PHYTOCHEMICAL ANALYSIS OF BIOACTIVE CONSTITUENTS FROM EDIBLE MYRTACEAE FRUITS

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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THE CITY UNIVERSITY OF NEW YORK

Abstract

PHYTOCHEMICAL ANALYSIS OF BIOACTIVE CONSTITUENTS FROM EDIBLE MYRTACEAE FRUITS

by

Kurt Allerslev Reynertson

Adviser: Professor Edward J. Kennelly

Many species of Myrtaceae cultivated throughout the tropics for their edible fruit can be found in local markets and processed into a variety of food products. Some are also used as traditional medicines in divergent practices from South America to Southeast Asia for inflammatory conditions and intestinal disorders, high blood pressure, diabetes, asthma, and as antimicrobials, antiscorbutics, carminatives, diuretics, and astringents.

Bioactivity-guided fractionation of the jaboticaba fruit (*Myrciaria cauliflora*) was performed and a novel depside, jaboticabin, together with 16 known compounds were isolated and identified by spectroscopic data interpretation or comparison with authentic standards. Jaboticabin and the related depside, 2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid, significantly inhibited chemokine interleukin (IL)-8 production before and after cigarette smoke treatment of cells. Jaboticabin was cytotoxic in the HT29 colon cancer cell line (IC_{50} = 65 μM), and 2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid was active against HCT116 colon cancer cells (IC_{50} = 30 μM). Both depsides also exhibited antiradical activity in the 1,1-diphenyl-2-

picrylhydrazyl (DPPH) assay (IC_{50} = 51.4 and 61.8 μM, respectively). The anthocyanins cyanidin 3-glucoside and delphinidin 3-glucoside, major constituents of the jaboticaba, showed good activity in these assays as well.

An HPLC-PDA method was developed to quantify the amounts of eight phenolic compounds (cyanidin 3-glucoside, delphinidin 3-glucoside, ellagic acid, kaempferol, myricetin, quercetin, quercitrin, and rutin) in 14 edible Myrtaceae fruits: *Eugenia aggregata*, *E. brasiliensis*, *E. luschnathiana*, *E. reinwardtiana*, *Myrciaria cauliflora*, *M. dubia*, *M. vexator*, *Syzygium cumini*, *S. curranii*, *S. jambos*, *S. javanicum*, *S. malaccense*, *S. samarangense*, and *S. samarangense* var. *Taiwan pink*. In addition, total phenolic content (TPC), total anthocyanin content (TAC), and antiradical activity was determined. TPC ranged from 3.57 to 101.17 mg gallic acid equivalents per g dry weight, TAC ranged from undetected to 12.13 mg/g, and antiradical activity, measured as DPPH IC_{50} ranged from very active (19.40 μg/mL) to inactive (388.69 μg/mL). A review of the ethnomedical, phytochemical, and chemotaxonomic literature has been conducted in the process of evaluating the bioactive constituents of these fruits.

Acknowledgments

Shortly after the beginning of my doctoral career, someone once said to me that pursuing a Ph.D. was one of the most selfish things a person can do. Indeed, it has taken considerable personal effort and focus, and I would not have been able to accomplish what I have without the support of many people. The love of my family has been essential, and I have Sheila and Ruby to thank for enduring the process with me. Their patience and love has been essential to my success. I also wish to thank my parents, who were always encouraging, as well as Soren, Kristina and my Danish family. Sheila's family has also been enormously supportive and encouraging. I am also deeply indebted to my old friends who have stuck by me through the years and kept me from disappearing completely into my research: Christopher Wilde, Marshall Weber, Mark Wagner, Jane LeCroy, Jason Fitzsimmons, and David Murphy among many others. In addition, we have been lucky to be part of a wonderful community of families in our neighborhood who have always been there to help.

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(Southwest Ranches, FL); Montgomery Botanical Center (Miami, FL); Chris Rollins, Director of the Fruit and Spice Park (Homestead, FL); Larry Shokman, Director of The Kampong, NTBG (Coconut Grove, FL); Dr. Jonathan Crane, Tropical Fruit Crop Extension Specialist, Tropical Research and Education Center (Homestead, FL); and the Fairchild Tropical Garden (Miami, FL).

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Much of this work was previously published in the *Journal of Natural Products*, the *Journal of Agricultural and Food Chemistry*, *Food Chemistry*, the *Journal of Ethnobotany Research and Applications* and the *Journal of Herbal Pharmacotherapy*. It has been presented at annual meetings of the Society for Economic Botany and the American Society of Pharmacognosy, two professional societies that made me feel comfortable and welcome in the world of research science.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

5-FU	5-fluorouracil
AP-1	activator protein-1
CHD	cardiovascular disease
COPD	chronic pulmonary obstructive disorder
COX	cyclooxygenase
CSE	cigarette smoke extract
DF	dilution factor
DMEM	Dulbecco's modified Eagle medium
DOP	dioctyl phthalate
DPPH	1,1-diphenyl-2-picrylhydrazyl
EGCG	epigallocatechin 3-gallate
EtOH	ethanol
FCR	Folin-Ciocalteu reagent
FBS	fetal bovine serum
FDA	Food and Drug Administration
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalents
HMBC	heteronuclear multiple bond correlation

HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum correlation
IC_{50}	50% inhibition concentration
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IL-1	interleukin-1
IL-8	interleukin-8
LC-MS	liquid chromatography-mass spectroscopy
LDL	low-density lipoprotein
LOD	limit of detection
LOQ	limit of quantification
LOX	lipooxygenase
LTB_4	leukotriene B_4
MAPK	mitogen-activated protein kinases
MeOH	methanol
MS	mass spectrometry
MW	molecular weight
NAPRALERT	Natural Products Alert
NF-κB	nuclear factor-κB
NMR	nuclear magnetic resonance
NTFP	non-timber forest product
PDA	photodiode array detector
PI-3K	phosphoinositide-3-kinase

Poly E	Polyphenon E
RDA	Recommended Daily Allowance
ROS	reactive oxygen species
SAE	small airway epithelial
SIM	selected ion monitoring
SPE	solid phase extraction
S/N	signal-to-noise
TAC	total anthocyanin content
TEAC	Trolox equivalent antioxidant capacity
TNF-α	tumor necrosis factor-α
TPC	total phenolic content
USDA	United States Department of Agriculture
USP	United States Pharmacopoeia
UV	ultraviolet

Chapter One:
Antioxidants, Anti-inflammatories, and Disease Chemoprevention

According to the Centers of Disease Control and Prevention, over half of all deaths in the United States for the year 2004 were caused by diseases of the heart and malignant neoplasms [1]. Oxidative damage and chronic inflammation play important causative roles in disease initiation and progression [2]. Diets rich in fruits and vegetables and low in cholesterol and fats are inversely correlated with the incidence of coronary heart disease (CHD), cancer, neurodegenerative diseases, and many chronic inflammatory conditions [*3-13*]. Natural antioxidants and anti-inflammatories from fruits and vegetables are implicated as protective constituents of these foods by providing a measure of protection that slows the processes of oxidative damage and mediates inflammation [*2, 8, 12, 14*].

This project describes the antioxidant and anti-inflammatory properties of several edible fruits produced by species in three genera (*Eugenia*, *Myrciaria,* and *Syzygium*) of the plant family Myrtaceae. A review of the ethnomedical, phytochemical, and chemotaxonomic literature has been conducted in the process of evaluating the bioactive constituents of these fruits. A qualitative and quantitative determination of antioxidant

and anti-inflammatory compounds responsible for this activity has also been conducted, and the results presented herein.

Oxidative Damage and Dietary Antioxidants

Free radicals and reactive oxygen species (ROS) are produced naturally through mitochondrial oxidative metabolism and are present in many environmental pollutants. Free radicals and ROS damage cell membranes, and oxidation of low-density lipoprotein (LDL) is a major factor in the promotion of CHD [*15-17*]. Carcinogenesis may also be initiated through oxidatively-induced DNA damage [*15, 16*]. Oxidative damage is balanced by endogenous antioxidants, but additional protection provided by nutritive and non-nutritive elements from food are critical in disease chemoprevention. Repeated damage caused by ROS throughout the span of a human life increases with time, and is a major cause of age-related cancers and other oxidatively-induced diseases.

Research in natural antioxidants is becoming increasingly important both in understanding the beneficial aspects of plant-based foods and in improving the quality of fatty foods. Antioxidants are routinely used by the industry to prevent the oxidation of food in storage and inhibit rancidity. The well-known vitamin antioxidants in food include ascorbic acid, β-carotene, and α-tocopherol. Many clinical and epidemiological studies have sought to demonstrate the efficacy of these vitamins in preventing a wide variety of diseases [*16, 18-22*]. However, some of these studies failed to show significant antioxidative protection *in vitro* [*21, 22*], which suggests that vitamins obtained via whole food or by a balanced diet may be more effective than supplements, possibly through synergistic interactions with other compounds.

Phenolics as Antioxidant Agents

There is now a strong consensus that flavonoids and related polyphenols are responsible for much of the antioxidant activity of fruits and vegetables [*11, 23, 24*]. Many fruits and vegetables are high in flavonoid content. Flavonoids impart color and taste to flowers and fruits, and it is estimated that humans consume between a few hundred milligrams and one gram of flavonoids every day [*11, 14*]. Green tea (*Camellia sinensis*) has received widespread research attention based in part on epidemiological evidence suggesting that cultures that use the beverage show lower incidence of oxidatively-induced disease. Tea is rich in pharmacologically active flavon-3-ols like epigallocatechin-3-gallate (EGCG); these catechins can account for 35-52% of green tea solid extract [*25*].

Flavonoids appear in blood plasma at pharmacologically active levels after eating flavonoid-rich foods, but do not accumulate in the body [*14, 26*]. Consuming flavonoids regularly increases longevity by reducing inflammation and contributing to the amelioration of atherosclerosis from CHD [*15*]. The range of flavonoid biological activity is large; in addition to scavenging free radicals and ROS, flavonoids have been shown to be anti-inflammatory, antiallergenic, antiviral, antibacterial, antifungal, antitumor, and antihemorrhagic [*27, 28*]. It has been hypothesized that flavonoids have been produced by plants for over one billion years, and that this continuous co-evolution with animals has led to the extraordinary diversity of biochemical and pharmacological activities in human systems [2]. Flavonoids inhibit a number of oxidative enzymes, including aldose reductase, α-glucosidase, xanthine oxidase, monooxygenase, lipoxygenase and

cyclooxygenase [*29, 30*]. Plant polyphenols interact with LDL, enriching and protecting it from oxidation when entering the bloodstream. The so-called "French Paradox" refers to the fact that despite the high fat content of the French diet, there is a lower incidence of CHD in France than in countries where fat intake is similar. This has been attributed to the high polyphenolic content of red wine and other fruits and vegetables prevalent in the French and "Mediterranean diet" [*15, 31*].

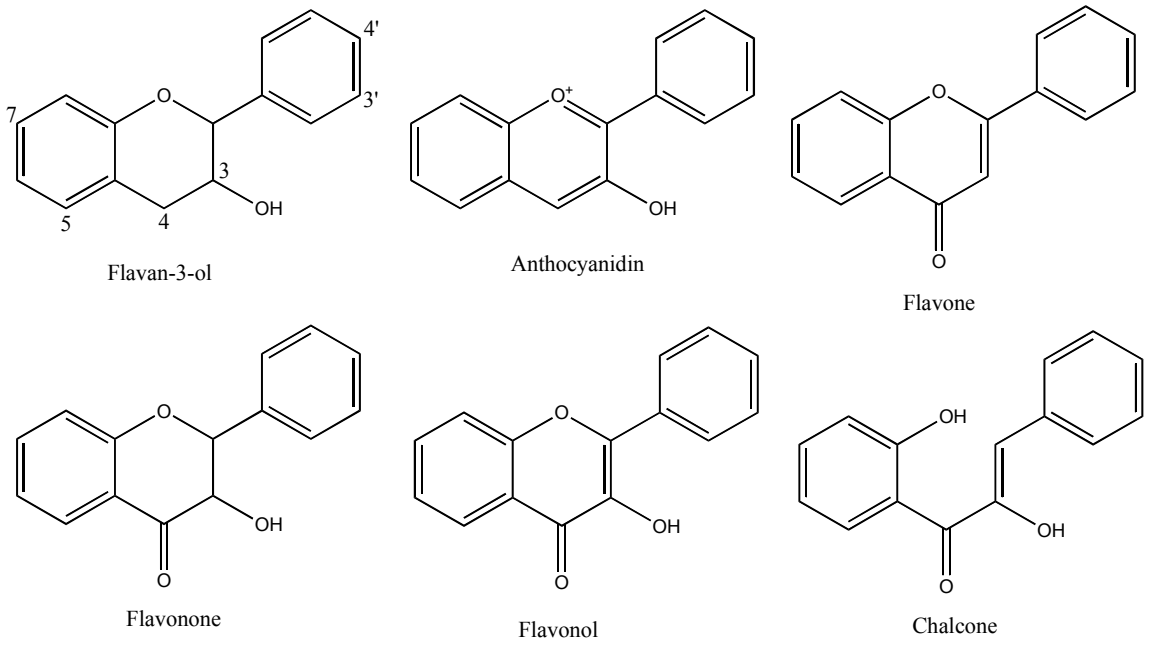


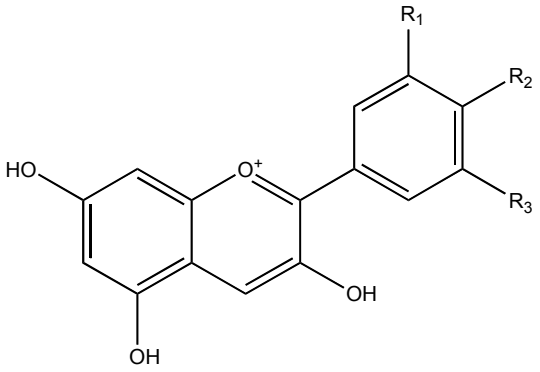
Figure 1.1. Chemical structures of the most common flavonoid subclasses.

There are over 4000 naturally occurring flavonoids in several subclasses (Figure 1.1). All have the same basic C_6-C_3-C_6 phenolic carbon skeleton. Flavonoids are ubiquitous in the higher plants and play an ecological as well as physiological role. The anthocyanins are the most important flower and fruit pigments; they attract pollinators and seed dispersers and protect plant tissues from ultraviolet (UV) radiation damage [*32*]. Some flavonoids mediate enzyme inhibition, act as antifeedants to herbivorous pests and defense compounds against infection, and play roles in photosynthesis, energy transfer, control of respiration, and the biosynthesis of toxic compounds [2]. Flavonols and isoflavones are responsible for the chemical signaling involved in legumous root node formation [*33-36*].

Studies suggest that the antioxidant potential of phenolics is mainly due to their ability to act as reducing agents [*31, 37, 38*]. It is well established that the efficacy of flavonoids as antioxidants stems from the number and position of the hydroxyl substitutions on the basic structure; an increase in number of hydroxyl groups is directly correlated with increasing activity, and the 3′,4′-dihydroxy substitution is significant [*39-41*].

Pigmented berries and other fruits like blueberries, cranberries, strawberries, grapes, and currants are of great interest for among the other phenolic compounds, they are rich in anthocyanins. Anthocyanins (Figure 1.2) are the glycosides of anthocyanidins, and contribute greatly to the orange, red and blue colors in fruits and flowers. Berries and other tropical fruits grow under conditions of high oxidative stress (intense sunlight and high heat), and produce anthocyanins and other protective phenolic compounds that inhibit lipid peroxidation and ultraviolet damage in plant tissues [*32*]. In humans,

anthocyanins have a strong antioxidant, anti-inflammatory, antimutagenic, and cancer chemopreventative activities [*42*].



Compound	R_1	R_2	R_3
Pelargonidin	H	OH	H
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Petunidin	OH	OH	OCH_3
Peonidin	OCH_3	OH	H
Malvidin	OCH_3	OH	OCH_3

Figure 1.2. Chemical structures of six common anthocyanidins.

Cytokines and Chronic Inflammation

Inflammation is characterized by the recruitment and accumulation of neutrophil leukocytes in biological tissues after injury or assault in a process called chemotaxis. Neutrophils are recruited from blood by a number of inflammatory chemotactic cytokines (chemokines). Chemokines are heparin-binding proteins circumscribed by four chemokine families, of which two have been extensively characterized. The two well-studied groups are distinguished by the position of the first two cysteines: either adjacent (CC chemokines), or separated by one amino acid (CXC chemokines).

