isolated for the first time from *C. kousa*. Also, it is hypothesized that the biosynthetic pathways for the production of flavonoids in *C. kousa* fruits during ripening was shut down in favor of the production of anthocyanins.

Introduction

Flavonoids including anthocyanins are responsible for the flower colors in many species. These compounds account for a variety of flower colors such as red, orange, pink, purple, blue and yellow (Shirely, 2001). Flavonoids constitute a diverse family of aromatic molecules that are derived from phenylalanine and malonyl-coenzyme A. The colorless flavonoids such as flavonols and flavones may affect the flower color through the formation of molecular complexes with anthocyanins by the process called copigmentation (Justesen et al, 1997).

Flavonoids comprise of several groups including chalcones, flavones, flavanols, flavandiols, anthocyanins, aurones and condensed tannins. Both flavonols and anthocyanins originate from the same substrate dihydroflavonol during their biosynthesis (Scheme 4.1). The dihydroflavonols represent as important targets in flavonoid synthesis. They act as intermediates for the production of anthocyanins through the action of dihydroflavonol 4- reductase (DFR) enzyme. The colorless flavonols are produced by the action of flavonol synthase (FLS) enzyme. FLS acts on dihydroflavonols by introducing an olefinic bond between C-2 and C-3 of the ring C in presence of cofactors 2-oxoglutarate, ascorbate and Fe^{2+} ions (Halto et al, 1993). However, the conversion of dihydroflavonols into anthocyanins involves at least 3 enzymatic steps beginning with a reduction at the 4-carbon position by dihydroflavonol 4-reductase (DFR). The competition between flavonol synthase (FLS) and DFR for common substrate may alter the flavonol-anthocyanin ratio in plants. By changing the flavonol- anthocyanin ratio, it is possible to produce the plants with different flower colors (Mol et al, 1998). Most plant tissues produce anthocyanins in response to external

stresses such as high light intensity and temperature. A low level expression of genes responsible for anthocyanin production may lead to the accumulation of flavonols by diverting dihydroflavonol to flavonols with FLS expression (Nielson et al., 2002). However, the control of FLS gene expression may direct flavonoid biosynthetic pathway either to anthocyanins or to flavonols in vegetative tissues. For example, in lisianthus (*Eustoma grandiflorum* Grise), the loss of FLS expression resulted in the loss of flavonol production and accumulation of dihydroflavonols without the overall increase in anthocyanin production. In contrast, tobacco plants showed three-fold increase in anthocyanin accumulation due to the loss of FLS activity (Nielson et al., 2002).

The genus *Cornus* contains many medicinal plants. Many of these species are used in traditional medicines in Asian countries. For example, *Cornus officinalis*, known as "Zhu Yu" or "Zao Pi" in Chinese medicine, was used as an astringent tonic for impotence, spermatorrhea, lumbago, vertigo and night sweats (www.ncsu.edu). The fruits of *C. officinalis* were used for antidiabetic preparations in China for several years. Fruits of *Cornus oblonga* have been used as a substitute for 'Zao Pi' (www.ncsu.edu). *Cornus kousa*, native to eastern Asia, commonly known as Korean or kousa dogwood, is an ornamental tree. It is a smaller tree with white or pink bracts and has dark green foliage late in the spring season. Its cultivars are increasingly used as landscape plants compared to the flowering dogwood *Cornus florida*, the widely used dogwood native to United States of America. This is mainly due to lack of disease and insect problems typically associated with *C. florida* (Trigiano et al., 2004). *C. kousa* bears colorful, attractive and edible fruits and are used for the production of wine in China (Seeram et al., 2002). Three anthocyanins, cyanidin 3-*O*-glucoside, delphinidin 3-*O*-glucoside, and

pelargonidin 3-*O*-glucoside were reported from *C. kousa* fruits (Du et al., 1974). Polyphenols β-glucogallin, (+)-catechin, (+)-gallocatechin and procyanidin B-3 were also identified from the callus of *C. kousa* (Kanji et al., 1993).

Anthocyanins are the major bioactive compounds reported in *Cornus* species. Total anthocyanin content of many *Cornus* species is 10-15 times higher than other fruits used as sources of anthocyanins (Shaiju et al., 2005). Very little is known about the compounds other than anthocyanins in *Cornus* fruits. Most reports were focused on the isolation, characterization and biological activities of compounds from *C. officinalis*. Our preliminary studies with water, methanol, and ethyl acetate extracts of *C. kousa* fruits showed promising lipid peroxidation and COX enzymes inhibitory activities. In this chapter, the bioassay guided isolation and characterization of compounds from ripened and matured green *C. kousa* fruits are presented.

