This is to certify that the

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ANTIOXIDANT AND ANTIINFLAMMATORY

COMPOUNDS IN TART CHERRIES

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HAIBO WANG

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ANTIOXIDANT AND ANTIINFLAMMATORY COMPOUNDS IN TART CHERRIES

BY

Haibo Wang

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ABSTRACT

ANTIOXIDANT AND ANTIINFLAMMATORY COMPOUNDS IN TART CHERRIES

By

Haibo Wang

Tart cherry production and processing is an important industry in Michigan. Michigan's tart cherry industry produces 75% of the nation's tart cherry supply, which is about 250-300 million pounds annually. Anecdotal reports indicate that consumption of tart cherries could alleviate arthritic- and gout-related pain and reduce the incidence of cardiovascular diseases. These beneficial effects may be associated with the anthocyanins, phenolics and flavonoids present in tart cherries. In order to evaluate these claims, the bioactive components in tart cherries were isolated using antioxidant-bioassay-directed fractionation and purification.

The water extracts of Montmorency and Balaton™ tart cherries yielded three anthocyanins. Also, the results indicated that both cultivars contained identical anthocyanins. The hydrolysis of the total anthocyanins and subsequent gas chromatographic (GC) and nuclear magnetic resonance (NMR) spectral analyses suggested that the anthocyanins in Montmorency and Balaton™ cherries were anthocyanin **1** [cyanidin-3-(2″-*O*-*β*-D-glucopyranosyl-6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside], anthocyanin **2** [cyanidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-

glucopyranoside] and anthocyanin **3** [cyanidin-3-*O*-β-D-glucopyranoside]. However, Balaton™ contains approximately six times more anthocyanin than Montmorency.

In another experiment, Balaton™ cherries were extracted sequentially with hexane, ethyl acetate and methanol. Antioxidant assays indicated that ethyl acetate and methanol extracts exhibited higher antioxidant activity than hexane extract. Further purification of the ethyl acetate extract yielded chlorogenic acid methyl ester and three novel compounds, 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid , 1-(3', 4'-dihydroxycinnamoyl)-cyclopenta-2,5-diol and 1-(3',4'-dihydroxycinnamoyl)-cyclopenta-2,3-diol. Similarly, the methanol extract yielded eight polyphenolic compounds, 5,7,4'-trihydroxyflavanone, 5,7,4'-trihydroxyisoflavone, chlorogenic acid, 5,7,3',4'-tetrahydroxyflavonol-3-rhamnoside, 5,7,4'-trihydroxyflavonol-3-rutinoside, 5,7,4'-trihydroxy-3'-methoxy-flavonol-3-rutinoside, 5,7,4'-trihydroxyisoflavone-7-glucoside and 6,7-dimethoxy-5,8,4'-trihydroxyflavone.

The antioxidant activities of the purified compounds were evaluated using the Fe^{2+}-induced lipid peroxidation assay. Commercial antioxidants propyl gallate, butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) and α-tocopherol were used as positive controls. The anthocyanins and their aglycones, cyanidin, 5,8,4'-trihydroxy-6, 7-dimethoxyflavone and caffeic acid analogues from Balaton™ were the most active antioxidant compounds. The antioxidant activities of these compounds were greater than α-tocopherol and comparable to the activities of BHT and TBHQ.

In the anti-inflammatory assay, which measured inhibition of the cycloxygenase (COX) activities of the prostaglandin endoperoxide H synthase-1 and –2 isozymes (hPGHS-1 and –2), cyanidin showed significant inhibitory activities against COX-1 and

COX-2 enzymes. The IC_{50} values of cyanidin against hPGH-1 and hPGH-2 were 90 and 60 μM, respectively. Similarly, the inhibitory activities on hPGHS-1 enzyme of the polyphenolics from Balaton™ tart cherries also were investigated. Genistein exhibited the highest COX-1 inhibitory activity at an IC_{50} value of 80 μM. Other flavonoids and isoflavonoids isolated from tart cherries have IC_{50} values greater than 400 μM concentration.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CD	Circular dichroism
COX	Cyclooxygenase
^{13}C NMR	Carbon nuclear magnetic resonance
DMSO	Dimethyl sulfoxide
DQFCOSY	Double quantum filtered correlated spectroscopy
dd	Doublet of doublet
EIMS	Electron impact ionization mass spectroscopy
FABMS	Fast atom bombardment mass spectroscopy
DPA-PA	3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
^{1}H NMR	Proton nuclear magnetic resonance
HPLC	High pressure lipid chromatography
LDL	low-density lipoprotein
LO	Lipoxygenase
MOPS	3-[N-morpholino] propanesulfonic acid
MPLC	Medium pressure lipid chromatography
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
ORD	Optical rotatory disperson
PDA	Photodiode array
PG	Prostaglandin
PGHS-1	Prostaglandin endoperoxide H synthase-1
PGSH-2	Prostaglandin endoperoxide H synthase-2
TBA	2-thiobarbituric acid
TBHQ	*tert*-butylhydroquinone
UV	Ultraviolet
δ	Chemical shifts
J	Coupling constant

INTRODUCTION

Tart cherry production and processing is an important industry in Michigan. Michigan's tart cherry industry produces about 75% of the nation's tart cherry supply. Michigan produces 250-300 million pounds of tart cherries annually, which are grown on a total of 36,300 acres and having an estimated value of 50-70 million dollars.

The original habitat of the red tart cherry (*Prunus cerasus* L.) was between Switzerland and the Adriatic Sea on the West and the Caspian Sea and somewhat northward on the East. Pomologists assumed that both sweet and red tart cherries originated in the same region and that the latter may have been derived from the former. The earliest records of the cultivation of the cherry indicated that it was first domesticated in Greece. The cherry was brought to America by the early colonists. Montmorency, a cultivar of tart cherry, which originated from France about 300 years ago, was first studied at the Michigan Agricultural Experimental Station in 1922.

Of the 270 named varieties of red tart cherries, there are only three varieties of red tart cherries grown in appreciable quantities, all of which are of European origin. They are Early Richmond (Kentish), Montmorency and English Morello. Montmorency represents 97% of the tart cherry acreage in the United States. Tart cherries are grown only in areas with favorable climate, soil and topography. In Michigan, tart cherries are grown in the western counties of the lower peninsula along Lake Michigan. In order to diversify the Montmorency monoculture, a new Hungarian cultivar, Balaton™, was introduced into the United States in 1984, and it has been tested in Michigan, Utah, and Wisconsin. Balaton™ produces fruits darker than Montmorency, and it may be used as a source of cherry anthocyanins.

Tart cherries can be used in many different ways. Tart cherry pits can be used as a source for natural benzaldehyde (Chandra and Nair, 1993). The oil from Montmorency cherry pits has potential for cooking or frying food (Chandra and Nair, 1993). The anthocyanins from Michigan tart cherries can provide a more efficient cherry colorant (Chandra et al., 1992). Anecdotal reports indicate that consumption of cherries could alleviate arthritic- and gout-related pain (Hamel, 1975) and reduce the incidence of cardiovascular diseases. Tart cherries also can be added to ground beef to make it "lean". Thiobarbituric acid (TBA) values for beef patties containing cherries were significantly lower when stored under refrigeration and kept for six months at -20°C than for patties which were cooked and then refrigerated for 24h (Liu et al., 1995).

These beneficial effects may be associated with the anthocyanins, phenolics and flavonoids present in tart cherries. Plant polyphenolics are multifunctional and can act as reducing agents, hydrogen-donating antioxidants, and singlet oxygen quenchers. In some cases, metal chelation properties have been proposed as well. The biological, pharmacological, and medicinal properties of the flavonoids have been reviewed extensively (Cody, et al, 1986; 1988). Flavonoids and other plant are reported to have multiple biological activities, in addition to their free radical scavenging activities. (Ho et al, 1992; Kinsella et al., 1993). These types of compounds were reported to have anticarcinogenic, antiinflammatory, antibacterial, immune-stimulating, anti-allergenic, antiviral, and estrogenic effects. They also can act as inhibitors of phospholipase A2, cyclooxygenase, and lipoxygenase (Brown, 1980, Middleton and Kandaswami, 1992; Mabry et al., 1982; Jovanvoic et al., 1992; Robak, et al., 1988; Sogawa, et al., 1993; Lindahl and Tagesson, 1993), glutathione reductase (Elliott, et al, 1992), and xanthine

oxidase (Chang et al., 1993). Their effects on a variety of inflammatory processes also have been reviewed (Gabon, 1979, 1986; Farkas et al., 1986; Welton et al., 1988). The flavonoids can significantly affect the function of the immune system. A number of flavonoid compounds can affect the activity of enzyme systems which are critically involved in the immune response, and the triggering of inflammatory processes. For example, quercetin and kaempferol exhibit extensive immune response activity (Lee et al., 1982; Middleton et al., 1981; 1984). Also, quercetin was shown to inhibit the mutagenic activity of benzo[α]pyrene(BP), a representative polynuclear aromatic hydrocarbon (PAH) carcinogen, in bacterial mutagenicity studies (Ogawa et al., 1985). Quercetin also was reported to inhibit many biochemical events associated with tumor promotion (Levy et al., 1984). Anthocyanins also were regarded as naturally occurring pigments with antiinflammatory (Vlaskovska et al., 1990) and antioxidant activities (Costantino et al., 1992; Gabor, 1988).

It is postulated that polyphenols, such as flavonoids, isoflavoniods, anthocyanins and anthocyanidins exist in tart cherries may act as antioxidants and possess other biological activities that are responsible for anecdotal health claims associated with tart cherry. In order to evaluate this hypothesis, we have investigated the active components in Montmorency and Balaton™ tart cherries and evaluated their biological activities using Fe^{2+}-induced lipid peroxidation and cyclooxygenase enzyme assays. Therefore, the objectives of this research are (1) Characterization and quantification of anthocyanins in Balaton™ and Montmorency tart cherries; (2) Isolation, purification and identification of bioactive polyphenolics and flavonoids in Balaton™ and Montmorency tart cherries using chromatographic and spectral methods, and (3) Determination of antioxidant and

antiinflammatory activities of polyphenolics and anthocyanins from Montmorency and Balaton™ tart cherries using Fe^{2+}-induced lipid peroxidation and cyclooxygenase enzymes assay.

This dissertation is organized into a series of chapters. Each chapter covers a specific aim of the study and is prepared as a manuscript with abstract, introduction, general experimental, results and discussion.

CHAPTER ONE

Literature review

INTRODUCTION

Cherry is a popular temperate fruit, which belongs to the genus *Prunus* and the family *Rosaceae*. Cherries are mainly of two types, sweet (*Prunus avium*) and tart (*P. cerasus*). Many important fruits are included in the *Prunus* genus, such as apricot, peach, and plum. They are widely used in traditional medicine as antipyretics and useful against thirst, leprosy and leucoderma (Chopra et al, 1956). The major chemical constituents in *Prunus* spp. are anthocyanins, polyphenols and organic acids. In this review, major chemical components with important biological activities from *Prunus* genus are summarized.

1. CHEMICAL CONSTITUENTS OF *PRUNUS* SPECIES

1.1 Anthocyanins

The anthocyanins belong to the flavonoid class of compounds consisting of a flavylium cation (Fig. 1.1). There are about 300 naturally occuring anthocyanins. The anthocyanin molecule consists of two or three portions; the aglycone moiety consisting of the flavylium cation, a group of sugars and, often a group of acylated functionalities. The 17 anthocyanidins reported so far are listed in Table 1.1 (Harborne and Grayer, 1988). The anthocyanins occur in nature as mono-, di-, and triglycosides. The typical sugar substitutions on anthocyandins are glucose, galactose, rhamnose, arabinose and xylose, respectively. The positions for the sugar substitutions are C_3, C_3', C_5, C_4', $C_5'$and C_7, respectively. Most of the sugar substitution present in anthocyanins are on the C_3 position

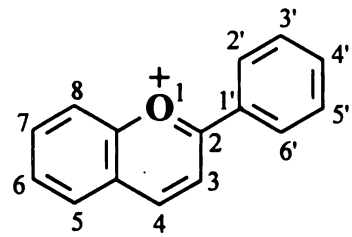


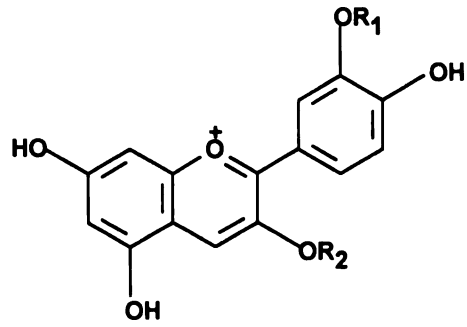
Fig. 1.1. Flavylium cation

Table 1.1. Aglycones reported in anthocyanins

		Substitution							
Name	Abbr.	3	5	6	7	3'	4'	5'	Color
Apigeninidin	Ap	H	OH	H	OH	H	OH	H	orange
Luteolinidin	Lt	H	OH	H	OH	OH	OH	H	orange
Triacetinidin	Tr	H	OH	H	OH	OH	OH	OH	red
Pelargonidin	Pd	OH	OH	H	OH	H	OH	H	orange
Aurantidin	Au	OH	OH	OH	OH	H	OH	H	orange
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	red
5-mecyandin	5MCy	OH	OMe	H	OH	OH	OH	H	red
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	red
Rosindin	Rs	OH	OH	H	OH	OMe	OH	H	red
6-OHcyanidin	6OHCy	OH	OH	OH	OH	OH	OH	H	red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	blue
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	blue
Malvidin	Mv	OH	OH	H	OH	OMe	OMe	OH	blue
Pulchellidin	Pl	OH	OMe	H	OH	OH	OH	OH	blue
Euopinidin	Eu	OH	OMe	H	OH	OMe	OH	OH	blue
Capensinidin	Cp	OH	OMe	H	OH	OMe	OH	OMe	blue
Hirsutidin	Hs	OH	OH	H	OMe	OMe	OH	OMe	blue

and are rarely found at C_3', C_5, C_4', C_5' and C_7 (Harborne and Grayer, 1988). Recent studies have indicated that anthocyanins can be acylated with coumaric, caffeic, ferulic, *p*-hydroxy benzolic, synapic acid and some aliphatic acids such as malonic, acetic, succinic, oxalic, and malic acid, respectively. However, aliphatic acylations in anthocyanins were not identified easily because of the instability of acyl linkages in methanolic HCl solution, which is a standard solvent used for pigment extraction. However, after acetic acid was first reported to be conjugated with grape pigment, many unacylated pigments reported previously were subsequently found to have acyl groups, such as in the petals of *Centaurea cyanus* (Tamura, 1983).

Tart cherries contain a variety of anthocyanins. Willstatter and Zollinger (1916) isolated a pigment from cherry skins, and named it as keracyanin. From Montmorency, Li and Wagenknecht (1956) isolated and characterized two anthocyanins, cyanidin 3-rhamnoglucoside and cyanidin 3-gentioside (mecocyanin) (Fig. 1.2). These two anthocyanins were confirmed by Markakis (1960). Harborne and Hall (1964) reported this triglycoside in seven other cultivars of tart cherries. Cyanidin 3-glucoside was reported as a minor pigment of Montmorency (Schaller and Von Elbe, 1968). In addition to cyanidin 3-glucoside and 3-glucosylrhamnoglucoside, cyanidin 3-rhamnoglucoside, cyanidin 3-sophoroside and peonidin 3-rhamnoglucoside were also identified in Montmorency cherries (Dekazos, 1970). Also, he quantified the content of seven anthocyanins in partially and fully matured Montmorency. Cyanidin 3-rhamnoglucoside and cyanidin 3-glucosylrhamnoglucoside were further confirmed by other authors (Von Elbe et al., 1968; Fischer and Von Elbe, 1970; Schaller et al., 1972). Chandra et al. (1992) reported peonidin-3-galactoside in Montmorency cherries using HPLC (Fig. 1.2).



Cyanidin 3-rutinoside: R_1=H, R_2= rhamnosylglucose

Cyanidin 3-gentioside: R_1=H, R_2= glucose (6 → 1)-glucose

Cyanidin 3-sophoroside: R_1= H, R_2= glucose (2 → 1)-glucose

Cyanidin 3-sambubioside: R_1= H, R_2= glucose (4 → 1)-glucose

Cyanidin 3-(2-glucosyl) rutinoside: R_1= H, R_2= 2-glucosylrutinoside

Cyanidin: R_1= H, R_2= H

Cyanidin 3-glucoside: R_1= H, R_2= glucose

Cyanidin 3-arabinoside: R_1= H, R_2= arabinose

Cyanidin 3-xylosylglucoside: R_1= H, R_2= xylosylglucose

Cyanidin 3-*p*-coumarylglucoside: R_1= H, R_2= *p*-coumarylglucose

Peonidin: R_1= CH_3, R_2= H

Peonidin 3-arabinoside: R_1=H, R_2=arabinose

Peonidin 3-rutinoside: R_1=CH_3, R_2=rutinoside

Peonidin 3-galactoside: R_1=CH_3, R_2=galactose

Fig. 1.2. Anthocyanins from *Prunus* species

From the varieties English Morello, Early Richmond and Meteor, Shrikhande and Francis (1973) identified cyanidin 3-glucosylrutinoside, cyanidin 3-sophoroside, cyanidin 3-rutinoside, peonidin 3-rutinoside, in addition to cyanidin 3-sambubioside or 3-xylosylglucoside (Fig. 1.2). Free cyanidin and peonidin were not detected. Cyanidin 3-gentiobioside and cyanidin 3-rutinoside were identified as the major anthocyanin pigments, and cyanidin 3-glucoside as a minor pigment in eight tart cherry varieties analyzed by electrophoresis and paper chromatography (Von Elbe et al., 1969). Cyanidin 3-gentioside in tart cherries was believed to be cyandin 3-sophoroside instead (Hong, V. and Wrolstad, R. E., 1990).

The sweet cherry, *Prunus avium L*, is used commercially as a table fruit. Color is the most important indicator of maturity and quality for cherries. Cyanidin 3-rhamnoglucoside and cyanidin 3-glucoside were identified from ripened sweet cherries (Li and Wagenknecht, 1958). Peonidin and two of its glycoside derivatives peonidin 3-glucoside and peonidin 3-rutinoside were found in 'Bing' cherries (Lynn and Luh, 1964). However, Only cyanidin derivatives and no peonidin glycosides were found in varieties of *P. avium* (Harborne and Hall, 1964). Peonidin 3-rutinoside was identified as the main pigment in 'Bigarreau Napoleon' cherries (Okombi, 1980).

Du et al. (1975) studied anthocyanins present in ornamental cherry, *P. sargentii, Rehd.*, and identified them as cyanidin 3-glucoside and cyanidin 3-diglucoside. The tomentosa cherry, *P. tomentosa Thunb.*, is a small, hardy tree or a very large shrub grown for ornamental purposes and for its globular, light red and slight hairy fruit. The fruits contain anthocyanins such as pelargonidin and cyanidin 3-rutinosides (Ishikura, 1975). The laurel cherry, *P. laurocerasus L.*, is an evergreen bush, seldom a small tree, native to

southeastern Europe and Iran, and grown for its ornamental value and black-purple fruit. The anthocyanins present in laurel cherry are peonidin 3-arabinoside and cyanidin 3-arabinoside (Fig. 2) (Tsiklauri, 1975). The European dwarf or ground cherry, *P. fruticosa Pall*, is a low-spreading bush. Only one anthocyanin was found in this fruit by Olden (1960), but its structure has not yet been elucidated.

Anthocyanins in other *Prunus* species also were characterized mainly as cyanidin glucosides. For example, the red color in apricot, *Prunus armeniaca* L., is due primarily to cyanidin 3-glucoside (Joshi et al., 1986). The pigment in peach, *Prunus persica*, is due to cyanidin 3-glucoside (Hayashi et al., 1963). Ishikura (1975) reported the presence of cyanidin-rutinoside and cyanidin 3-glucoside in peach as 10 and 90%, respectively.

The plum tree grows in temperate regions. The red pigments in ripe European plums are cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, and peonidin 3-rutinoside (Harborne and Hall, 1964; Hong and Wrolstad, 1990). From the Japanese *Salicina* plum (*Prunus salicina Lindl.*), Ishikura (1975) found only two cyanidin derivatives, cyanidin 3-glucoside and cyanidin 3-rutinoside.

1.2 Flavonoids and polyphenolics

The family of flavonoid compounds includes flavanol, flavanones, anthocyanidins, flavones, and flavonols. Along with the phenylpropanoids or hydroxycinnamic acid derivatives, flavonoids are found in almost every plant (Markham., 1988; Niemann., 1988; Giannasi., 1988). The biosynthetic pathway for individual flavonoids are shown in Fig. 1.3. The 5, 7-hydroxylation pattern of the A-ring is the most common one. Similarly, dihydroxylation at the 3′ and 4′ positions of the B ring is also common in flavones and flavonols, followed by those with a single B ring-hydroxyl

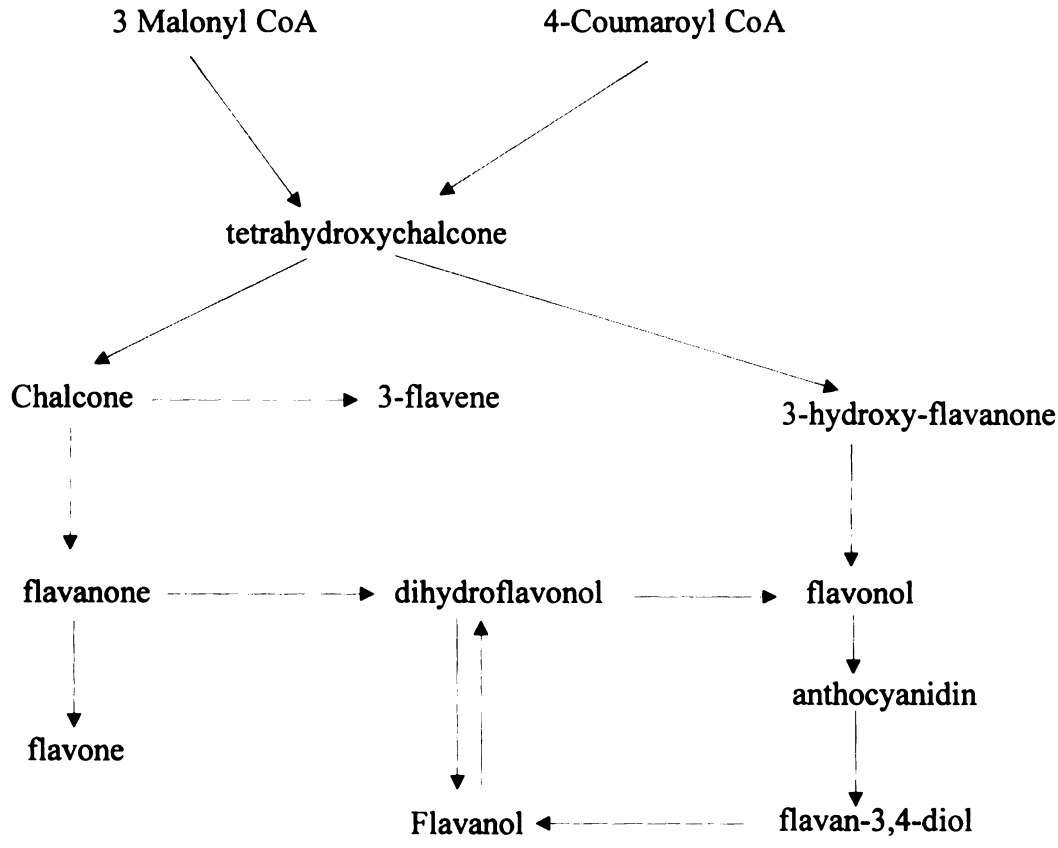
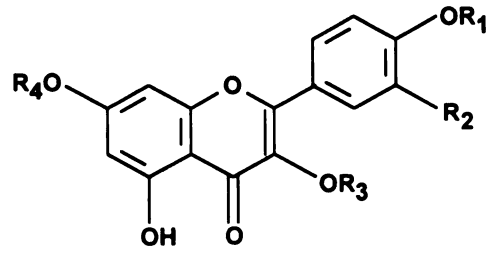


Fig. 1.3. Biosynthetic pathway for flavonoids

group at the 4′ position. Methylation of flavonoids hydroxyl groups can be occurred at any one of these positions. However, the preferred glycosylation site on the flavonoids is at C_3 and less frequently at C_5 and C_7, respectively. Recently, C_2', C_3', C_4 'and C_5' glycosides of flavonoids, were identified from several plants (Ibrahim et al., 1987). Glucose was the most common sugar moiety, but galactose, rhamnose, and xylose also were found. Another class of phenolic compounds, hydroxycinnamic acids, occur most frequently as simple esters with quinic acid or glucose. However, glycosylation often occurred at the acid group (Herrmann, 1989).

Schaller and Von Elbe (1972) reported the presence of polyphenolic components in Montmorency cherries and isolated six isomers of caffeoylquinic acid, four isomers of *p*-coumaroylquinic acid and two other free phenolic acids. In addition, two flavonols were identified as kaempferol 3-rhamnoglucoside and kaempferol 3-glucoside (Fig. 1.4). Olden and Nybon (1968), working with leaves of three varieties of cherries, separated the polyphenols and predicted the presence of the rutinosides and the 3-glucoside of quercetin and kaempferol (Fig. 1.4). Geissman (1956) indicated the presence of quercetin 3-glucoside in the leaves of *Prunus cerasus*. Shrikhande and Francis (1973) reported kaempferol 3-rhamnosylglucoside, quercetin-3-rhamnosylglucoside, quercetin 3-glucoside, quercetin 4'-glucoside and predicted the presence of three other phenolic glycosides kaempferol 3-rhamnoside-4'-galactoside, kaempferol 3-glucoside and kaempferol 4'-glucoside (Fig. 1.4). From the leaves of *Prunus cerasus*, Henning and Herrmann (1980) reported quercetin 3-O-rutinosyl-7, 3′-O-diglucoside (Fig. 1.4). Also, from the bark *of P. cerasus*, tectochrysin 5-glucoside, naringenin, prunin, sakuranetin, sakuranin, dihydrowogonin 7-glucoside, tectochrysin (Fig. 1.5), genistein, prunetin-4′-O-



Quercetin: R_1= H, R_2= OH, R_3=H, R_4=H

Quercetin 3-glucoside: R_1=H, R_2=OH, R_3= glucose, R_4=H

Quercetin 3-rhamnosylglucoside: R_1= H, R_2= OH, R_3= rhamnosylglucose, R_4= H

Quercetin 4'-glucoside: R_1= glucose, R_2= OH, R_3= H, R_4=H

Quercetin 3-rutinosyl-7,3'-diglucoside:
R_1= OH, R_2= glucosyl, R_3 = rhamnosylglucose, R_4= glucose

Kaempferol: R_1=H, R_2=H, R_3=H, R_4=H

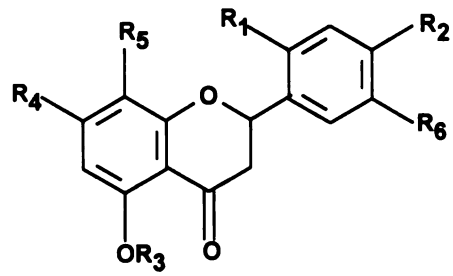
Kaempferol 3-glucoside: R_1= OH, R_2= H, R_3= glucose, R_4=H

Kaempferol 3-rutinoside: R_1=OH, R_2=H, R_3= rutinoside, R_4=H

Kaempferol 3-rhamnoside-4'-galactoside: R_1= galactose, R_2=H, R_3= rhamnose, R_4=H

Kampferol 4'-glucoside: R_1= glucose, R_2=H, R_3=H, R_4=H

Fig. 1.4. Flavonoids from cherries



Cerasinone: R_1= OCH_3, R_2= OCH_3, R_3= CH_3, R_4= OH, R_5= H, R_6= H

Naringenin: R_1= H, R_2= OH, R_3= H, R_4= OH, R_5= H, R_6= H

Tectochrysin: R_1= H, R_2= H, R_3= H, R_4= OCH_3, R_5= H, R_6= H

Tectochrysin 5-glucoside: R_1= H, R_2= H, R_3= glucose, R_4= OCH_3, R_5 = R_6= H

Prunin: R_1= H, R_2= OH, R_3= H, R_4= H, R_5= glucosyl, R_6= H

Sakuranin: R_1= H, R_2= OCH_3, R_3= glucose, R_4= OCH_3, R_5= H, R_6= H

Sakuranetin: R_1= H, R_2= OH, R_3= H, R_4= OCH_3, R_5= H, R_6= H

Pinostrobin: R_1= H, R_2= H, R_3= H, R_4= OCH_3, R_5= H, R_6= H

Pinostrobin 5-glucoside: R_1= H, R_2= H, R_3= glucose, R_4= OCH_3, R_5= H, R_6= H

Dihydrowogonin 7-glucoside: R_1= R_2 = R_3 = H, R_4= glucosyl, R_5= OCH_3, R_6= H

Persicogenin 3'-glucoside: R_1= R_2 = CH_3, R_3= H, R_4= CH_3, R_5 = H, R_6= glucosyl

Sakuranetin 5-xyloside: R_1= H, R_2= OH, R_3= glucose, R_4= OCH_3, R_5 = R_6= H

Isosakuranetin: R_1=H, R_2=OH, R_3=H, R_4= rhamnose, R_5=H, R_6=H

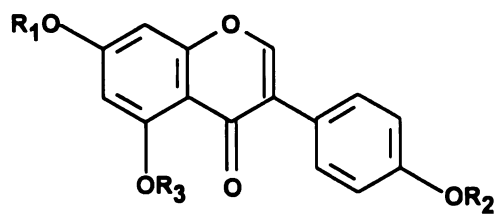
Naringenin 4'-methyl ester 7-xylose: R_1= R_3= H, R_2= OCH_3, R_4= xylose, R_5 = R_6 = H

Fig 1.5. Flavanone and flavanone glycosides in *Prunus* species

glucoside, prunetin, pinostrobin 5-β-D-glucoside, prunetin 5-glucoside, genistein 5-glucoside, pinostrobin (Fig. 1.6), apigenin 5-glucoside, chrysin, genkwanin 5-glucoside (Fig. 1.7) and neosakuranin (Fig. 1.8) were isolated and identified (Geibel et al., 1990; 1991; 1995; Ingham, 1983). Schwab et al (1990) identified benzyl-β-D-glucoside, 2-phenylethyl β-D-glucoside, 6-hydroxy-2, 6-dimethyl-octa-2 (E), 7-dienyl β-D-glucoside and 2-methoxy-4-(2-propenyl)phenyl β-D-glucoside (Fig. 1.9) from fruit pulp of tart cherry by HRGC, HRGC-MS and HRGC-FTIR experiments. Nagarajan (1977) identified 7-hydroxy-5,2′,4′-trimethoxyflavanone (cerasinone) (Fig. 1.5), 2′-hydroxy-2,4,4′,6′-tetramethoxychalcone (cerasidin) and 2′,4′-dihydroxy-2,4,6′-trimethoxychalcone (cerasin) (Fig. 1.8) from Montmorency cherries.