Interleukin (IL)-8 is a member of the CXC chemokine family, with a molecular weight of about 8 kD. A glutamic acid-leucine-arginine (ERL) sequence near the N-terminal found in IL-8 and related chemokines has been linked to the neutrophil chemotactic function. First identified in 1987, IL-8 is a prototypic inflammatory mediator that plays a key role in neutrophil recruitment.

Many stress factors and cellular stimuli induce production of IL-8, including tumor necrosis factor-α (TNF-α), IL-1, nuclear factor (NF)-κB, activator protein (AP)-1, hypoxia, acidosis, nitric oxide and cell density [*13, 43*]. The promoter region of the IL-8 gene contains a binding site for NF-κB, a redox-responsive transcription factor normally found in the cytoplasm. NF-κB is a sort of master switch for many pro-inflammatory mechanisms. Normally bound with the inhibitory protein IκB, NF-κB becomes activated when IκB is phosphorylated by IκB kinase [*44*].

IL-8 is not a constitutive peptide, rather it occurs in response to inflammatory stimuli [*45*]. Many different cell types can produce IL-8 when stimulated, including neutrophils themselves, the result being that they further intensify neutrophil recruitment to sites of inflammation in any type of tissue [*46*]. This sort of possible feedback loop may amplify and protract the inflammatory response. The fact that IL-8 is generated by tissue cells is important to the etiology of many different inflammatory conditions: rhinitis, bronchitis, pulmonary fibrosis, psoriasis, and inflammatory bowel disease [*44*].

Chronic inflammation has also been implicated in CHD, cancers, neurodegenerative diseases and chronic inflammatory conditions like chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, and chronic asthma. Elevated levels of IL-8 contribute to cancer progression by functioning as a mitogenic, angiogenic, and

motogenic factor [*43*]. Many tumor cells express IL-8 constitutively and the expression level appears to correlate with tumorigenic and metastatic potential.

Phenolics as Anti-inflammatories

Anti-inflammatory activity is not strictly correlated with antioxidant activity, even though there is some overlap. Flavonoids act on several different synergistic pathways as both antioxidants and anti-inflammatories. ROS can initiate transcription factors like NF-κB and AP-1, as well as signal-transduction pathways like mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI-3K) [*13*]. The activation of these pro-inflammatory mediators enhance transcription of downstream inflammatory chemokines [*47*]. By reducing the oxidative attack, flavonoids reduce the amount of activated NF-κB, which is directly tied to IL-8 production. They also inhibit the degradation of IκB, reducing the amount of activated NF-κB. It is not clear whether these are the direct mechanisms involved in flavonoid ability to inhibit IL-8 production, or whether they represent a complementary or synergistic mechanism acting to inhibit IL-8 chemokine production.

Antioxidant green tea polyphenols, especially EGCG, have shown an ability to inhibit IL-8 production in inflammatory airway diseases [*44*]. They also inhibit angiogenesis by decreasing DNA-binding ability of NF-κB by inhibiting phosphorylization and degradation of IκB [*48, 49*]. Luteolin has been shown to suppress TNF-α-induced IL-8 production in intestinal cells by inhibiting the phosphorylation of MAPK [*50*].

By inhibiting monooxygenase and lipoxygenase enzymes, flavonoids affect arachidonic acid metabolism, reducing eicosanoid production that can include pro-inflammatory prostaglandins and leukotrienes. Leukotriene B_4 is a potent chemoattractant involved in inflammation [2]. In addition, flavonoids modulate the function of many inflammatory cells, including T lymphocytes, B lymphocytes, natural killer cells, macrophages and neutrophils. A comprehensive review of flavonoid bioactivity by Middleton *et al*. describes some 35 different enzymatic pathways involved in inflammatory mediation that are affected by flavonoids, including protein kinase C, phospholipase A_2, aromatase, xanthine oxidase, aldose reductase, and many more [2].

Some of the most common and well-known anti-inflammatories are steroidal compounds. Steroids, however, often have unwanted effects on the endocrine system as hormonal modulators. Some chronic inflammatory conditions like COPD are virtually steroid-resistant, and it has been noted that non-steroidal anti-inflammatories that target chemokine pathways are needed as new therapies [*51-53*].

Chemotaxonomy and Selection of Study Plants

As early as 1897, Baker and Smith investigated the essential oils of *Eucalyptus* (Myrtaceae) and found a close connection between the chemistry of the oils and the taxonomy of the plants [*54*]. It has since been established that chemistry is very useful in plant systematics. Conversely, systematics can be used in the search for bioactive compounds, and flavonoids are considered excellent taxonomic guides [*55*]. While flavonoids are ubiquitous in the higher plants, certain subclasses of flavonoids can be taxa-specific. It is unusual for rare flavonoids to occur outside a group in which they have

been discovered. Glycosidic combinations may be highly specific within families, and some morphological characteristics have been linked to particular flavonoid patterns [*56*]. Some methylation patterns occur only in certain families [*57*].

Anthocyanins are produced in the vacuoles of plant cells, and can occur in any part of a plant; different parts of the same plant can have different anthocyanin pigments. The glycosylation pattern is often consistent in a plant, and correlates with systematic information [*56*]. Harborne found that species which do not conform to genera glycosidic patterns are exceptional in other respects as well. Some methylation patterns occur only in certain families[*57*]. Leaves and fruits tend to have simpler pigments than flowers, and there is an evolutionary trend toward more complex anthocyanin structures [*56*]. Complex pigments with several glycosylations are more stable to light degradation and enzymatic attack. The evolutionary trend towards complexity is paralleled by a trend toward the blue color, which in flowers is related to the color preference of insect pollinators.

Anthocyanin content of fruits tends to increase as the fruit matures, becoming complexed with metals and other flavonoids [*57, 58*]. The most common anthocyanidin is cyanidin, which occurs in 80% of permanently pigmented leaves, 69% of fruits and 50% of flowers [*57*]. The next two most common anthocyanidins are delphinidin and pelargonidin. As of 1995, only about 1000 species had been examined for anthocyanins, and only about one-fifth of those species have had the sugar groups fully described. This represents a tiny fraction of the 250,000 known species of angiosperm. Harborne believes that there must be a considerable number of new anthocyanin structures yet to be discovered [*56*].

The chemotaxonomic approach includes reviewing the literature relating to species that have been phytochemically examined. Information can be extrapolated to allied plants. Species closely related to plants containing known polyphenolic antioxidants are likely to have similar polyphenolic constituents. Therefore, the phytochemistry of one plant may serve as clues for related plants. The hypothesis is that antioxidant activity may "run in the family."

Plant Collection

Ethnobotanists regularly structure questionnaires to probe indigenous knowledge for the medicinal uses of plants. However, indigenous knowledge generally does not include a list of plants that help scavenge free radicals. Researchers looking for new antioxidants can, however, use ethnomedicinal information as a guide, paying special attention to plants that are used for illnesses or conditions that are ameliorated by compounds also linked to antioxidant activity. Polyphenolics are diverse in their biological activities, so ethnomedical information that hints at polyphenolic content was examined.

In an initial random screen of approximately 50 tropical fruits in our lab, extracts of *Eugenia uniflora* showed very good activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) antiradical assay similar to pure ascorbic acid [*59*]. Subsequently, the plants allied to *E. uniflora* were reviewed for ethnobotanical and phytochemical data. Several databases were queried, including Natural Products Alert (NAPRALERT), Chemical Abstracts, and Biological Abstracts. Some of the species queried had little or no

phytochemical or ethnobotanical data. Those that are widely used as food or medicine are more likely to have been phytochemically examined (i.e. guavas and cloves).

Several species were selected for analysis, and 14 have been collected and tested: *Eugenia aggregata* Kiaersk., *E. brasiliensis* Lam., *E. luschnathiana* Klotzsch ex O.Berg, *E. reinwardtiana* (Bloom) DC, *Myrciaria cauliflora* (Mart.) O.Berg, *M. dubia* (Kunth) McVaugh, *M. vexator* McVaugh, *Syzygium cumini* (L) Skeels, *S. curranii* (C.B.Rob.) Merr., *S. jambos* (L) Alston, *S. javanicum* Miq., *S. malaccense* (L)Merr. & L.M.Perry, *S. samarangense* (Bloom) Merr. & L.M.Perry, and *S. samarangense* var. *Taiwan pink*. These species are primarily cultivated for their edible fruits (Figure 1.3). Some of these species are also used medicinally, and prior phytochemical studies are not available in the literature. Table 1.1 summarizes the known phytochemistry and ethnobotany of the fruits.

Eugenia luschnathiana | *Myrciaria vexator* | *Syzygium samarangense* var. *Taiwan Pink*

Figure 1.3. Examples of three fruits in this study.

Several institutions in southern Florida dedicated to the propagation of tropical fruits generously permitted us to collect fruit. Collecting within the United States eliminates the need for international collection permits, and these institutions represent collections of plants that have been pre-selected and imported as edibles, medicinals, or both. By utilizing cultivated plants, re-collection of the same individual plants was possible over time. Fruit was collected from The Kampong (The National Tropical Botanical Gardens) in Coconut Grove, FL; The Rare Fruit and Vegetable Council of Broward County experimental garden in Southwest Ranches, FL; the Fruit and Spice Park and The University of Florida Tropical Resource and Education Center, both in Homestead, FL. Fruits were shipped frozen to the laboratory, where they were kept at –20° C until extraction. Voucher specimens have been deposited in The William and Lynda Steere Herbarium at The New York Botanical Garden (Bronx, NY).

Myrtaceae – The Myrtle Family

The Myrtaceae is a large, well-defined family, with about 140 genera and about 4000 species. Pantropical in occurrence, the family has a typical Gondwanan distribution, with centers of concentration in South America, Southeast Asia, and Australia. There are comparatively few occurrences in Africa. The capsular-fruited subfamily Leptospermoideae includes the well-known genus *Eucalyptus* from Australia. The fleshy-fruited subfamily Myrtoideae includes many economically important food and agriculture plants. Edible fruits and useful spices, including guava (*Psidium spp.*), clove (*Syzygium aromaticum*), allspice (*Pimenta dioica*), and bay rum (*Pimenta racemosa*) are all included in the subfamily Myrtoideae. Historically, the Myrtoideae was divided into three

subtribes, but most researchers now believe that this division is artificial. The old subtribe Eugeniinae represents many of the edible fruits, but molecular and morphological evidence supports the argument that this taxon is polyphyletic. A recent issue of *Plant Systematics and Evolution* [*60-65*] was devoted to the family, as the full taxonomic arrangement is still under debate.

The genus *Eugenia* is now generally considered the neotropical group, numbering around 1000 species, and the plants designated *Syzygium* are considered by many to be an Old World genus which falls into the *Acmena* clade, distinct from *Eugenia*. This suggests that the fleshy-fruit character has developed more than once in the family [*64, 65*]. The genus *Myrciaria* is closely allied with *Eugenia*. Many plants in these genera have multiple synonyms in one or more of these genera, which can add to taxonomic and identity confusion [*66-68*]. Defining field characteristics include the presence or absence of indument hairs and the type of and position of the embryo in the seed, making identification of non-fruiting species difficult.

Myrtoideae Phytochemistry and Ethnobotany

The whole family is characterized by leathery glandular leaves containing viscous aromatic terpenoid and polyphenolic substances and flowers with numerous stamens [*69*]. The edible fruits produced by the subfamily Myrtoideae are often described by their bright anthocyanin colors, including orange, red, purple, and black (dark purple). They are sweet to tart, and aromatic; many are somewhat astringent, indicating the presence of tannins. The taste is often described as somewhat acid. New shoot growth for many species is wine-colored [*66, 68*], suggesting a high anthocyanin content in the leaves as

well. Many known antioxidant flavonoids have been isolated from this group. Haron *et al*. [*70*] found that leaf extracts of 17 Neotropical and Paleotropical *Eugenia* species contained myricetin, quercetin was present in 71% and kaempferol was present in 24%. Ellagic acid, methylellagic acid, procyanidin, and prodelphinidin have been found in many species in the family [*71, 72*]. In dicots, ellagic acid is usually confined to certain families and plants containing trihydroxy flavonoids, just as caffeic and *p*-coumaric acid are found along with corresponding di- and monohydroxy flavonoids [*55*]. Theoduloz showed that flavonoids in the five species of the Myrtaceae tested inhibit xanthine oxidase activity [*73*]; Schmeda-Hirschman [*74*] credits this activity to the presence of the flavonoids quercitrin, quercetin, myricitrin, and myricetin.

Surinam cherry (*E. uniflora*) is widely regarded as one of the best tasting of the *Eugenia* species, and the fruits average about 3 cm in size. They have a characteristic ribbed appearance, and several cultivars have been developed with fruits ranging from orange to crimson to black [*66, 75*]. There is an extensive amount of literature documenting the ethnomedical uses of the leaves of Surinam cherry [*74, 76-80*], and most of the phytochemical work has subsequently focused on characterizing the essential oil of the leaves [*80*]. Ascorbic acid, β-carotene, and a few sesquiterpenes have been identified from the fruits [*77, 78*]. In unpublished studies from our lab, the Surinam cherry was found to contain cyanidin 3-glucoside, delphinidin 3-glucoside, dihydromyricetin, quercitrin, and quercetin 3-arabinoside [*81*]. Several well-known antioxidant flavonoids have been reported from leaf extracts, including myricetin, myricitrin, gallocatechin, quercetin, and quercitrin [*74*] as well as the tannins eugeniflorin D-1 and D-2 [*82*]. Popenoe notes that the Brazilians prepare a liqueur from the fruits, and

consider syrups and wines to have a medicinal value [*68*]. In Madeira, fruits of *E. uniflora* are eaten for intestinal troubles [*83*]. Fruits and leaves are also used for their astringent qualities, and to treat high blood pressure [*84*]. Water decoctions of *E. uniflora* leaves are used in Paraguay to lower cholesterol and blood pressure [*85*], and have a highly significant anti-inflammatory action [*79*]. Ferro showed that leaf extracts were slightly active on lipid metabolism, and may exert a protective effect on triglycerides and very low-density lipoprotein levels [*85*].

Other fruits in this subfamily are also colorful, with an extensive ethnobotanical and ethnomedical use that suggests a possible flavonoid content. *Syzygium jambos* fruit is used as a tonic for the brain and for liver problems, as an astringent, and digestive and diuretic [*75, 86*]. The leaves contain seventeen different flavonoids [*28, 87, 88*] and are used as an anesthetic, anti-inflammatory, and astringent, for apoplexy, asthma, bronchitis, cough, diabetes, dysentery, influenza, and rheumatism [*83*]. *Syzygium samarangense*, which is cultivated in India for its edible fruit [*89*], contains two flavonol glycosides as well as EGCG, epicatechin 3-*O*-gallate, and samarangenins A and B [*35*]. In Taiwan, the flowers, which contain tannins, are used to treat fever and halt diarrhea. Flowers also contain desmethoxymatteucinol, 5-*O*-methyl-4'-desmethoxymatteucinol, oleanic acid, and β-sitosterol [*75*]. The jaboticaba (*Myrciaria cauliflora*) is a popular edible in Brazil, much like grapes in the United States [*68*]. The fruits are a dark red to maroon-purple and black, and are used to make jam, tarts, strong wines, and liqueurs [*66*]. In addition, decoctions of the sun-dried skins have been used for a variety of inflammatory conditions, including hemoptysis, asthma, diarrhea, and tonsillitis. *E. aggregata* is a popular reddish-purple edible in Brazil, eaten fresh or used to make jams and jellies [*66*]

which has not been phytochemically examined. In preparing this study, the phytochemistry and ethnobotany of each plant was reviewed and noted, but those uses and compounds associated with the fruit only were given more emphasis, and are summarized in Table 1.1.

Overview of Research

This chapter provides the basic rationale for the research conducted on the edible fruits of several species of Myrtaceae. The second chapter presents an analysis of the phenolic antioxidant, anti-inflammatory, and cytotoxic constituents of one fruit in this taxon: the jaboticaba (*Myrciaria cauliflora*). In the course of this work, a new bioactive depside was isolated and identified using modern spectroscopic methods. In addition, 16 known compounds were identified from this fruit for the first time. The third chapter describes the analysis of over a dozen fruits related to the jaboticaba. An HPLC-PDA system is used to perform a quantitative analysis of eight antioxidant phenolic compounds present in these fruits. In addition, the total phenolic content, total anthocyanin content, and antioxidant activity are determined experimentally. The fourth and final chapter is a concise discussion of the results within the context of the current state of understanding of diet and human health.