Materials and Methods

All solvents used for isolation and purification were of ACS reagent grade (Aldrich Chemical Co., Inc., Milwaukee, WI). ^{1}H NMR spectra was recorded at 300 MHz on Varian INOVA and 500 MHz on VRX instruments. ^{13}C NMR spectra were recorded at 75 and 125 MHz instruments. Compounds were dissolved in $CDCl_3$, CD_3OD, and DMSO-d_6 and chemical shifts are given in parts per million (ppm) relative to $CDCl_3$, CD_3OD, and DMSO-d_6 at 7.24, 3.31, and 2.49 ppm, respectively for ^{1}H NMR, and 77.0, 49, and 39.5 ppm, respectively, for ^{13}C NMR. The silica gel used for Medium Pressure Liquid Chromatography (MPLC) was from Merck (35-70 μm particle size).

Thin Layer Chromatography (TLC) and Preparative Thin Layer Chromatography (P-TLC) plates (20 x 20, 500μm) were purchased from Analtech, Inc. (Newark, DE).

Plant Material: The red ripened fruits of *C. kousa* were collected on the campus of Michigan State University in August-September, 2002. The locations of trees were recorded in the Michigan State University Herbarium Plant Database. The green and matured *C. kousa* fruits were collected at 3934 E Sunwind Drive, Okemos, Michigan.

Extraction and Bioassay Guided Isolation of Compounds 12-18. The ripened fruits of *C. kousa* (1.6 kg) were pitted and the pulp was blended with water (1000 mL), and successively extracted with water, methanol, and ethyl acetate (500 mL x 3) to yield 124, 21.5, and 1.4 g of extracts, respectively.

The methanol extract (21.5 g) was stirred successively with ethyl acetate, methanol and water. The ethyl acetate and methanol soluble portion were combined based on TLC and evaporated under reduced pressure (1.3 g). This extract was applied to MPLC (silica) column and fractionated with $CHCl_3$:MeOH solvent system under gradient condition from 100% $CHCl_3$ to 100% MeOH. A total of 15 fractions were collected and each fraction was analyzed and combined based on TLC. Fractions 4 (33 g, 590 mL), and 5 (760 g, 130 mL) were combined based on TLC and purified by MPLC column (silica). The column was eluted, under gradient conditions, with 100% hexane to 100% acetone. A total of 10 fractions were collected. The evaporation of fraction 5 (60 mL) under reduced pressure afforded compound **12** (41.3 mg). The ^{1}H and ^{13}C NMR spectral data of compound **12** were identical to the published spectral data of ursolic acid (Seebacher et al., 2003). The fraction 2 resulting from the above column on repeated purification by MPLC (silica) column with hexane:acetone as the mobile phase afforded

compound **13** (24.0 mg). The ^{1}H and ^{13}C NMR spectral data of compound **13** were identical to the published spectral data of β-sitosterol (Sakuri and Rahmani, 1995). The ethyl acetate extract mainly contained compounds **12** and **13**, as confirmed by TLC, was not studied further.

The fresh and unripened green fruits of *C. kousa* (1.6 kg) were successively blended with methanol (1000 mL x 2) and ethyl acetate (1000 mL x 2), and centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10000g for 20 min at 4°C. The combined supernatants after evaporation under reduced pressure yielded methanol (80 g) and ethyl acetate (10 g) extracts, respectively. An aliquot of the methanolic extract (35.0 g) was dissolved in methanol (100 mL) and partitioned with hexane (150 mL x 3) to yield methanol (24.0 g) and hexane-soluble (10.0 g) fractions. A portion (5.1 g) of the methanol-soluble fraction was fractionated by MPLC (C-18) column using MeOH:H_2O as the mobile phase under gradient conditions from 10% MeOH to 100% MeOH. The fraction 2 (606 mg) from the above column was purified by C-18 column using MeOH:H_2O mobile system and subsequent purification by preparative thin layer chromatography afforded compound **14** (8.9 mg). The ^{1}H and ^{13}C NMR spectral data of compound **14** were identical with that of cornin (Tanaka et al., 2001). The fraction 6 was further fractionated by Prep-HPLC (X-terra® Prep MS C_{18}, 19 x 250, 10 μm) under isocratic conditions using 0.1% TFA/H_2O: CH_3CN (75:25) as the mobile phase. The flow rate was maintained at 3 mL/min and the peaks detected at 275nm. A total of six fractions were collected and further purification of fraction 5 by HPLC using 0.1% TFA/H_2O:CH_3CN (80:20) as the mobile phase afforded compounds **15** and **16**, respectively. The ^{1}H and ^{13}C NMR spectral data of compound **15** and **16** were in