Major phenolic compounds isolated from the sweet cherry, *Prunus avium L*, include: dihydrowogonin 7-glucoside, chrysin 7-glucoside, (-)-epicatechin, (+)-catechin (Fig. 1.10), kaempferol 3-rutinoside, 3-galactosyl-7-diglucoside, quercetin 3-rutinoside and 3-rutinosyl-4′-diglucoside, prunetin-4′-O-glucoside and neochlorogenic acid (Stohr, 1975; Ingham, 1983). Henning and Herrmann (1980) also reported quercetin 3-O-rutinosyl-7,3′-O-diglucoside, quercetin 3-O-rutinosyl-4-O-glucoside, and kaempferol 3-O-rutinosyl-4′-O-glucoside from sweet cherry. Prunin, kaempferol 3-O-rutinoside, kaempferol 3-O-glucoside, rutin, quercetin 3-O-glucoside, catechin and chlorogenic acid were characterized from *P. avium* leaves, (Bauer et al., 1989). Also, genistein and prunetin 5-glucosides were isolated from *P. avium* (Geibel et al., 1990; Khalin et al., 1989).

The fruits of native cherry species in the northern hemisphere, *P. jamasakura*



Prunetin: R_1= CH_3, R_2= H, R_3= H

Prunetin 4'-glucoside: R_1= CH_3, R_2= glucose, R_3= H

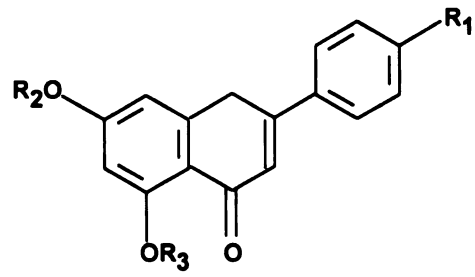
Prunetin 5-glucoside: R_1= CH_3, R_2= H, R_3= glucose

Genistein: R_1= H, R_2= H, R_3= H

Genistein 5-glucoside: R_1= H, R_2= H, R_3= glucose

Genistein 7-glucoside: R_1= glucose, R_2= H, R_3= H

Fig. 1.6. Isoflavonoids from tart cherries



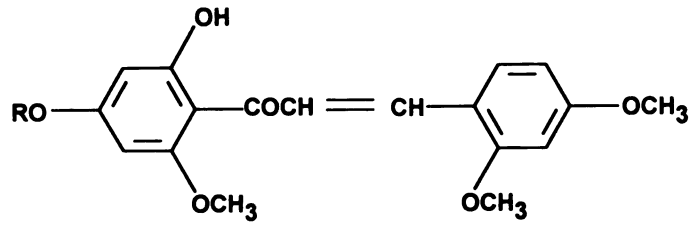
Chrysin: R_1= H, R_2= H, R_3= H

Genkwanin: R_1= OH, R_2= OCH_3, R_3= H

Genkwanin 5-glucoside: R_1= OH, R_2= OCH_3, R_3= glucose

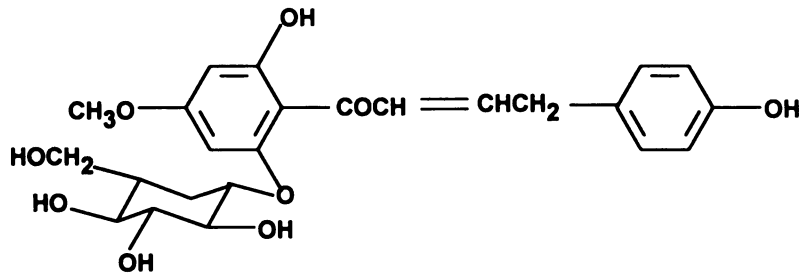
Apigenin 5-glucoside: R_1= OH, R_2= H, R_3= glucose

Fig. 1.7. Flavones from tart cherries



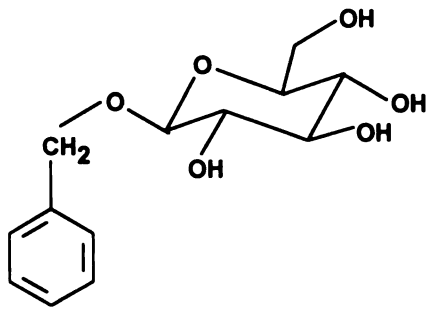
Cerasidin: R = CH_3

Cerasin: R = H

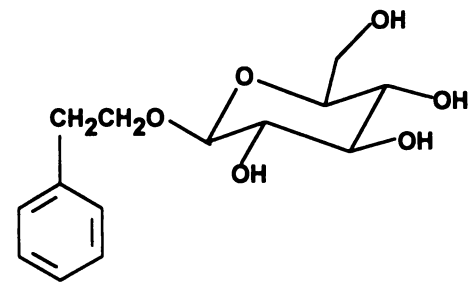


Neosakuranin

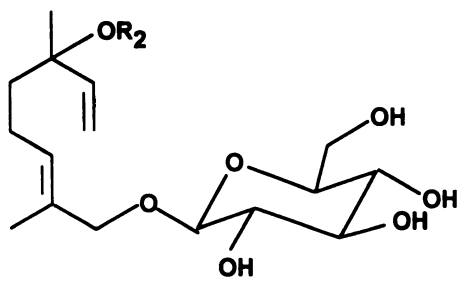
Fig. 1.8. Other phenolic compounds from tart cherries



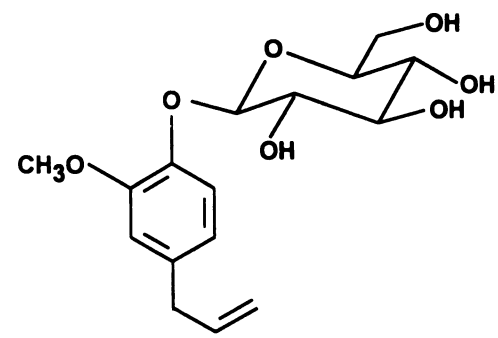
Benzyl beta-D-glucoside



2-phenylethyl beta-D-glucoside

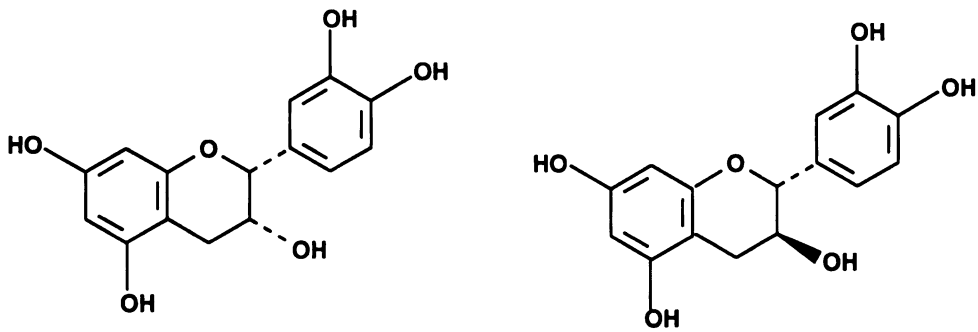


6-hydroxy-2,6-dimethyl-octa-2(E),7-diethyl beta-D-glucoside



2-methoxy-4-(2-propenyl)phenyl beta-D-glucoside

Fig. 1.9. Other glycosides from tart cherries

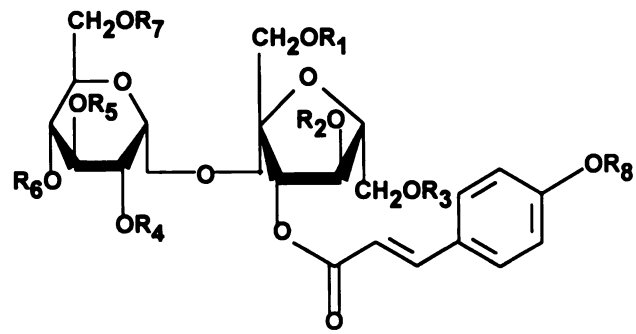


Catechin Epicatechin

Fig. 1.10. Proanthocyanidins from *Prunus* species

Sieb. and *P. maximowiczii Rupr* are not edible because of their bitter taste. From these two species, Shimazaki et al (1991) isolated prunasin, (-)-epicatechin, mandelic acid (Fig. 1.12), 1,6,2′,4′,6′-O-pentaacetyl-3-O-*trans-p*-coumaroylsucrose, 1,6,2′,3′,6′-O-pentaacetyl-3-O-*trans-p*-coumaroylsucrose, 1,6,2′,6′-O-tetraacetyl-3-O-*trans-p*-coumaroylsucrose, 6,2′,4′,6′-O-tetraacetyl-3-O-*trans-p*-coumaroylsucrose, 1,2′,6′-O-triacetyl-3-O-*trans-p*-coumaroylsucrose, 1,6,2′-O-triacetyl-3-O-*tran-p*-coumaroylsucrose and 6,2′,6′-O-triacetyl-3-O-*trans-p*- coumaroylsucrose (Fig. 1.11). The methanolic extract of the bark of *P. jamasakura* yielded sakuranin, neosakuranin, (±)eriodictyol, (±)-catechin, (–)-epicatechin, (±)-lyoniresinol, (2S)-5-β-D-xylopyranosyloxy-7-methoxy-4'-hydroxyflavanone (Fig. 1.5), (2,3-trans, 7'',8''-erythro)-5a,5b-dihydrobuddlenol B and (±)-(2,3-trans)-2,3-dihydro-2-[3',5'-dimethoxy-4'-(1'',3''-dihydroxy-5-benzofuranpropanol (sakuraresinol) (Fig. 1.13) (Yoshinari et al, 1990). Also, 1,6,2′,4′,6′-O-pentaacetyl-3-O-*cis-p*-coumaroylsucrose and 1,6,2′,6′-O-tetraacetyl-3-O-*cis-p*-coumaroylsucrose (Fig. 1.12) were identified from its bark (Yoshinari et al., 1990).

The concentration of the flavonols, flavan 3-ols, and phenolic acids contribute to the color and flavor characteristics of apricots. The phenolic compounds of apricots, *Prunus armeniaca* L., were quercetin 3-rutinoside and 3-glucoside; kaempferol 3-rutinoside and 3-glucoside (Henning and Hermann, 1980); (+)-catechin; (-)-epicatechin; chlorogenic, neochlorogenic, and crytochlorogenic acid; *cis*- and *tran*-3, 4-, and 5-*p*-coumaroylquinic acids; *cis*- and *tran*-3-,4- and 5-feruloylquinic acids; *p*-coumaric acid glucoside; ferulic acid glucoside; coumarin; and scopoletin (Mosel and Hermann, 1974; Möller and Herrmann, 1983).



1,6,2',4',6'-O-pentaacetyl-3-O-*trans* -*P* -coumaroylsucrose
R_1= Ac, R_2= H, R_3= Ac, R_4= Ac, R_5= H, R_6= Ac, R_7 = Ac, R_8= H

1,6,2',3',6'-O-pentaacetyl-3-O-*trans* -*P* -coumaroylsucrose
R_1= Ac, R_2= H, R_3= Ac, R_4= Ac, R_5= Ac, R_6= H, R_7= Ac, R_8= H

1,6,2',6'-O-tetraacetyl-3-O-*trans* -*P* -coumaroylsucrose
R_1= Ac, R_2= H, R_3= Ac, R_4= Ac, R_5= H, R_6= H, R_7 = Ac, R_8= H

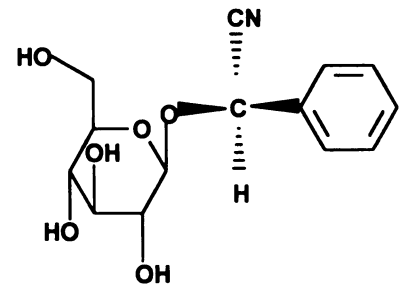
6,2',4',6'-O-tetraacetyl-3-O-*trans* -*P* -coumaroylsucrose
R_1= H, R_2= H, R_3= Ac, R_4= Ac, R_5= H, R_6= Ac, R_7 = Ac, R_8= H

1,2',6'-O-triacetyl-3-O-*trans* -*P* -coumaroylsucrose
R_1= Ac, R_2= H, R_3= H, R_4= Ac, R_5= H, R_6= H, R_7 = Ac, R_8= H

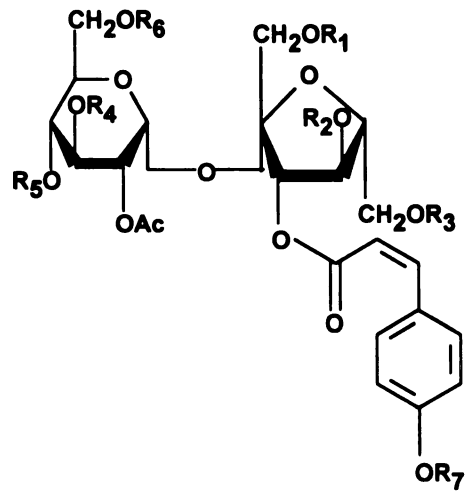
1,6,2'-O-triacetyl-3-O-*trans* -*P* -coumaroylsucrose
R_1= Ac, R_2= H, R_3= Ac, R_4= Ac, R_5= H, R_6= H, R_7 = H, R_8= H

6,2',6'-O-triacetyl-3-O-*trans* -*P* -coumaroylsucrose
R_1= H, R_2= H, R_3= Ac, R_4= Ac, R_5= H, R_6= H, R_7 = Ac, R_8 = H

Fig. 1.11. Bitter principles from *Prunus jamasakura* and *P. maximowiczii*



Mandelic acid



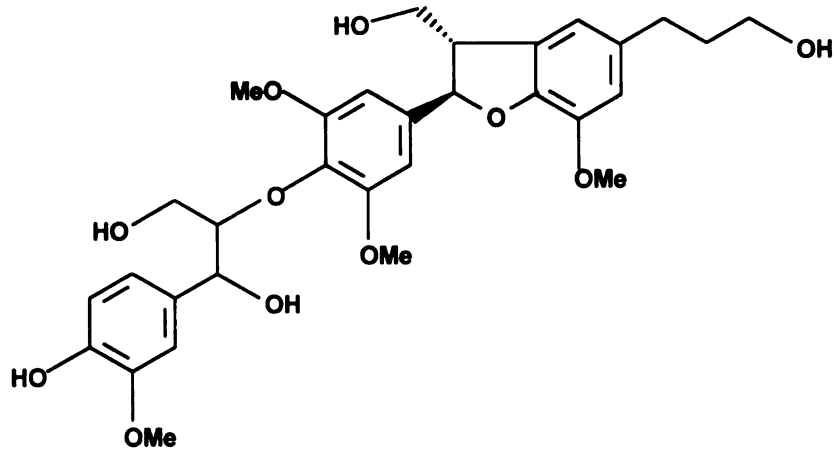
1,6,2',4',6'-O-pentaacetyl-3-O-*cis* -*p* -coumaroylsucrose

R_1= Ac, R_2= H, R_3= Ac, R_4= H, R_5= Ac, R_6= Ac, R_7 = H

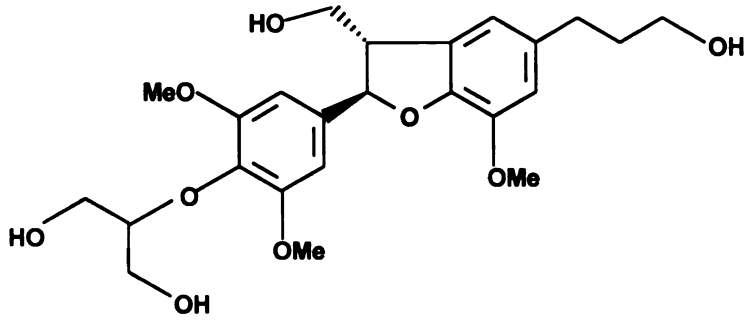
1,6,2',6'-O-tetraacetyl-3-O-*cis* -*p* -coumaroylsucrose

R_1= Ac, R_2= H, R_3= Ac, R_4= H, R_5= H, R_6 = Ac, R_7 =H

Fig. 1.12. Bitter principles from *Prunus jamasakura* and *P. maximowiczii*



(2,3-*trans*,7",8"-*erythro*)-5a,5b-dihydrobuddlenol B



(2,3-trans)-2,3-dihydro-2-[3',5'-dimethoxy-4'-(1",3"-dihydroxy-2"-propyloxy]
-3-hydroxymethyl-7-methoxy-5-benzofuranpropanol (Sakuraresinol)

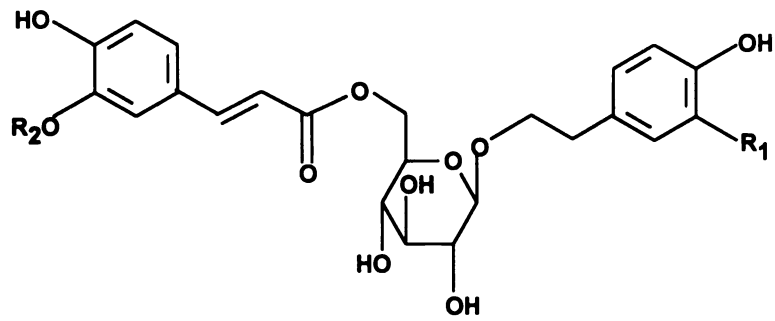
Fig. 1.13. Sesquilignan and neolignan from *Prunus jamasakura*

Kaempferol 3-sophoroside from the pollen and flavones from the seed coat have been reported from *P. amygdalus*. in addition to persicogenin 3'-glucosdie (5,3'-dihydroxy-7, 4'-dimethoxyflavanone 3'-glucoside) (Fig. 1.4) (Rawat et al., 1995). Peach fruit, *Prunus persica,* is rich in hydroxycinnamic acid derivatives, especially chlorogenic and neochlorogenic acid, flavan-3-ols, and flavonol (Henning and Herrman, 1980; Mosel and Herrmann, 1974).

In ripe European plums, phenolic compounds such as rutin, neochlorogenic and chlorogenic acids were found (Raynal et al., 1989). The plum tree (*Prunus domestica L.*,), which grows widely in the western temperate Himalayas and is cultivated for its fruit in India, yielded seven compounds from an alcohol extract of its heartwood, and these were identified as isosakuranetin (Fig. 1.5), prudomestin, dihydrokaempferide, naringenin, 5,7,4′-trihydroxy-3-methoxyflavanone, 3,5,7-trihydroxy-6,4′-dimethoxyflavanone, and 3,5,7-trihydroxy-8,4′-dimethoxyflavanone. Also, 4-O-methyl-phloracetophenone-6-O-glucoside, 4-O-methyl, phloracetophenone and 5,7-dimethoxy-6-hydroxycoumarin (fraxinol) were identified (Nagarajan et al., 1977). The bark of plum (*Prunus grayana Maxim.*) has been used as a crude drug for the treatment of coughs in Europe and America. Several phenylpropanoid glucoside isolated from the bark of this plant are 2-(4-hydroxyphenyl) ethyl-(6-O-feruloyl)-β-d-glucopyranoside (grayanoside A), (2-(3,4-dihydroxyphenyl)ethyl-(6-O-feruloyl)-β-D-glucopyranoside) (grayanoside B) and (2R)-[(6-O-caffeoyl)-β--D-glucopyranosyloxy]benzeneacetonitrile (grayanin) (Fig. 1.14). From the methanol extract of the bark of *P. grayana*, 2-(4-hydroxyphenyl)-ethyl-(6-O-caffeoyl)-β-D-glucopyranoside, 2-(3,4-dihydroxyphenyl)-ethyl-(6-O-caffeoyl)-β-D-glucopyranoside (Fig. 1.14), a bitter tannin-related compound, 3,4,5-trimethoxybenzoyl-

β-D-glucopyranoside, 2-(3′,4′-dihydroxyphenyl)-ethyl-β-D-glucopyranoside and 6-O-caffeoyl-β-D-glucopyranoside (Fig. 1.15) were reported (Shimomura et al., 1987; 1989). Similarly, the heartwood of this plant gave (+)-taxifolin, dehydrodicatechin, virgaureoside, henryoside, populine, 2′-β-D-glucopyranosyloxybenzyl 2-(6-O-benzoyl-β-D-glucopyranosyloxy) benzoate (pruyanaside A) and 2′-(6-O-benzoyl-β-D-glucopyranosyloxy) benzyl 2-β-D-glucopyranosyloxy-6-hydroxybenzoate (pruyanaside B) (Fig. 1.16) (Shimomura et al., 1987). Two new phenylpropanoid glucosyl esters, 6-O-caffeoyl-1-O-*p*-coumaroyl-β-D-glucopyranose and three known compounds: 6-O-*p*-coumaroyl-D-glucopyranose, 1,6-dicaffeoyl-β-D-glucopyranose (Fig. 1.17), 6-O-caffeoyl-D-glucopyranose and (2R)-[(6-O-caffeoyl)-β-D-glucopyranosyloxy] benzene acetonitrile were identified from *Prunus buergeriana* (Shimomura et al., 1988).

Prunus spinosa is distributed throughout Europe and the Middle East. From the extracts of flowers of *P. spinosa*, Kolodziej et al (1991) reported the range of natural dimeric A-type proanthocyanidins such as *ent*-epicatechin-(4α→8; 2α→O→7)-catechin and *ent*-epiafzelechin-(4α→8; 2α→O→7)-epicatechin, *ent*-epicatechin-(4α→8; 2α→O→7)-epicatechin, *ent*-epiafzelechin-(4α→8; 2α→O→7)-catechin and *ent*-epiafzelechin-(4α→8; 2α→O→7) epiafzelechin (Fig. 1.18). From the water extract of young branches of this plant, two proanthocyanidins were identified using circular dichroism (CD) and insensitive nuclei enhanced by polarization transfer (INEPT) techiques as mahuannin A and ent-epiafzelechin-(2α→ 7, 4α→8)-epicatechin (Fig. 1.18) (González et al., 1992)



2-(4-hydroxyphenyl)-ethyl-(6-O-caffeoyl)-beta-D-glucoside

R_1=H, R_2=H

2-(3,4-dihydroxyphenyl)-ethyl-(6-O-caffeoyl)-beta-D-glucoside

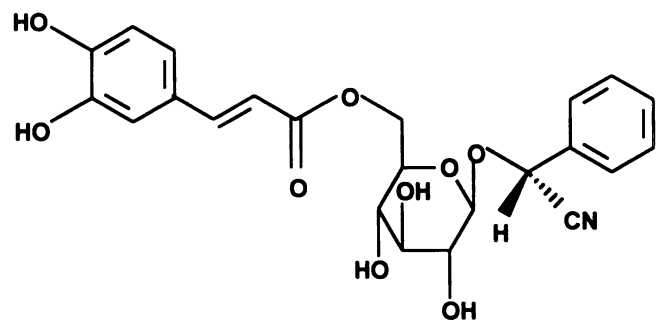
R_1= OH, R_2=H

2-(4-hydroxyphenyl)-ethyl-(6-O-feruloyl)-beta-D-glucoside

R_1=H, R_2= CH_3 (Grayanoside A)

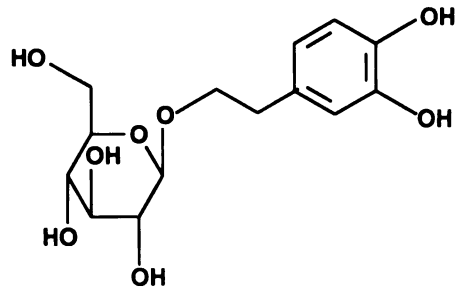
2-(3,4-dihydroxyphenyl)-ethyl-(6-O-feruloyl)-beta-D-glucoside

R_1= OH, R_2= CH_3 (Grayanoside B)

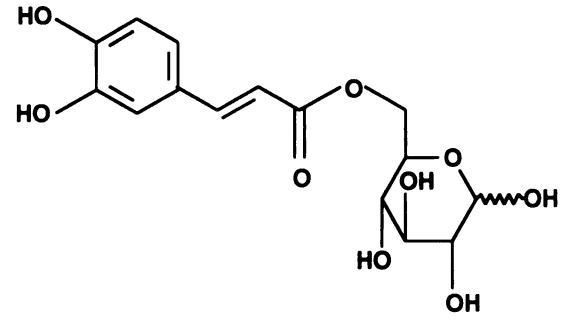


(2R)-[(6-O-caffeoyl)-beta-D-glucosyl]benzeneacetonitrile (Grayanin)

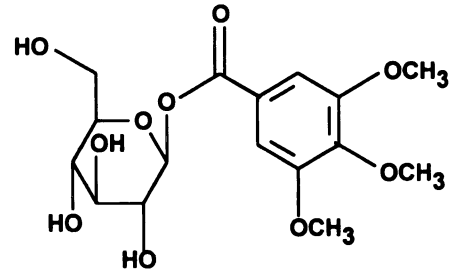
Fig. 1.14. Phenolic glucoside from *Prunus grayana*



2-(3,4-dihydroxyphenyl)-ethyl-beta-D-glucoside

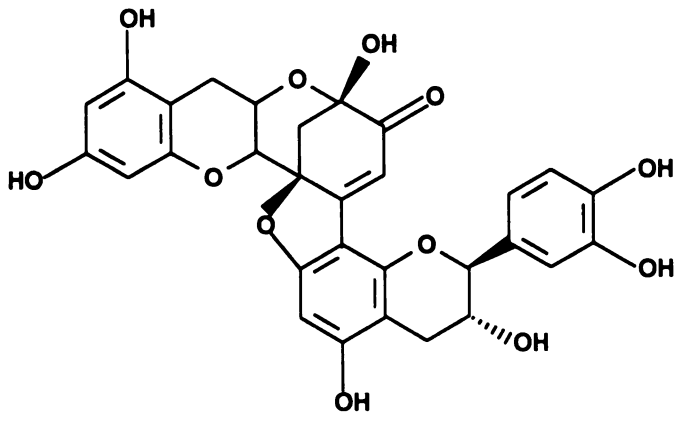


6-O-caffeoyl-D-glucoside

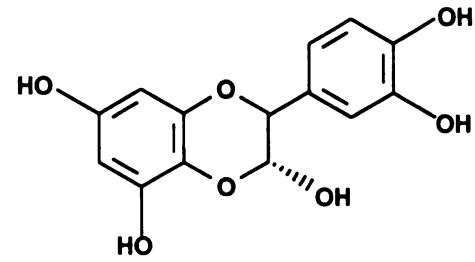


3,4,5-trimethoxybenzoyl-beta-D-glucoside

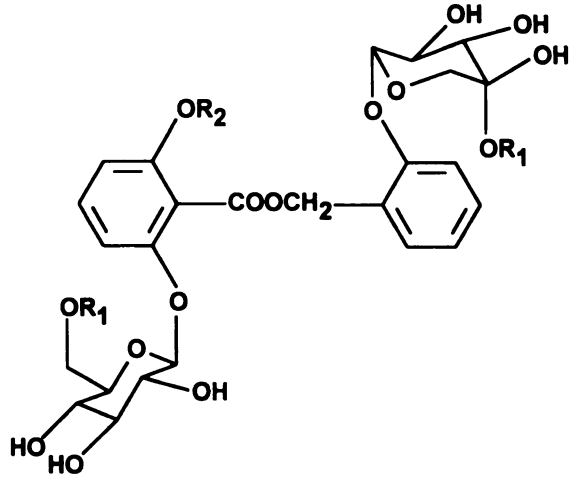
Fig. 1.15. Phenolic glucoside from *Prunus grayana*



Dehydrodicatechin A



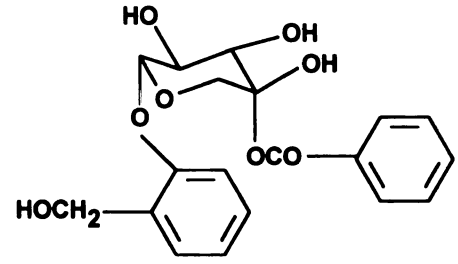
Taxifolin



Vigaureoside A: R_1= H, R_2= H

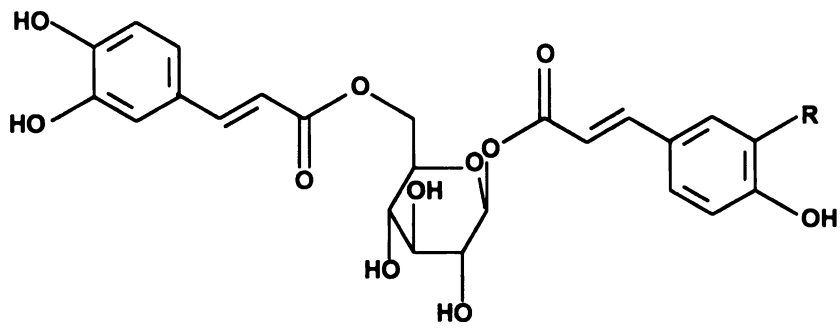
Henryoside: R_1= H, R_2= OH

Pruyanaside A: R_1= Benzoyl, R_2= H



Populine

Fig. 1.16. Phenolic compounds from *Prunus grayana*



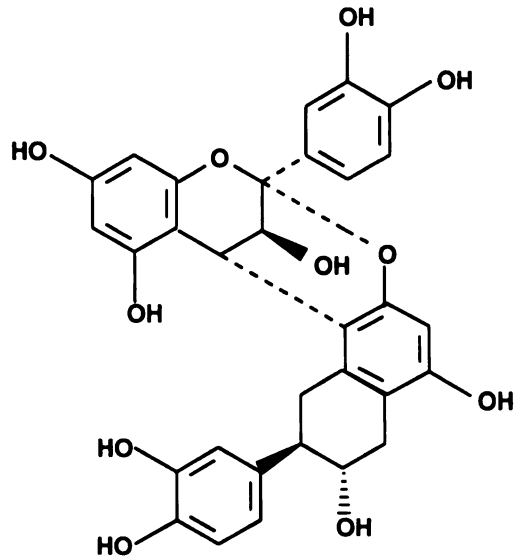
6-O-caffeoyl-1-O-p-coumaroyl-beta-D-glucose

R = H

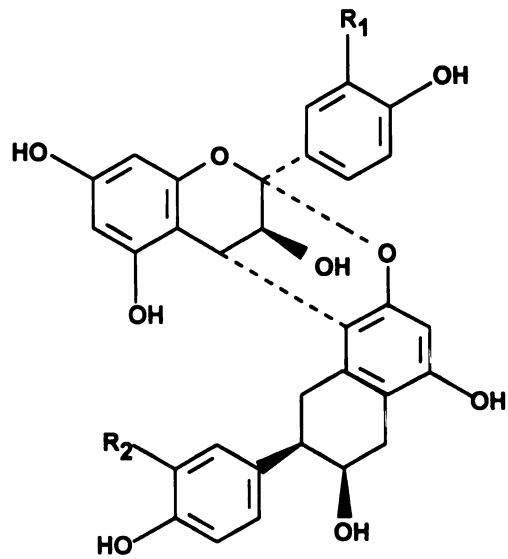
1,6-dicaffeoyl-beta-D-glucose

R = OH

Fig. 1.17. Phenylpropanoid glucosides from *Prunus buergeriana*



ent-epicatechin-(4a→8;2a→O→7)-catechin



ent-epicatechin-(4a→8;2a→O→7)-epicatechin
R_1=OH, R_2=OH
ent-epiafzelechin-(4a→8;2a→O→7)-epicatechin
R_1=H, R_2=OH
ent -epiafzelechin-(4a→8;2a→O→7)-epiafzelechin
R_1=H, R_2=H (mahuannin A)

Fig. 1.18. Proanthocyanidins from *Prunus* species

1.3 Other compounds present in *Prunus* species

Schaller and Von Elbe (1973) reported eight carotenoids in tart cherries (*P. cerasus*). They are phytofluene, α-carotene, β-carotene, cryptoxanthin, cryptoflavin, lutein, zeaxanthin and mutatoxanthin (Fig. 1.19). Tart cherries also contain several organic acids such as aspartic, citramalic, citric, fumaric, galacturonic, glyceric, glycolic, glucuronic, glutamic, glutaric, isochlorogenic, lactic, malic, malonic, phosphoric, quinic, shikimic, succinic and tartaric acids. In tart cherry, malic acid represents 75-95% of the total titrable acidity (Krishna et al., 1965). Also, Tanchev (1980) reported salicylic, cinnamic, gentisic, gallic, isoferulic and *p*-coumaric acids in tart cherries. The main sugars reported from the fleshy part of the tart cherry are fructose, α– and β-glucose. However, sorbitol, sucrose and inositol were detected in small quantities (Neubeller et al., 1977). The volatile compounds characterized from 'Bing' sweet cherry fruit were propanal, butanal, ethyl acetate, 2-propanol, ethanol, pentanal, butyl acetate, hexanal, 2-methyl butyl acetate, butanol, heptanal, butyl butyrate, butyl 2-methylbutyrate, ethyl hexanoate, 1-pentanol, hexyl acetate, octanal, 6-methyl 5-hepten-2-one, 1-hexanol, nonanal, hexyl 2-methyl butyrate, ethyl octanoate, acetic acid, 2-ethyl 1-hexanol, decanal, benzaldehyde, hexyl hexanoate and 2-methyl 2,4-pentanediol (Mattheis et al., 1992).