Table 1.1. Ethnobotanical and phytochemical information for 14 edible Myrtaceae fruits

Species	Vernacular Name	Fruit Color	Ethnobotanical Information	Previous Phytochemical Reports
Eugenia aggregata	Cherry of the Rio Grande	Reddish-purple	Brazil: eaten fresh, used for jams and jellies [*66*]	Catechin and epicatechin [*90*]
E. brasiliensis	Grumixama	Purple	Leaves and stem bark: gastrointestinal disorders and rheumatism [*91*]	NA
E. luschnathiana	Pitanga	Yellow	NA	NA
E. reinwardtiana	Australian Beach Cherry	Red	NA	NA
Myrciaria cauliflora	Jaboticaba	Purple	Brazil: eaten fresh, used for jam, tarts, strong wine and liqueur, as a treatment for hemoptysis, asthma, diarrhea dysentery and chronic inflammation of the tonsils.[7]	Fruit: Tannins [*75*]; six flavonols, five phenolic acids, two anthocyanins, two depsides, pyranocyanin B, and ellagic acid [*92*]
M. dubia	Camu-camu	Reddish	NA	Fruit: Cyanidin 3-glucoside, delphinidin 3-glucoside [*93*] terpenoids [*94*], carotenoids [*95*]; Leaves: ellagic acid derivatives [*96*].
M. vexator	Blue grape	Purple	NA	NA

Table 1.1. Ethnobotanical and phytochemical information for 14 edible Myrtaceae fruits (continued)

Species	Vernacular Name	Fruit Color	Ethnobotanical Information	Previous Phytochemical Reports
Syzygium cumini	Jamun	Purple	NA	Several flavonoids, ellagitannins and phenolic acids have been identified from fruits, seeds and aerial parts [*97-99*]
S. curranii	Lipote	Purple	NA	NA
S. jambos	Rose apple	Yellow	India: tonic for the brain and for liver problems, as an astringent, and digestive [*86*], distilled to make rosewater [*75*]	Fruit: Terpenoids [*100, 101*]; Leaves: flavonoids and ellagitannins [*28, 88, 102-104*]
S. javanicum	Java apple	Reddish	NA	NA
S. malaccense	Malay apple	Reddish	Leaves: antimicrobial, for high blood pressure, affects respiration [*75, 89*]	Leaves: four flavonoids [*105*]
S. samarangense	Wax jambu	Pink to red	India: eaten fresh; Malaya: greenish fruits are eaten raw with salt or cooked as a sauce [*75, 89*]	Fruit and leaves: several flavonoids and ellagitannins [*71, 106-108*] [*35, 108*]
S. samarangense var. *Taiwan pink*	Wax jambu	Red	NA	NA

NA, no literature available.

Chapter Two:
Antioxidant and Anti-Inflammatory Constituents from Jaboticaba (*Myrciaria cauliflora*) including the description of jaboticabin, a new bioactive depside

Introduction

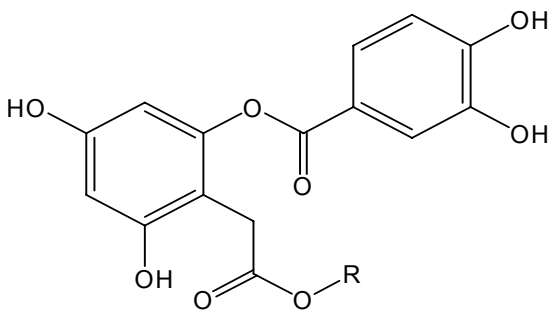
The jaboticaba (*Myrciaria cauliflora* (Mart.) O.Berg. [Myrtaceae]) is a small tree native to the Minas Gerais region of southern Brazil that is grown for the purple, grape-like fruits it produces. Traditionally, an astringent decoction of the sun-dried skins has been used as a treatment for hemoptysis, asthma, diarrhea, and gargled for chronic inflammation of the tonsils [*75*]. The fruit is 3-4 cm in diameter with one to four large seeds, borne directly on the main trunks and branches of the plant, lending a distinctive appearance to the fruiting tree. It has a thick, purple, astringent skin that covers a sweet, white, gelatinous flesh. Common in home gardens and Brazilian markets, jaboticabas are largely eaten fresh; their popularity has been likened to that of grapes in the United States [*68*]. Fresh fruit may begin to ferment three to four days after harvest, so they are often used to make jams, tarts, strong wines, and liqueurs.

In Brazil the fruit of several species, namely *M. jaboticaba* (Vell.) O.Berg, *M. tenella* (DC.) O.Berg, and *M. trunciflora* O.Berg, share the same common name [*68, 75, 109*]. The phytochemistry of these fruits has not been extensively reported in the

literature. The jaboticaba (no species distinguished) has been reported to contain tannins [*75*], and we previously reported the presence of cyanidin 3-glucoside (**3**) in *M. cauliflora* [*110*]. *M. jaboticaba* reportedly contains peonidin 3-glucoside and its aglycone [*111*], and the related camu-camu berry (*M. dubia*), an edible fruit known for its high levels of ascorbic acid, contains **3** and delphinidin 3-glucoside (**4**) as the main pigments [*93*].

Results and Discussion

As part of this project to investigate the antioxidant, anti-inflammatory, and potential cancer chemopreventive compounds from edible Myrtaceae fruits, the jaboticaba was selected for further analysis. Crude methanolic extracts were shown to have strong antiradical activity in the DPPH assay (IC_{50} = 35 μg/mL). Fruit extracts were subsequently subjected to activity-guided fractionation using the DPPH assay resulting in the isolation of a new depside, jaboticabin (**1**). In addition, the related depside 2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid (**2**), **4**, pyranocyanin B, quercetin, isoquercitrin, quercimeritrin, quercitrin, rutin, myricitrin, cinnamic acid, *o*-coumaric acid, gallic acid, protocatechuic acid (**5**), methyl protocatechuate, and ellagic acid were identified from this species for the first time (Figure 2.1).



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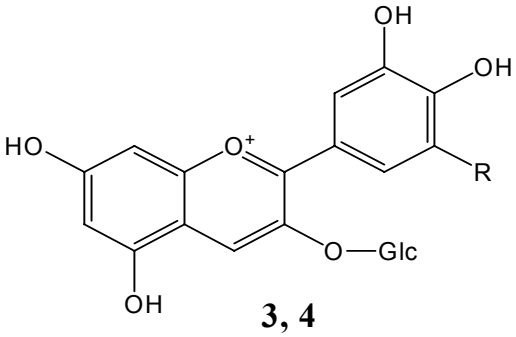

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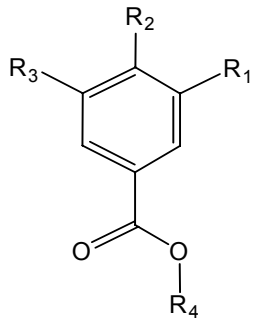

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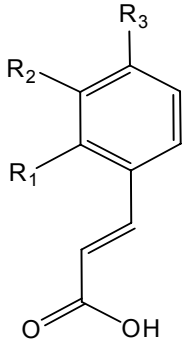

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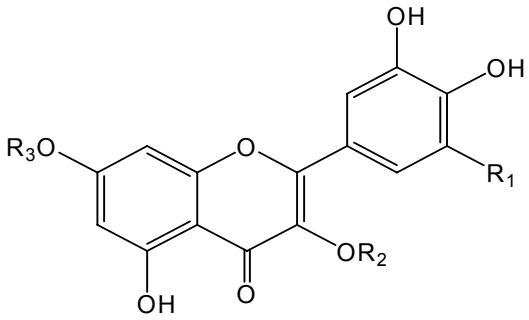

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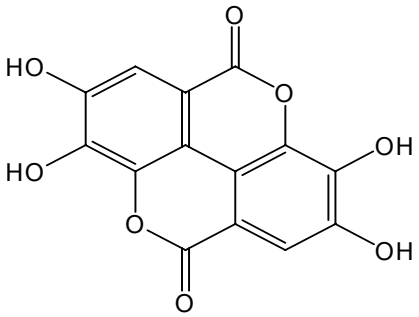

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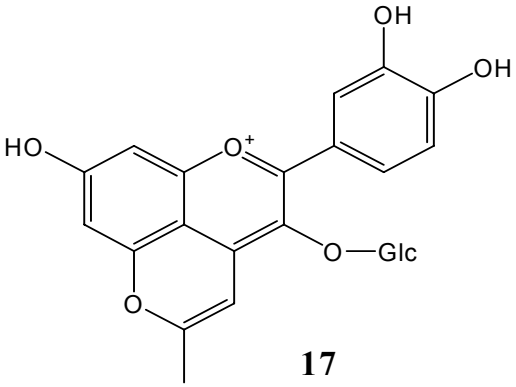

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	Compound	R
1	Jaboticabin	Me
2	2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-Trihydroxyphenylacetic acid	H
3	Cyanidin 3-glucoside	H
4	Delphinidin 3-glucoside	OH

	Compound	R_1	R_2	R_3	R_4
5	Protocatechuic acid	OH	OH	H	H
6	Methyl protocatechuate	OH	OH	H	Me
7	Gallic acid	OH	OH	OH	H

	Compound	R_1	R_2	R_3
8	Cinnamic acid	H	H	H
9	*o*-Coumaric acid	OH	H	H

		R_1	R_2	R_3
10	Quercetin	H	H	H
11	Quercitrin	H	rhamnose	H
12	Isoquercitrin	OH	glucose	H
13	Rutin	H	rutinose	H
14	Myricitrin	OH	rhamnose	H
15	Quercimeritrin	H	H	Glucose
16	Ellagic acid			
17	Pyranocyanin B			

Figure 2.1. Compounds from *Myrciaria cauliflora.*

Depsides are phenolic compounds composed of two or more monocyclic aromatic units linked by an ester bond. They are most often found in lichens, but have also been isolated from higher plants, including species of the Ericaceae, Lamiaceae, and Papaveraceae [*112-114*]. They have not been previously reported in the Myrtaceae. Depsides have antibiotic, anti-HIV, and antiproliferative activity [*115-117*]. As inhibitors of prostaglandin biosynthesis and leukotriene B_4 biosynthesis, depsides are potent non-steroidal anti-inflammatories [*118-121*].

Compound **1** was isolated as a reddish amorphous powder. The negative ESI mass spectrum showed a $[M - H]^-$ molecular ion of *m/z* = 333 (Figure 2.2). Positive HRESIMS gave a $[M + Na]^+$ molecular ion of *m/z* = 357.0581, corresponding to a molecular formula of $C_{16}H_{14}O_8$. The UV-Vis spectrum exhibited a peak at 267 nm with a shoulder at 298 nm, typical of a phenolic acid ester (Figure 2.3). The ^1H and ^{13}C NMR experiments were similar to literature values for **2** (Table 2.1), with an additional methoxy signal at δ 3.57 (3H, s, OCH_3-8) and δ 50.9 (OCH_3-8) [*114*]. NMR, UV-Vis, and mass spectra are presented in Figures 2.4 to 2.10. The position of the methoxy group was established through HMBC correlations between the proton signal at δ 3.57 (OCH_3-8) and 172.9 (C-8) (Figure 2.3). The C-1 attachment for the methyl ethanoate group followed from HMBC correlations; the methylene proton signal at δ 3.50 (H-7) showed HMBC correlations with C-1, C-2, C-6, and the carbonyl C-8. A detailed analysis of 1-D and 2-D NMR spectra and comparison with **2** confirmed the structure of jaboticabin (**1**) as methyl 2-[(3,4-dihydroxybenzoyloxy)-4,6-dihydroxyphenyl]acetate.

An ethanolic extract of jaboticaba fruits was analyzed by LC-MS in selected ion monitoring (SIM) mode to address concerns that **1** could be a methyl ester artifact from

the initial MeOH extraction. A $[M - H]^-$ molecular ion *m/z* = 333 with the same retention time as **1** was detected in ethanolic extracts, indicating that **1** is produced by the plant itself.

Table 2.1. ^{1}H and ^{13}C NMR spectral data for compounds 1 and 2 in CD_3OD

	1		**2**	
	δ_H	**δ_C**	**δ_H**	**δ_C**
C-1		105.7		106.1
C-2		157.0		157.1
C-3	6.16 (d, 2.4)[a]	99.6	6.16 (d, 2.4)	99.6
C-4		157.2		157.1
C-5	6.27 (d, 2.4)	100.7	6.27 (d, 2.4)	100.6
C-6		151.1		151.0
C-7		28.5		28.6
C-8		172.9		174.4
C-1′		120.3		120.3
C-2′	7.54 (d, 2.1)	116.4	7.59 (d, 2.1)	116.4
C-3′		145.0		145.0
C-4′		151.0		151.0
C-5′	6.88 (d, 7.8)	114.7	6.88 (d, 8.1)	114.7
C-6′	7.55 (dd, 2.1, 7.8)	122.9	7.56 (dd, 2.1, 8.1)	123.0
C-7′	3.50 (2H, s)	164.9	3.46 (2H, s)	164.9
OMe-8	3.57 (3H, s)	50.9		

[a]Coupling constants (*J*) given in parentheses.

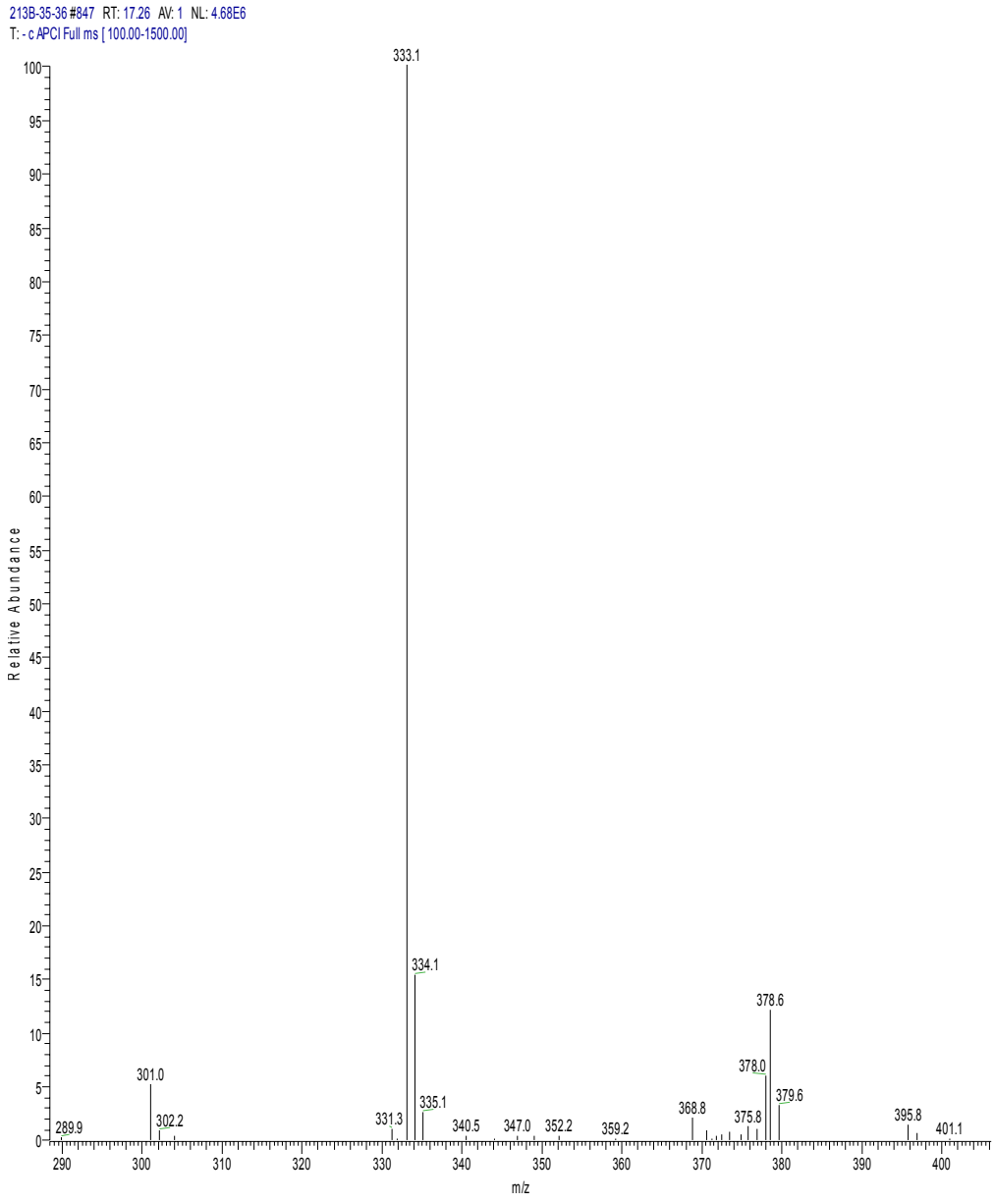


Figure 2.2. Negative ESI mass spectrum of jaboticabin.