agreement with kaempferol 3-*O*-rhamnoside and myricetin 3-*O*-rhamnoside, respectively (Markhem et al., 1982). The compound **17** was obtained by the purification of fraction 6 using the same mobile system mentioned above. The ^{1}H and ^{13}C NMR spectral data indicated that compound **17** was kaempferol 3-*O*-glucoside (Markhem et al., 1982)

Another portion of the methanolic extract (23.5 g) was dissolved in water (200 mL) and centrifuged. The supernatant was then fractioned by XAD-16 resin. The resin was washed with water (1000 mL x 2) and the adsorbed compounds were eluted with methanol (500 mL x 2) and the solution evaporated under reduced pressure to yield a residue (16.5 g). A portion (3.5 gm) of the was residue was further fractionated by MPLC (silica) using $CHCl_3$:MeOH as the mobile phase afforded six fractions. The fraction 2 obtained from the silica column was evaporated under reduced pressure and washed with $CHCl_3$ to afford compound **18** (2.4 mg). Compound **18** was identified as stenophyllin based on comparison of ^{1}H and ^{13}C NMR spectral data to those reported in the literature (Tanaka et al., 2001).

Results

The *Cornus* plants belong to the family Cornaceae, commonly known as dogwood, produce colorful and attractive flowers and fruits. The Japanese dogwood, *C. kousa* is a deciduous tree and it produces flowers during May-June and fruits during August-September. All *Cornus* plants including *C. kousa* are widely grown as ornamental plants. The edible *C. kousa* fruits are fleshy, round or oval in shape with an attractive red color (Seeram et al., 2002). We have compared the fresh weights of *C. kousa, C. mas, C. officinalis, C. controversa, C. alternifolia* and *C. florida* ripened fruits.

Ripened fruit of *C. kousa* weighed about 15.6 g per fruit and was the highest among all the *Cornus* fruits studied (Shaiju et al., 2005). Even though *C. kousa* fruits are edible and widely used for the production of wine in Asian countries, these fruits are not consumed in the USA.

The ripened *C. kousa* fruits were sequentially extracted with water, ethyl acetate and hexane. The methanol extract showed promising activity in the preliminary lipid peroxidation and cyclooxygenase enzyme inhibitory assays. The fractionation and purification of this MeOH extract afforded compounds **12** and **13** in addition to the anthocyanin, cyanidin 3-*O*-glucoside. The ethyl acetate extract of *C. kousa* contained mainly of compounds **12** and **13** based on TLC and hence was not analyzed further.

Since all *Cornus* fruits studied yielded primarily anthocyanins, we have investigated bioactive compounds in green fruits. For this, matured green *C. kousa* fruits were collected just before the ripening process. The methanol extract of green *C. kousa* fruit was partitioned with hexane to remove chlorophylls and fractionated to afford compounds **12-18** (Fig. 3).

Discussion

The fruits of *C. kousa*, commonly known as kousa dogwood, resemble to raspberries. The plant *C. kousa* is a deciduous shrub or a small tree of about 7-10 m in height. Its cultivars include 'Autumn Rose', 'Ballerina', 'Beni Fuji' and 'China Girl'. Many of these cultivars are produced by the hybridization of *C. kousa* and *C. florida*. The anthocyanins are primary bioactive constituents in its ripened fruits. We have recently reported several anthocyanins and its concentrations in *Cornus* fruits (Shaiju et

al, 2005). Anthocyanins cyanidin 3-*O*-glucoside and cyanidin 3-*O*-galactoside were characterized and quantified from *C. kousa* ripened fruits.

Other components isolated from *C. kousa* ripened fruits were compounds **12** and **13**. However, the analysis of unripened fruits afforded compounds **14-18** in addition to compounds **12** and **13** (Fig. 3). The concentration of **12** and **13** in ripened fruits was several times higher than in green fruits. It was interesting to note that *C. kousa* ripened fruits did not yield flavonoids but gave primarily anthocyanins cyanidin 3-*O*-galactoside and cyanidin 3-*O*-glucoside. This indicated that the biosynthetic pathway of flavonoids was regulated to a halt during the ripening process and favored the reaction which resulted in the accumulation of anthocyanins (Scheme 4.1).