The orange color in apricot, *Prunus armeniaca* L., is attributed to α- and β-carotene, phytofluene, phytoene, *cis*-β-and *cis*-α-carotene, cryptoxanthin, lutein, and other carotenoids (Curl, 1960). Molnar et al. (1987) isolated (3S, 5R, 6S)-5,6-epoxy-3-hydro-12′-apo-β-carotene-3,12′-diol (persicaxanthin) and periscachrome from peach.

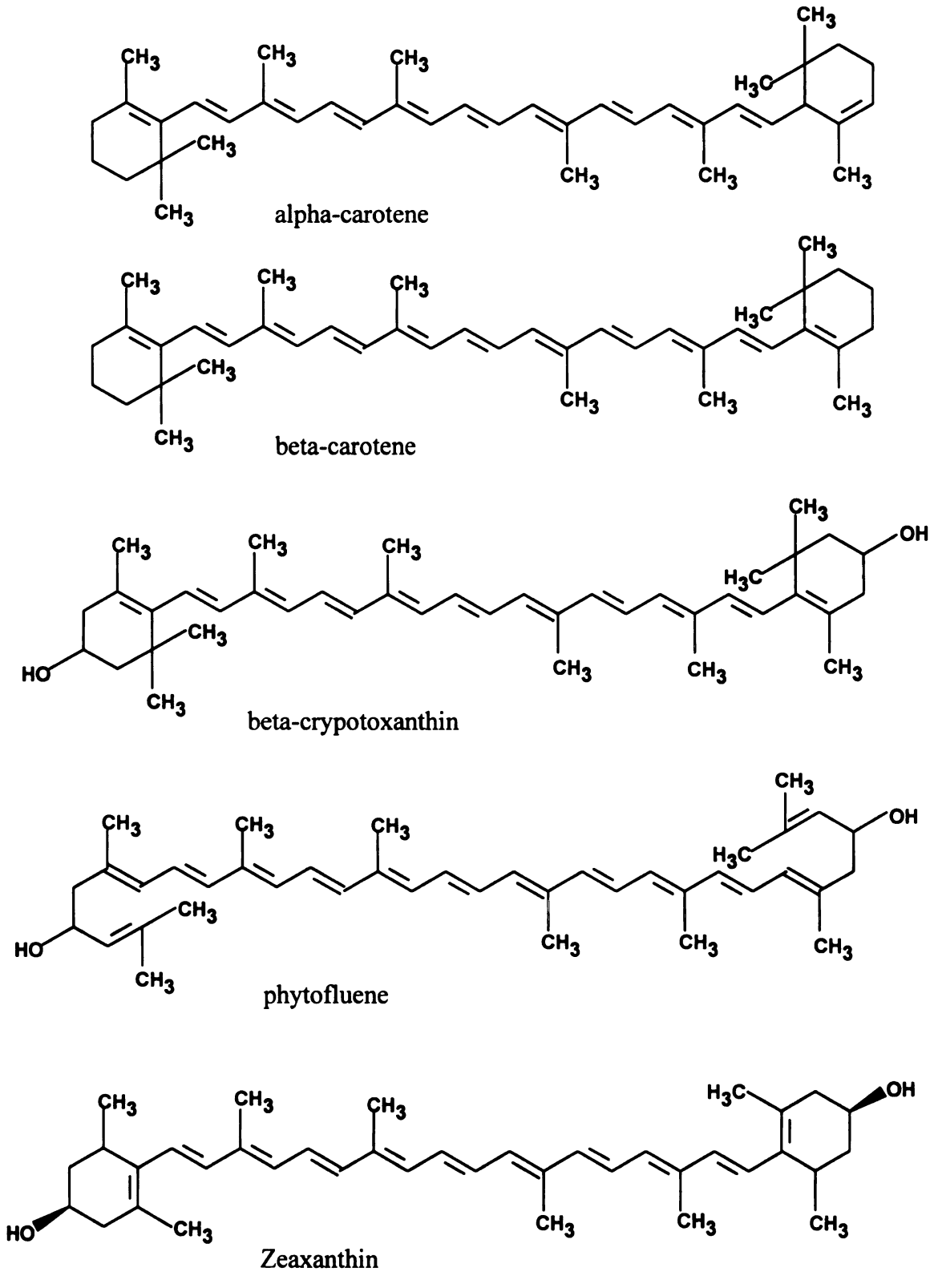


Fig. 1.19. Principle carotenoids in tart cherry

2. BIOLOGICAL ACTIVITY OF COMPOUNDS ISOLATED FROM *PRUNUS* SPECIES

Most *Prunus* species fruits contain anthocyanins, pro-anthocyanidins and other phenolic compounds which are important to human health. The flavonoids have long been considered to possess antiallergenic, antiinflammatory, antiviral and anticarcinogenic activities. Gabon (1986) verified the antioxidant effects of anthocyanins based on a number of external factors such as pH, the ratio of L-ascorbic acid and oxygen, reaction time and the nature of organic acids present.

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

The fruits of *Prunus* spp have been reported to possess antipyretic activity and to be useful against thirst, leprosy and leucoderma (Chopra et al., 1956). Anecdotal reports suggested that consumption of cherries could alleviate arthritic- and gout-related pain (Hamel and Chiltoskey, 1975). *P. spinosa* is distributed throughout Europe and the Middle East. Its ethnobotanic use is best known in Navarra, where infusions of its branches are used in the treatment of hypertension (Fernández, 1981). The flowers of *P. spinosa* were used in folk medicine in Navarra (Spain) as a mild purgative, diuretic, diaphoretic and depurative drug (Gessner, 1974). The biological activities of *P. spinosa* are due to the presence of numerous flavonoids including rutin and quercetrin (Rodriguez, 1986). The leaves are cited as having antidiabetic and antiasthmic properties.

Pharmacological studies of *P. spinosa L.* showed that intravenous injection of the aqueous extract produced a reduction in blood pressure. Further studies showed that the aqueous extract of the branches of *P. spinosa L.* diminished the response to histamine and epinephrine in guninea pig ileum (Rodriguez, 1986).

The effect of cherry-stalk (*Prunus avium*) extract on smooth muscle preparations was investigated. The extract, consisted of flavonoid components, was found to reverse the contractions of the rat and guinea pig uterus and counteracted oxytoxin-induced contractions (Hetényi and Vályi-Nacy, 1969). Also, they investigated the cardiovascular effects of cherry stalk extract. The extract exhibited the reduction of heart damage in frogs caused by quinine. Blazso and Gabor (1994) reported that the stalk extract of cherry showed good antiinflammatory activity induced with capsaicin or croton oil in the mouse ear. The European dwarf or ground cherry, *P. fruticosa Pall.*, a low, spreading bush, with intensely flavored, somewhat bitter fruits. It is used in Europe in specialty soft drinks. The fruits and leaves of *P. fruticosa* also are used as a source of an aromatic oil to flavor liquors (Olden, 1960).

Keishi-bukuryo-gan is a traditional Chinese herbal remedy used for the treatment of gynecological disorders such as hypermenorrhea, dysmenorrhea and infertility (Sakamoto et al., 1988). It contains five components: bark of *Cinnamomum cassia*, root of *Paeonia lactiflora*, seed of *Prunus persica*, carpophores of *Poria cocos* and root bark of *Paeonia suffruticosa*. The extract of *P. mume* fruits showed wormicidal and bactericidal activities (Rhee et al., 1981). The almond (*P. amygdalus*) shell extract showed antimicrobial activity (Sachdev, 1968). Similarly, the seed exhibited a hypoglycemic effect in albino rabbits (Teotia and Singh, 1997).

The Xiaoyu Pian (XYP), containing *P. persica*, *Carthamus tinctorius*, *Glycyrrhiza uralensis* as the major constituent, was used to treat patients with platelet aggregation defect. The results suggested that XYP could regulate the hemostatic action and the platelet aggregation function (Shen et al., 1994). Using the Ames/Salmonella/microsome assay, Yamamoto et al (1992) examined the antimutagenic effect of the hexane extract of apricot (*P. armeniaca L.*), peach (*P. persica Bat.)*, cherry (*P. avium L.*), plum (*P. salicina Lindle*) and almond (*P. dulcis Mill*) seeds. Hexane extracts of apricot and peach seeds inhibited the mutagenicity of benzo[α]pyrene (b[α]P), but those from cherry, plum and almond did not. The mutagenicities of 3-amino-1,4-dimethyl-5H-pyridol[4,3-β]indole (Trp-p-1) and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) also were inhibited by the extracts of apricot and peach. It is believed that the polyphenolic compounds of *Prunus* species act as antioxidants contributing to anticarcinogenic, cardioprotective and other related activities. A brief survey of the various biological activities of flavonoids, which may exist in the *Prunus* species including tart cherries, constitutes the rest of this chapter.

2.1 Antioxidant activity

Free radical formation is related to the normal metabolism of aerobic cells. The oxygen consumption inherent in cell growth leads to a series of oxygen free radicals, such as superoxide, hydroxyl, and lipid peroxides. This group of radicals may react with nucleic acids and proteins to produce oxidative reactions (Byers and Perry, 1992). Antioxidants are needed to prevent the formation and oppose the actions of reactive oxygen and nitrogen species such as nitric oxide (.NO), which are generated in vivo and

cause damage to DNA, lipids, proteins, and other biomolecules (Halliwell, 1996). Endogenous antioxidant defenses by superoxide dismutases, catalase, metal-binding proteins, and glutathione peroxidase are inadequate to prevent such damage completely. Therefore, the diet-derived antioxidants are important in maintaining health (Halliwell, 1996). Many dietary compounds have been proposed to have important antioxidant activities including, vitamins E, C and related plant pigments, such as flavonoids.

Flavonoids have a variety of biological effects in numerous mammalian cell systems, in vitro as well as in vivo. Recently, much attention has been paid to their antioxidant properties. Hanasaki et al (1994) compared the abilities of 15 flavonoids as scavengers of active oxygen (hydroxyl radical and superoxide anion. The phenolic compounds (+)-Catechin, (-)-epicatechin, 7,8-dihydroxy flavone and rutin exhibited an ·OH scavenging activity 100-300 times superior to that of mannitol, a typical ·OH scavenger. A major part of this inhibitory effect may be due to the suppression of xanthine oxidase activity by the flavonoids. (Hanasaki et al., 1994).

The antioxidant activity of a number of flavonoids in refined and bleached (RB) canola oil was compared with that of commonly used synthetic antioxidants, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) (Wannasundara and Shahidi, 1994). Among the flavonoids tested, myricetin, (-)epicatechin, naringenin, rutin, morin, and quercetin were superior to BHA and BHT in inhibiting the oxidation of canola oil. Therefore, natural flavonoids may have potential application for the stabilization of canola oil (Wanasundara and Shahidi, 1994).

Cao et al. (1997) investigated the antioxidant and prooxidant behavior of flavonoids and the related structure-activity relationships using the oxygen radical

absorbance capacity (ORAC) assay, which was carried out on the spectrophotometric centrifugal analyzer. The results indicated that flavones, isoflavones, and flavanones are antioxidants against peroxyl and hydroxyl radicals in the presence of Cu^{2+} activities (Cao et al., 1997). The higher numbers of hydroxyl substitutions produced stronger antioxidant (Cao et al., 1997). The other report also indicated that the flavonoids that contain multiple hydroxyl substitutions showed antiperoxyl radical activities several times stronger than Trolox, a water soluble alpha-tocopherol analogue (Chen et al., 1996). A single hydroxyl substitution at C_5 position provided no activity, whereas the di-OH substitutions at C_3'and C_4' were particularly important to the peroxyl radical absorbing activity of a flavonoid (Bors et al. 1990). The conjugation between rings A and B did not affect the antioxidant activity, but is very important for the copper-initiated prooxidant action of a flavonoid (Cao et al. 1997; Heilmann et al. 1995). The O-methylation of the hydroxyl substitutions inactivated both the antioxidant activities of the flavonoids (Cao et al. 1997). Tournaire et al (1993) also determined the structure-activity relationship of flavonoids by the rate constants of the chemical reaction of these flavonoids with O_2^- determined by near-IR singlet oxygen luminescence kinetic measurements. They found that the basic element of antioxidant activity was due to the conjugation of the B-ring to the 4-oxo via a C_2-C_3 double bond and the presence of a C_3 hydroxyl group (Tournaire et al., 1993).

According to the above studies, four groups were considered to be important for determining their radical scavenging and antioxidant capacity. They are (a) the *o*-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals, possibly through hydrogen bonding which participates in electron delocalization (Bors et al., 1990 a, b); (b) the C_2-C_3 double bond in conjugation with C_4-oxo function,

which are responsible for electron delocalization from the B-ring (Tournaire et al., 1993; Bors et al., 1990a); (c) the presence of both C_3 and C_5 hydroxyl groups for maximal radical-scavenging capacity and strongest radical absorption (Afanas's ev et al., 1989, De Whalley et al., 1990) and (d) the number of hydroxyl groups, a higher number providing maximal radical-scavenging potential and the stronger radical absorption (Bors et al., 1990; Cao al., 1997).

Saija et al (1995) studied the flavonoids quercetin, hesperetin, naringenin and rutin in in vitro experimental models. Quercetin, hesperetin, and naringenin interacted with dipalmitoylphosphatidylcholine (DPPC) liposomes causing different shifts of the main transition peak temperature (Tm) typical for DPPC liposomes (Saija et al., 1995). The results suggested that flavonoid capacity to modify membrane-dependent processes, such as free-radical-induced membrane lipoperoxidation, is related not only to their structural characteristics, but also to their ability to interact with and penetrate the lipid bilayers (Ioku et al., 1995).

Free radicals and antioxidants also are discussed widely in the clinical literature. Bindoli et al (1977) demonstrated that silymarin protected liver mitochondria and microsomes from lipid peroxidation. Also, quercetin and taxifolin showed similar protective action. Jha et al (1985) described the antiperoxidation action of isoflavonoids on rat microoxidation. Eight flavonoids were investigated for their antiperoxidative activities against lipid peroxidation induced in liver cell membranes either by nonenzymic (ascorbic acid-Fe^{2+} system, FeAs) or by enzymic methods (arachidonic acid, AA) (Galvez et al. 1995). When lipid peroxidation was induced by FeAs and AA, the

order of inhibitory potency for the different flavonoids assayed was different (Galvez et al. 1995).

These flavonoids also were tested for their influence on glutathione-related enzymes, which constitutes one of the principal physiological antioxidant systems. It was concluded that the antiperoxidative effect shown by most of the flavonoids was exerted without modifying these enzymes (Ahmad et al., 1990).

Low-density lipoproteins (LDL), mildly oxidized by copper ions or UV radiation exhibited a cytotoxic effect on cultured endothelial cells (Schmitt et al. 1995). A mixture of the three compounds rutin/ascorbic acid/alpha-tocopherol (4/4/1) exhibited a synergic antioxidant effect (Negre Salvayre et al., 1995). The protective effect of antioxidants was limited due to their own toxicity in the in vivo systems. The antioxidant mixture permitted a maximum cytoprotective effect when used in lower concentrations and helped to prevent the cytotoxicity (Negre Salvayre et al., 1995).

In searching for new drug candidates which could help bridge the gaps between free radical oxidations, pathophysiological responses, and pharmacological treatment, a series of flavonoids was screened (Ursini et al., 1994). The most interesting compound tested was 3′-hydroxyfarrerol. This compound was an effective inhibitor of microsomal lipid peroxidation induced by either adenosine 5′-diphosphate (ADP) or carbon tetrachloride (Ursini et al. 1994). Kandaswami and Middleton (1994) proposed that flavonoids react with peroxy radicals, thus bringing about the termination of radical reactions. A number of isomeric or chemically closely related C-methylated dihydrochalcones, was isolated from the fruit exudate of *Myrica gale L.* One of the compounds myrigalone B (2',6'-dihydroxy-4'-methoxy-3',5'-dimethyldihydrochalcone)

showed good activity in inhibition of lipid peroxidation induced by *tert*-butyl hydroperoxide or bromotrichloromethane in isolated rat hepatocytes. Also, this compound inhibited enzymatic lipid peroxidation in linoleic acid by soybean 15-lipoxygenase and peroxidation induced by Fe^{2+} ions in a cell free system with linolenic acid (Malterud et al., 1996). The biphenyl compound, 3,4,3',4'-tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl, and a flavonoid, eriodicytol, were isolated as antioxidant components from the leaves of *Thymus*. These compounds inhibited superoxide anion production in the xanthine/xanthine oxidase system. Mitochondrial and microsomal lipid peroxidation induced by Fe^{3+}-ADP/NADH or Fe^{3+}-ADP/NADPH also were inhibited by these compounds (Haraguchi et al., 1996). Natural antioxidants, such as flavonoids, may act as reducing agents, as free-radical scavengers, as complexers of prooxidant metals, and as quenchers of the formation of singlet oxygen. However, the most important activity of these compounds is credited to their primary antioxidant activity as free radical acceptors and/or as chain-breakers (Kandaswami and Middleton, 1994).

2.2 Anti-inflammatory activity

An inflammatory reaction is induced by one or several chemical or biological mediators such as arachidonic acid derivatives, prostaglandins [PG], leukotrienes [LT], thromboxanes [TX], vasoactive amines (histamine or serotonin), and oxygen free radicals (superoxide ion, $O_2^{\cdot-}$, or hydroxyl radicals, $^{\cdot}OH$) (Williams, 1983) (Fig. 1.20). The activity of flavonoids in antiinflammatory and antiallergenic responses was reviewed (Gabor, 1986). Also, recent studies revealed the antiinflammatory dose-dependent activity of

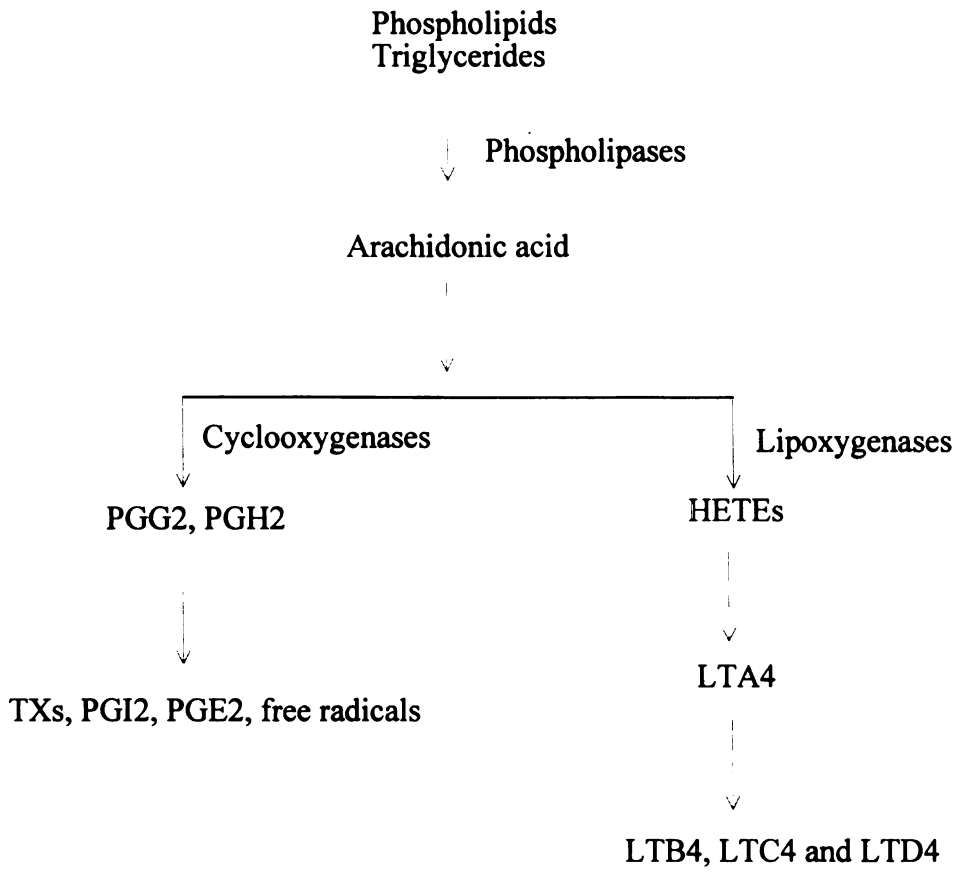


Fig. 1.20. Mechanism of inflammation

hesperidin, diosmin, and other flavonoids on the metabolism of arachidonic acid and histamine release (Gabor and Razga, 1991). These flavonoids significantly inhibited lysosomal enzyme secretion and arachidonic acid release from membranes by inhibiting lipoxygenase, cyclooxygenase, and phospholipase A_2 (Gabor, 1986). The arachidonic acid released from membrane phospholipids or other sources is metabolized by the 5-lipoxygenase (5-LO) pathway to leukotrienes (LTC_4, LTD_4, LTE_4 and LTB4) (Lewis and Austen, 1984).

Butenko et al. (1993) investigated antiinflammatory properties and inhibition of leukotriene C_4 biosynthesis in vitro by flavonoid baicalein, 5,6,7-trioxyflavone-7-O-β-D-glucoside. The antiinflammatory activity of baicalein was greater in the chronic inflammation model rat adjuvant arthritis than observed in the rat carrageenan-induced paw edema (Butenko et al., 1993). A study of 5-lipoxygenase (5-LO) inhibitory activity of baicalein on leukotriene C_4 (LTC_4) biosynthesis by rat resident peritoneal macrophages stimulated with calcium ionophore (A 23186) showed that baicalein significantly inhibited the LTC_4 production. These finding suggested that inhibition of the 5-LO pathway of arachidonic acid metabolism may be one of the mode of actions of baicalein's antiinflammatory activity (Butenko et al., 1993). Gil et al. (1994) tested four flavonoids for their influence on human recombinant synovial phospholipase A_2. They showed selectivity for quercetagetin, kaempferol-3-O-galactoside, scutellarein and scutellarein-7-O-glucuronide against phospholipase A_2, respectively. These flavonoids also inhibited 12-O-tetradecanoylphorbol-13-acetate-induced ear edema in mice with a potency comparable to that of indomethacin and carrageenan-induced mouse paw edema (Gil et al., 1994). The anti-inflammatory effects of Daflon, a micronized purified

flavonoid fraction containing 90 and 10% of diomin and hesperidin, respectively, were studied in in vivo and in vitro models (Jean and Bodinier, 1994). In a model study of inflammatory granuloma in the rat, Daflon (100 mg/kg, orally) considerably reduced edema formation and inhibited the synthesis of PGE_2, α-PGF_2 and TXB_2 (Jean and Bodinier, 1994). Intravenous injection of Daflon reduced the hyperglycemia induced by the injection of alloxen in rats. Similarly, the mechanism of antiinflammatory activities of Daflon's ability to scavenge active oxygen radicals was demonstrated in vitro using human neutrophils (Labrid, 1994).

Another flavonoid, apigenin, demonstrated potent antiinflammatory activity in carrageenan-induced rat paw edema and inhibited IL-1α-induced prostaglandin synthesis and TNF-α-induced IL-6 and IL-8 production (Gerritsen et al., 1995). Yamamoto et al (1984) studied the effect of several benzoquinones and flavonoids. Cirsiliol, 5,3′,4′-trihydroxy-6,7-dimethoxyflavone, proved to be a potent inhibitor of 5-LO derived from rat basophilic leukemia cells and guinea pig peritoneal polymorphonuclear leucocytes (PMN) (Yamamoto et al., 1984). Selected flavonoid inhibitors significantly suppressed the 5-LO activity and LT synthesis by sensitized, challenged guinea pig lung tissue. Cirsiliol had approximately 10-fold less activity against the 12-LO enzyme and negligible effect on cyclooxygenase (COX) from bovine vesicular gland (Yamamoto et al., 1984). Partially purified mouse epidermal cell lipoxygenase (LO) was inhibited potently by hydroxyflavones but not by flavone itself (Wheeler and Berry, 1986). The partially purified 5-LO of rat basophilic leukemia cell was also inhibited by Cirsiliol (Furukawa et al., 1984). Artonin E (5′-hydroxymorusin), a naturally occurring prenylflavone, was a potent and selective inhibitor of porcine leucocyte 5-LO (Reddy et al., 1991). Hepolaetin

(5,7,8,3′,4′-pentahydroxyflavone) proved to be a good inhibitor of rat peritoneal leucocyte 5-LO whereas it was inactive as a cyclooxygenase inhibitor (Moroney et al., 1988). Swies et al. (1984) found that ram seminal vesicle COX was stimulated by quercetin and several other flavonoids at high arachidonic acid substrate concentrations. However, quercetin showed inhibitory activities at low substrate concentrations.

The activity of cyclooxygenase was affected by specific flavonoids. Baumann et al. (1980) examined the effect of several flavonoids on arachidonic acid peroxidation. Luteolin, 3′,4′-dihydroxyflavone, morin, galangin and (+)-catechin were moderately active inhibitors of rat renal medulla COX enzyme. Landolfi et al. (1984) reported that flavone, chrysin, apigenin and phloretin depressed COX activity and inhibited platelet aggregation. They concluded that flavonoids offer important therapeutic potential for the treatment of a variety of inflammatory diseases such as involving an increase in leukocyte adhesion and trafficking (Landolfi et al., 1984). The effects of 24 flavonoid derivatives, reported as antiinflammatory, on lysosomal enzyme secretion and arachidonic acid release in rat neutrophils were also investigated (Tordera et al., 1994). Amentoflavone, quercetagetin-7-O-glucoside, apigenin, fisetin, kaempferol, luteolin and quercetin were the most potent inhibitors of β-glucuronidase and lysozyme release. Amentoflavone showed the highest potency for inhibition of β-glucuronidase release. Another flavonoid, 3-hydroxyflavone, was the only compound which exhibited a biphasic effect (Tordera et al., 1994). The flavonols fisetin, kaempferol and quercetagetin-7-O-glucoside, the flavones chrysin, apigenin and luteolin, as well as amentoflavone and naringenin, significantly inhibited arachidonic acid release from lipid membranes (Tordera et al., 1994). Hypolaetin-8-O-glucoside did not show significant effect on

lysosomal enzyme secretion, but it inhibited β-glucuronidase release in an experimental model of inflammation in rats (Barberan et al., 1987).

Some structural activity relationships are also studied for the inhibition of lysosomal enzyme release. Polyhydroxylated aglycones of the flavone or flavonol types and the presence of a free hydroxyl group at C_4', the keto group at C_4 position and the C_2-C_3 double bond increased the inhibition of lysosomal enzyme release (Tordera et al., 1994). However, the glycosylation or the introduction of a free hydroxyl at C_2' decreases the inhibitory activity (Tordera et al., 1994). The most active compounds were C_2-C_3 unsaturated, hydroxylated at C_7 and C_4' and with some additional hydroxy groups at C_5, C_3 or C_3' (Tordera et al., 1994). Limasset et al. (1993) measured the inhibitory activity of 34 flavonoids or related substances on the release of reactive oxygen species by stimulated human neutrophils. They reaffirmed that ring A (C_5 and C_7) and ring B (C_3' and C_4') dihydroxylation, ring C hydroxylation at C_3 and the presence of a methoxy group on ring B are important to produce high potency (Limasset et al., 1993).