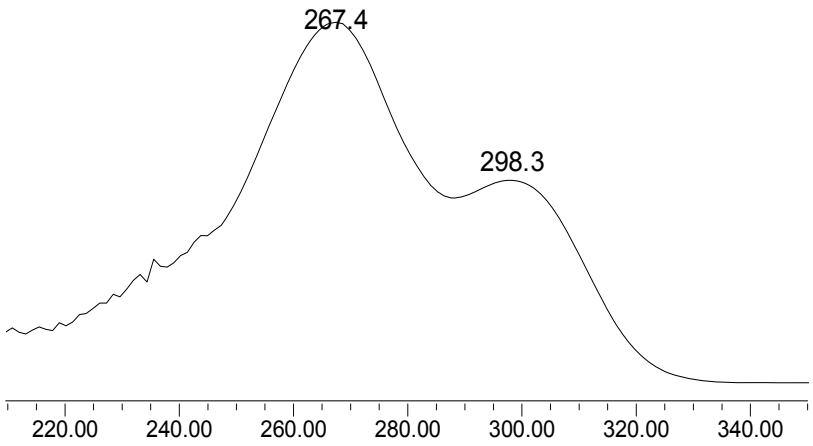


Figure 2.3. UV-Vis spectrum of jaboticabin.

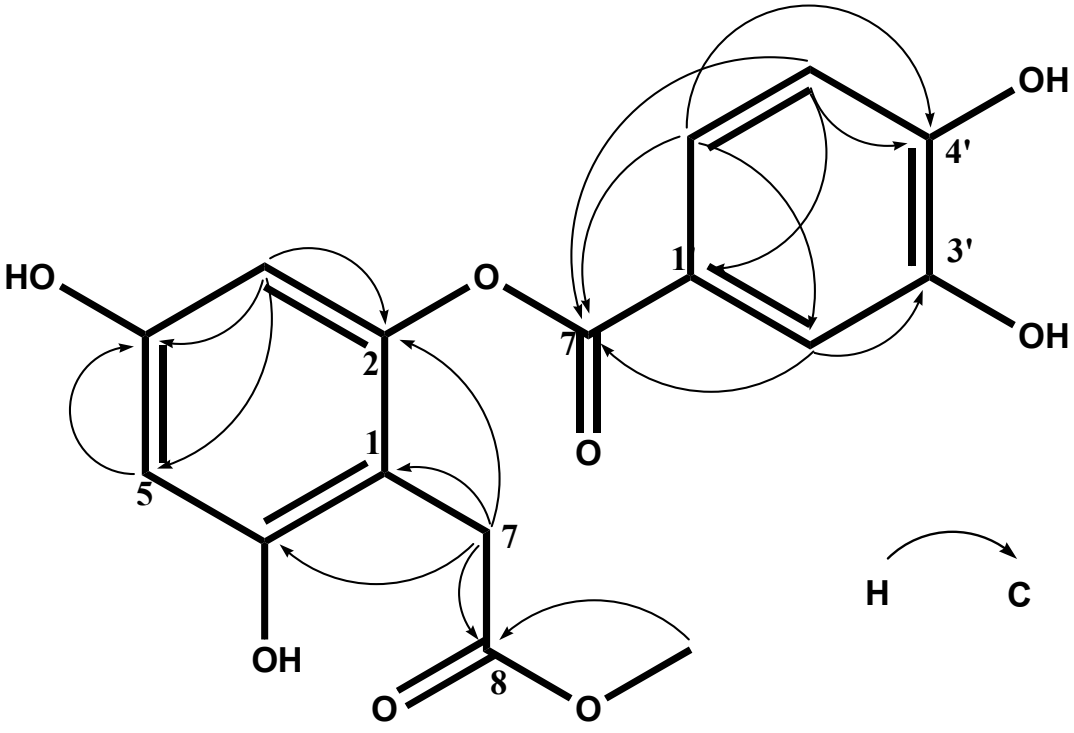


Figure 2.4 Major HMBC correlations for jaboticabin.

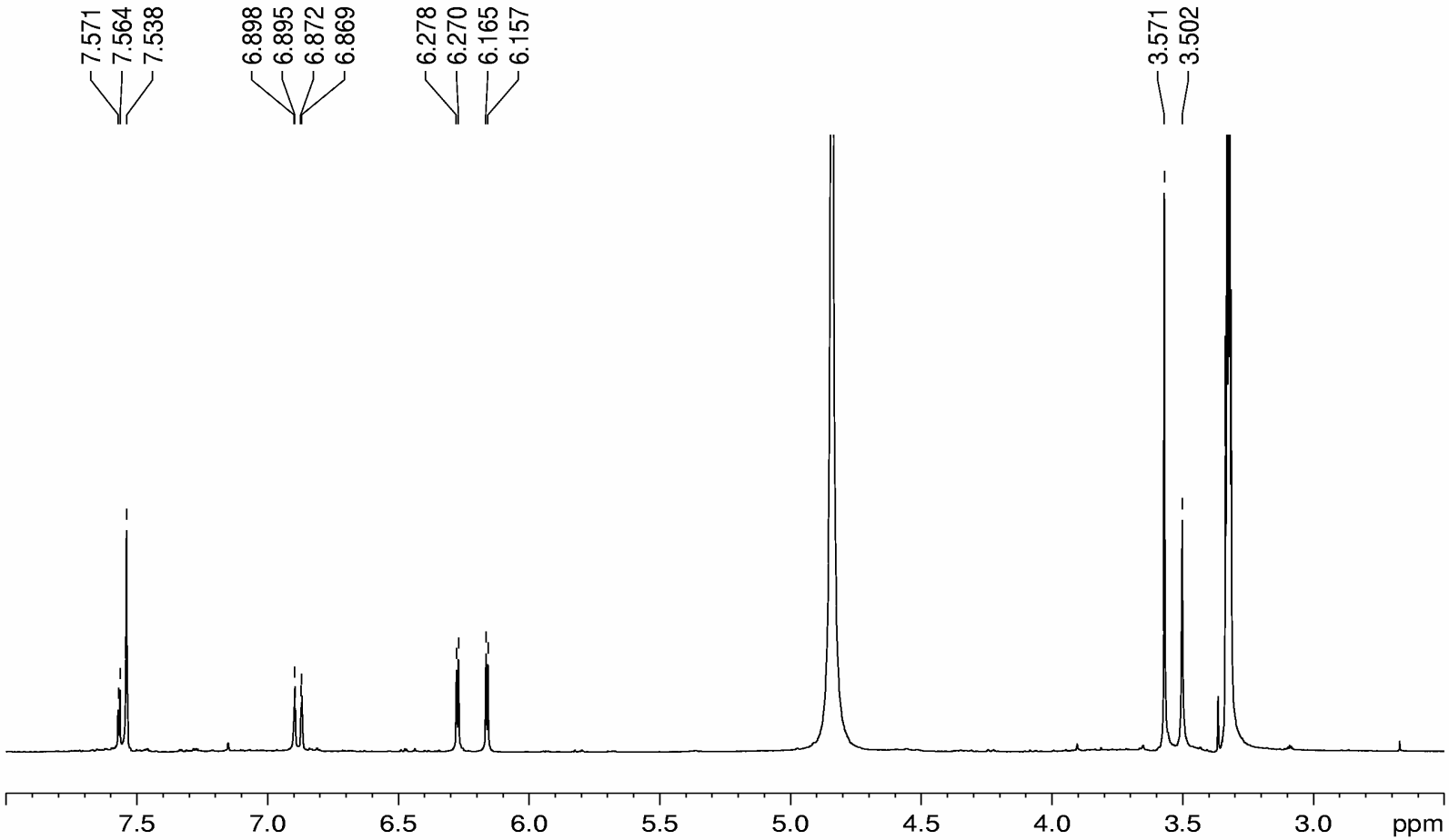


Figure 2.5. ^{1}H NMR (300.13 MHz) spectrum of jaboticabin in CD_3OD.

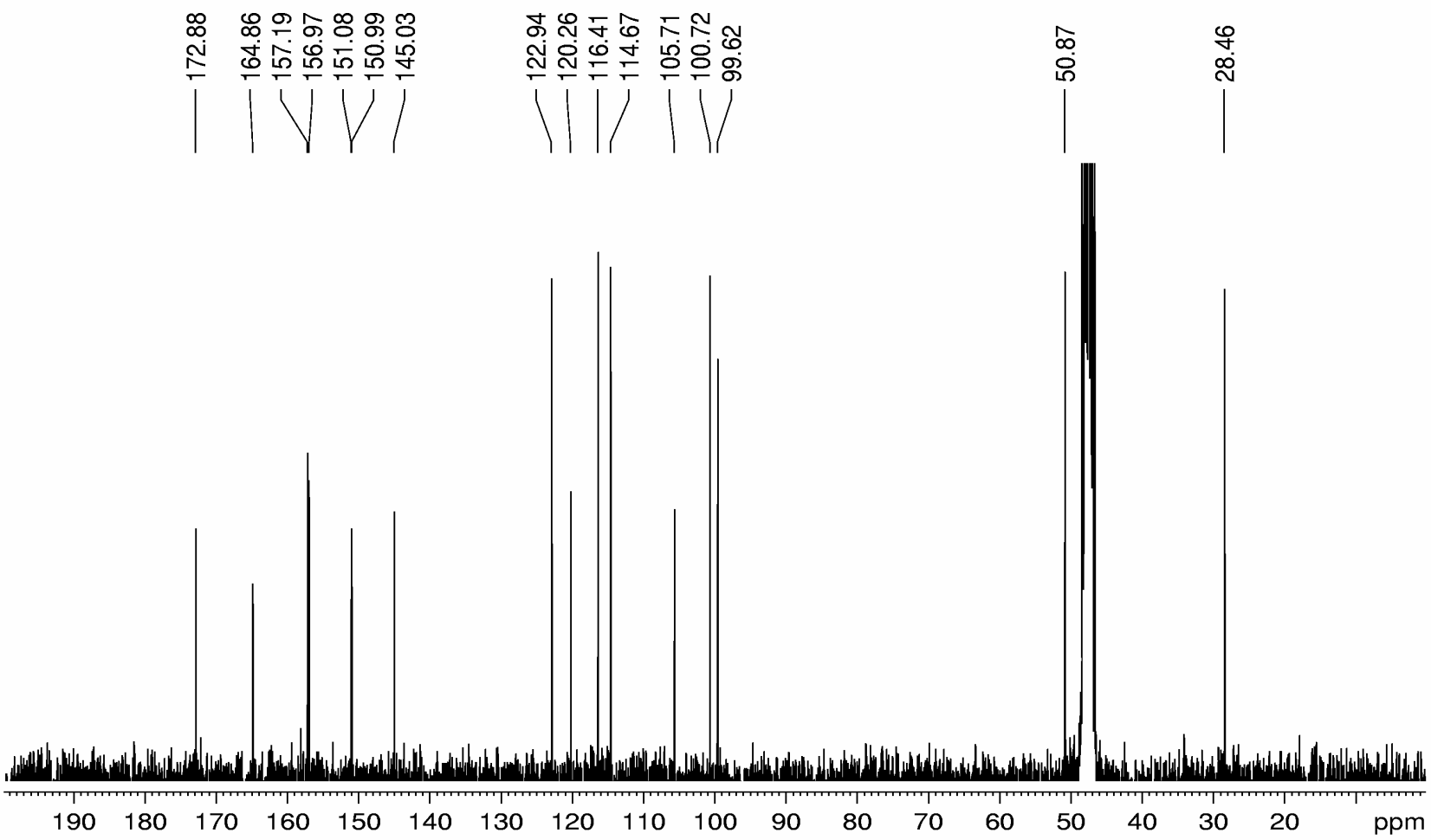


Figure 2.6. ^{13}C NMR (75.48 MHz) spectrum of jaboticabin in CD_3OD.

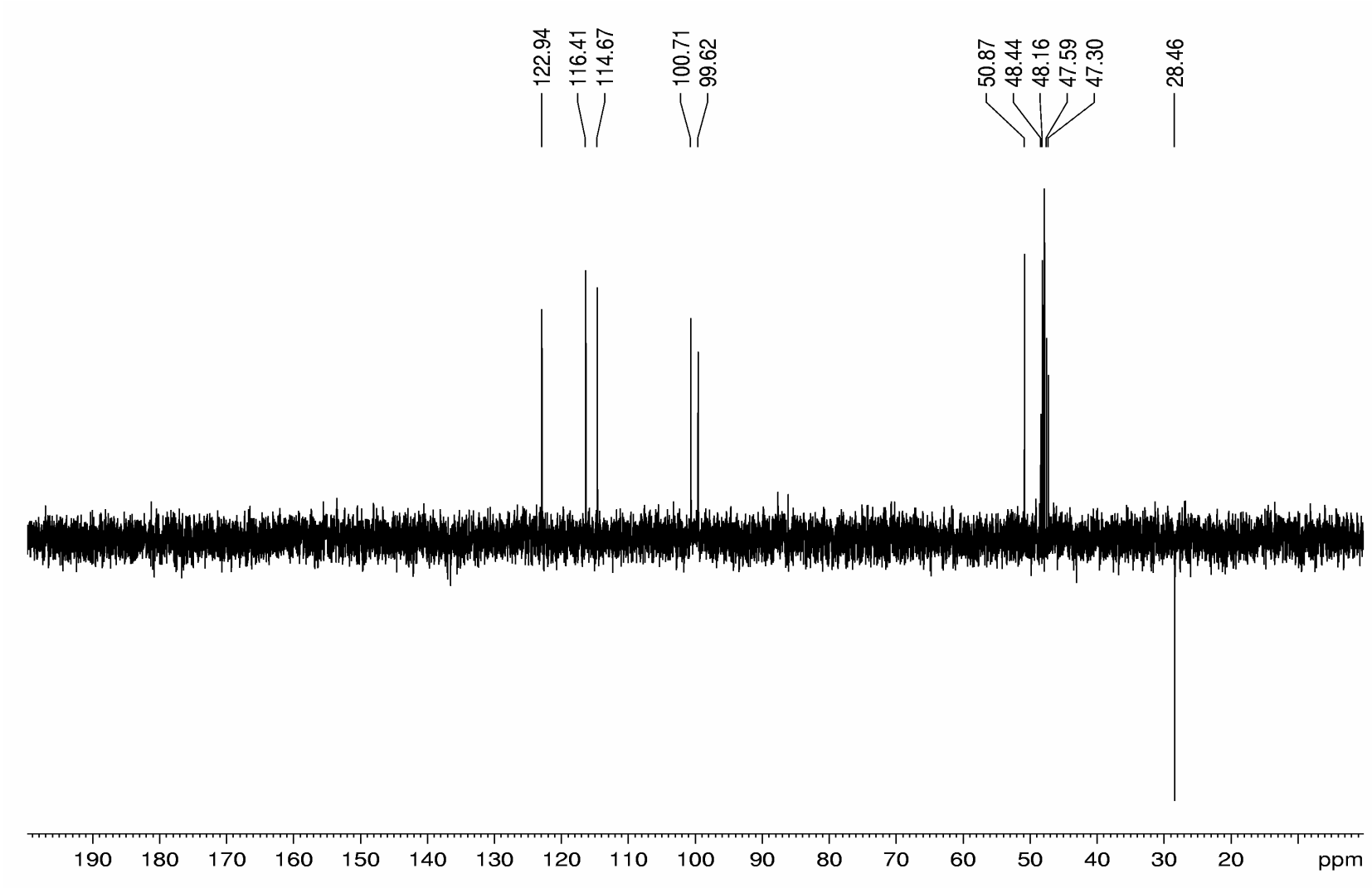


Figure 2.7. DEPT 135 (75.48 MHz) spectrum of jaboticabin in CD_3OD.