We also have studied the ripened fruits of *C. controversa* and *C. alternifolia* for bioactive compounds. Both compounds **12** and **13** were isolated and characterized from methanol and ethyl acetate extracts of *C. controversa* and identified in *C. alternifolia* ripened fruits. The aqueous extracts of *C. controversa* and *C. alternifolia* are shown to be excellent sources for delphinidin 3-*O*-glucoside (**1**) and delphinidin 3-*O*-rutinoside (**2**) (Shaiju et al, 2005). The total anthocyanin content in these fruits is also shown to be 20-25 times higher than other known natural sources of anthocyanins. Based on the absence of flavonoids in the ripened *Cornus* fruits, it is hypothesized that the flavonone-dihydroflavonol-anthocyanin biosynthetic route was dominant during ripening in *Cornus* fruits which then lead to the accumulation of anthocyanins (Scheme 1).

The anthocyanin accumulation in leaves and flowers depends on various factors such as nutrients, temperature, availability of water, and in particular, light (Shamir and Nissim, 1997). We have reported anthocyanin production in relation to light quantity in

Pennisetum setaceum Cvs. Rubrum and Red Riding Hood (Beckwith et al., 2004). When *P. setaceum* leaves were exposed to different light environmental conditions such as UV supplemental light in the green house, high-pressure sodium supplemental light in the green house, cool-white fluorescent light in the growth chamber and outside sunlight, the production of anthocyanin varied remarkably (Beckwith et al., 2004). The maximum level of anthocyanin detected was in plants grown under fluorescent light conditions. This indicated that both light intensity and temperature played a role in anthocyanin production and accumulation. That is, low temperature generally promoted anthocyanin production while high temperature inhibited its accumulation (Mazza and Miniati. 2000). Anthocyanin may acts as a protective shield to the photosynthetic machinery by absorbing UV radiation. The effect of UV light on anthocyanin accumulation was also studied in *Cotinus coggygria* (Shamir and Nissim, 1997). The exposure of *C. coggygria* to UV light between 300 -400 nm produced a significant accumulation of anthocyanins in its leaves.

Various enzymes, phenylalanine ammonia-lyase (PAL), chalcone synthase, flavanone 3- hydroxylase, flavanone 3′-hydroxylase and several glucosyltransferases are responsible for the biosynthesis of anthocyanins. However, phenylalanine ammonia-lyase is recognized as a critical enzyme for anthocyanin synthesis (Ebel and Hahlbroch, 1982). Anthocyanin accumulation during ripening of several fruits is directly related to the PAL activity (Aoki et al., 1970; Hyodo, H, 1971). The studies on the relationship between PAL and anthocyanin at different temperatures indicated that both anthocyanin synthesis and PAL activity increased at low temperatures (Tan, 1979). Fruits held at alternating temperatures of 6 and 18°C produced double the amount of anthocyanins