Flavonoids not only showed antiinflammatory activities, but also demonstrated protection against nonsteroidal antiinflammatory drug (indomethacin)-induced acute gastric damage. Blank et al (1997) reported that flavonoids could protect against acute gastric damage. The effects of 5-methoxyflavone and 5-methoxyflavanone on the gastric vasculature were compared both in vivo and in vitro on rat superior mesenteric arteries. Oral application of 5-methoxyflavone reduced indomethacin-induced macroscopic damage (Blank et al., 1997). However, the demage was not significantly reduced by 5-methoxyflavanone. Also, indomethacin-induced leukocyte adherence was inhibited to a greater extent by 5-methoxyflavone than by 5-methoxyflavanone (Blank et al., 1997).

This result suggested that the flavonoids such as 5-methoxyflavone could provide gastroprotection against nonsteroidal antiinflammatory drug-induced gastric damage (Blank et al., 1997).

Some authors further suggested that scavenging of reactive oxygen species and inhibition of arachidonic acid metabolism may be related (Dehmlow et al., 1996; Hoult and Paya, 1996; Jean and Bodinier, 1994)). The effects of the flavonoid silibinin on the formation of reactive oxygen species and eicosanoids by human platelets, white blood and endothelial cells were studied. The formation of leukotrienes through the 5-lipoxygenase pathway was strongly inhibited (Dehmlow et al., 1996). This indicated that the deleterious effects of HOCl that can lead to cell death, and those of leukotrienes that are important in inflammatory reactions, can be inhibited by silibinin.

There is no doubt that the flavonoids have profound effects on the function of immune and inflammatory cells as determined by a large number and variety of in vitro and in vivo studies. Ample evidence indicated that selected flavonoids, depending on structure, can affect (usually inhibit) secretory processes, mitogenesis, and cell-cell interactions including possible effects on adhesion molecule expression and function. Moreover, evidence indicated that certain flavonoids may affect gene expression and the effects of cytokines and cytokine receptors. One possible mechanism could be that flavonoids stimulate or inhibit protein phosphorylation and thereby regulate cell function. The effects of some flavonoids can certainly be attributed to their antioxidant and radical scavenging properties (Middleton and Kandaswami, 1992).

2.3 Anticarcinogenic properties of phenolic compounds

Recently flavonoids have attracted attention as potentially important dietary cancer chemoprotective agents (Hertog et al., 1993) and possible antitumor agents (Kandaswami et al., 1991). Flavonoids, due to their antioxidant properties and their ability to absorb UV light, may act in all stages of the carcinogenic process involving damage to the DNA (or initiation step), tumor growth (or promotion step), and invasion (or proliferative step). Due to their absorption of ultraviolet light, flavonoids can protect DNA from light damage. Recent experiments with plasmid DNA irradiated with UV-B light, showed the protective effect of naringenin and rutin against UV-induced DNA damage (Kootstra, 1994). In parallel, flavonoids are able to quench free radicals, which may promote mutations when they are generated in the vicinity of DNA. This radical scavenging ability is responsible for the protective effect of flavonoids observed in whole-body γ-ray irradiated mice (Shimoi et al., 1994). Flavonoids may also protect DNA by interacting directly with carcinogens that have escaped detoxification processes, as occurs in the chromosome aberration induced by bleomycin (Heo et al., 1994).

Quercetin has been shown to inhibit the mutagenic activity of benzo[α]pyrene (Bp), a representative polynuclear aromatic hydrocarbon (PAH) carcinogen, in bacterial mutagenicity studies (Ogawa et al., 1985). Castillo et al (1989) investigated the effect of quercetin on the in vitro and in vivo growth of two squamous cell carcinoma cell lines and a normal human lung fibroblast-like cell line. Quercetin caused inhibition of growth in both squamous cell carcinoma lines (Castillo et al., 1989). Effect on the fibroblast-like human lung cells was noted only at the high concentrations. Significant growth inhibition of squamous cell carcinoma was observed in implantable cell growth chambers retrieved 3 days after quercetin treatment. Quercetin appears to possess cytotoxic effect on

squamous cell carcinoma of head and neck origin both in vivo and in vitro (Castillo et al., 1989). The inhibitory effect of quercetin on malignant cells appears to be selective and dose-dependent. In addition, quercetin inhibited colon cancer in rats induced by azoxymethanol (Deschner et al., 1991).

Apigenin, another flavonoid, was reported to suppress 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-mediated tumour promotion in mouse skin (Huang et al., 1996). Flavonoids, bilirubin and myricetin, were capable of inhibiting the mutagenicities caused by 4-nitroquinoline 1-oxide and cigarette smoke (Camoirano et al., 1994). The intake of quercetin in experimental diet lowered the incidence of colon tumors in azoxymethanol treated rats (Deschner et al., 1991) as well as fibrosarcoma in mice induced by 20-methyl colanthrene (20-MC) (Elangovan et al., 1994). The possible mode of action of quercetin may be through its influence on the initiation and promotion phases of the carcinogenic processes coupled with the enhancement of detoxification process (Elangovan et al., 1994).

Non-melanoma skin cancer induced by solar UV is one of the most common cancers among humans (Bergfelt, 1993). Therefore, it is important to identify agents that can offer protection against this cancer. Katiyar et al (1997) evaluated the protective effects of silymarin, a flavonoid compound isolated from the milk thistle plant, against UVB radiation-induced nonmelanoma skin cancer in mice. The results indicated that silymarin can provide substantial protection against different stages of UVB-induced carcinogenesis, possibly via its strong antioxidant properties (Katiyar et al., 1997).

Flavonoids also display an antiproliferative effect on various human neoplasic cell lines such as myeloid and lymphoid leukemia cells (Piantelli, 1993), gastric cancer cells

(Yoshida et al., 1990), ovarian cancer cells (Scambia et al., 1990), prostate cancer cells (Peterson and Barnes, 1993), and squamous cell carcinoma (Kandaswami et al., 1991).

Flavonoids affect cell metabolism in various ways, either at the cell membrane level or on the intracellular enzymes. Flavonoid effects frequently include an inhibition of glycolysis, which is generally a very active metabolic pathway in tumor cells (Suolinna, 1975). The flavonoids may affect the activity of various enzymes involved in the transduction of mitogenic signals (kinases, phospholipases, and phosphodiesterases) and regulate other enzymes, which are critical for cell growth and proliferation. Most of the chemical carcinogens seem to require metabolic activation by DNA-reactive intermediates by cytochrome-P450-mediated mixed-function oxidase (MFOs) in order to exert their carcinogenic action (Dipple et al., 1984). The covalent binding of these reactive intermediates to cellular DNA, leading to adduct formation, is considered to be a critical event in the initiation of carcinogenesis (Miller, 1978).

Flavonoids may inhibit carcinogenesis by acting as 'blocking agents' (Wattenberg, 1983). Blocking agents can inhibit carcinogenesis by one of several possible mechanisms such as inhibiting the metabolic activation of the carcinogen, binding to cellular targets such as DNA, RNA and protein (Wattenberg, 1983). On the other hand, flavonoids may also inhibit tumor promotional events. Almost all of the polyphenolic compounds from plants, chlorogenic acid, caffeic acid, ferulic acid, alpha-tocopherol, catechins, carnosol, curcumin, curry, mustard and synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) possess several common biological and chemical properties (Wattenberg, 1985). These included antioxidant activity, the ability to scavenge active oxygen species and electrophiles, the

ability to inhibit nitrosation and to chelate metals, and the capability to modulate cellular enzyme activities. These compounds are likely to be able to inhibit various steps of tumor development in experimental animals and probably in humans (Huang and Ferraro, 1992).

2.4 Polyphenolics and cardiovascular diseases

Certain polyphenolic compounds have been shown to have an effects on blood platelet function, leukocyte function, blood coagulation, blood rheology, and ultimately thrombosis (Maalej, et al., 1997). Several studies indicated that certain flavonoids may have protective and therapeutic effects in coronary heart diseases. Knekt et al (1996) studied the association between dietary intake of flavonoids and subsequent coronary mortality. Finnish men and women between the age of 30-69 years from different parts of Finland and free from heart disease were investigated. The results indicated that people with very low intakes of flavonoids had higher risks of coronary disease. Hertog et al (1993) assessed the flavonoid intake of 805 men aged 65-84 years in 1985. Flavonoid intake was inversely associated with mortality from coronary heart disease and showed an inverse relation with incidence of myocardial infarction (Hertog et al., 1993). In order to determine whether flavonoid intake explains differences in mortality rates from chronic diseases between populations. Results indicated that average flavonoid intake may partly contribute to differences in coronary heart disease mortality across populations (Hertog et al., 1995).

In France and other Mediterranean areas, red wine is regularly consumed with meals. Red wine and grape juice inhibited platelet activity. The reason is that red wine

and grape juice contain a wide variety of naturally occurring compounds including tannins, anthocyanins, and phenolics including flavonols and flavones. The antithrombotic effect of red wine and grape juice may be due to the flavonoids common in some vegetable, fruits, and herbs such as tea (Demrow et al., 1995). Besides having anti-thrombotic properties, flavonoids are also antioxidants that prevent lipid oxidization known to contribute to atherosclerosis. Studies have demonstrated that the consumption of flavonoid rich foods and beverages may have protective effects against the development of coronary artery disease and may decrease the risk of myocardial infarction due to their platelet inhibitory and antioxidant effects (Folts et al., 1997).

In vitro experiments showed that flavonoids inhibit the oxidation of low-density lipoprotein (LDL) and reduce thrombotic tendencies (Maalej et al., 1997). There is ample evidence that free-radical oxidation of LDL plays an important part in atherogenesis. Flavonoids are scavengers of free radicals such as superoxide anions and peroxy radicals and thus can interrupt radical chain reactions. The oxidation of low-density lipoprotein (LDL) is thought to be a key step in the development of atherosclerosis (Mosca et al., 1997). The catechin oligomers, the procyanidin dimers and trimers were extracted, isolated and purified from grapes seeds (Kovac et al., 1992). These compounds were tested for their inhibition of LDL oxidation, along with other monomeric wine phenolics. Thus, the numerous phenolic compounds found in wine are potent antioxidants in inhibiting LDL oxidation in vitro (Kerry and Abbey, 1997). In vitro, some flavonoids inhibit the oxidative modification of LDL by macrophages, mainly by inhibiting the generation of hydroperoxides and protecting the α-tocopherol present in lipoprotein

oxidation. It is possible that flavonoids reduce the rate of oxidized compound, thus inhibiting the growth of atherosclerotic complications (Hertog et al., 1993).

2.5 Antiviral effects of flavonoids

Naturally occurring flavonoids with antiviral activity have been recognized in the past, but only recently they are investigated for their antiviral activity. Quercetin, morin, rutin, dihydroquercetin (taxifolin), dihydrofisetin, leucocyanidin, pelargonidin chloride, apigenin, catechin, hesperitin and naringenin have been reported to possess antiviral activity against 11 types of viruses (Selway, 1986).

Mucsi and Pragai (1985) demonstrated the inhibitory effect of four flavonoid compounds on virus multiplication and their influence on the intracellular cyclic AMP (cAMP) levels in cell cultures. Quercetin and quercitrin reduced the yields of human (alpha) herpesvirus 1 (HSV-1) and Suid (alpha) herpesvirus 1 (pseudorabies virus), but hesperidin and rutin had no effect. Further, quercetin and quercitrin elevated the intracellular level of cAMP, whereas hesperidin and rutin did not alter the cAMP level. Both antiviral activity and cAMP-enhancing effect were dependent on the concentrations of the flavonoids. This study suggested that a relationship existed between the antiviral effect and the cAMP-enhancing activity of flavonoids.

The flavonoid quercetagetin 3'-methylether isolated from flower and leaf extracts of *Centaurea rupestris* L. revealed a strong antiviral activity when inoculated simultaneously with tomato bushy stunt virus in two *Nicotiana* species (Rusak et al., 1997). Also, the flavonoids have the ability to interfere with the initiation of virus infection (Rusak et al., 1997). Critchfield et al (1997) reported that transcription from the

integrated provirus is inhibited by members of two distinct classes of compounds, the flavonoids and the benzothiophenes, via an unknown mechanism, possibly involving a cellular factor (Sandoval and Carrasco, 1997). Ro-090179 (Ro), a flavonoid isolated from the herb *Agastache rugosa*, induced the specific swelling and disruption of the Golgi complex and strongly inhibited poliovirus infection. Also, Ro provoked the swelling and the disruption of the stacked cisternae and trans-Golgi elements without affecting the cis-most Golgi cisternae (Sandoval and Carrasco, 1997). Isoscutellarein-8-methylether (5,7,4'-trihydroxy-8-methoxyflavone) from the roots of *Scutellaria baicalensis* was studied on the single-cycle replication of mouse-adapted influenza viruses A/Guizhou/54/89 (H3N2 subtype) and B/Ibaraki/2/85 in Madin-Darby canine kidney (MDCK) cells. The agent suppressed replication of these viruses from 6 to 12 h after incubation in a dose-dependent manner (Nagai et al., 1995). Results suggested that this compound inhibited the replication of A/Guizhou and B/Ibaraki viruses at least partly by inhibiting the fusion of viral envelopes with the endosome/lysosome membrane which occurs at the early stage of the virus infection cycle (Nagai et al., 1995). Baicalin, 7-D-glucuronic acid-5,6-dihydroxy-flavone, was purified from the plant *Scutellaria Baicalensis*. The inhibitory effect of BA against human immunodeficiency virus (HIV-1) infection and replication has been studied in vitro (Li et al., 1993). The enzymatic activity of purified recombinant HIV-1/RT was inhibited by baicalin. The anti-HIV-1 activity of baicalin was also observed in cultures of primary human peripheral blood mononuclear cells infected with HIV-1 in vitro (Li et al., 1993). Baicalin was also found to inhibit human T cell leukemia virus type I (HTLV-I) and reverse transcriptase activity

in HTLV-I-infected cells as well as the activity of purified reverse transcriptase from Moloney murine leukemia virus and Rous-associated virus type 2 (Baylor et al., 1992).

SP-303, a natural plant flavonoid polymer, was found to have antiviral activity against two strains of type 1 herpes-type simplex virus (Barnard et al, 1993). The mode of antiviral action of this biopolymer was through inhibition of virus penetration into cells (Barnard et al., 1993). SP-303 was also found to have antiviral activity against respiratory syncytial virus (RSV) in plaque reduction assays and cytopathic-effect-inhibition assays (Barnard et al., 1993). In vivo experiments, the SP-303 was evaluated against experimentally induced influenza A (H1N1) virus infections in mice. Mice receiving SP-303 by SPA exhibited consistent but reversible hypothermia immediately after termination of treatment (Sidwell, 1994).

Lophirone A, a biflavonoid, inhibited Epstein-Barr virus (EBV) activation (Murakami et al., 1991). Three structurally related flavonoids, chrysin, acacetin, and apigenin were found to inhibit HIV expression (Critchfield et al., 1996). These findings indicated that flavonoids can inhibit HIV-1 activation via a novel mechanism, and that these agents are potential candidates for therapeutic strategies aimed at maintaining a cellular state of HIV-1 latency (Critchfield et al., 1996). Naturally occurring flavones, baicalein, quercetin, quercetagetin and myricetin and two catechins, (-)-epicatechin gallate and (-)-epigallocatechin gallate, were isolated from the tea (*Camellia sinensis*) are known inhibitors of reverse transcriptase (Nakane and Ono, 1990). They were shown to induce mammalian topoisomerase II-dependent DNA-cleavage in vitro. The flavones differed from the catechins in causing the unwinding of duplex DNA, but both classes of compound induced enzymic DNA breakage at the same sites on DNA (Austin et al.,

1992). The structural basis for the antiviral activity of natural flavonoids was examined by Wleklik et al (1988). Hydroxylations at positions C_3', C_4', C_3, C_5 and C_7 was associated with highest antiviral actvity. Isoquercitrin, 3,3',4',5,7-pentahydroxyflavone-3-β-O-glucoside, an antiviral agent from *Waldsteinia fragarioides* (Rosaceae) was active against herpes simplex type 1 virus (Abou Karam and Shier, 1992). Substituted γ-chromones were found to weakly inhibit HIV-1 proteinase, an important enzyme in the replication and processing of the AIDS virus. Chromones bearing hydroxyl substituents and a phenolic group at the 2-position (flavones) were the most active compounds (Brinkworth et al., 1992).

Plant flavonoids are a large group of naturally occurring phenylchromones found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine. Several hundred milligrams are consumed in the average western diet everyday (Hertog et al., 1993). A variety of in vitro and in vivo experiments have shown that selected flavonoids possess antiallergic, anti-inflammatory, antiviral and antioxidant activities. Moreover, particular flavonoids have been shown to exert significant anticancer activity including anticarcinogenic activities. Certain flavonoids possess potent inhibitory activity against a wide array of enzymes such as protein kinase C, protein tyrosine kinases, and phospholipase A-2. These results suggested that plant flavonoids may possess health promoting and disease-preventing attributes when ingested as dietary supplements.

CHAPTER TWO*

Quantification and Characterization of Anthocyanins in Balaton™ Tart Cherries

ABSTRACT

The anthocyanin contents of Balaton™ and Montmorency cherries were compared. The results indicate that both cherries contain identical anthocyanins. However, Balaton™ contains approximately six times more anthocyanins than does Montmorency. Also, hydrolysis of the total anthocyanins and subsequent gas chromatography (GC) and nuclear magnetic resonance (NMR) experiments with the resulting products indicated that both varieties contain only one aglycone, cyanidin. This observation contrasts with existing reports of the presence of peonidin glycosides in Montmorency cherry. Results of the present study suggest that the anthocyanins in Balaton™ and Montmorency cherries are anthocyanin **1** [cyanidin-3-(2′′-*O*-*β*-D-glucopyranosyl-6′′-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside], anthocyanin **2** [cyanidin-3-(6′′-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside] and anthocyanin **3** [cyanidin-3-*O*-*β*-D-glucopyranoside].

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INTRODUCTION

Prunus cerasus L. (Rosaceae), cv. Montmorency is the major tart cherry (commercially) grown in the United States. In order to diversify the Montmorency monoculture, a new Hungarian cultivar, Balaton™ tart cherry, was introduced into the United States in 1984, and has been tested in Michigan, Utah, and Wisconsin. Balaton™ produces fruits darker than Montmorency, and may be used as a source for cherry anthocyanins.

Natural pigments like anthocyanins were regarded as the index of quality in tart cherries (Mazza and Miniati, 1993). In addition, recent studies have demonstrated the strong antioxidant activity of anthocyanins such as cyanidin-3-glucoside (Tsuda et al., 1994). Antioxidants are commonly used to increase the shelf life of food products by preventing, or at least delaying, the onset of lipid peroxidation (Tsuda et al., 1994). Natural antioxidants may play an important role in the prevention of carcinogenesis. Dietary antioxidants may be effective against the preoxidative damage in living systems (Halliwell and Gutteridge, 1989; Osawa et al., 1990).

Early studies revealed that Montmorency cherry contains cyanidin -3-gentiobioside and cyanidin -3-rutinoside (Li and Wagenknecht, 1956). Cyanidin-3-glucosylrutinoside was also found in six out of seven tart cherry varieties analyzed by Harborne and Hall (1964). Cyanidin-3-glucoside is reported as a minor pigment in Montmorency cherries (Schaller and Von Elbe, 1968; Chandra et al., 1992). Dekazos

(1970) reported anthocyanin pigments in Montmorency cherry as peonidin-3-rutinoside, peonidin and cyanidin along with cyanidin-3-sophoroside, cyanidin-3-rutinoside and cyanidin-3-glucoside (Schaller and Von Elbe, 1968). However, cyanidin-3-glucosylrutinoside as well as cyanidin-3-glucoside, cyanidin-3-sophoroside and cyanidin-3-rutinoside were identified as the main pigments in tart cherries. Using high performance liquid chromatography (HPLC) retention values, Chandra et al. (1992) reported that cyanidin-3-sophoroside and cyanidin-3-glucoside were the major and minor anthocyanins, respectively, in the Michigan-grown Montmorency cherries. Similarly, cyanidin-3-xylosylrutinoside was detected as a minor pigment in Montmorency cherry (Shrikhande and Francis, 1973). In addition to the comparison of anthocyanins in Balaton™ and Montmorency, this chapter describes the isolation of anthocyanins in Balaton™ and Montmorency cherries and their characterization by NMR, GC and mass spectroscopic (MS) methods.

MATERIALS AND METHODS

Cherry fruits. Pitted and frozen Montmorency and Balaton™ tart cherries were obtained from commercial growers (Traverse City, Michigan) through the Cherry Marketing Institute, Inc. (Dewitt, Michigan). The cherries were flushed with nitrogen in freezer bags prior to their storage at -20 °C.

General experimental. ^{1}H NMR, ^{13}C NMR and DQF COSY spectra were recorded on Varian 500 and 300 MHz spectrometers using CD_3OD/DCl (μL) solution at

25 °C. All chemical shifts are given in ppm relative to CD_3OD (3.3ppm). GC analyses were performed on an HP 5890 II (Hewlett Packard, Palo Alto, California) using a DB-17 (30 m × 0.313 mm × 0.25 μm, J & W Scientific, Palo Alto, California) column. The temperature program used was: 150 °C, initial temperature held for 5 min, and then increased to 210 °C at 5 °C min^{-1}, maintained for 5 min, and finally to 270 °C at 5 °C min^{-1}. The injection port temperature was maintained at 250 °C. The flame ionization detector temperature was 300 °C and the carrier gas was helium at a linear flow velocity of 4 cm s^{-1} with a 1:70 split ratio. Fast atom bombardment-mass spectroscopy (FAB-MS) was carried out on a double focusing mass spectrometer in a glycerol matrix using Xe as reactant gas.

HPLC conditions for anthocyanin analysis. All sample extracts (20 μL each) were analyzed on Chemcopak and Capcellpak C-18 columns (10×250 mm, 5 μm) (Dychrom; Sunnyvale, California). The mobile phase (4% aqueous H_3PO_4 /CH_3CN (80:20 v/v) was used under isocratic conditions at a flow rate of 1.5 mL min^{-1}. The anthocyanins were detected at 520 nm using a Waters PDA detector. Anthocyanins **1-3**, 0.5mg each, were weighed and dissolved in 1 mL of H_2O/CH_3CN (1:1). The solutions were prepared by the serial dilution of the respective stock solutions to afford 0.25, 0.20, 0.10, 0.05, 0.025 and 0.0125 mg/mL concentrations, respectively. Quantification of anthocyanins were carried out using a Millennium 2010 chromatography manager (Waters Associates, Milford, Massachusetts).

Isolation of crude anthocyanins from tart cherries. The pitted cherries (400 g each of Balaton™ and Montmorency) were homogenized separately for 10 min using a Kinematica CH-6010 (Roxdale, Ontario, Canada) homogenizer and centrifuged (Model RC5C, Sorvall Instruments, Hoffman Estates, Illinois) at 10000 *g* for 10 min at 4°C to separate insoluble materials from the supernatant. The supernatant (400 mL each) was applied to a XAD-2 (100 g, amberlite resin, mesh size 20-50; Sigma Chemical Co., St. Louis, Missouri) column, which was prepared as described by Chandra et al (1993). The column was washed with H_2O (9 L) until the colorless washings gave a pH of about 7. The adsorbed pigments were then eluted with methanol (500 mL). The red methanolic solution was concentrated at 50 °C *in vacuo*, and the aqueous solution was lyophilized to yield an amorphous red anthocyanin powder, 0.86 and 0.54 g, respectively, for Balaton™ and Montmorency samples.

HPLC Analysis of anthocyanins in cherries. Pitted cherries (100 g) were homogenized and centrifuged as described above. The supernatant was decanted and adjusted with H_2O to a final volume of 250 mL in a volumetric flask. An aliquot of 1 mL of this solution was passed through a preconditioned C-18 Sep-Pak cartridge (Waters Associates, Milford, Massachusetts). The adsorbed pigments were then washed with 2 mL of water followed by 1 mL of H_2O/ CH_3CN (1:1). The eluate was stored at -20 °C prior to HPLC analysis.

Purification of anthocyanins 1-3. The crude anthocyanins from Balaton™ were fractionated by C-18 medium pressure liquid chromatography (MPLC) to produce pure

anthocyanins. Both Balaton™ and Montmorency showed identical HPLC profiles (Fig. 2.1). The anthocyanin mixture (350 mg) was dissolved in water (2 mL), injected into the C-18 column (40 X 500 mm) and eluted with 4% H_3PO_4 :CH_3CN (80:20). Four fractions, I: 125 mL, II: 100 mL, III: 100 mL and IV: 275 mL, were collected and evaporated under reduced pressure. The H_3PO_4 from these fractions was removed by passing each fraction through preconditioned C-18 Sep-Pak (Waters Associates) with methanol, followed by 10% methanol. The adsorbed pigment was washed with 5 mL water to remove the acids and then eluted with 5 mL of H_2O/ methanol (1:1) to afford pure anthocyanins. The yield of anthocyanins from fractions I-IV were 53, 24, 133 and 64 mg, respectively. HPLC analysis of these fractions revealed that fraction I was pure and contained only anthocyanin **1**. Fraction II contained anthocyanins **1** and **2**, fraction III had anthocyanins **2** and **3** and fraction IV contained anthocyanin **3** with other phenolics as indicated by their HPLC profiles (Fig. 2.2).

Since fractions II and III from MPLC contained all three of the anthocyanins, 40 mg of II and 30 mg of III were purified further by HPLC on Capcellpak C-18 column (10× 250 mm, 5 μm) to yield pure anthocyanins **2** and **3**. Peaks were detected using a PDA detector at 520 and 283 nm, respectively. The mobile phase (4% aqueous H_3PO_4 : CH_3CN , 83:17 v/v) was used under isocratic conditions at a flow rate of 2.0 mL/min. Respective anthocyanin fractions from HPLC purification of fractions II and III were combined, dried under reduced pressure and purified further using C-18 Sep-Pak to remove H_3PO_4. The weights of pure anthocyanins **1-3** were 5.7, 8.9 and 2.9 mg, respectively.

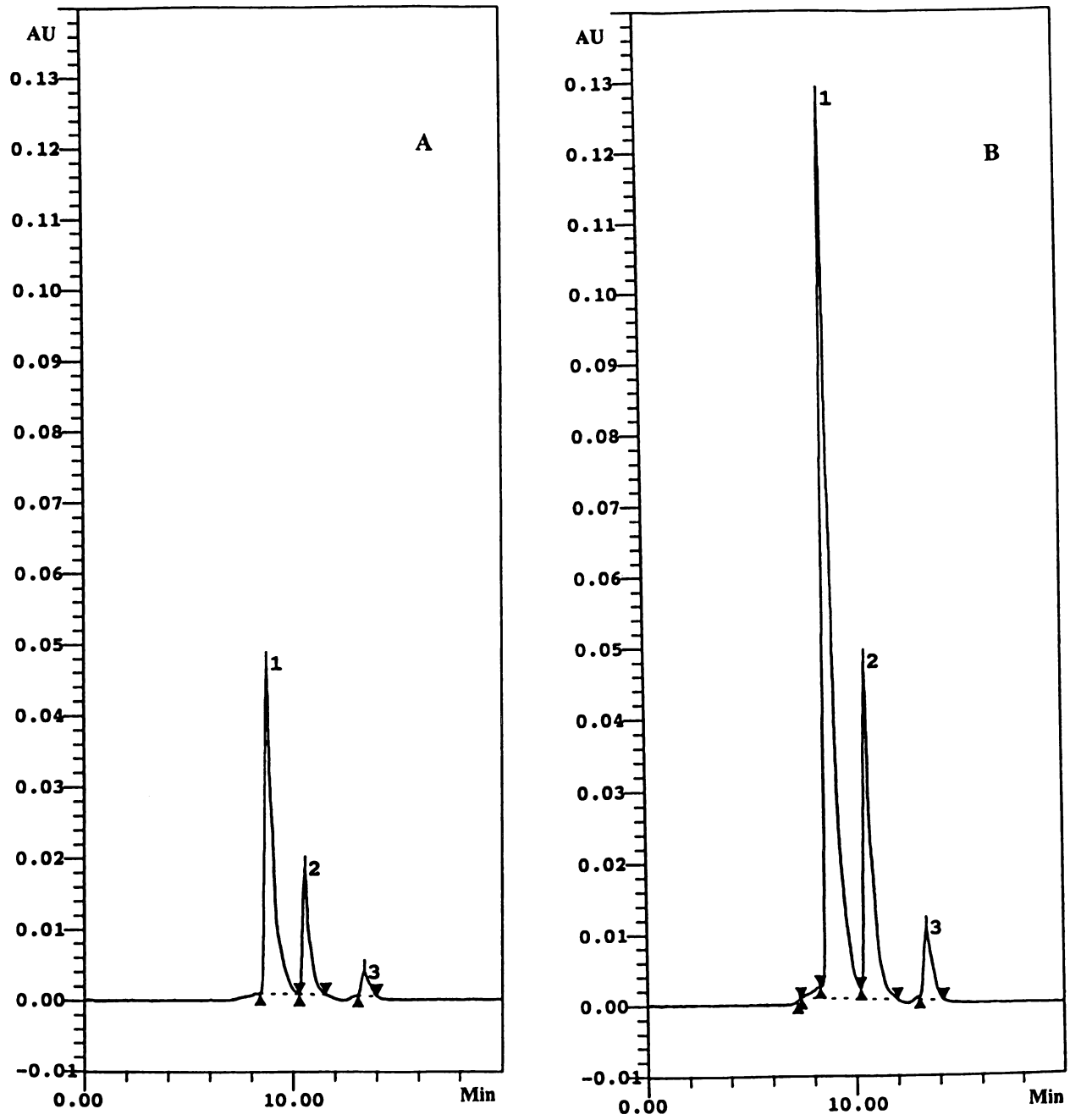
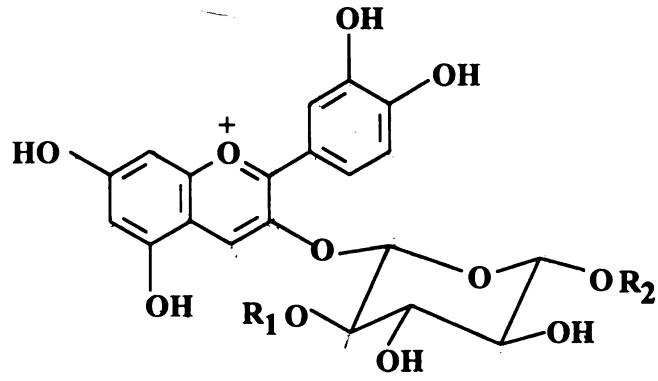


Fig. 2.1. HPLC profile of Montmorency (**A**) and Balaton™ (**B**) cherry extracts: **1**: Cyanidin-3-glucosylrutinoside; **2**: Cyanidin-3-rutinoside; **3**: cyanidin-3-glucoside.