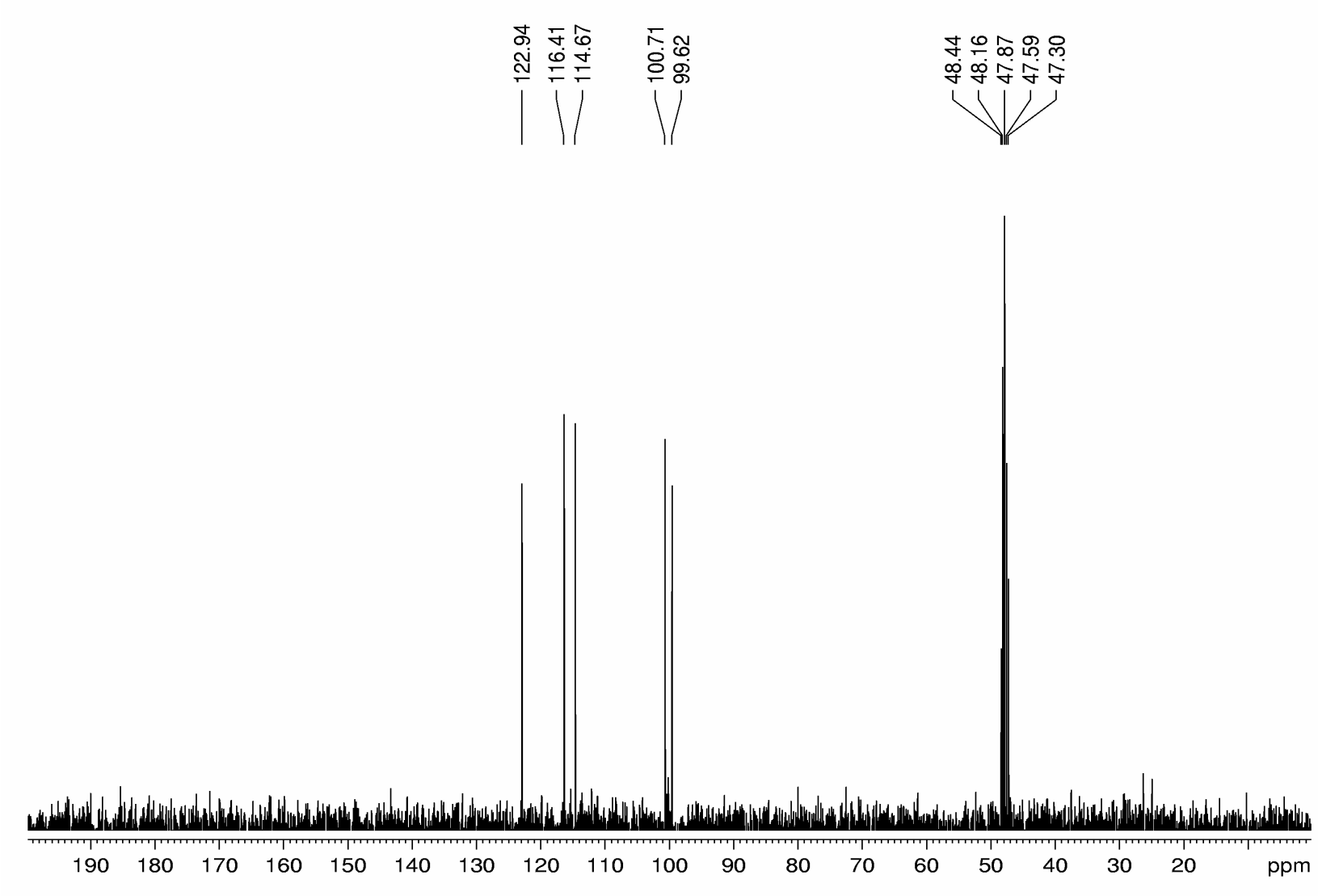


Figure 2.8. DEPT 90 (75.48 MHz) spectrum of jaboticabin in CD_3OD.

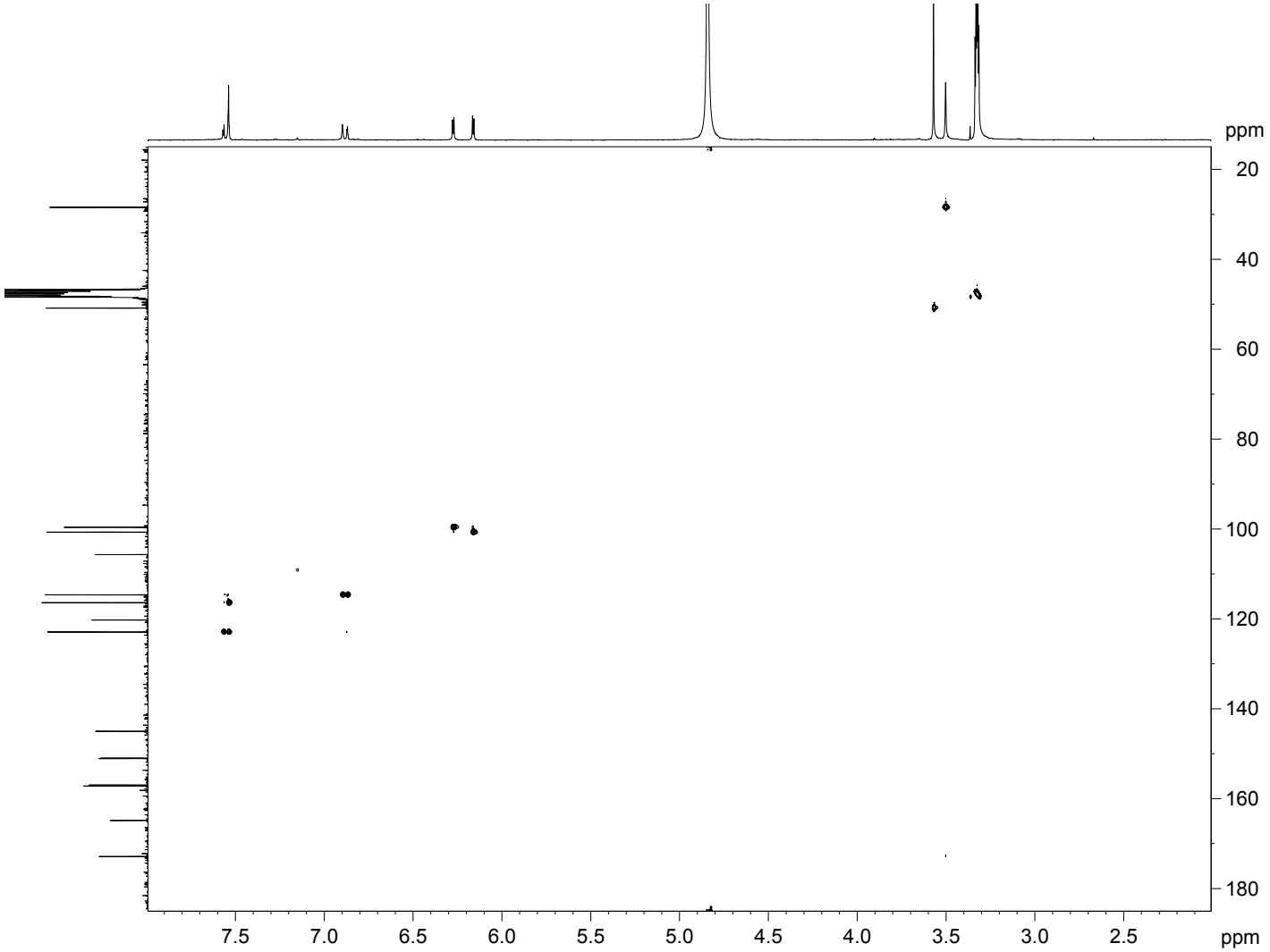


Figure 2.9. HSQC (300.13 MHz) spectrum of jaboticabin in CD_3OD.

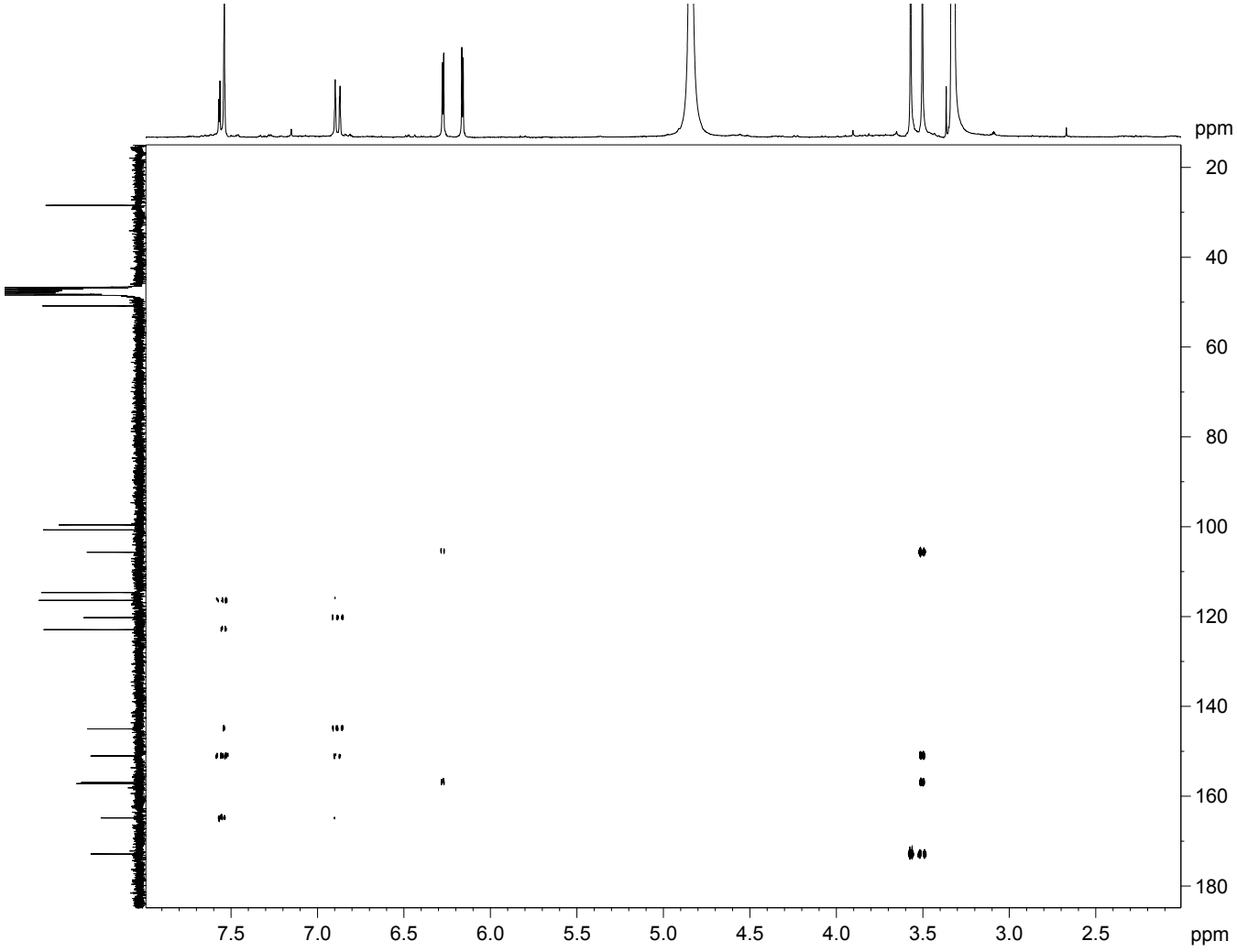


Figure 2.10. HMBC (300.13 MHz) spectrum of jaboticabin in CD_3OD.

Compounds **2** – **5**, pyranocyanin B, methyl protocatechuate, ellagic acid, quercimeritrin, and quercitrin were isolated and identified by comparison of spectroscopic measurements to published literature values [*114, 122-127*]. Quercetin, isoquercitrin, rutin, myricitrin, cinnamic acid, *o*-coumaric acid, and gallic acid were identified by comparison of retention time, UV-Vis, and MS spectral data to authentic standards.

Depsides **1** and **2** exhibited antiradical activity in the DPPH assay, colon cancer cell cytotoxicity, and significantly inhibited chemokine interleukin (IL)-8 production in human small airway epithelial (SAE) cells before and after treatment with cigarette smoke extract (CSE) (Table 2.2). Compound **1** decreased IL-8 production in untreated SAE cells by 81.3%, and decreased production in SAE cells treated with 5% CSE by 47.3%. Compound **2** inhibited IL-8 production by 74.9% in untreated SAE cells and 70.3% in treated SAE cells. Compound **5**, structurally similar to one moiety of the depsides, also inhibited IL-8 production, but not to the same degree as the depsides (40.7% and 45.0% in treated and untreated cells, respectively). This indicates that either the dimeric structure or the phluoroglucinol moiety, with or without the methyl ethanoate group, is important for activity.

The anthocyanins **3** and **4**, major constituents of jaboticaba fruits, also displayed significant activity against IL-8 production in SAE cells. IL-8 was not detected in SAE cells treated with compound **4**, which caused a 96% reduction of IL-8 production in SAE cells treated with CSE; compound **3** inhibited IL-8 production by 65.3% and 36.4%, respectively. Compounds **1** – **5** were more effective at blocking IL-8 production in

untreated SAE cells than catechin, and **2** and **4** were more effective than catechin at blocking cigarette smoke-induced inflammation.

Airway epithelial cells play a crucial role in chronic airway inflammatory diseases, and are an important target for therapeutic intervention [*128*]. IL-8 is a chemotactic cytokine (chemokine) implicated in some cancers and a wide range of chronic inflammatory conditions, including rheumatoid arthritis, and heart and lung diseases [*52, 53, 115, 129*]. IL-8 is a powerful chemoattractant for neutrophils; the recruitment and accumulation neutrophils is a hallmark of inflammation. The ability of compounds **1** – **5** to reduce IL-8 production suggests an important anti-inflammatory action of these compounds. Similar compounds have been shown to inhibit the initiation of related inflammatory pathways, including NF-κB, TNF-α, AP-1, and LTB_4 biosynthesis [*2, 13, 43, 44, 118*]. Chronic obstructive pulmonary disease (COPD) is a complex lung disease characterized by irreversible airflow obstruction due to chronic inflammation, and characterized by an accumulation of neutrophils in lung cells and tissues. COPD includes chronic obstructive bronchiolitis (fibrosis and obstruction of small airways) and emphysema (permanent enlargement of the airspaces distal to the terminal bronchioles accompanied by destruction of lung parenchyma). It has been noted that non-steroidal anti-inflammatories that target chemokine pathways are needed as new therapies for COPD, as it is generally considered steroid-resistant [*51-53*]. The demonstration that jaboticaba depsides, phenolic acids, and anthocyanins can reduce inflammation secondary to smoke exposure could provide a novel therapeutic role for these compounds in COPD.

The cytotoxicity of **1**, **2**, and **4** is comparable to IC_{50} values for 5-fluorouracil (5-FU), a drug used for colon cancer treatment, epigallocatechin gallate (EGCG), and Polyphenon E (Poly E), a standardized decaffeinated green tea extract [*130, 131*] (Table 2.2). Compound **1** is cytotoxic against HT29 colon cancer cells (IC_{50} = 65 μM), and **2** is cytotoxic against HCT116 colon cancer cells (IC_{50} = 30 μM). Consistent with published literature, **4** was more cytotoxic than **3** [*132, 133*]. Compound **4** showed good activity against both the HCT116 and SW480 cell lines (IC_{50} = 12 and 20 μM, respectively), while **3** inhibited 50% cell growth only at the 100 μM range. Pyranocyanin B was not significantly cytotoxic against any of the colon cancer cell lines tested. Compounds **1** – **4** also exhibit good antiradical activity in the DPPH assay (Table 2.2).

The anthocyanins are a group of well-studied phenolic compounds with antioxidant, anti-inflammatory, antimutagenic, and cancer chemopreventative activities [*42*]. In one study, it was shown that UVB-exposed HaCaT keratinocytes pretreated with **3** were protected from UVB-induced inflammation, inhibiting NF-κB and AP-1 activation and IL-8 mRNA expression [*134*].

These experiments demonstrate that jaboticaba anthocyanins and depsides exhibit good antiradical activity, cytotoxicity, and inhibit IL-8 production in both untreated SAE cells and those treated with pro-inflammatory CSE. Depsides from foods and botanicals are less well-studied than the anthocyanins, possibly as a result of their limited distribution in higher plants, and this is the first report of their ability to inhibit IL-8 production and cytotoxicity against colon cancer cells. The jaboticaba is rich in anthocyanins, phenolic acids, flavonoids, and contains depsides with antiradical, anti-inflammatory and cytotoxic activity, and therefore it may be a good candidate for

development in larger-scale agriculture and has the potential to be developed as a functional food.

Table 2.2. Cytotoxicity and antiradical IC_{50} activity

Compound	IC_{50} (μM)				IL-8 inhibition[a]	
	HT29	HCT116	SW480	DPPH	Untreated SAECs	Treated with 5% CSE
1	65	>100	nt[b]	51.4	81.3%	47.3%
2	>100	30	nt	61.8	74.9%	70.3%
3	~100	~70	~100	28.4	65.3%	36.4%
4	~100	12	20	26.3	nd[c]	96.0%
5-FU	46.1	45.1	53.0			
Gallic acid				30.0		
Catechin					nc[d]	60.3%

[a]IL-8 inhibition was measured at the 100 μM level; [b]nt = not tested; [c]nd = no detected; [d]nc = no change).