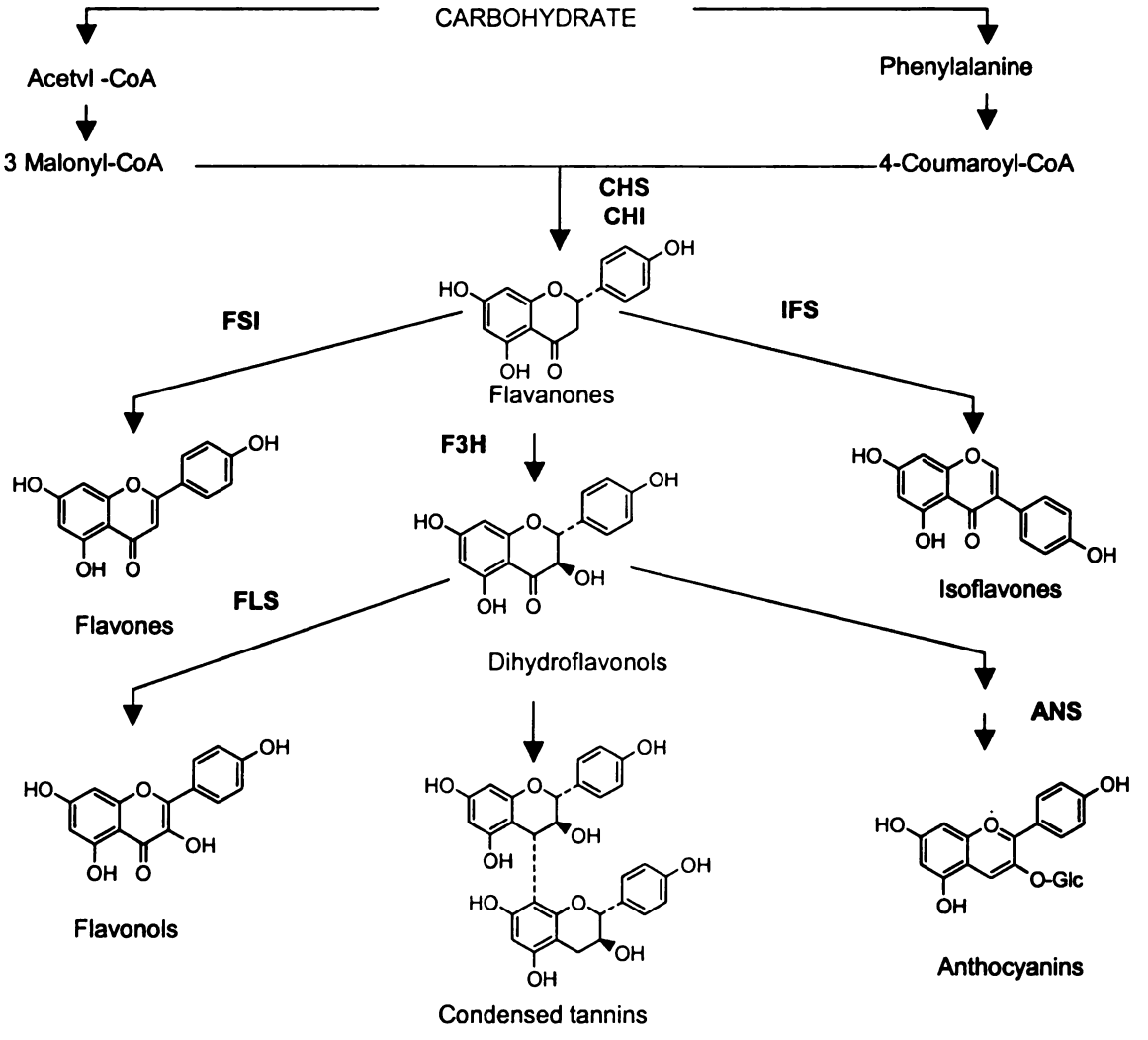
compared to the fruits at 18 °C. The PAL activity was also stimulated by ethylene, low nutrient level, light, water condition, sugar content and fruit ripening. A report by Faragher and Broheir indicated that anthocyanin accumulation increased with the rise of ethylene level in ripening of fruits due to the stimulation of PAL activity (Faragher and Brohier, 1984). It was also hypothesized that low temperature may promote the anthocyanin production by reducing the activity of gibberellins (Saure, 1990).

Sugars are essential for the synthesis of anthocyanins. Fructose, glucose, lactose, maltose, and sucrose stimulate the synthesis of anthocyanins (Vestrheim, 1970). The catabolism of glucose though pentose phosphate pathway (PPP) has been associated with anthocyanin production in many fruits (Faust, 1965). Many reports indicate that anthocyanin production increase several times during the ripening of the fruits. For example, total anthocyanin in ripened raspberry fruits was four fold higher than in unripened fruits. In the case of 'Montmorency' sour cherries, total anthocyanin content increased from 2 to 43.6 mg per100 g fresh wt during ripening (Gross, 1987).

A recent report of gene expression studies of flavonoid biosynthesis in lisianthus (*Eustoma grandiflorum* Grise) indicated that phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI) and flavonoid 3′ hydroxylase (F3′-OH) were the important enzymes expressed during bud development (Nielson et al., 2002). During the floral development, two new enzymes, dihydroflavonol 4- reductase (DFR) and flavonoid 3′5′- hydroxylase (F3′5′ –OH), were expressed in addition to PAL, CHS and CHI although both CHS and CHI were expressed in lower levels (Nielson et al., 2002). This indicated that during the early flower development of lisianthus, there was

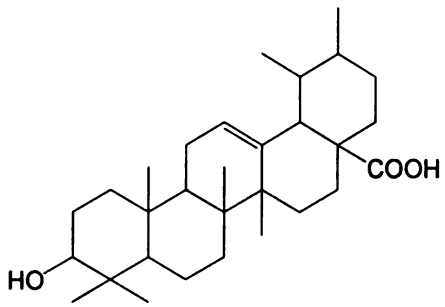
flavonoid accumulation and in later stages the pathway shifted more towards the accumulation of anthocyanins. A similar scenario is possible in *Cornus* plants as well.