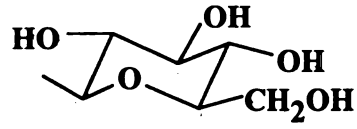
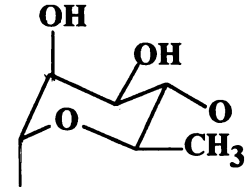
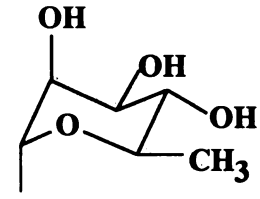
	R_1	R_2
Anthocyanin 1		
Anthocyanin 2	H	
Anthocyanin 3	H	H

Fig. 2.2. Anthocyanins **1-3** from Balaton™ and Montmorency tart cherries

Crude anthocyanins from Montmorency (500 mg) were also fractionated by C-18 MPLC as in the case of Balaton™. Three bands with red color were collected as fractions I, II and III and removal of solvents at reduced pressure afforded 10, 30 and 20 mg of anthocyanins, respectively. Fraction I was pure and contained only anthocyanin **1**. Fractions II and III were not pure by HPLC analysis and contained anthocyanins **1-3**.

The purified anthocyanins are red amorphous powders. Complete assignments of ^{1}H- and ^{13}C-NMR spectra of pure anthocyanins **1** [Cyanidin-3-(2"-*O*-*β*-D-glucopyranosyl-6"-*O*-*α*-L-rhamnosyl-*β*-D-glucopyranoside], **2** [Cyanidin-3-(6"-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside)] and the ^{1}H-NMR of **3** [Cyanidin-3-*β*-Dglucopyranoside] are given in Tables 2.1 and 2.2, respectively. ^{13}C-NMR on pure anthocyanin **3** was not performed due to its low yield.

Cyanidin, the aglycone. The crude anthocyanin powder from Balaton™ (55 mg) was hydrolyzed with 3 M HCl (15 mL) for 1 h at 100 °C. The red solution was cooled to room temperature and stirred with 1-butanol (20 mL). The mixture was extracted with water (3 × 20 mL). The combined water extracts were evaporated to dryness at reduced pressure to yield the sugars (30 mg). The red butanol layer was evaporated to dryness (24.3 mg). The residue was purified by silica gel preparative TLC using the solvent system, ethyl acetate : formic acid: 2 M HCl , 85: 6: 9. The single red band at R_f 0.28 was eluted with MeOH, evaporated under reduced pressure and afforded a red amorphous powder, cyanidin (11.2 mg). Similarly, pure anthocyanins (0.5 mg each) were hydrolyzed also to obtain their respective sugars for GC analysis. The aglycones from anthocyanins

1-3 and the crude anthocaynin gave identical R_f values and HPLC retention times. Also, all aglycones showed identical ^1H- and ^{13}C-NMR spectra (Tables 2.1 and 2.2).

Characterization of sugars by GC analysis. The sugar standards rhamnose, fructose, galactose, glucose and the internal standard phenyl-β-D-glucoside (E. Merck, Darmstadt, Germany) (1 mg each) and the sugars obtained from the hydrolysis of crude and pure anthocyanins (**1-3**) (1 mg each) were reacted separately with 30 mg/mL hydroxylamine HCl in dry pyridine (2 mL). The resulting oximes were then reacted with 1.0 mL hexamethyldisilazane (HMDS) and 0.1 mL trifluoroacetic acid (TFA) to yield their silyl derivatives. The samples were then analyzed by GC using an autosampler. Sugars from anthocyanins **1**, **2** and **3** were identified by comparing with the retention times of sugar standards. The retention times were 6.97, 9.83, 10.58, 10.90, 19.82 min, respectively, for rhamnose, fructose, galactose, glucose and phenyl-β-D-glucoside. The GC analysis of sugars yielded from the hydrolysis of anthocyanins showed that anthocyanin **1** contained a 2:1 ratio of glucose and rhamnose. Anthocyanin **2** showed a 1:1 ratio of glucose and rhamnose and anthocyanin **3** afforded only glucose. Similarly, the GC analysis of sugars from the crude anthocyanin powder indicated that it contained only rhamnose and glucose at a ratio of 2:4, respectively.

RESULTS AND DISCUSSION

Lyophilization of 100 g each of Balaton™ and Montmorency cherries afforded 17.1 and 14.7 % of dry weights, respectively. The concentrations of the sugars and acids

in Balaton™ were about 50% more than those in Montmorency cherries (data not presented). Similarly, total anthocyanin concentration in Balaton™ cherry is about six times greater than those in Montmorency cherry based on anthocyanin concentrations in fractions obtained from MPLC and HPLC purifications.

Prior to the isolation of anthocyanins for spectral characterization, both Balaton™ and Montmorency cherries were analyzed by HPLC under identical conditions. HPLC profiles of the cherry extract showed that there are two major and one minor anthocyanins in both varieties as indicated by retention times 8.76, 10.58, 13.38 min, respectively, for anthocyanins **1-3** (Fig. 2.1). Also, it was evident from the marked difference in the red color between these two cherries and from HPLC profiles (Fig. 2.1) that Montmorency contained relatively smaller amounts of anthocyanins compared to Balaton™.

The crude anthocyanins were fractionated and purified by C-18 MPLC and HPLC, respectively, to afford pure anthocyanins for spectral studies. Purification of 500 mg crude Montmorency anthocyanins from XAD-2 yielded 60 mg of anthocyanins **1-3** (Fig. 2.2) compared to 391.43 mg from Balaton™. This indicates that crude anthocyanins from Montmorency obtained from the XAD-2 contained a higher percentage of other organic compounds.

The presence of cyanidin and respective sugar moieties in anthocyanins **1-3** were confirmed by the comparison of their ^{1}H- and ^{13}C-NMR chemical shifts with published

data (Gläßgen et al, 1992; Stack and Wray, 1989). We have determined the relative configuration and nature of the sugars in anthocyanins **1** and **2** by DQFCOSY and from the vicinal and geminal ^{1}H-^{1}H coupling constants. The ^{1}H NMR spectrum of **1** (Table 2.1) gave signals for three anomeric protons that appeared at δ 5.43, 4.76 and 4.64, respectively, for glucose (attached to the aglycone), glucose and rhamnose . Also, the presence of β-D-glucosidic linkage in **1** was confirmed by the large coupling constants for the anomeric protons (Table 2.1). The signal at δ 4.64 ppm corresponded to the anomeric proton of an L-rhamnopyranose and the 1.8 Hz coupling constant indicated an α-glycosidic linkage.

The ^{13}C NMR chemical shifts observed for anthocyanins in Balaton^{TM} and Montmorency were similar to the published data (Agrawal et al., 1989). The C-7 resonated at very low field 170.4 ppm compared to the rest of the oxygenated aromatic carbons in anthocyanins. The oxygen cation in ring C is responsible for the downfield shift of C-7 carbon. The ^{13}C NMR signal for C-5 carbon in **1** at δ 69.8 confirmed the rhamnosyl moiety with an α-linkage to the glucose (Agrawal, 1992).

The downfield shift of the C-2'' proton in **1** relative to the C-2'' signal of **2** (Table 2.2) was due to the glucosylation and indicated a 1,2 linkage between the two glucose units. Similarly, the downfield shift of C-6'' proton in the ^{1}H-NMR spectrum of **1** (Table 1.1) relative to the C-6 proton signal of glucose was due to the rhamnose moiety and indicated a 1,6 linkage between the glucose and rhamnose. Anthocyanin **1** gave a molecular ion at *m/z* 758 $[M+H]^+$ and the base peak at *m/z* 596 $[M+H-C_6H_{10}O_5]$ in the

Table 2.1. ^{1}H NMR chemical shifts in ppm for anthocyanins **1-3** and Cyanidin in CD_3OD/DCl (J in Hz)

Proton	Anthocyanin 1	Anthocyanin 2	Anthocyanin 3	Cyanidin
H-4	8.89 s	8.92 s	8.98s	8.62s
H-6	6.67 d(1.96)	6.69 d(1.95)	6.71 d(1.95)	6.65 d(1.95)
H-8	6.90 d(1.96)	6.91 d(1.95)	6.98 d(1.95)	6.90 d(1.95)
H-2'	8.00 d(2.24)	8.02 d(2.23)	8.05 d(2.23)	8.11 d(2.23)
H-5'	7.06 d(8.66)	7.01 d(8.65)	7.07 d(8.65)	7.02 d(8.66)
H-6'	8.18 dd(8.66, 2.24)	8.27 dd(8.65, 2.23)	8.29 d(8.65, 2.23)	8.17dd(8.66,2.23)
H-1"	5.43 d(7.29)	5.29 d(7.53)	5.40 d(7.50)	
H-2"	4.05 dd(9.08, 7.29)	3.67 dd(9.06, 7.53)	3.67 dd(9.00,7.50)	
H-3"	3.77 dd(9.28, 9.08)	3.55 dd(9.22,9.06)	3.55dd(9.22,9.00)	
H-4"	3.50 dd(9.53, 9.28)	3.34 dd(9.49,9.22)	3.34 dd(9.50, 9.22)	
H-5"	3.72 ddd(9.53, 6.41, 1.76)	3.71 m	3.71 m	
H-6a"	4.04 dd(12.23, 6.41)	4.05 dd (11.90, 6.31)	3.91 dd(11.90, 6.30)	
H-6b"	3.61 dd(12.23, 1.76)	3.62 dd (11.90, 1.62)	3.68 dd(11.90, 1.62)	
H-1'''	4.76 d(7.74)	4.65 d(1.67)		
H-2'''	3.19 dd(9.08, 7.74)	3.80 dd(3.35, 1.67)		
H-3'''	3.33 dd(9.08, 9.28)	3.63 dd(9.49, 3.35)		
H-4'''	3.23 t(9.28)	3.41 dd (9.49,9.21)		
H-5'''	2.92 dt(9.28, 3.97)	3.54 dd(9.21, 6.14)		
H-6'''	3.44 d(3.97)	1.15 d(6.14)		
H-1''''	4.65 d(1.54)			
H-2''''	3.78 dd(3.31, 1.54)			
H-3''''	3.60 dd(9.50, 3.31)			
H-4''''	3.27 dd(9.28, 9.50)			
H-5''''	3.56 dd(9.28, 6.18)			
H-6''''	1.14 d(6.18)			

Table 2.2. ^{13}C NMR chemical shifts in ppm for anthocyanins **1,2** and their aglycone (Cyanidin) in CD_3OD

Carbon	Anthocyanin 1	Anthocyanin 2	Cyanidin
C-2	164.3	164.3	162.6
C-3	145.2	145.6	146.5
C-4	136.1	136.6	134.1
C-5	159.1	159.1	157.9
C-6	103.5	103.5	103
C-7	170.4	170.4	168.8
C-8	95.2	95.3	94.7
C-9	157.6	157.6	156.9
C-10	113.2	113.2	113.5
C-1'	121.2	121.2	121.9
C-2'	118.6	118.4	117.9
C-3'	147.4	147.4	147.3
C-4'	155.7	155.8	155.1
C-5'	117.6	117.5	117.2
C-6'	128.3	128.4	127.1
C-1"	104.9	103.5	
C-2"	82.3	74.7	
C-3"	77.2	77.4	
C-4"	71.2	71.2	
C-5"	77.9	78	
C-6"	67.6	67.8	
C-1'''	101.9	102.1	
C-2'''	75.9	71.9	
C-3'''	77.7	78.1	
C-4'''	70.8	73.9	
C-5'''	77.7	69.7	
C-6'''	62.3	17.9	
C-1''''	102.2		
C-2''''	71.8		
C-3''''	72.4		
C-4''''	73.9		
C-5''''	69.8		
C-6''''	17.9		

FAB MS also indicated the presence of cyanidin, two glucose and one rhamnose moieties in **1**. Therefore, anthocyanin **1** is confirmed to be cyanidin-3-(2′′-*O*-*β*-D-glucopyranosyl-6′′-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside).

^{1}H NMR spectrum of **2** (Table 2.1) showed signals for two anomeric protons at δ 5.29 (J= 7.53 Hz) and 4.64 (J = 1.67). This indicated the presence of a *β*-D- glucose because all vicinal coupling constants were 7.53~11.9ppm. The doublet (J = 6.14 Hz) at 1.15 ppm of a methyl group confirmed one of the sugars as rhamnose in **2**. The small coupling constant of 1.67 Hz for the anomeric proton suggested an *α*-rhamnosyl linkage. The C-6′′ proton of glucose at 5.5 ppm indicated a 1,6 linkage between the glucose and rhamnose. The FAB-MS of **2** gave the molecular ion at *m/z* 596 $[M+H]^+$ and confirmed its structure as cyanidin-3-(6′′-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside).

The ^{1}H NMR of anthocyanin **3** (Table 2.1) revealed only a single glucose moiety attached to the aglycone cyanidin. The structure of **3** was confirmed to be cyanidin-3-*β*-D-glucoside. Hydrolysis of crude anthocyanins and TLC of resulting products as well as ^{13}C NMR data showed that the only aglycone present in both Balaton™ and Montmorency cherries was cyanidin.

Our results suggest that there are only three identical anthocyanins present in both Balaton™ and Montmorency cherries. The yields of spectroscopically pure anthocyanins **1-3** in 100 g of fresh Balaton™ and Montmorency cherries were 14.99, 6.20; 6.18, 0.97; 2.42, 0.35 mg, respectively. The amount of anthocyanins isolated from Montmorency in

our studies show that it is lower than the reported yields (Dekazos, 1970). However, this may be due to varying environmental and nutritional factors. An important point to note is that when anthocyanins are monitored by HPLC at 520 nm, other phenolic compounds which absorb at 283 nm are ignored. We have isolated at least four phenolic compounds co-eluted with the anthocyanins and detected at 283 nm.

Chandra et al. (1992) reported that Montmorency cherries grown in Michigan contain only cyanindin-3-sophoroside and cyanidin-3-glucoside. These results were confirmed by matching their retention times to those of the anthocyanins present in an authentic sample of blackberry juice described by Hong and Wrolstad (1990a, b). Also, earlier reports indicated that there are peonidin-3-glycoside and peonidin-3-galactoside present in Montmorency cherry (Chandra et al., 1992). However, our present study indicates that Montmorency contains the same number of anthocyanins found in Balaton™ cherries and were identical. This is the first report of the characterization of anthocyanins from Balaton™ cherries and the spectral characterization of anthocyanins from Montmorency cherries.

CHAPTER THREE*

Antioxidant compounds from the ethyl acetate extract of tart cherries (*Prunus cerasus*)

ABSTRACT

As indicated by a Fe (II)-induced liposome peroxidation bioassay, the ethyl acetate extract of tart cherries (*Prunus cerasus* L. Rosaceae) was found to have strong antioxidant activity. Purification of this extract afforded chlorogenic acid methyl ester (**1**) and three novel compounds, 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid (**2**), 1-(3', 4'-dihydroxycinnamoyl)-cyclopenta-2, 5-diol (**3**) and 1-(3',4'-dihydroxycinnamoyl)-cyclopenta-2, 3-diol (**4**), as determined by their spectral data. At a 20 μM concentration, the antioxidant activities of compounds **3** and **4** were comparable to the antioxidant activities of caffeic acid, whereas compound **1** showed activity similar to chlorogenic acid. Also, these compounds showed antioxidant activities similar to the commercial antioxidants *tert*-butylhydroquinone and butylated hydroxytoluene. However, compound **2** was not active when tested at a 100 μM concentration.

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INTRODUCTION

Consumers are now including phytonutrients in their diet with the notion that antioxidant compounds may reduce the incidence of cancer and aging in humans. Free radicals are implicated in a number of pathological processes including aging, inflammation, reoxygenation of ischemic tissues, atherosclerosis, and cancer (Halliwell and Gutteridge, 1990). Free radicals such as hydroxyl radicals ($OH^{\cdot}$) initiate a chain reaction which causes lipid peroxidation, damage to enzymes and DNA, and cell death. Naturally occurring antioxidant components such as flavonoids, can stabilize highly reactive and potentially harmful free radicals (Graf, 1992). Many common foods contain non-nutritional components such as flavonoids and are considered to reduce the incidence of chronic diseases (Cody et al., 1988; Tanaka et al., 1993). The antioxidant phenolic compounds are reported to remove free radicals and protect the structural integrity of cells and tissues (Hertog et al., 1993).

Preliminary antioxidant assays revealed good antioxidant activities in the MeOH and EtOAc extracts of Balaton™ tart cherries (*Prunus cerasus* L. Rosaceae) while hexane extracts showed little or no activity. Anecdotal reports indicating that consumption of tart cherries can alleviate the pain of gout and arthritis (Hamel., 1975) prompted us to investigate tart cherries for biologically active compounds. We have evaluated the antioxidant efficacy of *P. cerasus* constituents using a Fe (II)-induced peroxidation of liposome antioxidant bioassay (Arora and strasburg, 1997). In this paper, we report the isolation and identification of three novel antioxidant compounds **(2-4)** from the EtOAc extracts of tart cherries.

EXPERIMENTAL SECTION

General Experimental procedures. Commercial antioxidant TBHQ and BHT were used as positive controls. TBHQ was purchased from Eastman Chemical Products Inc., Kingsport, Tennessee. BHT was purchased from National Biochemicals Corporation, Cleveland, Ohio. Silica gel (60 mesh, 35-70 μm) used for medium pressure liquid chromatography (MPLC) was purchased from E. Merck, Darmstadt, Germany. TLC plates (GF Uniplate, Analtech, Inc., Newark, DE), after developing, were viewed under 254 and 366 nm. For preparative high pressure liquid chromatography (HPLC) (LC-20, Japan Analytical Industry Co., Tokyo, Japan) purification, two Jaigel-ODS, A-343-10 (20 mm ×250 mm, 10 μm, Dychrom, Santa Clara, CA) columns were used in tandem. Peaks were detected using a model D-2500 Chromato-integrator connected with a UV detector. ^{1}H-, ^{13}C-, DQFCOSY and HMQC NMR spectra were recorded on a Varian Unity 500 and an Inova 300 MHz spectrometers at 25°C and referenced to the residual proton solvent resonance, CD_3OD at 3.30 and 49.0 ppm and DMSO-d_6 at 2.49 and 39.5 ppm, for ^{1}H- and ^{13}C NMR, respectively. Fast atom bombardment mass spectroscopy (FABMS) were obtained on a JEOL JMS-HX110 using a glycerol matrix and EIMS spectra were obtained on JEOL JMS-AX505 mass spectrometers. Circular dichroism (CD) and Optical rotatory dispersion (ORD) measurements were carried out using a JASCO J-710 CD-ORD spectropolarimeter (Japan Spectroscopic Co., Hachioji city, Tokyo, Japan). For CD/ORD measurements, test compounds were dissolved in methanol (0.2 mg mL^{-1}), and CD/ORD were determined under the following conditions: scan mode (wavelength), bandwidth (0.5 nm), sensitivity (50 m deg), response (1 s), wavelength range (200-400 nm for CD and 200-800 nm for ORD), step resolution (1

nm), scan speed (200 nm min^{-1}), and accumulation (1). Nitrogen (99.99%) was generated by a nitrogen generator model NG-150 (Birtley Co., Durham, England) at the rate of 15 L min^{-1}. UV spectra of compounds, in MeOH, were measured on a Shimadzu UV-visible spectrophotometer (Kyoto, Japan).

Plant Material. Pitted and individually quick frozen (IQF) Balaton™ cherries (*Prunus. cerasus* L. Rosaceae), which were collected July, 1995, were obtained from commercial growers (Traverse City, MI) and supplied by the Cherry Marketing Institute, Inc. (Dewitt, MI).

Extraction and Isolation. IQF Balaton™ tart cherries (2 kg) were lyophilized at 10°C and yielded 342 g of dried cherries. The Balaton™ dried cherries (340 g) were milled and extracted with hexane (500 mL × 3), ethyl acetate (500 mL × 3) and methanol (500 mL × 3) to yield 0.71, 2.53 and 198.9 g of extracts, respectively.

The ethyl acetate extract of Balaton™ cherries (1.75 g) was fractionated by silica gel (100 g) MPLC using $CHCl_3$ and MeOH under gradient conditions starting with 100% $CHCl_3$ and ending with 100% MeOH. Fractions 1-4 (125 mL each, $CHCl_3$), 5-8 (100 mL each, $CHCl_3$-MeOH, 8:1), 9-12 (100 mL each, $CHCl_3$-MeOH, 4:1), and 12-16 (150 mL each, MeOH) were collected and combined after TLC analysis (silica gel plates developed with MeOH-$CHCl_3$, 16:1, for fractions 1-8 and MeOH-$CHCl_3$-HCOOH, 1:4:0.2, for fractions 9-16), to yield 85, 134, 330, 910 and 225 mg each of fractions A-E, respectively. Fractions A and B did not show antioxidant activity. Fractions C-E were further purified for antioxidant compounds.

Fraction C (250 mg) was purified by preparative silica TLC using MeOH-$CHCl_3$-HCOOH (4:1:0.2) as the mobile phase to yield compounds **1** (10.1 mg, R_f = 0.50) and **2**

(8.9 mg, R_f , 0.67). ^{1}H- and ^{13}C- NMR spectra of compound **1** was identical to the published data of chlorogenic acid methyl ester (Rumbero-Sanchez and Vazquez, 1991)

Compound 2: White solid; IR (film) ν_{max} 3316, 1728, 1590, 1406 cm^{-1}; UV λ_{max} (MeOH) 218 (3.04), 253 (3.42), 289 (3.66) nm; CD/ORD measurements gave straight lines indicating that compound **2** was obtained as a racemic mixture; ^{1}H NMR (CD_3OD) δ 7.40 (1 H, d, *J* = 7.32 Hz, H-6'), 7.22 (1H, t, *J* = 7.57 Hz, 7,32 Hz, H-4'), 7.00 (1H, t, *J* = 7.57 Hz, 7.32 Hz, H-5'), 6.86 (1H, d, *J* = 7.57 Hz, H-3'), 4.19 (1H, m, H-2), 2.80 (2H, m, H-3); ^{13}C NMR (DMSO-d_6): δ 178.6 (C-1), 141.6 (C-2'), 133.4 (C-6'), 128.4 (C-4'), 123.8 (C-5'), 121.3 (C-1'), 109.2 (C-3'), 73.4 (C-2), 48.6 (C-3); FABMS, *m/z* 183 (4) $[M+H]^+$.

Compound 3: Fraction D (900 mg) was purified using a preparative HPLC with the mobile phase being MeOH-H_2O (30:70) at a flow rate of 3 mL/min, to yield compound **3** (R_t = 58 min, 9.4 mg): pale yellow oily compound; IR (film) ν_{max} 3351, 2926, 1669, 1599, 1379, 1267, 1076 cm^{-1}; UV λ_{max} (MeOH) 206 (3.99), 215 (4.00), 243 (3.83), 299 (3.86), 325 (3.89) nm; ORD (m deg) 336 (75), 316 (-44), 298 (-40), 260 (35), 240 (-22) and 216 (52) nm; ^{1}H- NMR (DMSO-d_6) δ 7.45 (1H, d, *J* = 15.9 Hz, H-7'), 7.01 (1H, d, *J* = 1.8 Hz, H-2'), 6.96 (1H, dd, *J* = 8.1 Hz, 1.8 Hz, H-6'), 6.75 (1H, d, *J* = 8.1 Hz, H-5'), 6.19 (1 H, d, *J* = 15.9 Hz, H-8'), 5.17 (1H, m, H-1), 3.82 (1H, m, H-2), 3.54 (1H, m, H-5), 1.83 (4 H, m, H-3, H-4); ^{13}C NMR (DMSO-d_6) δ 166.1 (C-9'), 148.2 (C-3'), 145.5 (C-4'), 144.4 (C-7'), 125.7 (C-1'), 121.1 (C-6'), 115.8 (C-5'), 115.0 (C-8'), 114.6 (C-2'), 70.9 (C-2, C-5), 67.5 (C-1), 35.2 (C-3, C-4); FABMS *m/z* 281 (2) $[M+H]^+$; EIMS *m/z* 180 (93), 163 (100), 145 (20).

Compound 4: Fraction E (225 mg) was purified by preparative HPLC. The mobile phase was MeOH-H_2O (40:60) at a flow rate of 4ml/min. Sub-fractions 1 (180 mg), 2 (10.8 mg), 3 (8.4 mg), 4 (8 mg), and 5 (10 mg) were collected. sub-fraction 1 was not active and contained malic acid, as confirmed by its ^{1}H NMR spectrum. Fraction 2 (10.8 mg) was the most active and hence purified again by HPLC under the same conditions to yield compound **4** (R_t = 34 min, 9.4 mg); oily compound; IR (film) ν_{max} 3372, 1692, 1603, 1277, 1184, 1074 cm^{-1}; UV λ_{max} (MeOH) 203 (3.95), 215 (3.94), 243 (3.76), 299 (3.81) and 327 (3.90) nm; ORD (m deg) 314 (-58), 288 (-61) and 234 (-61) nm; ^{1}H NMR (CD_3OD) δ 7.58 (1H, d, *J* = 15.9 Hz, H-7'), 7.04 (1H, d, *J* = 1.8 Hz, H-2'), 6.93 (1 H, dd, *J* = 8.2 Hz, 1.8 Hz, H-6'), 6.76 (1H, d, *J* = 8.2 Hz, H-5'), 6.30 (1H, d, *J* = 15.9 Hz, H-8'), 5.35 (1H, m, H-1), 4.14 (1H, m, H-3), 3.64 (1H, dd, J=8.3 Hz, 3.1 Hz, H-2), 2.15 (2H, m, H-4), 2.15 (1H, m, H-5a), 1.95 (1 H, m, H-5b); ^{13}C NMR (CD_3OD) δ 169.0 (C-9'), 149.4 (C-3'), 146.8 (C-4'), 146.8 (C-7'), 128.0 (C-1'), 122.9 (C-6'), 116.5 (C-5'), 115.8 (C-8'), 115.1 (C-2'), 74.8 (C-2), 73.0 (C-1), 68.3 (C-2), 41.5 (C-5), 36.7 (C-4); FABMS *m/z* 281 (2) $[M+H]^+$; EIMS *m/z* 180 (34), 163 (100).

Methylation of Compound 2: *N*-Nitroso-*N*-methylurea (1.5 g) was slowly added to 100 mL of 25% KOH and 100 mL diethyl ether mixture at 0°C and reacted for about 1 h. The yellow ether layer containing CH_2N_2 was separated using a separatory funnel (500 mL) and washed with cold water (100 mL) to remove excess KOH. Compound **2** (4 mg) was dissolved in methanol and mixed with excess CH_2N_2 reagent (5 mL) in ether. The reaction mixture was kept at room temperature for 1 h. The solvent was then evaporated to afford compound **5** (4 mg). Compound **5**: White solid; ^{1}H NMR (CD_3OD) δ 7.35 (1H, d, *J* = 7.32 Hz, H-6'), 7.25 (1H, dd, *J* = 7.57 Hz, 7.32 Hz, H-4'), 7.01 (1H, dd, *J* = 7.57

Hz, 7.32 Hz, H-5'), 6.87 (1H, d, J = 7.57 Hz, H-3'), 4.49 (1H, dd, J = 7.32 Hz, 4.88 Hz, H-2), 2.80 (1H, d, J = 12.45 Hz, 4.88 Hz, H-3a), 2.69 (1H, dd, J = 12.45 Hz, 7.32 Hz, H-3b), 3.69 (3H, s, OCH_3), 3.47 (3H, s, $COOCH_3$).

Antioxidant Assay. All the buffers were stored in Chelex 100 to remove metal ions. A mixture containing 5 μmol of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 15 nmol of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (Molecular Probes, Inc., Eugene, OR) was dried under vacuum. The resulting film was suspended in 500 μL of buffer (NaCl, 0.15 M; EDTA 0.1 mM; MOPS 10 mM) and was then subjected to 10 freeze-thaw cycles in an ethanol/dry ice bath. The suspension was passed 29 times through a polycarbonate membrane with a pore size of 100 nm using a LiposoFast extruder (Avestin, Inc., Ottawa, Canada). Liposomes (200 nmol) were suspended in 2 mL of buffer (100 mM NaCl, 50 mM HEPES pH 7.0) and peroxidation was initiated by addition of 4 nmol of Fe^{2+}. Control samples contained no added Fe^{2+} or test compound. Anthocyanins, BHT, propyl gallate or α-tocopherol were added to final concentrations of 2 μM. Fluorescent intensity of this lipid suspension was monitored for a period of 21 min with or without test compounds, immediately following addition of Fe^{2+}, using a SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL). The values of relative fluorescence were determined by dividing the fluorescence value at a given time point by that at t = 0 min. The decrease in relative fluorescence intensity with time indicates the rate of preoxidation. The percent inhibition of the lipid oxidation was calculated using the equation: Percent Inhibition $=\{[(F_{rel})_{PI}- (F_{rel})_{Fe} /[(F_{ref})_C - (F_{rel})_{Fe}]\}\times 100$, where: $(F_{rel})_{PI}$ = relative fluorescence for the Fe (II) and test samples at the end of 21 min, $(F_{rel})_C$ =

relative fluorescence for the control sample at 21 min, and $(F_{rel})_{Fe}$ = relative fluorescence for the Fe (II)-containing sample at the end of 21 min (Arora and Strasburg, 1997).

RESULTS AND DISCUSSION

Ethyl acetate extracts of dried Balaton™ tart cherries were separated by medium pressure liquid chromatograpgy (MPLC), preparative TLC and HPLC to yield compounds **1-4**. Both the ^{1}H and ^{13}C NMR spectral analysis of compound **1** revealed that the chemical shifts observed were identical to the published spectral data of the known compound, chlorogenic acid methyl ester (Rumbero-Sanchez and Vazquez, 1991).