Materials and Methods

General Experimental Procedures

UV-Vis spectra were measured on a Perkin-Elmer Lambda 35 UV/VIS spectrometer. NMR experiments were collected on a Bruker Avance AV300 NMR spectrometer operating at 300.13 MHz for ^{1}H and 75.48 MHz for ^{13}C using standard Bruker software. Mass spectra were obtained on a ThermoFinnigan LCQ utilizing both ESI and APCI in the positive and negative modes. HRESIMS was performed on a Micromass Q-TOF Ultima mass spectrometer. HPLC was done on a Waters 2695 using a Phenomenex Aqua column (250 x 4.6 mm, 5 μm) and monitored using a Waters 996 PDA scanning from 240 to 600 nm. Column chromatography was accomplished using Sephadex LH-20 (Pharmacia, 25-100 μm), reversed-phase C18 silica gel (J. T. Baker, 40 μm), and Diaion HP-20 (Mitsubishi, Japan). Separations were monitored using silica gel 60 F254 and RP18 F254 TLC plates (1 mm thickness, EM Science, Germany). Quercetin, rutin, cinnamic acid, *o*-coumaric acid, gallic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO). Isoquercitrin and myricitrin were previously isolated in our laboratory [*135*].

Plant Material

Fruits of *M. cauliflora* were collected at the Fruit and Spice Park in Homestead, FL, immediately frozen and shipped by overnight courier on dry ice to the laboratory, where they were kept in cold (-20 °C) dark storage until processed. A voucher specimen (Reynertson 39) was prepared, identified, and deposited at the Steere Herbarium of The New York Botanical Garden (Bronx, NY).

Extraction and Isolation Procedures

Deseeded fresh fruits (6.2 kg) were homogenized in a blender with MeOH, extracted exhaustively and concentrated *in vacuo* at temperatures not exceeding 40 °C to give a thick syrup that was diluted with water. The aqueous solution was separated over Diaion HP-20 and eluted using H_2O, MeOH, and acetone. The MeOH fraction was concentrated to give a residue (52 g). A portion (44 g) of that residue was subjected to Sephadex LH-20 column chromatography (~125 g) in amounts of 8, 11, 12, and 13 g and eluted with formic acid-water-MeOH (1:9:10). Fractions from all four columns were recombined to give 8 fractions (A-H). Fractions D (3.74 g) and E (111 mg) were chromatographed over Sephadex LH-20 and eluted using MeOH-formic acid (9:1). The recombined fraction A1 was then separated in a smaller Sephadex LH-20 column (12 g) using an isocratic system of acetonitrile-water. Fractions $A1_{35\text{-}59}$ were recombined as fraction A2 and subjected to reversed-phase C18 column chromatography (6 g) using 10% formic acid-acetonitrile (95:5 to 50:50; 5% gradient; 20 mL each eluant, fractions of 3 mL). Fractions $A2_{12\text{-}27}$ were recombined and subjected to final purification using Sephadex LH-20 and water-acetonitrile to give 16 mg of **1**. Compound **2** was isolated according to a similar scheme to give 10 mg.

Approximately 50 g of freeze-dried fruits were extracted in EtOH and subjected to LC-MS SIM analysis to address concerns that **1** might be a methyl ester artifact of **2** following extraction in MeOH. Analysis was performed in negative ESI mode, using a gradient of 0.1% formic acid (A) and acetonitrile (B) from 95% A to 50% A over 30 min, monitoring $[M - H]^-$ *m/z* 332 to 334.

Jaboticabin (methyl 2-[(3,4-dihydroxybenzoyloxy)-4,6-dihydroxyphenyl]acetate, 1)

Reddish amorphous solid (MeOH); UV (MeOH) λ_{max} (log ε) 267.5 (3.35), 298.5 (3.16) nm; ^{1}H NMR (CD_3OD, 300.13 MHz) δ 7.55 (1H, dd, J = 2.1, 7.8 Hz, H-6′), 7.54 (1H, d, J = 2.1 Hz, H-2′), 6.88 (1H, d, J = 7.8 Hz, H-5′), 6.27 (1H, d, J = 2.4 Hz, H-5), 6.16 (1H, d, J = 2.4 Hz, H-3), 3.57 (3H, s, OCH_3-8), 3.50 (2H, s, H-7); ^{13}C NMR (CD_3OD, 75.48 MHz) δ 172.9 (C, C-8), 164.9 (C, C-7′), 166.4 (C-2′), 157.2 (C, C-4), 157.0 (C, C-2), 151.1 (C, C-6), 151.0 (C, C-4′), 145.0 (C, C-3′), 122.9 (CH, C-6′), 120.3 (C, C-1′), 114.7 (CH, C-5′), 105.7 (C, C-1), 100.7 (CH, C-5), 99.6 (CH, C-3), 50.9 (OCH_3-8), 28.5 (CH_2, C-7); ESIMS *m/z* 333 $[M - H]^-$ ($C_{16}H_{14}O_8$), HRESIMS *m/z* 357.0581 $[M + Na]^+$ (calcd for $C_{16}H_{14}O_8Na$, 357.0586).

DPPH Antiradical Assay

The DPPH assay was performed on extracts, fractions, and purified compounds and described in detail in Appendix B. Briefly, test material was resuspended in DMSO, and ethanolic DPPH (400 μM) was used in the reaction mixture. Serial dilutions were combined with the DPPH solution in a 96-well microtiter plate and incubated for 30 min at 37 ºC. The change in absorbance at 517 nm was measured to calculate a DPPH IC_{50}. DMSO was used as a negative control and gallic acid was used as a positive control (IC_{50} = 30.0 ± 2.9 μM).

IL-8 Immunoassay

Human SAE cells were cultured according to supplier instructions (Clonetics, CA) and maintained in a controlled atmosphere of air-5% CO_2 at 37 °C. Confluent SAE cells at passages 4-8 were used for experiments.

Cigarette smoke extract (CSE) was prepared using a modified protocol [*136*]. Briefly, a Barnet vacuum pump operating at constant flow was used to draw the smoke of one unfiltered 2R1 reference cigarette (University of Kentucky) through 25 mL of Dulbecco's phosphate-buffered saline. This solution (100% CSE) was adjusted to pH 7.4, filtered, diluted with small airway growth medium to a final concentration of 5%, and added to the cells immediately.

Cells were treated with 5% CSE or pure compounds (100 μM), or pretreated with pure compounds 30 min prior to 5% CSE exposure. After 24 h, measurement of human IL-8 in cell culture supernates was performed by ELISA (R&D Systems Inc., MN). Statistical analyses were performed by Student's t-test (two-sided) using the JMP Statistics software package (SAS Institute Inc., NC) and defined at the 5% level.

Cytotoxicity Assays

Colon cancer cell lines HT29, HCT116, and SW480 (10,000 cells) were plated into 24-well plates in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). After 24 h, cells were treated with six concentrations (1, 5, 10, 30, 50, and 70 μM) of compounds and incubated for 72 h under DMEM containing 1% FBS. The plates were washed with PBS once and the attached cells were collected by tripsinization. The numbers of cells were counted using a Coulter Counter (Beckman Coulter Co., CA) as previously described [*137*]. EGCG and Poly E were used as positive controls (HT29 IC_{50} = 27 μg/mL and 22 μg/mL, respectively).

Chapter Three:
Quantitative Analysis of Antiradical Phenolic Constituents of 14 Edible Myrtaceae Fruits

Introduction

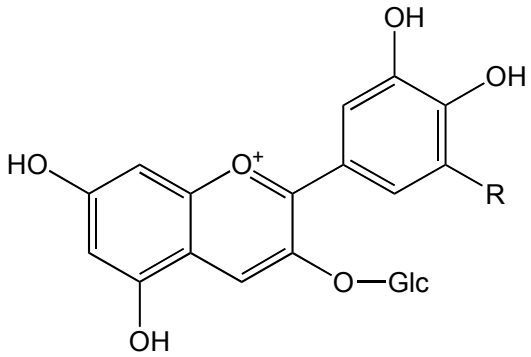
Quantitative studies investigating the phenolic content and antioxidant potential of edible fruits are useful in understanding the role that these factors play in health and disease chemoprevention. With over half the deaths in the United States attributable to heart disease and cancer [1], and the continued evidence that plant-based diets are inversely correlated with these diseases [*6, 9-12, 138, 139*], a comprehensive understanding of the phytochemicals in edible fruits and vegetables is crucial to developing better diets and healthier lives. The biological damage from inflammation and oxidative assault is cumulative, and incidence of disease increases over a lifetime. A measure of protection is provided by diets high in foods that supply beneficial phytochemicals. While the benefits of many popular foods and beverages (i.e. green tea [*25, 44, 48, 49*] and wine [*2, 15, 31, 140*]) have been documented, there are thousands of "underutilized" fruits and vegetables that may confer as good or better chemoprevention than those well-studied foods.

The plant family Myrtaceae is pan-tropical, with concentrations in South America, Southeast Asia, and Australia. The fleshy-fruited subfamily Myrtoideae

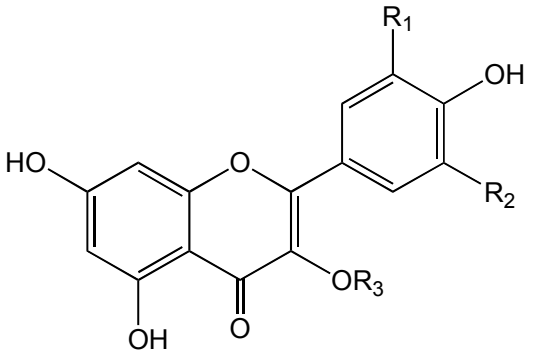
includes many economically important food plants, agricultural crops, and ornamentals, specifically the genus *Myrtus* (myrtle), spices such as clove (*Syzygium aromaticum*), allspice (*Pimenta dioica*), and bay rum (*Pimenta racemosa*), and the fruits of *Psidium* (guavas). Recently, the camu-camu berry (*Myrciaria dubia*) has become important as a functional food, and can be found on the shelves of many health food stores as a dietary supplement due to the high levels of ascorbic acid in the fruit. The subfamily Myrtoideae is large, however, and produces many fruits with a potential for agricultural development and NTFP projects in tropical areas.

Many edible Myrtaceae species are cultivated by rare fruit enthusiasts in the continental United States. This chapter describes the analysis of 14 edible fruits from 13 species of *Myrciaria*, *Eugenia*, and *Syzygium*: *Eugenia aggregata*, *E. brasiliensis*, *E. luschnathiana*, *E. reinwardtiana*, *Myrciaria cauliflora*, *M. dubia*, *M. vexator*, *Syzygium cumini*, *S. curranii*, *S. jambos*, *S. javanicum*, *S. malaccense*, *S. samarangense*, and *S. samarangense* var. *Taiwan pink*.

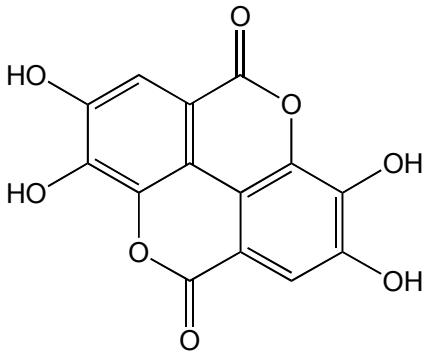
Eight phenolic compounds, cyanidin 3-glucoside, delphinidin 3-glucoside, ellagic acid, kaempferol, myricetin, quercetin, quercitrin, and rutin (Figure 3.1) have been quantified by HPLC-PDA. In addition, the total phenolic content (TPC) and total anthocyanin content (TAC) were determined, and each extract was evaluated for antiradical activity using the DPPH assay.



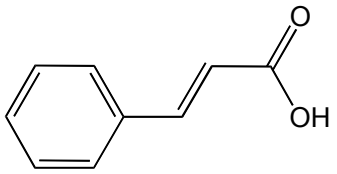
(1) Cyanidin 3-glucoside: R = H
(2) Delphinidin 3-glucoside: R = OH



(4) Kaempferol: R_1 = H; R_2 = H R_3 = H
(5) Myricitrin: R_1 = OH; R_2 = OH R_3 = rhamnose
(6) Quercetin: R_1 = OH; R_2 = H R_3 = H
(7) Quercitrin: R_1 = OH; R_2 = H R_3 = rhamnose
(8) Rutin: R_1 = OH; R_2 = H R_3 = rutinoside



(3) Ellagic acid



(9) Cinnamic acid

Figure 3.1. Compounds quantified (1-8) or identified (9) in the 14 edible Myrtaceae fruits.

To the best of our knowledge, the phenolic constituents of *Eugenia brasiliensis*, *E. luschnathiana*, *E. reinwardtiana*, *Myrciaria vexator*, *Syzygium curranii*, *S. javanicum*, and *S. samarangense* var. *Taiwan pink* have not been reported in the literature, despite their widespread consumption in the tropics. Table 3.1 summarizes the literature with an emphasis on the phenolic constituents of the fruits. The leaves in this taxon are typically fragrant, and most of the published phytochemical work on these species concerns those aromatic terpenoids [*28, 100, 101, 104*].

The species tested are mostly red to purple drupes 2-4 cm in diameter, although some species produce larger or less pigmented fruit. These species are often cultivated in home gardens throughout the tropical world, and can often be found in local markets. They are primarily eaten fresh, and can be used to make jams, desserts, wines, liquors, and vinegars. In addition to their use as food, many of these fruits have been used in divergent traditional medical practices for a variety of illnesses and conditions. Most notably, the seeds of the jamun (*S. cumini*) are an important Ayurvedic medicine for diabetes. The rose apple (*S. jambos*) has been used in India as a tonic for the brain and for liver problems, as an astringent, and digestive [*86*], and distilled to make rosewater [*75*]. In Brazil, *E. brasiliensis* leaves have been used for gastrointestinal disorders and rheumatism [*75*], and the jaboticaba fruit (*M. cauliflora*) has been used as a treatment for hemoptysis, asthma, diarrhea, and chronic inflammation of the tonsils. Other related Myrtoideae fruits not analyzed here have been used for several inflammatory conditions, including sore throat, high blood pressure, ringworm, and as an antimicrobial, antiscorbutic, carminative, diuretic, and astringent [*68, 75, 83, 84, 89, 141*].

Table 3.1. The fruit color, vernacular name, and previously reported phenolic constituents of selected edible Myrtaceae fruits

Species	Vernacular Name	Fruit Color	Know Phenolic Content
Eugenia aggregata	Cherry of the Rio Grande	Reddish-purple	Catechin and epicatechin [*90*]
E. brasiliensis	Grumixama	Purple	ND[a]
E. luschnathiana	Pitanga	Yellow	ND
E. reinwardtiana	Australian Beach Cherry	Red	ND
Myrciaria cauliflora	Jaboticaba	Purple	Tannins [*75*]; several flavonols, phenolic acids, anthocyanins, and depsides, pyranocyanin B, and ellagic acid [*92*]
M. dubia	Camu-camu	Reddish	Cyanidin 3-glucoside, delphinidin 3-glucoside [*93*] terpenoids [*94*]
M. vexator	Blue grape	Purple	ND
Syzygium cumini	Jamun	Purple	Several flavonoids, ellagitannins and phenolic acids have been identified from fruits, seeds and aerial parts [*97-99*]
S. curranii	Lipote	Purple	ND
S. jambos	Rose apple	Yellow	ND
S. javanicum	Java apple	Reddish	ND
S. malaccense	Malay apple	Reddish	ND
S. samarangense	Wax jambu	Pink to red	Several flavonoids and ellagitannins [*71, 106-108*]
S. samarangense var. *Taiwan pink*	Wax jambu	Red	ND

[a]ND = no phenolic literature was found.

Results and Discussion

Dark-colored fruits have generated considerable interest recently as a rich source of phenolic antioxidants. Blueberries, cranberries, strawberries, grapes, cherries, and other temperate fruits have been the subject of many studies and analyses [*124, 142-144*]. In this report, we have analyzed and quantified the concentration of several antioxidative and anti-inflammatory flavonols, phenolic acids, and anthocyanins from 14 underutilized Myrtaceae fruits. The anthocyanins, strong antioxidants and anti-inflammatories, with antimutagenic and cancer chemopreventative activities [*42, 92*], are the most abundant compounds among those quantified and are largely responsible for the bright colors of these fruits.