Our studies with *C. kousa* fruits indicated that flavonoids are the major compounds in matured green fruits. The flavonoids accumulation in early stages of fruit development could be for protecting the plants from reproductive tissue damage. The high flavonols production may be due to the shift of biosynthetic pathway in favor of flavonols due to the over expression FLS gene. Contrary, ripened *Cornus* fruits showed high amount of anthocyanins and no flavonoid was detected in any of its species. This could be due to the shift of biosynthetic pathway in favor of anthocyanins with the expression of DHR. Molecular experiments to determine the up or down regulation of genes responsible for flavonoid and anthocyanin production in *Cornus* fruits should be carried out. Also, such experiments will be useful to further enhance the production of anthocyanins in *Cornus* and possibly in other edible fruits.

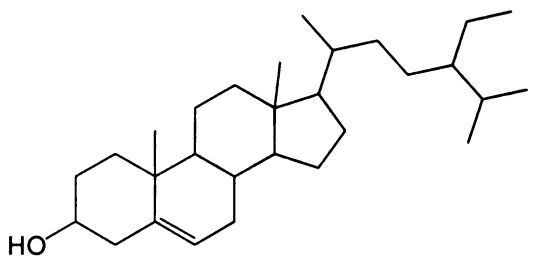


Scheme 1

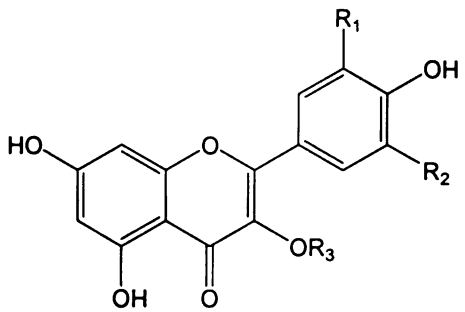
Scheme 4.1. Reported flavonoid and anthocyanin biosynthetic pathway. **CHS** Chalcone synthase, **CHI** Chalcone isomerase, **FSI** Flavone synthase, **IFS** Isoflavone synthase, **FLS** Flavanol synthase, **F3H** Flavanone 3-hydroxylase, and **ANS** anthocyanidin synthase (Harbone, 1988., Taiz and Zieger, 1998)