Compound **2** was obtained as a white solid. The molecular formula of this compound was determined as $C_9H_{10}O_4$ by FABMS. The ^{1}H NMR spectrum revealed two aromatic protons that appeared as doublets at δ 7.40 and 6.86, respectively. Another two aromatic protons in the molecule appeared as triplets at δ 7.22 and 7.00, respectively. This indicated that there is an *ortho*-substituted aromatic moiety in the molecule. The multiplets at δ 4.19 and 2.80 were assigned to oxygenated methine and methylene moieties, respectively. The ^{13}C NMR spectrum of **2** supported these assignments in addition to a carbonyl carbon at δ 178.6. The structure of this compound was further confirmed by the formation of product **5** from **2** by methylation. Methylation of **2** by CH_2N_2 yielded one unit each of $-OCH_3$ and $-COOCH_3$. These data confirmed the presence of a phenolic OH and a COOH in **2**. Therefore, the NMR confirmation of the identity of compound **2** as 2-hydroxy-3-(*o*-hydroxyphenyl)-propanoic acid is in agreement with the methylation data. Circular dichroism (CD) studies of **2** showed that it

is a racemic mixture as evident from a straight line in the CD spectrum. To our knowledge, this is the first report of this compound as a natural product.

Compound **3** was obtained as a pale yellow gum. The ^{1}H NMR spectrum of **3** indicated two olefinic proton signals appeared as doublets at δ 7.45 and 6.19, respectively. A coupling constant of 15.9 Hz for these two protons suggested that they are *trans* oriented. The signals appeared at δ 7.02, 6.97 and 6.75 were assigned to aromatic protons of a 3, 4-dihydroxylcinnamoyl group, respectively, and were similar to the chemical shifts of chlorogenic acid. The peaks at m/z 180 and 163 in the EI-MS of **3** confirmed that it contained a caffeic acid moiety. The signals at δ 5.17, 3.82, 3.54 were assigned to three oxygenated protons, one (δ 5.17) esterified, as well as multiplets at δ 1.83 integrating for four protons of two methylene groups. These oxymethines appeared at δ 70.9 (× 2) and 67.5, respectively, in the ^{13}C NMR spectrum. Compound **3** showed only one carbonyl carbon at 166.1 ppm. The fact that compound **3** has only one carbonyl carbon and no quaternary carbon around δ 70.9 suggested that the caffeic acid moiety was not connected to a quinic acid moiety but a cyclopentane-2,5-diol moiety. From these spectral data, the structure of compound **3** was assigned as 1-(3', 4'-dihydroxylcinnamoyl)-cyclopenta-2, 5-diol.

Compound **4** was obtained as a colorless oily product. The ^{1}H NMR spectrum of **4** revealed a 3, 4-dihydroxylcinnamoyl moiety as in compound **3**. However, the two multiplets in **4**, appearing at δ 2.15 and 1.95 were assigned to two methylene groups, respectively. The DQFCOSY experiment showed that two CH_2 protons in **4** were correlated and adjacent to each other and also coupled to other hydrogens. The ^{13} C NMR spectrum of this compound revealed that there were only one carbonyl carbon, eight

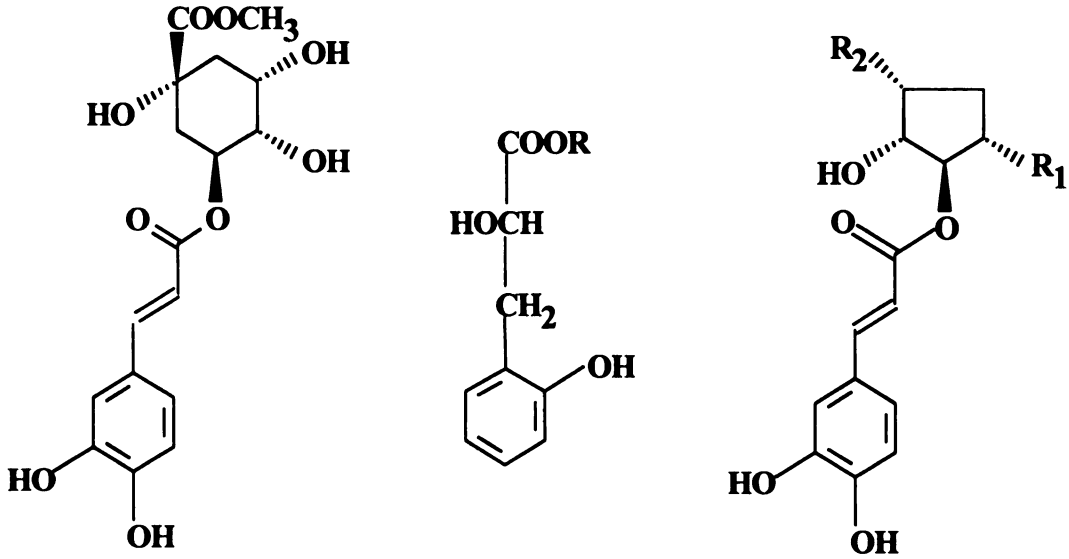
methine carbons, and two methylene carbons. Three of the methine carbons at δ 74.8, 73.0, and 68.3 were oxygenated and showed correlations to three methine protons at δ 3.64, 5.35, and 4.14, respectively, as evident from the HMQC spectrum. Also, five other methine carbons at δ 115.1, 116.5, 122.9, 146.8, and 115.8 showed correlations to three aromatic protons appearing at δ 7.04, 6.76, 6.93 and two olefinic protons at 7.58 and 6.30 ppm, respectively. Therefore, compound **4** was assigned as 1-(3′, 4′-dihydroxylcinnamoyl)-cyclopenta-2, 3-diol.

CD measurements of compounds **3** and **4** did not show absorption maxima or minima. This seems to be because the cyclopentane moieties in **3** and **4** do not absorb in the UV region. However, both of these compounds gave observable peaks in their ORD spectra. Compounds **3** and **4** are novel.

The antioxidant activity of compounds **1-4** was determined by fluorescence spectroscopy (Arora and Strasburg, 1997) and the activity was compared with caffeic acid, ferulic acid, chlorogenic acid, *p*-hydroxycinnamic acid, and two commercial antioxidants, *tert*-butylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT), each at a 20 μM concentration. In this assay, the lipid peroxidation was initiated by Fe^{2+} and the rate of decrease of fluorescence intensity reflected the rate of lipid peroxidation. The inhibitory activities of Fe^{2+}-induced lipid peroxidation in the large unilamellar vesicles (LUVs) for compounds **3** and **4** were about 80% at 20 μM. Compound **1** showed about 50% inhibitory activity. However, **2** did not show antioxidant activity even tested at a 100 μM concentration. The assay results showed that *p*-hydroxycinnamic acid is a weak antioxidant when compared to ferulic acid. However, the caffeic acid analogues, compounds **3** and **4**, showed the highest antioxidant activity in this assay. The percent

inhibition of lipid peroxidation for TBHQ and BHT were > 90% at 20 μM concentration (Fig. 2.1).

The variation in antioxidant activity among caffeoyl esters is dependent on the hydroxyl substitution of the aryl ring. More than one hydroxyl substitution in the aryl ring enhanced the antioxidant activity. Introduction of a second hydroxyl group in the *ortho* position, as in caffeic acid, also enhanced the antioxidant activity. Methylation of the hydroxyl group in the *ortho* position of caffeic acid, as in ferulic acid, resulted in a decrease of antioxidant activity. This result is in agreement with published studies on the effects of hydroxycinnamates on the autoxidation of fats and lipids (Shihidi and Wanasundara, 1992). Our data suggest that caffeic acid is the best antioxidant, followed by compounds **4** and **3**, chlorogenic acid, and chlorogenic acid methyl ester (**1**). This trend in activity may be due to the difference in hydrophilicity or chelation properties of these compounds. It is interesting to note that the antioxidant activities of the novel caffeic acid analogues, **3** and **4**, are comparable to the commercial antioxidants BHT and TBHQ at the concentration tested.



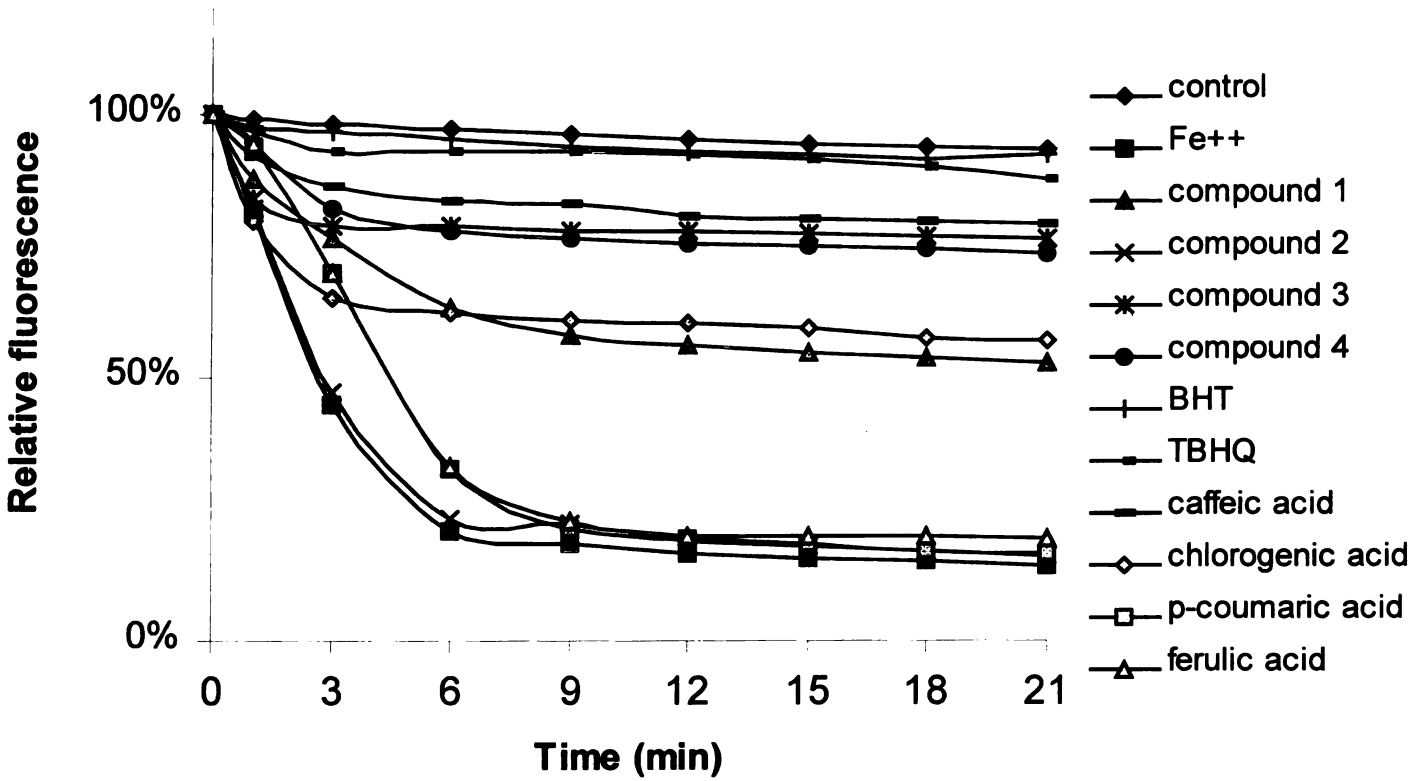
1

2 R = H
5 R = CH_3

3 R_1 = OH, R_2 = H
4 R_1 = H, R_2 = OH

Fig. 3.1. Structure of compounds isolated from ethyl acetate fraction

Fig. 3.2. Antioxidant activities of compounds **1**, **3** and **4** and some commercial antioxidants at 20μM concentration. The antioxidant activity of compound **2** was measured at 100 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative intensity represents the fluorescence intensity at a given time divided by the initial intensity at the start of the assay. Values represent the means of duplicate measurements.


Relative fluorescence
100%
50%
0%
0
3
6
9
12
15
18
21
Time (min)
control
Fe++
compound 1
compound 2
compound 3
compound 4
BHT
TBHQ
caffeic acid
chlorogenic acid
p-coumaric acid
ferulic acid

CHAPTER FOUR*

Antioxidant polyphenolics from the methanol extract of tart cherries (*Prunus cerasus*)

ABSTRACT

Montmorency and Balaton™ tart cherries were lyophilized and sequentially extracted with hexane, ethyl acetate and methanol. Methanolic extracts of dried Balaton™ and Montmorency tart cherries (*Prunus cerasus*) inhibited lipid peroxidation induced by Fe^{2+} at 25 ppm concentration. Further partitioning of this methanol extract with EtOAc yielded a fraction, which inhibited lipid peroxidation by 76 % at 25 ppm. Purification of this EtOAc fraction afforded eight polyphenolic compounds 5,7,4'-trihydroxyflavanone (**1**), 5,7,4'-trihydroxyisoflavone (**2**), chlorogenic acid (**3**), 5,7,3', 4'-tetrahydroxyflavonol-3-rhamnoside (**4**), 5,7,4'-trihydroxyflavonol 3-rutinoside (**5**), 5,7,4'-trihydroxy-3'methoxyflavonol-3-rutinoside (**6**), 5,7,4'-trihydroxyisoflavone-7-glucoside (**7**) and 6,7-dimethoxy-5,8,4'-trihydroxyflavone (**8**), as characterized by ^{1}H- and ^{13}C NMR experiments. The antioxidant assays revealed that compound **8** is the most active, followed by quercetin 3-rhamnoside, genistein, chloregenic acid, naringenin and genistin, at 10 μM concentrations.

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INTRODUCTION

The Montmorency (*Prunus cerasus*) variety constitutes more than 95% of tart cherry cultivations in Michigan and USA. However, Balaton™ tart cherry (*P. cerasus*), a new tart cherry cultivar, is being planted to replace Montmorency in several Michigan orchards. Anthocyanin contents of Montmorency and Balaton™ tart cherries have been reported (Wang et al., 1997; Chandra et al., 1993). However, a detailed investigation of other phenolic compounds in Balaton™ tart cherry was not carried out before.

Flavonoids, a group of polyphenolic compounds, are widely distributed and have been reported to act as antioxidants in biological systems (Morel et al., 1993). Flavonoids are considered to have antioxidant activity similar to α-tocopherol, vitamin E. It is one of the most common and active naturally occurring antioxidant compounds in food because of its activity in both hydrophilic and lipophilic systems (Kühnau, 1976).

Kaempferol-3-rutinoside and kaempferol-3-glycoside were reported in the fruits of Montmorency cherries (Von Elbe, 1970). Geissman (1956) indicated the presence of quercetin 3-glucoside in the leaves of *P. cerasus*. Also, various kaempferol and quercetin glucosides were identified from Montmorency cherry (Shrikhande and Francis, 1973). From the bark *of P. cerasus*, tectochrysin 5-glucoside and genistein 5-glucoside, pinostrobin, naringenin, prunin, sakuranetin, sakuranin, dihydrowogonin 7-glucoside, chrysin, tectochrysin, genistein, prunetin and prunetin 5-glucoside were reported (Geibel et al., 1990; 1991; 1995). Isomers of caffeoylquinic acid, *p*-coumaroylquinic acids, caffeic and ferulic acids were characterized from Montmorency tart cherry (Schaller and Von Elbe, 1970). Similarly, Schwab et al (1990) reported benzyl-β-D-glucoside, 6-

hydroxy-2, 6-dimethyl-octa-2 (E), 7-dienyl beta-D-glucoside and 2-methoxy-4-(2-propenyl)phenyl beta-D-glucoside from Montmorency cherry pulp.

Recently, meat products containing tart cherries are available to the consumers. Researchers have found that cooked low-fat ground beef with approximately 12 % of tart cherries had less rancidity development (Liu et al., 1995). Also, the addition of cherry fruits to ground beef before frying significantly inhibited the formation of heterocyclic aromatic amines (HAAs) (Britt et al., in press). The mechanism of this protective action may be involved in the potential antioxidant flavonoids and other polyphenolics present in cherries. Until now, researchers have not investigated the antioxidant compounds in Balaton™ and Montmorency tart cherries. In this chapter, the isolation, identification and efficacy of antioxidant polyphenolic compounds from Balaton™ and Montmorency tart cherries are described.

MATERIALS AND METHODS

Cherry Fruits. Pitted and frozen Montmorency and Balaton™ tart cherries were obtained from commercial growers (Traverse City, MI) through the Cherry Marketing Institute, Inc. (Dewitt, MI). The cherries were flushed with nitrogen in freezer bags prior to their storage at -20°C.

General Experimental. Silica gel (60 mesh size, 35-70 μm) was purchased from E. Merck, New Jersey. TLC plates (GF uniplate, Analtech, Inc., Newark, DE) after developing were viewed at 254 and 366 nm, respectively. For preparative high pressure liquid chromatography (HPLC) (LC-20, Japan Analytical Industry Co., Tokyo) purification, two JAIGEL-ODS, A-343-10 (20mm ×250mm, 10 μm, Dychrom, Santa

Clara, CA) columns were used in tandem. Peaks were detected using a UV detector equipped with model D-2500 Chromato-integrator (Hitachi, Tokyo). ^{1}H-, ^{13}C-, DQFCOSY and HMQC NMR spectra were recorded on a Varian UNITY 500 and an INOVA 300 MHz spectrometers at 25°C. All chemical shifts are given in parts per million relative to CD_3OD and DMSO-d_6 at 3.30, 49.0 ppm and 2.49 and 39.5 ppm, respectively. Fast atom bombardment mass spectroscopy (FABMS) were obtained on a JEOL JMS-HX110 using a glycerol matrix and Electrom impact ionization mass spectroscopy (EIMS) spectra were obtained on JEOL JMS-AX505 Mass Spectrometers.

Antioxidant Assay: All the buffers were stored in Chelex 100 to remove metal ions. A mixture containing 5 μmol of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 15 nmol of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (Molecular Probes, Inc., Eugene, OR) was dried under vacuum. The resulting film was suspended in 500 μL of buffer (NaCl, 0.15 M; EDTA 0.1 mM; MOPS 10 mM) and was then subjected to 10 freeze-thaw cycles in an ethanol/dry ice bath. The suspension was passed 29 times through a polycarbonate membrane with a pore size of 100 nm using a LiposoFast extruder (Avestin, Inc., Ottawa, Canada). Liposomes (200 nmol) were suspended in 2 mL of buffer (100 mM NaCl, 50 mM HEPES pH 7.0) and peroxidation was initiated by addition of 4 nmol of Fe^{2+}. Control samples contained no added Fe^{2+} or test compound. Anthocyanins, BHT, propyl gallate or α-tocopherol were added to final concentrations of 2 μM. Fluorescent intensity of this lipid suspension was monitored for a period of 21 min with or without test compounds, immediately following addition of Fe^{2+}, using a SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL). The values of relative

fluorescence were determined by dividing the fluorescence value at a given time point by that at t = 0 min. The decrease in relative fluorescence intensity with time indicated the rate of preoxidation. The percent inhibition of the lipid oxidation was calculated using the equation, Percent Inhibition $=\{[(F_{rel})_{Pl}- (F_{rel})_{Fe}]/[(F_{ref})_C - (F_{rel})_{Fe}]\}\times 100$, where: $(F_{rel})_{Pl}$ = relative fluorescence for the Fe^{2+} and test samples at the end of 21 min, $(F_{rel})_C$ = relative fluorescence for the control sample at 21 min, and $(F_{rel})_{Fe}$ = relative fluorescence for the Fe^{2+}-containing sample at the end of 21 min (Richman et al., 1997; Arora and Strasburg, 1997).

Extraction of cherries. Dried Balaton™ tart cherries (200 g) were ground and extracted sequentially with hexane, ethyl acetate and methanol (500 mL × 3) and the solvents were evaporated under reduced pressure at 40°C to yield crude extracts 0.42, 1.48 and 116.3g, respectively. Similarly, dried Montmorency tart cherry yielded crude extracts 0.29, 0.74 and 125.4 g, respectively.

The methanol extract of Balaton™ tart cherries (116.3 g) was dissolved in water (300 mL) and extracted with ethyl acetate (300 ml × 3). The ethyl acetate extract was evaporated to dryness under reduced pressure to yield fraction I (5. 3g). The aqueous layer was evaporated under reduced pressure to remove ethyl acetate, and applied to an XAD-2 column (100 g, Amberlite resin, mesh size 20-50, Sigma Chemical CO., St. Louis, MO), which was prepared as described by Chandra et al (1993). The column was then washed with distilled water (3 L) until the colorless washing gave a neutral pH. The adsorbed pigments were then eluted with methanol (500 mL). The red methanolic solution was concentrated at 40°C and the aqueous solution was then lyophilized to yield fraction II (3.5g). Since the major components of this fraction were anthocyanins, similar

to the components in water extract of Montmorency and Balaton™ cherries (Wang et al., 1997), this fraction was not further purified.

Purification of fraction I. The crude solvent extracts from Montmorency and Balaton™ cherries, fractions I and II from the methanol extract of Balaton™ tart cherry were bioassayed for antioxidant activity (Fig. 4.1). It was evident that fraction I from Balaton™ cherries contained the most active antioxidant compounds. Therefore, fraction I was further purified for antioxidant compounds. Fraction I (5.3 g) was chromatographed by MPLC (200 g) using solvent system $CHCl_3$ and methanol gradient starting with $CHCl_3$-MeOH (16:1, v/v, 1 L), $CHCl_3$-MeOH (8:1, v/v, 800 mL), $CHCl_3$-MeOH (4:1, v/v, 1 L) and finally with MeOH (1L). Sixteen fractions were collected and monitored by silica TLC plates using $CHCl_3$-MeOH (10:1) and $CHCl_3$-MeOH-HCOOH (4:1:0.1) as developing solvents. The fractions were combined to yield fractions A-F; 740, 2500, 466, 386, 418 and 370 mg, respectively. Fractions A and B showed only weak antioxidant activity and hence, was not further purified for antioxidant compounds.

Compounds **1** and **2**: The fraction C (427 mg) was further purified on preparative silica gel TLC plates (20 ×20 cm, 500 microns) and developed with $CHCl_3$-MeOH (15:1). The antioxidant band (26.2 mg), which showed very strong UV fluorescence at λ_{366} and λ_{254}, was repeatedly purified by preparative TLC using acetone-$CHCl_3$ (1:6) as the mobile phase. This yielded compounds **1** (R_f = 0.48, 2.4 mg) and **2** (R_f = 0.46, 2.4 mg).

Compound **1**: ^{1}H NMR (DMSO-d_6): δ12.15 (1H, s, 5-OH), 10.80 (1H, s, 7-OH), 9.60 (1H, s, 4′-OH), 7.32 (2H, d, J=8.5 Hz, H-2′, H-6’), 6.81 (2H, d, J=8.5 Hz, H-3′, H-5′), 5.90 (2H, s, H-6, H-8), 5.43 (1H, dd, J=12.7 Hz, 2.8 Hz, H-2), 3.26 (dd, J=17.1Hz,

12.7 Hz, H-3_{ax}), 2.69 (dd, J=17.1Hz, 2.8 Hz, H-3_{eq}); ^{13}C NMR (DMSO-d_6): δ196.6 (C-4), 166.8 (C-7), 163.4 (C-5), 162.1 (C-9), 157.8 (C-4′), 129.4 (C-1′), 128.8 (C-2′, C-6′), 115.6 (C-3′, 5′), 102.2 (C-10), 96.1 (C-6), 95.4 (C-8), 78.9 (C-2), 42.4 (C-3).

Compound **2**: ^{1}H NMR (DMSO-d_6): 12.98 (1H, s, 5-OH), 10.92 (1H, s, 7-OH), 9.60 (1H, s, 4′-OH), 8.26 (1H, s, H-2), 7.38 (2H, d, J=8.2 Hz, H-2′, H-6′), 6.82 (2H, d, J=8.2 Hz, H-3′, H-5′), 6.39 (1H, d, J=1.95 Hz, H-8), 6.21 (1H, d, J=1.95 Hz, H-6); ^{13}C NMR (DMSO-d_6): δ 180.6 (C-4), 164.4 (C-7), 163.6 (C-5), 158.1 (C-9), 157.5 (C-4′), 154.4 (C-2), 130.6 (C-2′, 6′), 122.8 (C-3), 121.7 (C-1′), 115.5 (C-3′, C-5′), 104.9 (C-10), 99.3 (C-6), 94.2 (C-8).

Compound **3**: Fraction D (155 mg) was purified by HPLC using CH_3CN-H_2O (25:75) as the mobile phase at a flow rate of 4 ml/min to yield active compound **3** (27.2 mg, R_t = 52 min). ^{1}H NMR (DMSO-d_6): δ7.45 (1H, d, J=15.9 Hz, H-7′), 7.00 (1H, d, J=2.0 Hz, H-2′), 6.95 (1H, dd, J=8.4 Hz, 2.0 Hz, H-6′), 6.75 (1H, d, J=8.4 Hz, H-5′), 6.18 (1H, d, J=15.9 Hz, H-8′), 5.16 (1H, m, H-5), 3.85 (1H, m, H-3), 3.53 (1H, m, H-4), 2.02-1.83 (4H, m, H-2, H-6); ^{13}C NMR (DMSO-d_6): 176.1 (COO^-), 166.1 (C-9′), 148.2 (C-3′), 145.6 (C-4′), 144.4 (C-7′), 125.7 (C-1′), 121.1 (C-6′), 115.8 (C-5′), 115.1 (C-8′), 114.6 (C-2′), 72.9 (C-4), 71.2 (C-1), 71.0 (C-3), 67.3 (C-5), 38.8 (C-2), 35.1 (C-6).

Compounds **4**, **5**, **6** and **7**: Fraction E (418 mg) was purified by HPLC using CH_3CN-H_2O (30:70) as mobile phase at flow rate of 4 ml/min to yield compounds **4** (R_t = 64 min, 11 mg), **5** (R_t = 64 min, 8.6 mg), **6** (R_t = 71 min, 13 mg) and **7** (R_t = 84 min, 3.8 mg), respectively. Compound **4**: ^{1}H NMR (DMSO-d_6): δ7.62 (1H, d, 2.2, H-2′), 7.58 (1H, dd, 8.6, 2.2, H-6′), 6.70 (1H, d, 8.6, H-5′), 6.10 (1H, d, 2.0, H-8), 5.96 (1H, d, 2.0, H-6), 4.96 (1H, s, H-1′′), 3.82-3.22 (H-2′′-H-5′′), 1.15 (3H, d, 6.1, H-6′′).

Compound **5**: ^{1}H NMR (DMSO-d_6): δ7.94 (2H, d, J=8.8 Hz, H-2′, H-6′), 6.85 (2H, d, J=8.8 Hz, H-3′, H-5′), 6.34 (1H, s, H-8), 6.12 (1H, s, H-6), 5.24 (1H, d, 7.3, H-1″), 4.35 (1H, s, H-1‴), 3.51(d, 10.5 Hz, H-6″), 3.37 (1H, m, H-4″), 3.28 (1H, m, H-5″), 3.27 (1H, m, H-3‴), 3.22 (1H, dt, J=9.3 Hz, 6.0 Hz, H-5‴), 3.22 (1H, m, H-3″), 3.13 (1H, m, H-4‴), 3.13 (1H, d, J=5.1 Hz, H-2‴), 3.01 (1H, dd, J=9.6 Hz, 7.3 Hz, H-2″), 0.94 (3H, d, J=6.0 Hz, H-6‴); ^{13}C NMR (DMSO-d_6): δ177.2 (C-4), 161.0 (C-7), 159.9 (C-5), 156.9 (C-9), 156.8 (C-2), 156.8 (C-4′), 133.4 (C-3), 131.1 (C-2′), 131.1 (C-6′), 121.3 (C-1′), 115.2 (C-3′), 115.2 (C-5′), 103.6 (C-10), 101.7 (C-1″), 100.9 (C-1‴), 99.4 (C-6), 94.2 (C-8), 76.4 (C-3″), 75.8 (C-5″), 74.2 (C-2″), 71.8 (C-4‴), 70.6 (C-3‴), 70.4 (C-2‴), 70.0 (C-4″), 68.4 (C-5‴), 67.1 (C-6″), 17.9 (C-6‴); FABMS *m/z* 594 $[M+H]^+$, *m/z* 617 $[M+Na]^+$; EIMS *m/z* (% rel. Int.) 286 (100);

Compound **6**: ^{1}H NMR (DMSO-d_6); δ7.81 (1H, d, J=2.0 Hz, H-2′), 7.48 (1H, d, J=8.4 Hz, 2.0, H-5′), 6.88 (1H, d, J=8.4 Hz, H-6′), 6.34 (1H, s, H-8), 6.12 (1H, s, H-6), 5.39 (1H, d, J=7.3 Hz, H-1″), 4.38 (1H, s, H-1‴), 3.82 (OCH_3), 3.67 (1H, d, J=10.5, H-6″), 3.37 (1H, m, H-4″), 3.31 (1H, dd, J=6.4 Hz, 5.1 Hz, H-3‴), 3.28 (1H, m, H-5″), 3.22 (1H, m, H-3″), 3.22 (1H, dt, J=9.3, 6.0 Hz, H-5‴), 3.13 (d, 5.1, H-2‴), 3.13 (1H, m, H-4‴), 3.05 (1H, dd, J=9.6 Hz, 7.3 Hz, H-2″), 0.95 (3H, d, J=6.0 Hz, H-6‴); ^{13}C NMR (DMSO-d_6): δ177.2 (C-4), 161.0 (C-7), 159.9 (C-2), 159.9 (C-5), 156.7 (C-9), 133.2 (C-3), 121.2 (C-1′), 113.4 (C-2′), 99.3 (C-6), 94.2(C-8), 103.6 (C-10), 149.4 (C-3′), 147.0 (C-4′), 115.3 (C-5′), 122.4 (C-6′), 101.5 (C-1″), 101.1 (C-1‴), 76.4 (C-3″), 76.0 (C-5″), 74.3 (C-2″), 71.8 (C-4‴), 70.6 (C-3‴), 70.4 (C-2‴), 70.2 (C-4″), 68.5 (C-5‴), 67.1 (C-6″), 55.9 (OCH_3), 17.9 (C-6‴); FABMS, *m/z* 624 $[M+H]^+$, 647 $[M+Na]^+$; EIMS (% rel. Int.) *m/z* 316 (100).

Compound **7**: ^{1}H NMR (DMSO-d_6): δ 8.41 (1H, s, H-2), 7.40 (2H, d, J=8.7 Hz, H-2′, H-6′), 6.82 (2H, d, J=8.7 Hz, H-3′, H-5′), 6.72 (1H, d, J=2.1 Hz, H-8), 6.46 (1H, d, J=2.1 Hz, H-6), 5.05 (1H, d, J=7.5 Hz, H-1″), 3.91-3.30 (5H, H-2″-6″); ^{13}C NMR (DMSO-d_6): 181.7 (C-4), 164.2 (C-7), 163.8 (C-5), 158.4 (C-9), 155.8 (C-2), 155.8 (C-4′), 131.3 (C-2′, C-6′), 123.7 (C-3), 123.7 (C-1′), 116.3 (C-3′, C-5′), 101.0 (C-10), 100.7 (C-1″), 100.1 (C-6), 95.9 (C-8), 78.4 (C-5″), 77.6 (C-3″), 74.2 (C-2″), 70.7 (C-4″), 61.8 (C-6″).