Total Phenolic Content, Total Anthocyanin Content and Antiradical Activity

Many of the fruits analyzed are high in both total phenolics and anthocyanins. Results for TPC, TAC, and antiradical activity are displayed in Table 3.2. The TPC for most species fell within the range of 3.57 (*S. javanicum*) to 44.12 mg GAE/g (*M. vexator*). The TPC for *M. dubia* (camu-camu), however, is over double the TPC for *M. vexator*, and is an outlier at 101.17 mg GAE/g. This may be due to interference from ascorbic acid [*145*], as the camu-camu berry is known to contain one of the highest levels of ascorbic acid of any fruit [*146*]. TAC values range from undetected (*E. luschnathiana* and *S. jambos*) to 12.13 mg/g equivalents (*S. curranii*). Antiradical activity ranges from very active (*M. cauliflora*, 19.40 μg/mL) to inactive (388.69 μg/mL, *S. cumini*). *M. cauliflora* is the most active fruit extract in the DPPH assay, with only a slightly higher

than average TPC (31.63 mg GAE/g) and TAC (2.78 mg/g). *S. currani* is the highest in anthocyanin content according to both the TAC method and HPLC method. In our analysis, TAC did not correlate strongly with antiradical activity ($\gamma^2 = 0.1147$), possibly because the anthocyanins make up only a portion of the overall phenolic profile. TPC was more closely correlated with antiradical activity, but did not reach significance ($\gamma^2 = 0.4542$). Inspection of the UV-Vis and mass spectra of unidentified peaks in the chromatograms indicates that many of these species contain ellagitannins and flavonoids that may influence both the TPC and antioxidant activity.

Table 3.2. Results of antiradical DPPH assay (IC_{50} in μg/mL), TAC (mg C3G/g dry weight), and TPC (mg GAE/g dry weight)

	DPPH	TAC	TPC
E. aggregata	84.64 ± 2.63	1.26 ± 0.45	25.34 ± 1.54
E. brasiliensis	42.73 ± 5.92	8.37 ± 0.23	24.80 ± 1.06
E. luschnathiana	38.01 ± 4.86	nd[a]	21.99 ± 0.21
E. reinwardtiana	109.75 ± 8.31	0.08 ± 0.03	9.25 ± 0.78
M. cauliflora	19.40 ± 0.28	2.78 ± 0.17	31.63 ± 0.39
M. dubia	57.19 ± 5.61	tr[b]	101.17 ± 0.25
M. vexator	38.64 ± 2.40	6.84 ± 0.36	44.12 ± 1.21
S. cumini	388.69 ± 35.98	6.33 ± 0.10	9.95 ± 1.26
S. curranii	33.42 ± 2.52	12.13 ± 0.53	39.63 ± 0.77
S. jambos	91.99 ± 8.24	nd	8.69 ± 0.57
S. javanicum	81.39 ± 6.24	$0.09 \pm 4.0 \times 10^{-3}$	3.57 ± 0.24
S. malaccense	269.28 ± 7.66	tr	8.58 ± 0.12
S. samarangense	77.51 ± 4.19	0.07 ± 0.03	18.04 ± 0.70
S. samarangense var. *Taiwan pink*	157.29 ± 13.02	1.35 ± 0.10	23.84 ± 0.30

[a]nd = none detected; [a]tr = trace (< 0.1mg/g).

Standard Curves, Limits of Detection (LOD) and Quantification (LOQ), and Recovery

All standard curves had a correlation coefficient (γ^2) value > 0.9998. The minimum LOD and minimum LOQ for each compound are given in Table 3.3. *Syzygium malaccense* was chosen as a representative species for the recovery experiment because the experimental matrix being analyzed (lyophilized, deseeded fruit) was the same for each species. When using a relatively small amount of material (1 g), the constituents of *S. malaccense* were not sufficiently concentrated to interfere with recovery analysis. Using the same analytical methods as the HPLC-PDA quantification, it was determined that the average recovery of cyanidin 3-glucoside was 113.2 ± 12.6%; ellagic acid was 80.1 ± 9.5%; and rutin was 96.1 ± 9.6%.

Table 3.3. Minimum limits of detection and quantification, retention time and mass fragment of each standard compound

	LOQ (ng)	LOD (ng)	Rt (HPLC-PDA)	$[M - H]^-$
cyanidin 3-glucoside[a]	10.0	3.0	13.7	447[b]
ellagic acid	8.3	2.5	18.8	609
kaempferol	11.0	4.0	37.9	285
myricetin	17.8	4.5	29.2	317
quercetin	16.4	4.5	22.9	447
quercitrin	[c]	[c]	21.5	463
rutin	25.0	8.0	19.5	301

[a]Cyanidin 3-glucoside was quantified at 520 nm, all others were quantified at 254 nm. [b]$[M - 2H]^-$. [c]The LOD and LOQ were not determined for quercitrin.

HPLC-PDA Quantification

Chromatograms of the standard mixture and the 14 fruit extracts are presented in Figure 2. In our HPLC analysis, cyanidin 3-glucoside (**1**) is the most abundant compound in *E. aggregata*, *E. brasiliensis*, *M. cauliflora*, *M. vexator*, *S. curranii*, *S. malaccense*, and *S. samarangense* var. *Taiwan pink* (Table 3.4). It was detected or quantified in all but the two species that do not produce fruit colored dark purple or red; the orange fruit of *E. luschnathiana* and yellow fruit of *S. jambos* have no detectable anthocyanins by HPLC-PDA or TAC measurement. *Eugenia brasiliensis*, *M. vexator*, and *S. curranii* have far greater levels of **1** (10.24, 13.13, and 9.56 mg/g, respectively) than the next-highest species (*M. cauliflora*, 4.33 mg/g). The purple-skinned fruits *E. brasiliensis*, *E. aggregata*, *M. cauliflora*, *M. vexator*, *S. curranii*, and *S. cumini* contain both **1** and delphinidin 3-glucoside (**2**) and are unsurprisingly the highest in TAC (Table 3.2). The only reddish fruit that contains both **1** and **2** is *M. dubia*; the remaining red to pink fruits *E. reinwardtiana*, *S. malaccense*, *S. samarangense*, and *S. samarangense* var. *Taiwan pink* contain only **1**. *Syzygium curranii* has far higher levels of delphinidin 3-glucoside than any other fruit in the analysis (4.83 mg/g). *Syzygium cumini*, which is used by Ayurvedic practitioners in India as an antidiabetic medicine [*147*], has more **2** than **1** based on the HPLC analysis, and the HPLC chromatogram indicates that the fruit contains several delphinidin glycosides. Compound **2** is a potent antiradical compound (DPPH IC_{50} = 26.3 μM), so it is remarkable that *S. cumini* fruit pulp is essentially inactive in the DPPH assay. However, while the fruit may contain many delphinidin glycosides, the TPC (9.95 mg/g) was below average for the fourteen fruits tested, and

comparable with other fruits with low antiradical activity (IC_{50} > 100). Seed extracts of *S. cumini*, the part most often used in Ayurvedic medicine, were previously shown to have high levels of total phenolics and good activity in the Trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) antioxidant assays [*148*].

Quercetin (**6**) and its glycosides are prevalent in the fourteen study fruits. These flavonols are important dietary constituents, with many chemopreventive activities [*27*]. The concentration of **6** is < 0.01 mg/g only in *S. malaccense*. *Syzygium curranii* and *M. dubia* had levels of **6** approximately six times higher (0.28 and 0.24 mg/g, respectively) than the other test fruits. Quercitrin (**7)** was also detected in every species, from < 0.01 mg/g (*S. cumini*) to 0.18 mg/g (*S. samarangense* var. *Taiwan pink*). *Syzygium curranii* has levels of **7** (0.17 mg/g) similar to *S. samarangense* var. *Taiwan pink*. Rutin (**8**), detected in every fruit but *S. jambos*, was found at the highest levels in *E. aggregata* (0.48 mg/g).

Ellagic acid (**3**) and its derivatives are also abundant in this taxon [*108, 149*]. It is likely that ellagic acid derivatives are present in many of these extracts, and contribute considerably to the overall TPC and antiradical activity. In our analysis, **3** was identified and quantified. The three *Myrciaria* species contain the highest levels of **3** (*M. cauliflora*, 0.52; *M. dubia*, 0.45; and *M. vexator*, 0.64 mg/g). It was found in every species except *E. aggregata*, *E. reinwardtiana*, and *S. samarangense* var. *Taiwan pink*. Generally, it was the most abundant compound by HPLC-PDA analysis in the red, orange, and yellow-fruited species (*E. luschnathiana*, *S. javanicum*, *S. jambos*, *S. samarangense*, and *M. dubia*).

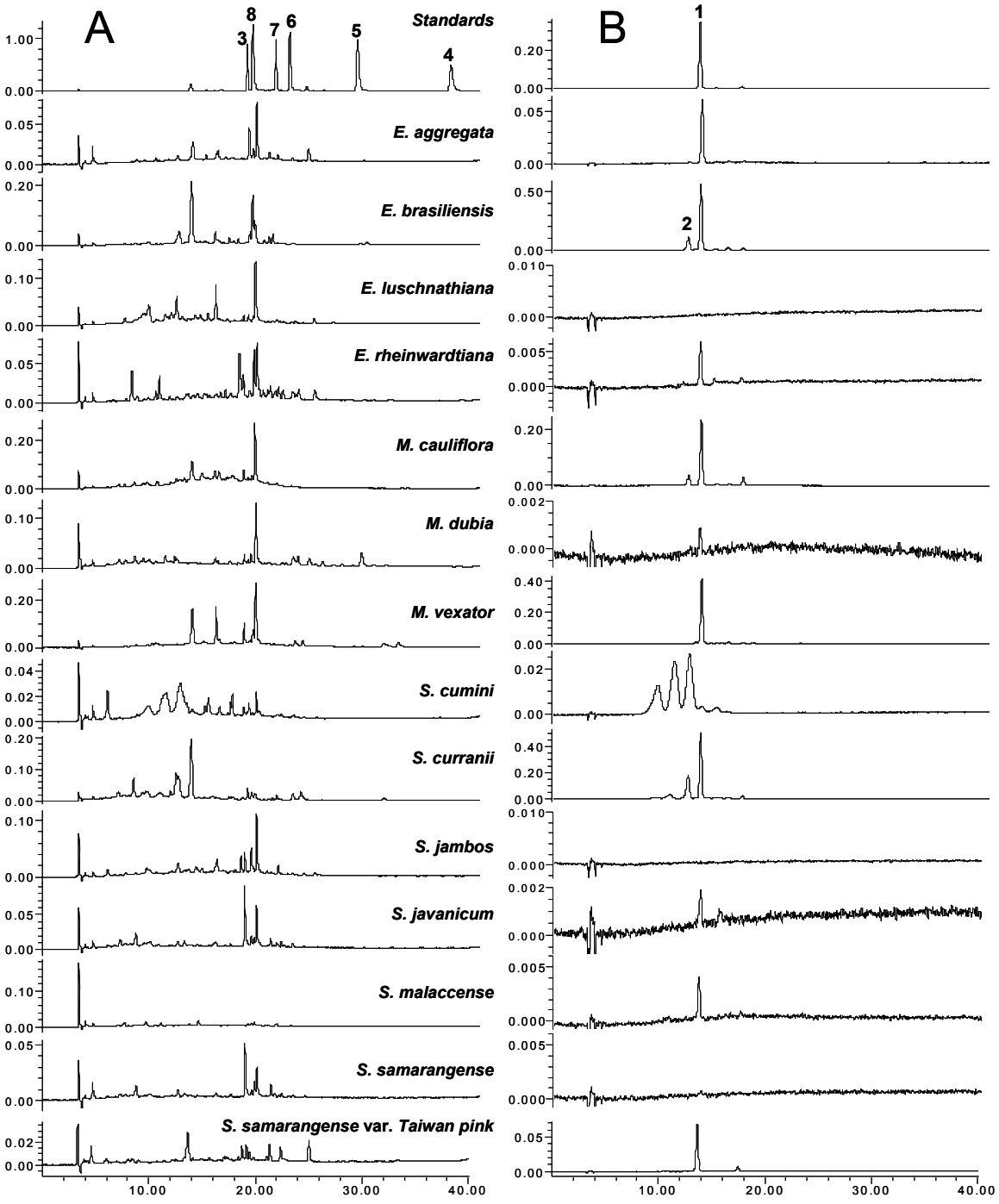


Figure 3.2. HPLC-PDA chromatograms at (A) 254 nm and (B) 520 nm of the standard mixture and methanolic extracts of 14 edible Myrtaceae fruits. Compounds are numbered as follows: cyanidin 3-glucoside (1**), delphinidin 3-glucoside (**2**), ellagic acid (**3**), kaempferol (**4**), myricetin (**5**), quercetin (**6**), quercitrin (**7**), and rutin (**8**).**

Myricetin (**5**) was found in each of the *Eugenia* and *Myrciaria* species tested but *M. dubia*, while among the *Syzygium* species, it was detected only in *S. curranii* and *S. samarangense* at levels below 0.01 mg/g. Kaempferol (**4**) was detected in *E. brasiliensis*, *E. luschnathiana*, and *S. curranii*, but at levels below 0.01 mg/g. *M. cauliflora*, *S. malaccense*, and *S. jambos* were also found to contain *t*-cinnamic acid (**9)** by HPLC-PDA, but it was not quantified.

With cancers and heart disease the leading causes of death in the United States, studies indicate that diets high in naturally occurring antioxidants and anti-inflammatories are important as a first-line strategy of chemoprevention [*3-6*]. The evolution and development of the human species has primarily occurred in parallel with a hunter-gatherer diet, one that is high in fruits and vegetables and low in saturated fat. It is estimated that 65 to 70% of the Paleolithic diet was plant-based. This diet was especially high in lean protein, unsaturated fats, fiber, vitamins, minerals, antioxidants, and other beneficial phytochemicals [*7*]. Physiologically, we are nearly identical to our Paleolithic ancestors, and it has been suggested that the Paleolithic diet might be considered a paradigm or standard for modern humans from an evolutionary perspective [*150*].

This project has demonstrated that edible fruits in the Myrtaceae are a rich source of biologically active phenolic compounds, similar to other well-studied berries and fruits [*144*]. Anthocyanins, flavonoids, phenolic acids, and tannins primarily play ecological roles to attract pollinators and seed disbursers, and act as antifeedants and defense compounds against microbial infection [*2*]. Myrtaceae fruits are regularly grown and consumed in tropical parts of the world, and this report suggests that many of these fruits

have the potential for development as functional foods. Tropical fruits grow under conditions of high oxidative stress from intense sunlight and heat, and these phenolic compounds inhibit lipid peroxidation and ultraviolet damage in plant tissues [*32*] in addition to protecting mammalian systems. Most of these species can be grown in southern Florida, California, and Hawaii, where much of the produce supplied to the rest of the continental United States is grown. The camu-camu is currently marketed as a dietary supplement, and many products are already on the market. It primarily grows in flooded plains in the Peruvian Amazonian Basin, and may be a valuable non-timber forest product for local communities. *Syzygium curranii*, a fruit native to the Philippines, is of particular interest, as it has high levels of many of the phenolics tested. The DPPH antiradical activity of the fruit extract is half that of pure ascorbic acid, and the TPC is higher than most of the fruits tested, comparable with many common fruits and berries [*37, 144*].