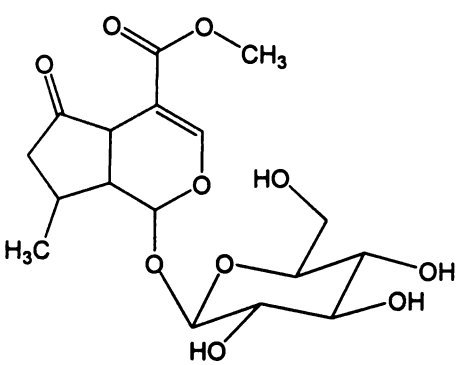
12



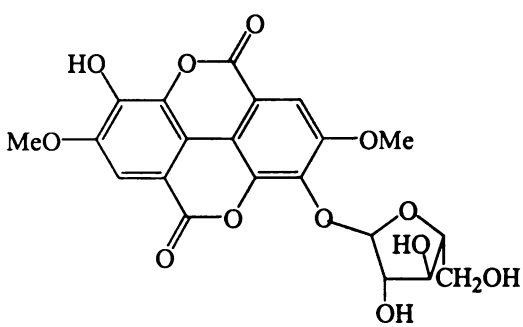
13



Compound	R_1	R_2	R_3
15	H	H	Rha
16	OH	OH	Rha
17	H	H	Glc

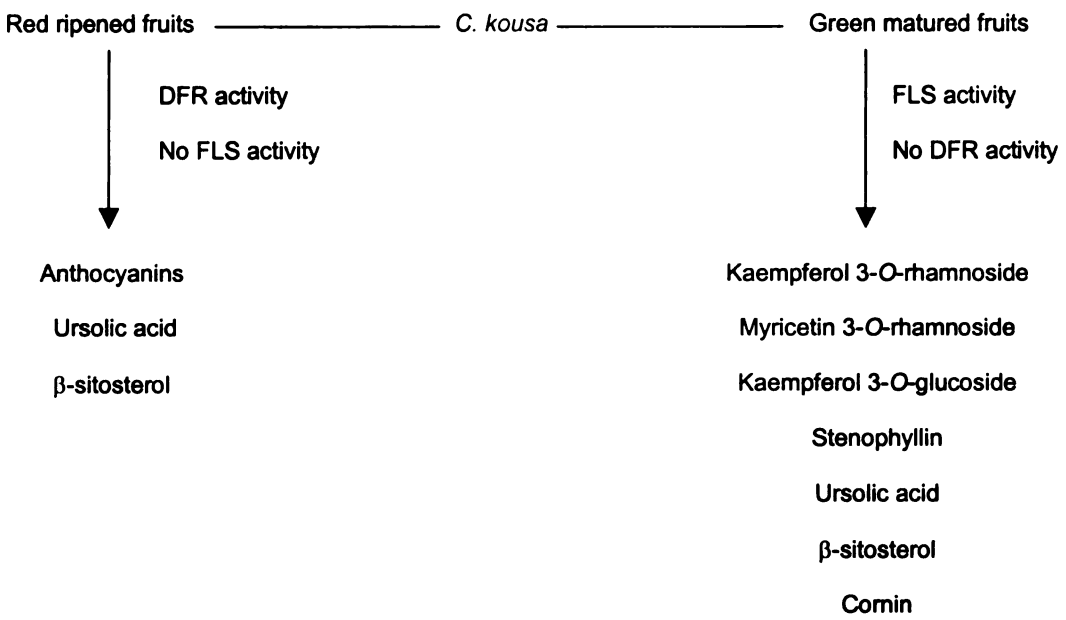


14



18

Figure. 4.1 Structures of compounds **12-18** isolated from *C. kousa* fruits.



Scheme 4. 2. Compounds isolated from *C. kousa* fruits

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

The genus *Cornus* have been used for thousands of years in many parts of the world especially in China and Japan. *Cornus* fruits are mainly used as a medicinal agent for the treatment of diseases associated with liver and kidney. The fruits of *C. officinalis* have been used for antidiabetic preparation in China for centuries. Even though *Cornus* species has reported several medicinal properties, very little is known about its bioactive compounds. A detailed investigation of botany, chemistry, biological and pharmacological activities of *Cornus* spp. conducted earlier studies is presented in **Chapter 1**. Based on the detailed literature review it was determined that *C. mas, C. officinalis, C. controversa, C. alternifolia, C. kousa* and *C. florida* fruits should be investigated further for bioactive compounds with potential human health benefits.

The ripened fruits of *C. mas, C. officinalis, C. controversa, C. alternifolia, C. kousa,* and *C. florida* were collected from Michigan State University campus in August-September 2002 and 2004. The matured green fruits of *C. kousa* were collected from 3934 E Sunwind Drive, Okemos, Michigan in August 2004. The anthocyanins in these fruits were isolated, characterized and quantified by various chromatographic and spectroscopic techniques. The isolation, characterization and quantification of anthocyanins in these *Cornus* spp. fruits are presented in **Chapter 2**. The quantification results of anthocyanins, delphinidin 3-*O*-glucoside (**1**), delphinidin 3-*O*-rutinoside (**2**), delphinidin 3-*O*-galactoside (**3**), cyanidin 3-*O*- galactoside (**4**), pelargonidin 3-*O*-

galactoside (**5**) and cyanidin 3-*O*-glucoside (**6**), indicated that *C. controversa, C. alternifolia* and *C. mas* are the excellent sources for these anthocyanins. The amount of delphinidin 3-*O*-glucoside (**1**), delphinidin 3-*O*-rutinoside (**2**), and cyanidin 3-*O*-glucoside (**6**) in *C. alternifolia* and *C. controversa* were 8.21, 8.44 and 0.02 mg; and 7.74, 5.92, and 0.02 mg/g of fresh fruits, respectively. Similarly, delphinidin 3-*O*-galactoside (**3**), cyanidin 3-*O*- galactoside (**4**), and pelargonidin 3-*O*-galactoside (**5**) in *C. mas* were 0.47, 1.66 and 1.62; and 0.15, 0.21, and 0.78 mg/g of fruits, respectively. Anthocyanin content in these species are 20-25 times higher than other major fruit sources of anthocyanins.