Compound **8**: Fraction F (370 mg) was purified by preparative TLC (20 × 20 cm, 500 μm) using MeOH-$CHCl_3$-H_2O (1:2:0.1, v/v) as the mobile phase. Six bands were collected and eluted with MeOH to yield bands I-VI: 9.6, 5.8, 14.5, 55.6, 131.6 and 56.2 mg, respectively. The active band, VI (56.2 mg), was further purified by preparative TLC using MeOH-$CHCl_3$ (1:8, v/v) as the mobile phase and yielded compound **8** (R_f = 0.62, 17 mg). UV λ_{max} (MeOH): 209, 222 (sh), 290 (sh) and 303 nm; UV λ_{max} (MeOH + $AlCl_3$): 209, 235, 229, 321 and 360 nm; UV λ_{max} (MeOH +$AlCl_3$ + HCl): 209, 233, 289, 320 and 355nm; UV λ_{max} (MeOH + NaOAc): 210 and 305nm; ^{1}H NMR (CD_3OD): δ7.95 (2H, d, J=8.5 Hz, H-2', H-6′), 6.92 (2H, d, J=8.5 Hz, H-3′, H-5′), 6.64 (1H, s, H-3), 4.02 (3H, s, OCH_3), 3.91 (3H, s, OCH_3); ^{13}C NMR (CD_3OD): δ 184.8 (C-4), 166.7 (C-2), 162.9 (C-4'), 149.2 (C-7), 146.4 (C-9), 142.9 (C-5), 137.8 (C-6), 132.2 (C-8), 129.8 (C-2′, C-6′), 123.3 (C-1′), 117.0 (C-3′, C-5′), 107.9 (C-10), 103.5 (C-3), 62.0 (OCH_3), 61.3 (OCH_3).

RESULTS AND DISCUSSION

The methanol extract was partitioned with ethyl acetate to yield fraction I. The aqueous portion was purified on an XAD-2 column to yield fraction II (Wang et al., 1997). Preliminary antioxidant assay on these fractions and the crude methanol extract revealed that fraction I had the best antioxidant activity, followed by fraction II and methanol extract (Fig. 4.1). Fraction II contained anthocyanins **1-3** and hence was not purified further. Our preliminary antioxidant assay revealed that the percent inhibitions of Fe^{2+} induced lipid peroxidation of hexane, EtOAc and MeOH extracts of Balaton™ tart cherry were 9.9, 28.3 and 13.6%, respectively. Similarly, the percent inhibitions of hexane, EtOAc and MeOH extracts of Montmorency tart cherry were 11, 26.3 and 14.3%, respectively. The percent inhibitions of fraction I and II from methanol extract of Balaton™ tart cherry were 75.7 and 67.3%, respectively. Since Montmorency and Balaton™ cherry extracts gave an identical chromatographic profiles, only Balaton™ extracts were further studied for antioxidant compounds. This was mainly due to larger quantities of the extracts available from Balaton™ compared to Montmorency. Also, Balaton™ is the new variety of tart cherry grown commercially in several of Michigan cherry orchards.

Purification of fraction I by MPLC, TLC and HPLC afforded compounds **1-8**. Compounds **1** and **2** gave identical ^{1}H and ^{13}C NMR spectral data to that of naringenin and genistein, respectively (Harborne, 1994; Agrawal, 1989). The spectral data of **3** were identical to the ^{1}H and ^{13}C NMR spectral data of an authentic sample of chlorogenic acid. Similarly, compounds **4** and **7** were confirmed to be quercetin 3-rhamnoside and genistein 7-glucoside, respectively, by comparison of their ^{1}H- and ^{13}C NMR spectral data (Kosuge et al., 1985; Ohta et al., 1980).

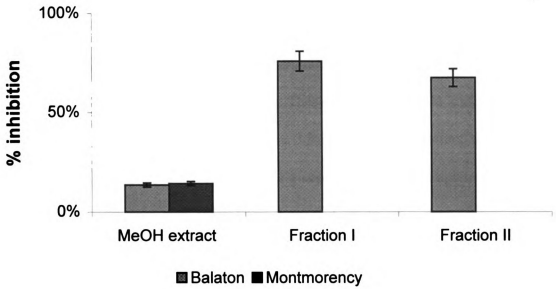


Fig. 4.1. Inhibitory effects of methanol extracts from Balaton™ and Montmorency tart cherries and fractions I and II from Balaton™ cherry on Fe^{2+}-induced large unilaminar vesicles (LUVs) peroxidation at 25 ppm concentrations. Fraction I contains compounds **1-8** and fraction II contains anthocyanins **1-3**. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative intensity represents the fluorescence intensity at a given time divided by the initial intensity at the start of the assay. Data presented are mean of duplicate experiments (X ± SD)

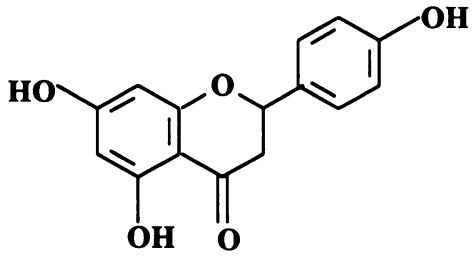
The FABMS and EIMS revealed a molecular formula of $C_{27}H_{30}O_{15}$ for compound **5**. The ^1H NMR spectrum of compound **5** gave signals for two anomeric protons that appeared at δ5.24 and 4.35, respectively. These were assigned to anomeric protons of glucose and rhamnose, respectively. The 7.3 Hz coupling constant for the anomeric proton at δ5.24 indicated a β-linkage of a glucose moiety to the aglycone. The doublet appeared at 0.94 ppm was assigned to a methyl group of a rhamnose sugar moiety. Therefore, The doublet at δ 4.35 corresponded to the anomeric proton of an L-rhamnopyranose. Also, the small coupling constant of <1Hz for this proton indicated an α-glycosidic linkage. The DQF-COSY spectrum of compound **5** helped to confirm the assignment of all protons in **5**. The HMQC spectrum was used to assign the carbon signals in compound **5** and further confirmed glucose and rhamnose moieties in **5**. The appearance of C-6 at 67.1 ppm for the glucose moiety, which was about 5 ppm further downfield than the normal chemical shift value of C-6 in glucose, indicated that rhamnose moiety was attached to the C-6 of the glucose moiety. The ^1H and ^{13}C NMR spectra of **5** indicated a kaempferol aglycone functionality (Markham et al., 1978). The FAB-MS of **5** gave a molecular ion at m/z 594 and an ion at m/z 617 indicating an $[M+Na]^+$. The EI-MS gave a base peak at m/z 286, which corresponded to a kaempferol moiety. Therefore, compound **5** was assigned as kaempferol 6"-O-α-L-rhamnopyranosyl-β-D-glucopyranoside (Fig 4.2).

The molecular formula of compound **6** was determined as $C_{31}H_{32}O_{16}$ by FABMS and EIMS, respectively. ^1H and ^{13}C NMR spectra indicated that compound **6** contained identical sugar moieties and linkages as in compound **5**. The only difference was that

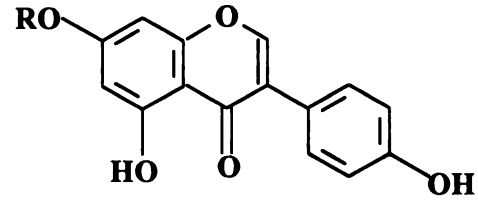
compound **6** showed the presence of a methoxy group. As in **5**, H-8 and H-6 in compound **6** appeared at δ 6.34, 6.12, respectively. The chemical shifts at δ7.81, 7.48 and 6.88 were assigned to protons in B ring and indicated that one methoxy group was at 3' or 4' position. The HMBC spectrum of **6** suggested that this methoxy group was attached to 3' position. The FAB-MS of **6** gave a molecular ion at m/z 624 and an ion at m/z 647 indicating an $[M+Na]^+$. Also, the EI-MS showed a base peak at m/z 316, which corresponded to 3'-methoxy kaempferol moiety. Therefore, compound **6** was assigned as rhamnazin 6"-O-α-L-rhamnopyranosyl-β-D-glucopyranoside. This is the first report of the isolation of this compound from tart cherries (Fig. 4.2).

A flavonoid structure was revealed for compound **8** from its ^1H and ^{13}C NMR spectral data. Compound **8** showed a carbon signal at δ 184.8. This indicated that it is a flavone with a hydroxyl group at C-5 (Agrawal, 1989). The ^1H NMR spectrum of **8** showed the presence of two aromatic protons each at δ7.95 and 6.92, respectively, which were assigned to H-2', H-6' and H-3', H-5', respectively. In addition, there were two OCH_3 groups appeared at 62 and 61.3 ppm, respectively. The UV spectra of **8** in methanol before and after addition of aluminum chloride followed by HCl were comparable to the published value of 5,8-dihydroxy-6,7-dimethoxyflavone (Barberán et al, 1985). Further comparison with published ^1H and ^{13}C NMR spectral data of related compounds (Horie et al, 1995), compound **8** was assigned to be 6,7dimethoxy-5,8, 4'-trihydroxyflavone. Like compound **6**, this is the first report of compound **8** from tart cherries (Fig. 4.2).

Compounds 1, 2, 3 4, 7 and 8 were assayed at 10 μm concentrations for antioxidant activity. The inhibitory effect of flavonoids on Fe^{2+} lipid peroxidation was

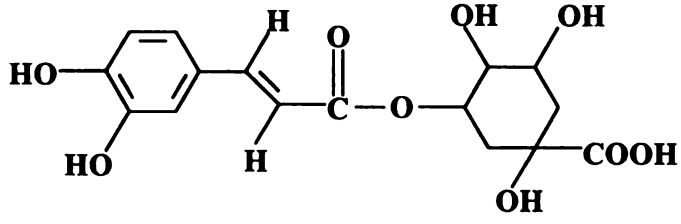


1

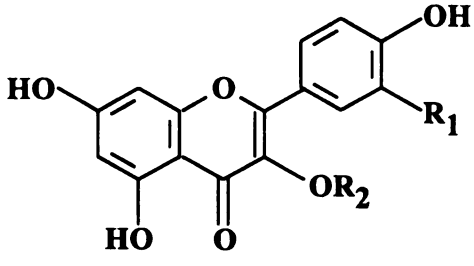


2: R=H

7: R=Glucose



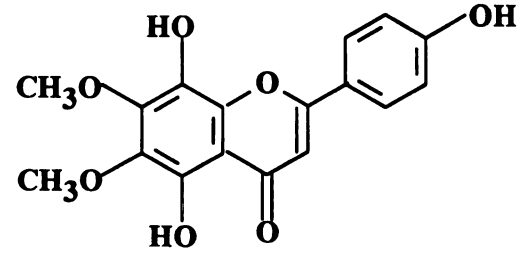
3



4: R_1=OH, R_2=rhamnose

5: R_1=H, R_2=rutinose

6: R_1=OCH_3, R_2=rutinose



8

Fig. 4.2. Structures of Compounds 1-8

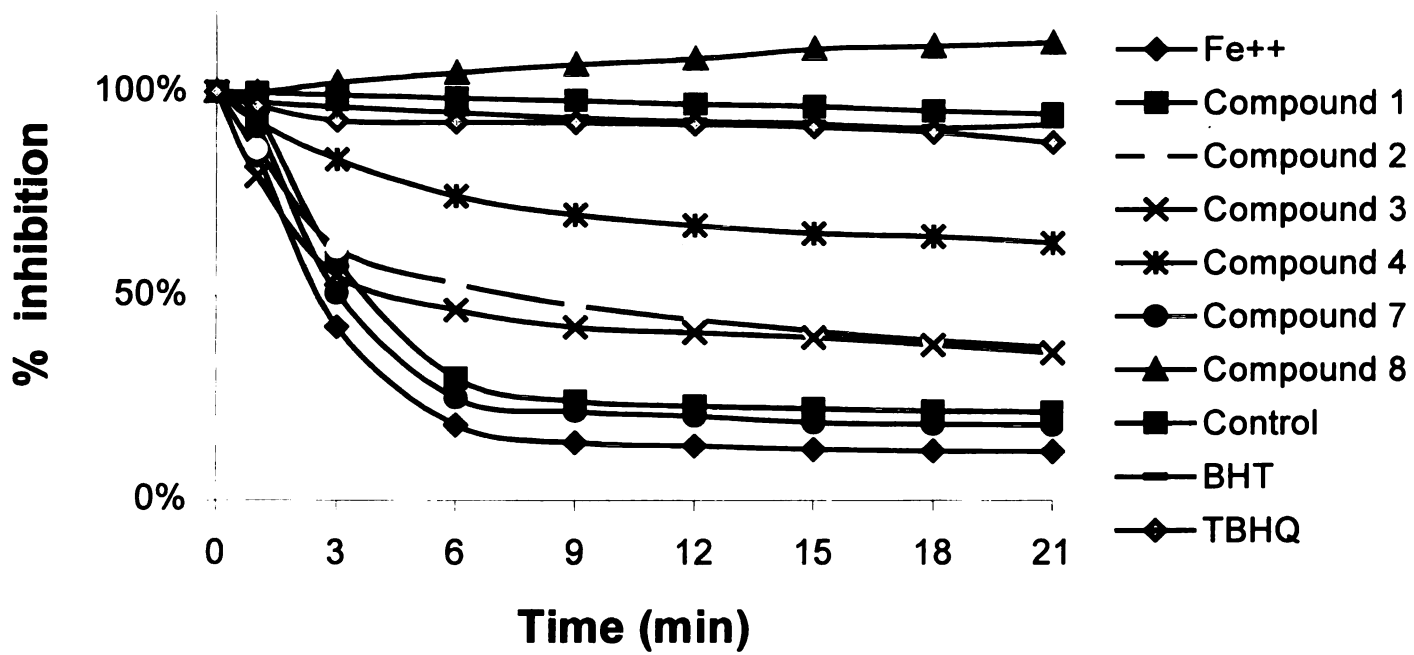
attributed due to their ability to chelate Fe^{2+} with the formation of inert complexes that are unable to initiate peroxidation (Afanas'ev et al., 1989). Additionally, the Fe^{2+} complexes of flavonoids are considered to retain their free radical scavenging activities, therefore, can scavenge the free radical intermediate in lipid peroxidation. Also, flavonoids can act as free radical scavengers. The antioxidant activity of compound **8** was superior to the antioxidant activities of **1**, **2**, **4** and **7** at 10 μm concentrations studied (Fig. 4.3).

Earlier reports suggested that the presence of *ortho*-dihydroxyl groups on the B ring (Bors et al. 1990), a hydroxyl group at position 3 on the C ring (Afanas's et al., 1989; Mora et al., 1990) and a double bond at C_2-C_3 in conjugation with a 4-oxo functional group (Bors et al., 1990) are considered to be essential for effective radical scavenging by the flavonoids. Even though compound **8** does not posses a 3-hydroxyl group and has only one hydroxyl group on the B ring, the antioxidant activity of **8** is higher than quercetin 3-rhamnoside. Quercetin 3-rhamnoside contains an *ortho*-dihydroxyl group in the B ring in addition to a 3-hydroxyl group and a double bond at C_2-C_3 in conjugation with a 4-oxo functional group. The enhanced antioxidant activity of compound **8** was probably due to the hydroxyl and two methoxy groups in ring A. Arora et al (1997) reported that 7,8 -dihydroxyflavone showed similar antioxidant activity to quercetin, though it lacked any substitution on the B-ring and at 3-position. Watanabe (1998) compared the antioxidant activity of (±) catechin and (±) epicatechin with rutin and quercetin on the basis of the inhibition. The peroxyl radical-scavenging activities of these compounds were investigated by measuring the inhibition of hydroperoxidation of methyl linoleate initiated by a radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile)

(AMVN). The results indicated that (±) catechin and (±) epicatechin showed similar antioxidant activities to that of quercetin, even though they have C_2-C_3 saturated bond and no 4-oxo functional group (Rice-Evans et al., 1996).

In order to evaluate which component contributed to the highest antioxidant activity in fraction I, a mixture of compounds **1-8** was prepared, according to the ratio of their weight extracted from fraction I. The antioxidant activities of this mixture was determined at 25 ppm concentrations, which contained compounds **1-8** 0.6, 0.6, 14, 2.3, 1.1, 2.6, 0.8 and 3.5 ppm, respectively (Fig. 4.4). The antioxidant activities of individual components at 0.6, 0.6, 14, 2.3, 1.1, 2.6, 0.8 and 3.5 ppm concentrations, respectively, were also measured (Fig. 4.4) and compared with the mixture at 25 ppm concentrations. Results indicated that the mixture of compounds **1-8** gave 89.6 % of inhibition on Fe^{2+} induced lipid peroxidation, while compounds **1**, **2**, **3**, **4**, **5-6**, **7** and **8** showed 5.1, 5.4, 76.7, 20.5, 16, 11 and 110 % of inhibition, respectively. Compounds **8** and **3** were the most active components in the mixture and probably in fraction I. Interestingly, the sum of the antioxidant activity of individual compounds is higher than that of the mixture of these eight compounds. This suggested that in our assay system, some of the purified compounds are more effective inhibitors of lipid peroxidation when tested alone.

Fig. 4.3. Antioxidant activities of compounds **1**, **2**, **3**, **4**, **7**, **8** and commercial antioxidants TBHQ and BHT at 10 μM concentrations. Data represent the means of duplicate experiments.


% inhibition
100%
50%
0%
0
3
6
9
12
15
18
21
Time (min)
Fe++
Compound 1
Compound 2
Compound 3
Compound 4
Compound 7
Compound 8
Control
BHT
TBHQ

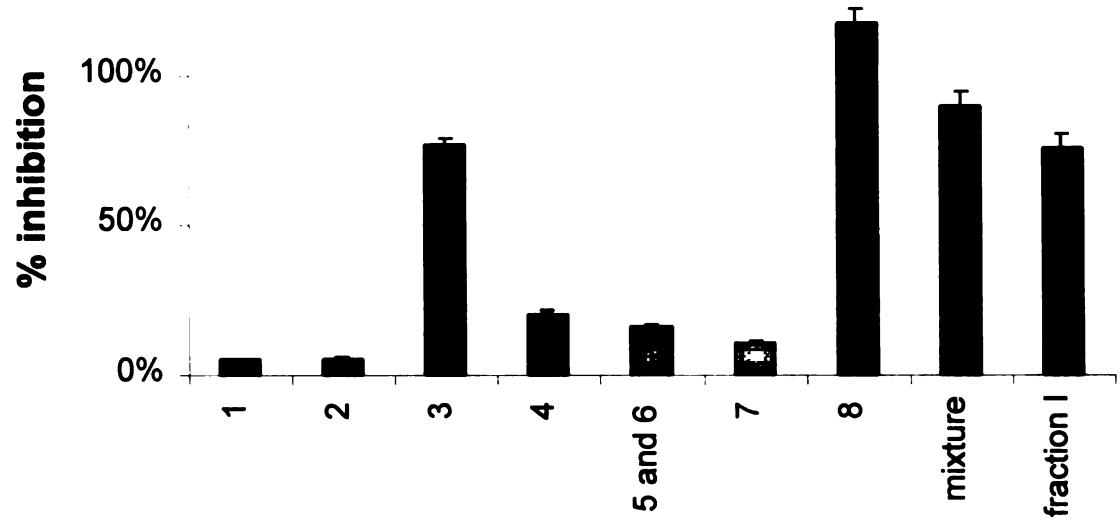


Fig. 4.4: Percent inhibition of compounds **1-8** and their mixture on Fe^{2+} induced LUVs peroxidation. The mixture of compounds **1-8** was prepared, according to the ratio of their weight extracted from fraction I. The mixture and fraction I were tested at 25 ppm concentrations. The mixture at 25 ppm concentrations contained compounds **1-8** 0.6, 0.6, 14, 2.3, 1.1, 2.6, 0.8 and 3.5 ppm, respectively. Similarly, compounds **1-8** were assayed independently at 0.6, 0.6, 14, 2.3, 1.1, 2.6, 0.8 and 3.5 ppm concentrations, respectively. Data presented are mean of duplicate experiments (X ± SD).

CHAPTER FIVE*

Antioxidant and anti-inflammatory activities of anthocyanins and their aglycone from tart cherries

ABSTRACT

Cherries are a rich source of anthocyanins which may possess nutraceutical and phytoceutical properties. We have isolated anthocyanins and cyanidin from tart cherries and evaluated their antioxidant and antiinflammatory efficacies in vitro. The anthocyanins and cyanidin isolated from tart cherries exhibited comparable antioxidant and antiinflammatory activities to commercial products. The inhibition of lipid peroxidation of anthocyanins **1-3** and their aglycone, cyanidin, were 39, 70, 75 and 57%, respectively, at 2 μM concentrations. The antioxidant activities of anthocyanis and cyanidin were comparable to the antioxidant activities of BHA and superior to vitamin E at 2 μM concentrations. In the anti-inflammatory assay, which measured prostaglandin H endoperoxide synthase-1 (PGHS-1) and and its isoform (PGHS-2) inhibitory activities, cyanidin gave IC_{50} values of 90 and 60 μM for PGHS-1 and PGHS-2 enzymes, respectively. The positive controls aspirin, naproxen and ibuprofen, gave IC50 value of 1050, 11 and 25 μM against PGHS-1 enzyme.

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*Will be Submitted to *J. Nat. Prod.*

INTRODUCTION

Public interest in phytoceuticals to inhibit chronic diseases and aging is gathering momentum. Reactive oxygen species such as hydroxyl ($OH^{\cdot}$) and peroxyl radicals ($ROO^{\cdot}$), and the superoxide anion ($O_2^{\cdot}$) are constantly produced as a result of metabolic reactions in living systems (Halliwell and Gutteridge, 1990). Living systems are protected from oxidative damage by these reactive species by enzymes such as superoxide dismutase and glutathione peroxidase, and by antioxidant compounds such as ascorbic acid, tocopherols, and carotenoids (Sies, 1997). However, when free radical production exceeds the antioxidant capacity of the organism, these radical species attack lipids, proteins, and DNA thus damaging structural integrity and function of cell membranes, enzymes and genetic material (Byers and Perry, 1992). A growing body of evidence indicates that various pathological conditions including cardiovascular disease, arthritis, various cancers, and Alzheimer's disease are associated, at least in part, with the damaging effects of uncontrolled free radical production (Byers and Perry, 1992).

Many foods contain non-nutritive components such as flavonoids and other phenolic compounds which may provide protection against chronic diseases through multiple effects which are as yet poorly understood (Tanaka et al., 1993). These compounds may act as antioxidants by reacting with free radicals and thus interrupting the propagation of new free radical species, or by chelating metal ions such as Fe^{2+} which catalyze lipid oxidation to alter their redox potentials. In addition, it has been shown that antioxidant supplements can significantly improve certain immune responses (Hertog et al., 1993).

Consumption of cherries was reported to alleviate arthritic pain and gout (Hamel, 1975), although there is no evidence for the active components and mode of action. These beneficial effects may be partially associated with the abundance of anthocyanins, the glycosides of cyanidin. Anthocyanins have been investigated as antioxidant substances (Costantino et al., 1992). Anthocyanins isolated from the seed coat of red beans and other sources inhibited lipid peroxidation (Tamura and Yamagami, 1994; Tsuda et al., 1994; Kanner et al., 1994). Several anthocyanins were investigated for their antioxidant activity on human low density lipoprotein and lecithin-liposome systems (Satue-Gracia et al., 1997). Commercial meat products containing cherry tissue are now available to the consumers. Cooked low-fat ground beef with approximately 12 % of tart cherries showed less rancidity development (Liu et al., 1995). The addition of cherry tissue to ground beef before frying significantly inhibited the formation of heterocyclic aromatic amines (HAAs) (Britt et al., in press). HAAs are dietary compounds that are formed naturally during the cooking of muscle foods and are thought to arise from reactions involving creatine or creatinine, sugars and amino acids (Skog, 1993). In order to determine the nature of compounds that are responsible for these activities, we have studied antioxidant (Arora and Strasburg, 1997) and anti-inflammatory (Meade et al., 1993) activities of anthocyanins and its aglycone, cyanindin.

MATERIALS AND METHODS

General experimental

Materials. Arachidonic acid and Ovine Prostaglandin H Synthase-1 (PGHS-1) were purchased from Oxford Biomedical Research (Oxford, MI, USA). hPGHS-2

enzyme was obtained from Dr. David Dewitt (Department of biochemistry, Michigan State university). Anthocyanins **1-3** were purified from Balaton™ tart cherry by HPLC and were identified by ^{1}H- and ^{13}C NMR spectral data. Naproxen, ibuprofen and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Liposome Assay: All the buffers were stored in Chelex 100 to remove metal ions. A mixture containing 5 μmol of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 15 nmol of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (Molecular Probes, Inc., Eugene, OR) was dried under vacuum. The resulting film was suspended in 500 μL of buffer (NaCl, 0.15 M; EDTA 0.1 mM; MOPS 10 mM) and was then subjected to 10 freeze-thaw cycles in an ethanol/dry ice bath. The suspension was passed 29 times through a polycarbonate membrane with a pore size of 100 nm using a LiposoFast extruder (Avestin, Inc., Ottawa, Canada). Liposomes (200 nmol) were suspended in 2 mL of buffer (100 mM NaCl, 50 mM HEPES pH 7.0) and peroxidation was initiated by addition of 4 nmol of Fe^{2+}. Control samples contained no added Fe^{2+} or test compound. Anthocyanins, BHT, propyl gallate or α-tocopherol were added to final concentrations of 2 μM. Fluorescent intensity of this lipid suspension was monitored for a period of 21 min with or without test compounds, immediately following addition of Fe^{2+}, using a SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL). The values of relative fluorescence were determined by dividing the fluorescence value at a given time point by that at t = 0 min (Arora and Strasburg, 1997).

Cyclooxygenase assay: Cyclooxygenase activities were measured PGHS-1 enzyme vesicles (*ca.* 5 mg protein/mL in 0.1 M TrisHCL, pH 7.4), a homogeneous

protein purified from ram seminal. Cyclooxygenase assays were performed at 37°C by monitoring the initial rate of O_2 uptake using an O_2 electrode (Yellow Springs Instrument Inc., Yellow Springs, Ohio). Each assay mixture contained 3.0 ml of 0.1M TrisHCL, pH adjusted to 7 by the addition of 6M HCl, 1 mM phenol, 85 μg hemoglobin and 10 μmol arachidonic acid. Reactions were initiated by adding 5 to 25 μg of microsomal protein in a volume of 15-50 μL. Instantaneous inhibition was determined by measuring the cyclooxygenase activity initiated by adding aliquots of microsomal suspensions of PGHS-1 or PGHS-2 (10 μM O_2/min cyclooxygenase activity/aliquot) to assay mixtures containing 10 μM arachidonate and various concentrations of the test substances (100-1100 μM). The IC_{50} values represent the concentrations of the test compound that gave half-maximal activity under the standard assay conditions.

RESULTS AND DISCUSSION

About 20 fresh cherries (@ 100 g fresh tissue) contain 125 to 250 mg of anthocyanin, depending on the variety (Wang et al., 1997). The anthocyanins were assayed for antioxidant activity using the method developed by Arora and Strasburg (1997). This assay is based on the reaction of a fluorescent probe inserted into a phospholipid with free radicals generated by a pro-oxidant such as Fe^{2+}. Oxidative and reductive decomposition of peroxides, which are mediated by transition metal ions, can amplify the peroxidation process. As the reaction proceeds, the fluorescent probe is degraded and the signal declines. In the presence of an antioxidant, the rate of fluorescence decrease is reduced. Antioxidant activities of anthocyanins **1**, **2** and **3** and

of the aglycone cyanidin (Fig. 5.1) compared favorably with the commercial antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Fig. 2). All were better than α-tocopherol. At 2 μM concentrations, the extent of peroxidation of the sample containing α-tocopherol was indistinguishable from that of the Fe^{2+}-containing sample with no added antioxidant (Fig. 5.2).

The aglycone of anthocyanins, cyanidin, has higher efficacy than its glycosides suggesting that the antioxidant activity of anthocyanins is due to their aglycone moiety. Anthocyanins **1-3** contain 3, 2, and 1 sugar residues, respectively, and explained the lowest antioxidant activity observed for antocyanin **1** (Fig 5.2). The number of sugar residues at C_3 position seem to be very important for antioxidant activity. The smaller the number of sugar units at 3-position, the higher the antioxidant activity. Bors et al (1990) reported that the stability of aryloxyl radical affected the antioxidant activities of compounds and may give rise to pro-oxidant effects. The antioxidant activity of cyanidin may depend on the stability of its aryloxyl radical. The mode of inhibition of oxidation by polyphenolics in general is not clear. However, some of the suggested mechanisms are: a) chelating ions via the *ortho*-dihydroxy phenolic structure (Afanas'ev et al., 1989); b) scavenging lipid alkoxyl and peroxyl radicals by acting as chain breaking antioxidants (Bors et al., 1990), and c) as hydrogen donors (Morel et al., 1994). The *ortho*-dihydroxy substitution in the B ring of anthocyanins and cyanidin is important to stabilize the resulting free radical through the 3'and 4'-OH moieties. *Ortho*-dihydroxy groups in anthocyanins can chelate the metal ion to prevent iron-induced lipid peroxidation. Also, the unsaturated C ring, which participates in electron delocalization, is an important factor as well (Bors et al., 1990).