Although both anthocyanins and anthocyanidins were reported as potent antioxidant and anti-inflammatory agents, anticancer and insulin secretion activities of these compounds were not studied earlier. A detailed investigation of biological activities of anthocyanins and anthocyanidins were discussed in **Chapter 3**. Based on the studies conducted as part of my thesis research project, it was determined that both anythocyanins and anthocyanidins exhibited good anticancer and insulin secretion activities in addition to lipid peroxidation and cyclooxygenase enzyme inhibitory activities. At 50 μg/mL, delphinidin 3-*O*- glucoside (**1**) and delphinidin 3-*O*- rutinoside (**2**) inhibited lipid peroxidation by 71 and 68%, respectively. Similarly, they inhibited COX-1 enzymes by 39 and 49% and COX-2 enzyme by 54 and 48%, respectively, at 100 μg/mL. Anthocyanins, delphinidin 3-*O*- glucoside and delphinidin 3-*O*-rutinoside, and the anthocyanidin malvidin showed potent cell proliferation inhibitory activities when tested against human cancer cell lines, AGS (gastric), CNS (central nervous system, SF-268), HCT-116 (colon), NCI-H460 (lung), and MCF-7 (breast). Delphinidin 3-*O*-

glucoside (**1**) displayed 50% growth inhibition (IC_{50}) at 21, 25, 50, 60, and 75 μg/mL, against colon, breast, lung, central nervous system (CNS), and stomach human tumor cell lines, respectively. Similarly, IC_{50} values for delphinidin 3-*O*- rutinoside (**2**) were 38, 30, 76, 100, and 100 μg/mL against colon, breast, lung, central nervous system (CNS), and stomach cell lines, respectively. Anthocyanins delphinidin 3-*O*- glucoside (**1**) and cyanidin 3-*O*- glucoside (**6**), and anthocyanidin pelargonidin (**9**) also showed promising insulin secretion activity when tested against rodent pancreatic β cells at 4 and 10 mM glucose concentrations.

A detailed study of all the non-pigmented fractions of *C. kousa, C. controversa,* and *C. alternifolia* fruits was discussed in **Chapter 4**. Bioassay-guided isolation, purification and characterization of methanol, ethyl acetate and hexane extracts of *C. kousa* ripened and unripened fruits afforded ursolic acid (**12**), β-sitosterol (**13**), cornin (**14**), flavanoids, kaempherol 3-*O*- rhamnoside (**15**), myricetin 3-*O*-rhamnoside (**16**), and kaempherol 3-*O*- glucoside (**17**), and stenophyllin (**18**). The compounds ursolic acid (**12**) and β-sitosterol (**13**) were also identified in the fruits of both *C. controversa* and *C. alternifolia*. This is the first report of these compounds from *C. kousa* fruits.

My research on various *Cornus* fruits for bioactive compounds indicated that anthocyanins are the major active compounds present in all *Cornus* fruits. The evaluation of several compounds yielded from *Cornus* fruits in my study confirmed that anthocyanins and anthocyanidins were the most active in inhibiting lipid peroxidation, cyclooxygenase enzymes and the growth of human tumor cell lines. Also, it suggested that *Cornus* fruits containing high levels of these anthocyanins are powerful insulin

secretagogues and may be useful for the prevention of type-2 diabetes. The bioassay results also explained the anecdotal health claims associated with *Cornus* fruits.

The in vitro cell proliferation studies with various human cancer cell lines suggest that anthocyanins in *C. alterniflolia* and *C. controversa* fruits may be useful in the prevention of certain tumor progression. Because *C. alterniflolia* and *C. controversa* fruits have high amount of health beneficial anthocyanins, it is possible that these plants could be cultivated as alternate crops to yield fruits for tumor cell growth inhibitory and insulin secretion anthocyanins and anthocyanidins. However, in vivo studies and clinical evaluation of these compounds must be carried out to further validate the in vitro results.

Ornamental plants, both indigenous and introduced, provide a tremendous resource for value added products and functional foods. Anecdotal information on many of these species suggests the application of these plants not only in human health but also in the diversification of agricultural production. The *Cornus* spp. is already in mainstream horticulture production as landscape ornamentals. They also have similar botanical traits to horticultural crops in food production. For example, *C. mas* and *C. officinalis* can be easily integrated into tart cherry ('Montmorency' and 'Balaton®') production systems. Trees can be managed the same, *C. mas* and *C. officinalis* require minimal chemical inputs, and fruits can be harvested with the same equipment in the fall after the demands of cherry production has ceased. Production of *C. mas* while providing an economically valued crop can extend a production season without disrupting the current production system. The same is true for *C. alternifolia* and *C. controversa*; these species could be integrated into small fruit production systems. Ornamental plants production in the United States is an untapped resource for phytochemicals and

functional foods. Ornamental species can make a seamless transition into cropping systems with the value added benefits. *Cornus* spp. is one example where investigation, examination, and analysis offer therapeutic options in human health.

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