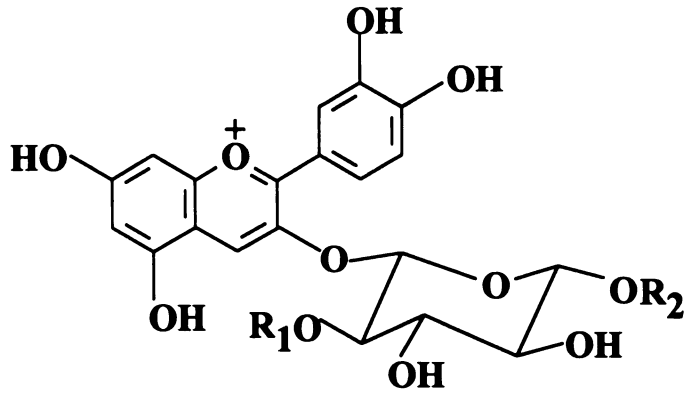
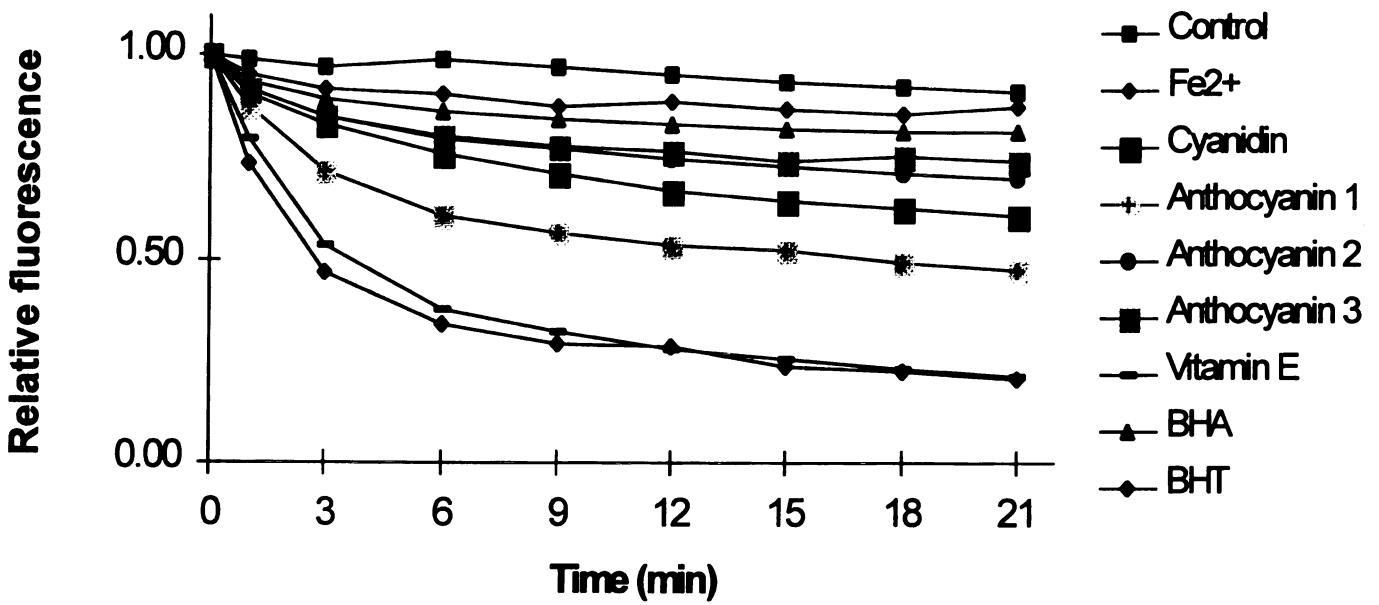


Fig. 5.1. Anthocyanins in Montmorency and Balaton™ cherries. Anthocyanin **1**, R_1=glucose, R_2= rhamnose; Anthocyanin **2**, R_1=H, R_2= rhamnose; Anthocyanin **3**, R_1= R_2=H

Fig. 5.2. The antioxidant efficacy of anthocyanins and commercial antioxidants in a liposomal model system. Oxidation was initiated by the addition of ferrous ions. In the presence of test compounds, the rate of decay of fluorescence was decreased. Control samples contained no added Fe^{2+} and Fe^{2+} contains no added test compounds. Other samples contained Fe^{2+} plus 2 μM test compound.


Relative fluorescence
1.00
0.50
0.00
0
3
6
9
12
15
18
21
Time (min)
Control
Fe2+
Cyanidin
Anthocyanin 1
Anthocyanin 2
Anthocyanin 3
Vitamin E
BHA
BHT

The anti-inflammatory assays (Meade et al., 1993) are based on the measurement the prostaglandin endoperoxide H synthase -1 and -2 isozymes (hPGHS-1, and -2) ability to convert arachidonic acid to prostaglandins (PG) products. Cyanidin showed good PGHS-1 and –2 inhibitory activities with IC_{50} values of 90 and 60 μM, respectively (Fig. 5.3, Fig. 5.4). In a preliminary experiment, the crude anthocyanins, **1-3**, gave hPGHS-1 and hPGHS-2 activity at 33 ppm concentrations or above. However, pure anthocyanins **1-3** showed little or no activity against hPGHS-1 and hPGHS-2 (data not shown). Higher concentrations of anthocyanins **1** and **2**, on the contrary, increased the activity of enzyme. This is probably due to the ability of anthocyanins **1** and **2** to act as oxygen carriers at high concentration and enhance the oxygen uptake in this assay. The positive controls used in this experiment were aspirin, naproxen and ibuprofen, respectively. Aspirin had an IC_{50} value of 1050 μM each against hPGHS-1 and hPGHS-2 enzymes (Fig. 5.3). Naproxen and ibuprofen gave IC_{50} values of 11 and 25 μM against hPGHS-1 enzyme, respectively (Fig. 5.3). The aglycone cyanidin inhibited hPGHS-1 and hPGHS-2 enzymes at 90 and 60 μM, respectively. The ratio of IC_{50} values for hPGHS-1/hPGSH-2 was about 0.56 (Fig. 5.4). For measurements of time-dependent inhibition of their enzyme activities by cyanidin, hPGHS-2 isozyme suspension was pre-incubated at 37°C with 15 μM of cyanidin (one fourth of the concentration of IC_{50}), and added to oxygen electrode chamber with arachionic acid substrate to initiate the PGHS emzyme reaction. Our results suggested that the rate of inhibition of PGSH-2 did not change with time. Further experiments should be carried out to determine the arachidonic acid metabolites in order to understand the mechanism of the action of anthocyanins and cyanidin on PGHS-1 and -2 enzymes.

The specific inhibition of PGHS-2 enzyme will be a major advance in anti-inflammatory therapy since it significantly reduce the adverse effects of non-steriodal antiinflammatory drugs (NSAIDs) treatment (Copeland et al., 1994). It is generally believed that ulcerogenic and other adverse properties of NSAIDs result from the inhibition of PGHS-1 whereas the therapeutically desirable effects come from inhibition of PGHS-2 enzyme (Masferrer et al., 1994). These results suggest that dietary tart cherry anthocyanins may possess numerous health benefits. Our experiments using anthocyanins and cyanidin isolated from tart cherries in model systems indicate that they possess antioxidant activity which is comparable to commercial antioxidants. Similarly, cyanidin, which may be metabolized from anthocyanins in in vivo system, showed better response than aspirin in the inflammatory assays. The antioxidant and anti-inflammatory properties of anthocyanins and cyanidin suggested that consumption of cherries containing these compounds may be beneficial in protection against chronic diseases.

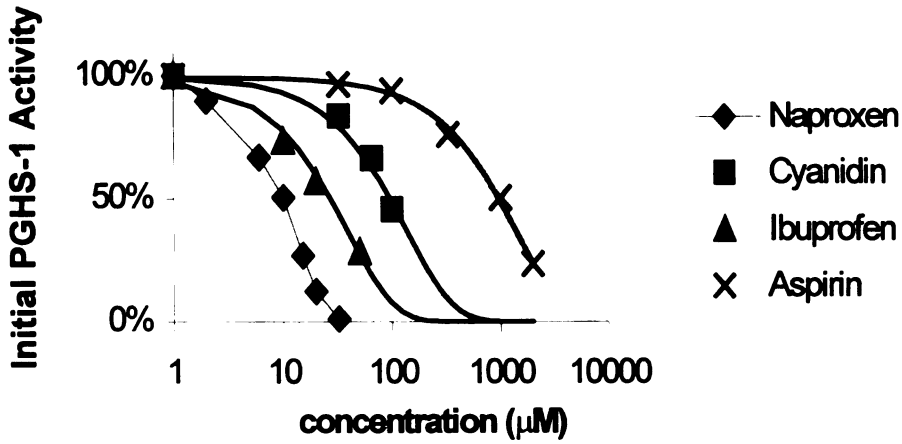


Fig. 5.3. Dose response curve for the inhibition of the human PGHS-1 enzyme by cyanidin. The anti-inflammatory properties of cyanidin were estimated by its ability to inhibit the cyclooxygenase activity of the prostaglandin H endoperoxide synthase (PGHS-1) enzyme. Cyanidin has an IC_{50} value of 90 μM for PGHS-1 enzyme, while the non-steroidal antiinflammatory drugs, aspirin, naproxen and ibuprofen had IC_{50} of 1050, 11 and 25 μM, respectively.

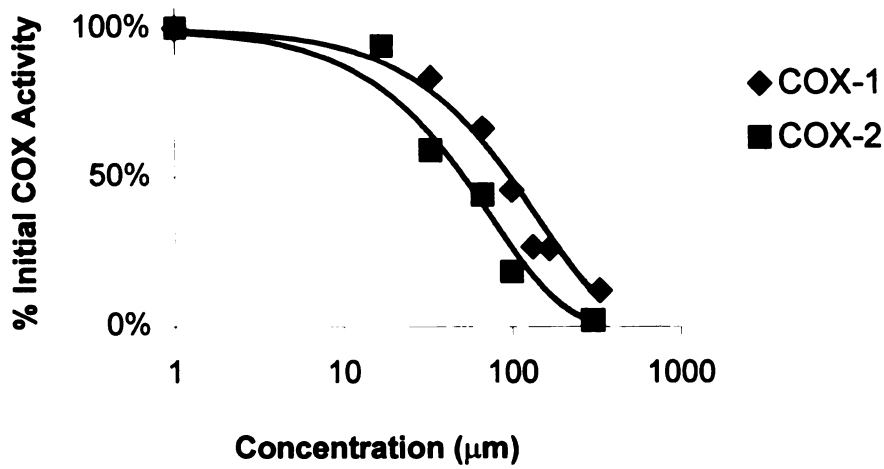


Fig. 5.4. Dose response curve for the inhibition of COX-1 and COX-2 enzymes by cyanidin. Cyanidin has IC_{50} values of 90 and 60 μM for COX-1 and COX-2 enzymes, respectively.

CHAPTER SIX*

Cyclooxygenase active bioflavonoids from Balaton™ tart cherry and their structure activity relationships

ABSTRACT

Five flavonoids, naringenin, quercetrin, 5,8,4'-trihydroxy-6,7-dimethoxyflavone, kaempferol 3-rutinoside and 3'-methoxy kaempferol 3-rutinoside, and two isoflavonoids, genistein, genistin, isolated from Balaton™ tart cherry were assayed for Cyclooxygenase-1 (COX-1) activity. Genistein showed the highest COX-1 inhibitory activity among the isoflavonoids tested with an IC_{50} value of 80 μM concentrations. Kaempferol showed the highest COX-1 activities among the flavonoids tested with an IC_{50} value of 180 μM concentrations. The structure-activity relationships of flavonoids and isoflavonoids revealed that hydroxyl groups at C_4', C_5 and C_7 in isoflavonoids were essential for a better COX-1 inhibitory activity. Also, the double bond between C_2 and C_3 in flavonoids are important for COX-1 enzyme inhibition. However, hydroxyl group at C_3' position in flavonoids decreased the COX-1 inhibition.

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*Will be submitted to *Planta Medica*

INTRODUCTION

The cyclooxygenase enzyme, prostaglandin endoperoxide H synthase (PGHS), has been widely used as a tool for investigating the anti-inflammatory effects of plant products (Bayer et al., 1989; Goda et al., 1992; Wagner, 1990; Abad et al., 1994). Cyclooxygenase (COX) enzyme is the pharmacological target site of the nonsteroidal anti-inflammatory drugs (NSAIDs) (Humes et al., 1981; Rome and Lands, 1975). There are two isozymes of cyclooxygenase that catalyze the first step in prostaglandin synthesis: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Hemler, et al., 1976; Dewitt et al., 1993). It is hypothesized that selective COX-2 inhibitors are mainly responsible for anti-inflammatory activities (Masferrer et al., 1994). Flavonoids have been widely investigated as anti-inflammatory substances. Arturson and Johsson (1975) first reported the activity of several semisynthetic derivatives of rutin on the prostaglandin (PG) synthase enzyme. After that, a series of flavonoids and other phenolic compounds were tested for cyclooxygenase and lipoxygenase inhibitions (Baumann et al., 1980). The structural features for cyclooxygenase (COX) and lipoxygenase (LOX) inhibitions were investigated. The 5,7-dihydroxyflavone galangin with an IC_{50} of 5.5 μM, was found to be the most active cyclooxygenase inhibitory flavonoid (Wurm et al., 1982). Flavonoids with an *ortho*-dihydroxy moiety in ring A or B were stronger inhibitors than those with a free 3-OH group (Wurm et al, 1982; Baumann et al., 1980). Certain prenylated flavonoids, such as morusin, were also active, because of their higher lipophilicity (Kimura et al., 1986).

In flavonols, hydroxylation at C_3 is a sufficient condition, especially if the compound possesses another hydroxyl at 5 position. In any case, the C_2-C_3 double bond,

which determines the coplanarity of the heterorings, appears to be a major determinant of COX activity (Wurm et al., 1982). These strucutural requirements have been confirmed in other systems. Also, unsubstituted flavone is a good COX inhibitor (Mower et al., 1984; Landolfi et al., 1984; Welton et al., 1986). Kalkbrenner et al (1992) further studied the effects of 37 flavonoids on prostaglandin endoperoxide synthase enzyme. Nonplanar flavans were more potent inhibitors than planar flavones and flavonols. Flavones with an *ortho*-dihydroxy structure in the B ring and flavonols with hydroxyl groups at C_5 and C_7 in A ring were potent prostaglandin endoperoxide synthase inhibitors. Most of the flavanones studied did not cause significant COX inhibition, except for the flavanone-3-ol, silibinin (Kalkbrenner et al., 1992).

Flavones and flavanones with 3,7,4′ hydroxyl groups are potent inhibitors of 5-LOX (Welton et al., 1986). The catechol structure has been proposed as an important feature for the inhibition of this enzymes (Welton et al., 1986). Also, the presence of hydroxyl at C_5 increased the COX activity (Kimura et al., 1985). A study of 5-lipoxygenase inhibitory activity of baicalein (5,6,7-trioxyflavone) on leukotriene C_4 (LTC_4) biosynthesis showed that this compound significantly inhibited LTC_4 production with an IC_{50} of 9.5 μM (Butenko et al 1993). However, the anti-inflammatory activity of isoflavonids has been rarely investigated.

Montmorency and Balaton™ tart cherries were reported to alleviate arthritic- and gout-related pain in addition to antioxidant activities. The obvious antioxidant activity of flavonoid components in cherry may be responsible for the beneficial effect of chronic diseases, including inflammatory disorders. In order to evaluate anecdotal claims associated with tart cherries, we purified anthocyanins and polyphenolics from Balaton™

cherries and evaluated their anti-inflammatory activity. Balaton™ tart cherry is a new cultivar being planted in Michigan orchards to replace Montmorency cultivar. In this chapter, we have evaluated seven flavonoids and isoflavonoids from methanol extract of Balaton™ tart cherry for COX-1 enzyme inhibitory activity and compared their structure activity relationships with structurally related compounds.

MATERIALS AND METHODS

Materials. Arachidonic acid and Ovine Prostaglandin H Synthase-1 (PGHS-1) were purchased from Oxford Biomedical Research (Oxford, MI, USA). Genistein, genistin, naringenin, quercetrin, 5,8,4'-trihydroxy-6,7-dimethoxyflavone, kaempferol 3-rutinoside and 3'-methoxy kaempferol 3-rutinoside were purified from Balaton™ tart cherry by HPLC and were identified by ^{1}H- and ^{13}C NMR spectral data. Daidzein and formononetin were purchased from Research Plus, Inc. (Bayonne, New Jersey, USA). Biochanin A, kaempferol, quercetin, naproxen, ibuprofen and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Luteolin was purchased from Adams Chemical. Co (Round Lake, IL, USA).

For measuring the effects of flavonoids and isoflavonoids, each sample was dissolved in DMSO to yield 40mM stock solution. The stock solution was further diluted to the desired concentrations according to the COX-1 inhibitory activity of each tested compound.

Anti-inflammatory assay: Cyclooxygenase activities were measured PGHS-1 enzyme, a homogeneous protein purified from ram seminal vesicles. PGHS-1 enzyme (*ca.* 5mg protein/ml in 0.1 M Tris HCl, pH 7.4) were prepared and assayed on the same

day. Cyclooxygenase assay was performed at 37°C controlled by a circulation bath (Model-1166, VWR Scientific Products, Chicago, IL) by monitoring the initial rate of O_2 uptake using an 5357 Oxygen electrode (INSTECH Laboratory, Plymouth Meeting, PA) (Meade et al., 1993).

Each assay mixture contained 600 μL of 0.1 M Tris-HCl, pH 8.0, 1 mM phenol, 17μg hemoglobin and 10μM arachidonate and were mixed in a microchamber (INSTECH Laboratory, Plymouth Meeting, PA). Reactions were initiated by adding 1 to 5 μg of microsomal protein (5 μL). Instantaneous inhibition of PGH Synthase isozymes were determined by measuring the cycloxygenase activity initiated by adding aliquots of microsomal suspensions of PGHS-1 in the assay mixtures containing 10 μM arachidonate and various concentrations of test compounds. The IC_{50} values represent the concentrations of inhibitor that gave half-maximal activity under the standard assay conditions. The kinetics of the enzyme activity was monitored by Biological Oxygen Monitor (YSI model 5300, Yellow Springs Instrument CO., Inc., Yellow Springs, Ohio) and collected in Quicklog Data Acquistion and Control computer software (Strawbeey tree Inc., Sunnyvale, CA).

RESULTS AND DISCUSSION

The COX-1 activity was determined by monitoring O_2 uptake using an O_2 electrode. The conversion of arachidonic acid to prostaglandins were initiated by adding enzyme preparations. One unit of cyclooxygenase represents the oxygenation of 1 nmol of arachidonate/min under the standard assay condition by the COX enzyme. This is a modification of the assay reported by Dewitt et al (1990). K_m values for arachidonate

conversion to PGH were determined using arachidonate concentrations ranging from 2 to 50 μm. We have used 10 μM arachidonate for cyclooxygenase-1 assay, because this substrate concentration was high enough to give near-maximal cyclooxygenase activity and low enough to permit the detection of enzyme inhibition by lipophilic inhibitors (Meade et al., 1993). This methodology can also be used for cyclooxgenase-2 assay. Three known COX inhibitors, aspirin, ibuprofen and naproxen, were selected as positive controls. We compared COX-1 inhibition of flavonoids, kaemperol, quercetin, luteolin, quercetin 3-rhamnoside, 5,8,4'-trihydroxy-6,7-dimethoxyflavone, kaempferol 3-rutinoside, 3'-methoxy kaempferol 3-rutinoside and naringenin, and five isoflavonoids, genistein, genistin, daidzein, formononetin and biochanin A to the positive control used.

COX-1 inhibitory activity of each compound at different concentrations was calculated by comparing the tangent of O_2 uptake curves of test compounds with that of blank control. For each isoflavonoids and flavonoids, IC_{50} values (50% inhibitory concentrations) were calculated by linear regression analysis. The half-maximal inhibitory concentrations of aspirin, ibuprofen and naproxen, flavonoids and isoflavonoids are listed in Table 2.1.

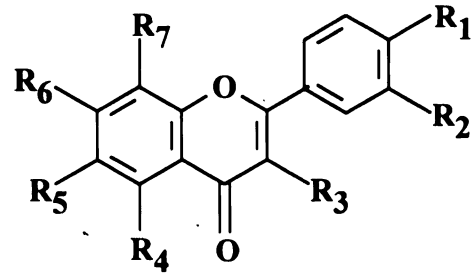
Among the flavonoids tested, kaempferol showed the highest COX-1 inhibition, followed by luteolin, quercetin, naringenin and quercetin 3-rhamnoside. In quercetin, compared to kaempferol, the presence of a hydroxyl group at C_3' position decreased the COX-1 inhibitory activity (Fig. 6.1). Kaempferol and quercetin showed varying COX inhibitory activitities under different assay conditions. (Hoult et al.,1994; Moroney et al., 1988; Kalkbrenner et al., 1992). A substitution at C_3 positions also important for the COX-1 inhibitory activity. Glucosylation of hydroxyl group at C_3 position removed the

COX-1 inhibitory activity. Comparing the COX-1 inhibitory activity of flavones (luteolin) with their corresponding flavonols (quercetin), it can be concluded that the absence of a hydroxyl group at C_3 slightly enhanced the inhibitory effect on COX-1 enzyme. The quercetin 3-rhamnoside, which is not active in our studies, possessed antiinflammatory activity in vivo (Sanchez de Medina et al., 1996). It is speculated that glucoside might exhibit a COX inhibitory effect when it was metabolized to quercetin in in vivo system. The double bond between C_2 and C_3, which determines the coplanarity of the heteroring C, was essential for a higher inhibitory activity. Also, the saturation of C_2-C_3 double bond dramatically decreased the COX-1 inhibitory effect as in the case of naringenin (Fig. 6.1). This result is consistent with published reports (Wurm et al, 1982; Kalkbrenner et al., 1992). If the multiple numbers of hydroxyl and methoxyl substitution were present in A ring, the COX-1 activity was also decreased as in the case of 6,7-dimethoxy-5,8,4'-trihydroxyflavone.

Among the isoflavonoids (Fig. 6.2), genistein showed the highest COX-1 inhibitory activity. When the 7-hydroxyl group in the A ring of genistein was glycosylated (genistin), the COX-1 activity was dramatically decreased. The hydroxyl group in C-4' position was also essential for the COX-1 inhibitory activity. When 4'-OH group in genistein was methylated, the inhibitory effect of the methyl ester of genistein, biochanin A, decreased constantly (Fig. 6.2). Similarly, when 4'-OH group in daidzein was methylated, the COX-1 activity decreased dramatically. The 5-OH group in isoflavonoids is also an important factor for COX-1 inhibitory effect. The COX-1 inhibitory effect decreased when the C_5 hydroxyl group in gensitein was removed to yield daidzein. These results indicated that C_4', C_5 and C_7 hydroxyl groups in isoflavonoids are

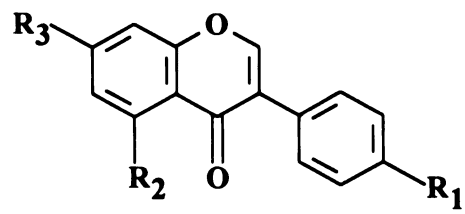
essential for COX-1 inhibitory activity. Comparison of genistein with that of kaempferol indicates that the ring B substitution at C_3 position enhanced COX-1 inhibitory effect.

In addition to COX-1 inhibition, these isoflavonoids and flavonoids also showed good antioxidant activity as demonstrated in chapter four. The biological activities of these compounds may offer health benefits such as prevention of chronic diseases in human. Also, it suggested that the bioflavonoids present in tart cherries may be partially responsible for the anecdotal health claims such as the reduction of arthritic and gout related pain when cherries or cherry products are consumed.



Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7
quercetin	OH	OH	OH	OH	H	OH	H
kaempferol	OH	H	OH	OH	H	OH	H
luteolin	OH	OH	H	OH	H	OH	H
quercetrin	OH	OH	rhamnose	OH	H	OH	H
kaempferol 3-rutinoside	OH	H	rutinose	OH	H	OH	H
3'-methoxy kaempferol 3-rutinoside	OH	OMe	rutinose	OH	H	OH	H
5,8,4'-trihydroxyl-6,7-dimethoxyflavone	OH	H	H	OH	OMe	OH	OMe

Fig. 6.1. Structure of flavonoids tested for COX-1 inhibitory activity



compound	R_1	R_2	R_3
genistein	OH	OH	OH
genistin	OH	OH	glucose
biochanin A	OMe	OH	OH
daidzein	OH	H	OH
formononetin	OMe	H	OH

Fig. 6.2. Structure of isoflavonoids tested for COX-1 inhibitory activity

Table 6.1. IC_{50} Values (μM) of flavonoids, isoflavonoids, aspirin, ibuprofen and naproxen on cyclooxygenase-1 enzyme

Compound	IC_{50}
Aspirin	1050
ibuprofen	11
naproxen	23
naringenin	> 800
luteolin	300
quercetin	350
quercetrin	not active
Kaempferol 3-rutinoside	not active
3'-methoxy kaempferol 3-rutinoside	not active
5,8,4'-trihydroxy-6,7dimethoxyflavone	not active
kaempferol	180
genistein	80
genistin	>400
daidzein	400
biochanin A	350
formononetin	> 800

SUMMARY AND CONCLUSIONS

It is generally believed that many common foods contain non-nutritional components such as flavonoids and anthocyanins that are considered to reduce the incidence of chronic diseases. Tart cherry is claimed to have various health benefits. In order to evaluate the anecdotal claims, we have investigated the active components from aqueous, ethyl acetate and methanol extracts of Montmorency and Balaton™ tart cherries and determined their anticancer, antioxidant and anti-inflammatory activities.

Three anthocyanins, anthocyanin **1** [cyanidin-3-(2''-*O*-*β*-D-glucopyranosyl-6''-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside], anthocyanin **2** [cyanidin-3-(6''-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside] and anthocyanin **3** [cyanidin-3-*O*-*β*-D-glucopyranoside, were identified in the aqueous extracts of both Montmorency and Balaton™ tart cherries by antioxidant assay-directed fractionation and purification. However, Balaton™ tart cherry contained approximately six times more anthocyanins than does Montmorency tart cherries.

The anthocyanins and their aglycones, cyanidin, were further tested for antioxidant activity using Fe^{2+}-induced lipid peroxidation. The antioxidant activities of anthocyanins and cyanidin are comparable to the commercial antioxidants butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) and superior to vitamin E at 2 μM concentrations. The number of sugar moieties at C_3 position is an important factor for the variation of antioxidant activity observed for anthocyanins.

The anti-inflammatory activities of anthocyanins and cyanidin were assayed on prostaglandin endoperoxide H synthase-1 (PGHS-1) and -2 (PGHS-2) enzymes. Results suggested that anthocyanins did not show PGHS-1 and PGHS-2 inhibitory activities.

However, the aglycone cyanidin demonstrated PGHS-1 and PGHS-2 inhibitory activities at IC_{50} values of 90 and 60 μM, respectively. The positive controls, aspirin, ibuprofen and naproxen had IC_{50} values of 1050, 11 and 25 μM, respectively, against COX-1 enzyme. The anthocyanins, which are not active in in vitro assays, could be metabolized to cyanidin in biological systems and hence can act as an anti-inflammatory agent. The anthocyanins are the most abundant class of compounds in cherries and hence have the potential to substantiate the anecdotal claims such as the reduction of arthritic and gout related pain and incidence of cardivascular diseases.

From the ethyl acetate extract of Balaton™ tart cherry, three novel compounds, 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid, 1-(3', 4'-dihydroxycinnamoyl)-cyclopenta-2,5-diol and 1-(3', 4'-dihydroxycinnamoyl)-cyclopenta-2,3-diol were identified by ^{1}HNMR, ^{13}C NMR, DQCOCY, HMQC, FABMS and EIMS experiments. The inhibitory activities of lipid peroxidation of 1-(3', 4'-dihydroxycinnamoyl)-cyclopenta-2,3-diol and 1-(3', 4'-dihydroxycinnamoyl)-cyclopenta-2,5-diol were 79 and 75 %, respectively, at 20 μM concentrations. However, 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid did not show activity at 100 μM. The antioxidant activities of these compounds were compared with caffeic acid analogues. It is concluded that the 3, 4-dihydroxy functionality on the aromatic ring is essential for the antioxidant activities of caffeic acid analogues. Also, the smaller the ester group, the better was the antioxidant activity. These phenolic compounds did not show anti-inflammatory activity when tested at 1000 μM concentrations.

From the methanol extract of Balaton™ tart cherry, five flavonoids, 5,7,4'-trihydroxyflavanone, quercetin 3-rhamnoside, kaempferol 3- 6"-O-α-L-

rhamnopyranosyl-β-D-glucopyranoside, rhamnazin 6"-O-α-L-rhamnopyranosyl-β-D-glucopyranoside and 5,8,4'-trihydroxy-6,7-dimethoxyflavone and two isoflavonoids, 5,7,4'-trihydroxyisoflavone and 5,7,4'-trihydroxyisoflavone 7-glucoside, were identified by spectral methods. Both rhamnazin 6"-O-α-L-rhamnopyranosyl-β-D-glucopyranoside and 5,8,4'-trihydroxy-6,7-dimethoxyflavone are reported from tart cherries for the first time. 5,8.4'-trihydroxy-6,7-dimethoxyfalvone was found to be the most active antioxidant phenolic compound isolated from Balaton™ cherry. The antioxidant activity of 5,8.4'-trihydroxy-6,7-dimethoxyflavone is better than that of *tert*-butylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT) at 10 μM concentrations.

These compounds were further tested for hPGSH-1 (COX-1) inhibitory activity and compared with structurally related compounds. Genistein is the most active COX-1 inhibitor among the compounds isolated from Balaton™ tart cherry. The OH groups at C_4', C_5 and C_7 in isoflavonoids are the essential functionality for COX-1 inhibitory activity. In the case of flavonoids, C_2-C_3 unsaturation determined the coplanarity of the heteroring and is essential for the increased COX-1 inhibitory activity. Similarly, the OH groups at C_3' and C_7 hydroxyl groups are also important factors for COX-1 inhibitory activity. Flavonoids with high number of substitutions in A ring, such as 5,8,4'-trihydroxy-6,7-dimethoxyflavone, did not exhibit COX-1 inhibitory activity. It is possible that the phenyl group, ring B, substituted at C_3 may have enhanced the COX-1 inhibitory effect.

Our study indicated that a number of compounds present in tart cherries have antioxidant and prostaglandin endoperoxide H synthase (COX) enzymes inhibitory activities in vitro system. The antioxidant or free-radical scavenging action and the

enzyme inhibitory actions of anthocyanins and flavonoids present in tart cherries could account for many other pharmacological activities, such as antiallergic, antiviral, anticancer including anticarcinogenic activities and prevention of cardiovascular disease and aging. Therefore, it can be stipulated that the consumption of cherries containing these compounds may be beneficial and protect human against various chronic diseases.

In order to verify these in vitro results, extensive human trials using cherries and cherry extracts should be carried out. Similarly, bioavailability of these cherry compounds in human should be studied to determine the mechanism of activity as demonstrated in our in vitro studies.

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