Table 3.4. Quantification of common phenolics in 14 edible Myrtaceae fruit determined by HPLC-PDA

	Cyanidin 3-glc	Delphinidin 3-glc[a]	Ellagic acid	Myricetin	Quercetin	Quercitrin	Rutin
E. aggregata	1.42 ± 0.17[b]	0.04 ± 4.7 x 10^{-3}	ND[c]	0.04 ± 3.6 x 10^{-3}	0.04 ± 4.3 x 10^{-3}	0.06 ± 7.8 x 10^{-3}	0.48 ± 0.04
E. brasiliensis	10.24 ± 0.42	2.58 ± 0.13	0.26 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
E. luschnathiana	ND	ND	0.40 ± 0.01	Tr[d]	0.04 ± 2.98 x 10^{-3}	0.07 ± 2.31 x 10^{-3}	0.18 ± 0.01
E. reinwardtiana	0.04 ± 2.2 x 10^{-3}	ND	ND	0.01 ± 0.7 x 10^{-3}	0.03 ± 0.6 x 10^{-3}	0.05 ± 5.6 x 10^{-3}	0.04 ± 5.6 x 10^{-3}
M. cauliflora	4.33 ± 0.24	0.81 ± 0.06	0.52 ± 0.22	0.02 ± 2.6 x 10^{-3}	0.04 ± 7.6 x 10^{-3}	0.11 ± 0.03	0.21 ± 0.02
M. dubia	0.02 ± 3.2 x 10^{-3}	Tr	0.45 ± 0.04	ND	0.24 ± 0.02	0.06 ± 7.2 x 10^{-3}	0.13 ± 7.0 x 10^{-3}
M. vexator	13.13 ± 3.17	0.29 ± 0.06	0.64 ± 0.26	0.03 ± 4.4 x 10^{-3}	0.08 ± 0.02	0.05 ± 0.02	0.11 ± 0.02
S. cumini	0.14 ± 0.02	1.61 ± 0.50	0.03 ± 6.2 x 10^{-3}	ND	0.01 ± 8.0 x 10^{-3}	Tr	0.13 ± 0.02
S. curranii	9.56 ± 0.84	4.83 ± 0.41	0.09 ± 0.01	Tr	0.28 ± 9.0 x 10^{-3}	0.17 ± 0.02	0.32 ± 0.01
S. jambos	ND	ND	0.05 ± 0.02	ND	0.01 ± 0.3 x 10^{-3}	0.03 ± 4.0 ± x 10^{-3}	ND
S. javanicum	0.01 ± 1.4 x 10^{-3}	ND	0.05 ± 0.02	ND	0.01 ± 2.2 x 10^{-3}	0.02 ± 0.9 x 10^{-3}	Tr
S. malaccense	0.02 ± 1.5 x 10^{-3}	ND	0.01 ± 1.7 x 10^{-3}	ND	Tr	0.02 ± 0.01	0.02 ± 1.4 x 10^{-3}
S. samarangense	Tr	ND	0.09 ± 0.01	Tr	0.02 ± 2.2 x 10^{-3}	0.02 ± 6.0 x 10^{-3}	Tr
S. samarangense var. *Taiwan pink*	1.56 ± 0.10	ND	ND	ND	0.07 ± 0.02	0.18 ± 6.6 x 10^{-3}	0.16 ± 0.02

[a]Amount of compound per dry weight of plant material expressed as mg/g; [b]mean ± SD (n = 6). [c]nd, not detected; [d]tr, amount was less than 0.01 mg/g.

Materials and Methods

General Experimental Procedures

HPLC was performed on a Waters Alliance 2695 using a Phenomenex Aqua column (250 x 4.6 mm, 5 μm) and monitored using a Waters 996 PDA scanning from 220 to 600 nm. UV-Vis spectra for total anthocyanin content [*96*] measurements were determined using a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer. A Molecular Devices Versamax tunable absorbance detector was used for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) antiradical assay and total phenolic content measurements (TPC). Quercetin, rutin, quercitrin, kaempferol, myricetin, ellagic acid, DPPH, and 2N Folin-Ciocalteu reagent (FCR) were purchased from Sigma. Cyanidin 3-glucoside and delphinidin 3-glucoside were isolated from *Myrciaria cauliflora*; purity was established by HPLC-PDA, LC-MS, and NMR analysis [*92*].

Plant Material

Ripe fruits of *Eugenia aggregata*, *E. brasiliensis*, *E. luschnathiana*, *E. reinwardtiana*, *Myrciaria cauliflora*, *M. vexator*, *Syzygium cumini*, *S. curranii*, *S. jambos*, *S. javanicum*, *S. malaccense*, *S. samarangense*, and *S. samarangense* var. *Taiwan pink* were collected at the Fruit and Spice Park (Homestead, FL), Rare Fruit and Vegetable Council of Broward County experimental garden (FL), The Kampong (Coconut Grove, FL), and the University of Florida Tropical Resource and Education Center (Homestead, FL). Fruits were frozen and shipped by overnight courier on dry ice to the laboratory, where they were kept in cold (-20 °C) dark storage until processed. Voucher specimens were prepared, identified, and deposited at the Steere Herbarium of The New York

Botanical Garden (Bronx, NY). Dried, powdered camu-camu fruit (*M. dubia*) was supplied by Essential Living Foods (Los Angeles, CA).

Extraction and Sample Preparation

Approximately 10 g of freeze-dried fruit was extracted three times in approximately 200 mL MeOH with 1% formic acid for 1 h each in a sonicator. Extracts were combined and concentrated under reduced pressure at temperatures not exceeding 40 ºC and brought up to 100 mL total. Aliquots (2 to 4 mL) were prepared for each experiment and stored at – 20 ºC until analyzed. Aliquots analyzed by HPLC and the DPPH antiradical assay were dried, resuspended in 1 to 2 mL water, and subjected to solid phase extraction (SPE), eluting sequentially with water and MeOH. The MeOH fractions were collected, dried, and dissolved in DMSO for the DPPH assay or MeOH-formic acid (9:1) for HPLC analysis.

Quantitative HPLC-PDA Analysis

Following SPE, two aliquots of each fruit extract were analyzed in triplicate (n = 6). HPLC-PDA analysis was performed using a gradient solvent system of 1% formic acid (A) and acetonitrile (B) as follows: 90% to 75% A over 30 min; 75% to 40% from 30 to 45 min at a flow rate of 1 mL/min. Results were monitored from 210 to 600 nm. Sample concentrations ranged from 1.5 to 7 mg/mL. Peak area for each compound quantified in the test samples was integrated from the HPLC-PDA chromatogram using Waters Empower software and the concentration was calculated using the equation for each standard curve.

Standard Curves, Limits of Detection and Quantification, Recovery

Stock solutions of cyanidin 3-glucoside, ellagic acid, rutin, quercitrin, quercetin, myricetin, and kaempferol were prepared in MeOH with 10% formic acid, and combined to make a standard mixture. This stock mixture was diluted to make six standard solutions (ranging from 0.95 to 887.5 μg/mL) for HPLC-PDA quantification. Peak area for each standard was integrated from HPLC-PDA chromatograms at 520 nm for the anthocyanins and 254 nm for all other compounds using Waters Empower software and plotted against concentration to create a linear curve. Delphinidin 3-glucoside was quantified based on cyanidin 3-glucoside equivalents.

Minimum limits of detection (LOD) at a signal-to-noise (S/N) ratio of 3:1 and minimum levels of quantification (LOQ) at a S/N ratio of 10:1 were determined experimentally for the standard compounds (Table 3.3).

The percent recovery for the analytical method was calculated for three representative compounds (cyanidin 3-glucoside, ellagic acid, and rutin). Approximately 0.1 mg of each compound was added to 1 g of freeze-dried *S. malaccense* fruit, and the material was extracted, prepared, and analyzed following the same method used for HPLC-PDA quantification.

Total Phenolic Content

Analysis was performed by the Folin-Ciocalteu method [*37, 151*]. This method was adapted and optimized for our lab and described in greater detail in Appendix C. Three aliquots were analyzed in triplicate (n = 9). Samples or gallic acid (100 μL) and 1 mL FCR (diluted 10-fold) were mixed in a vortexer and incubated for 5 min at room temperature (22 ºC) prior to the addition of 1 mL 10% Na_2CO_3 solution. This mixture

was then allowed to stand for 90 min at room temperature, and the absorbance was determined at 765 nm. TPC was calculated as gallic acid equivalents (GAE) per g of dry weight.

Total Anthocyanin Content

Anthocyanin content was determined by the pH-differential method [*152*]. Pigment concentration is calculated and expressed as cyanidin 3-glucoside equivalents using the formula:

$$\text{TAC (cyanidin 3-glucoside equivalents, mg/g DW)} = \frac{A \times MW \times DF}{\varepsilon \times 1}$$

where A $(A_{520\ nm} - A_{700\ nm})$pH 1.0 − $(A_{520\ nm} - A_{700\ nm})$pH 4.5; MW (molecular weight) = 449.2 g/mol; DF = dilution factor; 1 = cuvette pathlength in cm; ε = 26,900 L/mol/cm molar extinction coefficient for cyanidin 3-glucoside.

DPPH Antiradical Assay

The DPPH assay was performed on extracts, fractions, and purified compounds and described in detail in Appendix B. Briefly, test material was resuspended in DMSO, and ethanolic DPPH (400 μM) was used in the reaction mixture. Serial dilutions were combined with the DPPH solution in a 96-well microtiter plate and incubated for 30 min at 37 ºC. The change in absorbance at 517 nm was measured to calculate a DPPH IC_{50}. DMSO was used as a negative control and gallic acid was used as a positive control (IC_{50} = 30.0 ± 2.9 μM).

Chapter Four:
Conclusions and Discussion

Natural products are rich sources of bioactive compounds, and play an important role in the development of new drugs. Between 1981 and 2002, 62% of the new and approved drugs for cancer were compounds with natural origins. For infectious diseases, the number is still higher, at 75%. As for anti-inflammatory drugs, 26% were natural product-derived compounds. Overall, 67% of the 1031 new drugs approved for all diseases during this time period were compounds that were not of purely synthetic origin. They were either entirely from natural sources, modified natural products, or compounds based on a pharmacophore derived from a natural product [*153*].

While these statistics stress the importance of natural products in the development of drugs, they do not describe the continuous dietary influence of small molecules and other bioactive chemical entities from plants as foods, herbs, spices, and dietary supplements. To determine the effect of dietary natural products, we turn to epidemiological evidence, which suggests that a "Mediterranean diet" rich in plant foods is inversely correlated with many deadly diseases [*8-13*]. This correlation is due in part to high levels of fiber and lower levels of fat intake, but as the "French Paradox" indicates, much of the chemopreventative action of a diet rich in fruits and vegetables is due to the intake of bioactive natural products in plants. The "French Paradox" refers to the fact that

despite the high fat content of the French diet, there is a lower incidence of heart disease in France than in countries where fat intake is similar. Some have attributed this to the high polyphenolic content of red wine and other fruits and vegetables prevalent in the French and "Mediterranean diet" [*15, 31*]. In general, there is a consensus in the medical and nutritional community that diets high in fruits and vegetables are inversely correlated with many degenerative diseases [*5, 7-13*].

Of particular importance are the flavonoids and other phenolic compounds which can minimize the biological damage from oxidative assault and inflammation. Flavonoids have been produced by plants for over one billion years, and that continuous co-evolution with animals has likely contributed to the extraordinary diversity of biochemical and pharmacological activities in human systems [2]. Flavonoids have been documented to have anti-inflammatory, antioxidant, antiallergic, antifungal, antihemorrhagic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities [*2, 27, 28*]. Of primary interest to this study is their ability to act as chain-breaking antioxidants, prevent the oxidation of LDL, inhibit several oxidative enzymes and inflammatory mechanisms, and act as chemopreventative agents in regards to heart disease, cancers, inflammatory conditions, and some neurodegenerative diseases [*3-6*].

The evolution and development of the human species has primarily occurred in parallel with a hunter-gatherer diet, one that is high in fruits and vegetables and low in saturated fat. The greatest shift away from this diet began with the advent of modern agricultural practices approximately 10,000 years ago; more recently, the age of industrialization and global commerce has further changed our diets and amount of physical activity and exercise. It is estimated that 65 to 70% of the Paleolithic diet was

plant-based. This diet was especially high in lean protein, unsaturated fats, fiber, vitamins, minerals, antioxidants, and other beneficial phytochemicals [*7*]. Physiologically, we are nearly identical to our Paleolithic ancestors, and it has been suggested that the Paleolithic diet might be considered a paradigm or standard for modern humans from an evolutionary or genomic perspective [*150*]. Many of the USDA recommended daily allowances (RDA) for dietary nutrients are similar to those amounts believed to have been represented in a Paleolithic diet [*150*]. Approximately 95% of the human genome was naturally selected during the Late-Paleolithic era (50,000 – 10,000 BC), and little evolutionary change has taken place in the genome over the last 10,000 years [*154*]. It is therefore believed that a thorough understanding of the phytochemistry of plant foods will contribute greatly to human health and disease chemoprevention.

This project has demonstrated that the edible fruits in the Myrtaceae are a rich source of biologically active compounds, including depsides, anthocyanins, flavonoids, phenolic acids, and ellagitannins. With cancers and heart disease the top causes of death in the United States [1], the evidence shows that diets high in naturally occurring antioxidants and anti-inflammatories are important as a first-line strategy of chemoprevention [*5, 7-13*]. Edible fruits in the Myrtaceae are regularly grown and consumed in tropical parts of the world, and are highly regarded for their many uses. Those uses range from ornamental to food, food products, spices, dietary supplements, and traditional medicines. Many of these fruits have the potential for further development as new fruits or functional foods.

Almost all the species described in this project can be found growing in southern Florida, California, or Hawaii. The camu-camu is difficult to cultivate outside the flooded

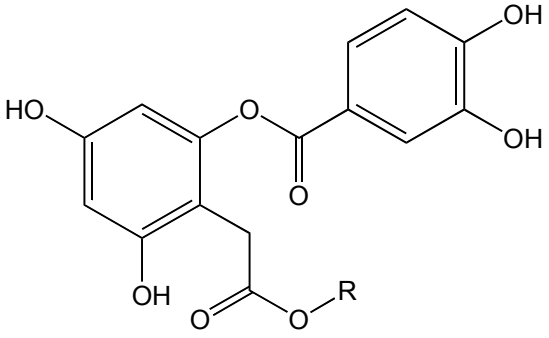
plains of the Peruvian and Brazilian Amazon Basin where it grows naturally. The trunk of the tree is generally submerged in seasonally flooded areas, and at times the lower branches are even submerged. This species has proven to be a valuable non-timber forest product (NTFP) because it produces a fruit that is extremely high in ascorbic acid while growing in locations that are difficult to otherwise utilize for agriculture. This provides for a local economy based on the sustainable harvest of a product with a market – namely the health food and nutraceuticals consumers.

The jaboticaba is a prolific fruit-bearing shrub in southern Florida, and this project has shown that it contains depsides, unusual phenolic compounds with potential for development as anti-inflammatories and COPD therapeutics. In addition to the depsides, which occur as minor constituents of the fruit, there is a high concentration of anthocyanins, particularly cyanidin 3-glucoside and delphinidin 3-glucoside. These two anthocyanins are common flower and fruit pigments, and yet their antioxidant and anti-inflammatory activity is significant. They are strong antiradical compounds in the DPPH assay, with IC_{50} concentrations below 30 μM. Of the 14 fruits tested (Chapter Three), jaboticaba extracts were the most active in the DPPH assay (IC_{50} =19.4 μg/mL). The jaboticaba also contains many flavonols and phenolic acids with documented antioxidant and anti-inflammatory activities. Chapter Two demonstrates that jaboticaba compounds can inhibit the production of IL-8, a potent chemokine neutrophil attractant. Neutrophil recruitment is one of the hallmarks of inflammation, and the ability of jaboticaba phenolics to inhibit the production of IL-8 is a significant anti-inflammatory mechanism. In addition, some depsides are very potent non-steroidal anti-inflammatories in that they inhibit the biosynthesis of prostaglandin and LTB_4 [*118-121*]. These pro-inflammatory

eicosanoid mediators are the result of arachidonic acid metabolism, which has been shown to be affected by flavonoids that inhibit COX and LOX enzymes [2]. In addition to these phenolics, there are many ellagitannins in jaboticaba that remain unidentified. Ellagitannins are responsible for much of the beneficial effects of strawberries [*155*] and pomegranate juice [*156-158*].

Given the diverse and interesting phytochemistry of the jaboticaba, other underutilized Myrtaceae species should be considered for further investigations. This project analyzed 14 related fruits for antioxidant composition, and compares the total phenolic content, total anthocyanin content and antioxidant activity of these species, as well as a quantitative analysis of several common phenolic antioxidants. These results show that many of these species are rich sources of some phenolics, and are potentially healthy additions to the human diet.

Appendix A:
Primary Chemical Structures in the Text



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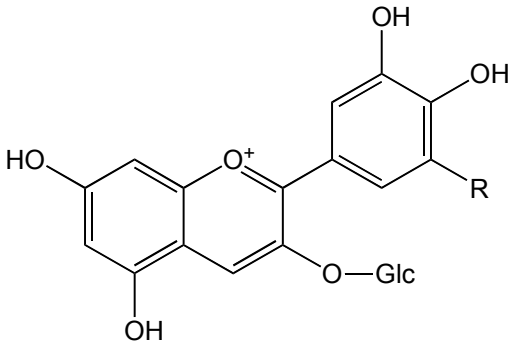

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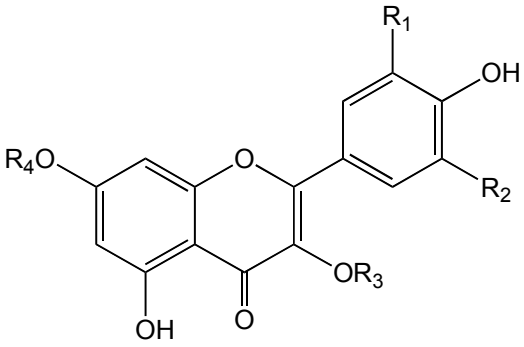

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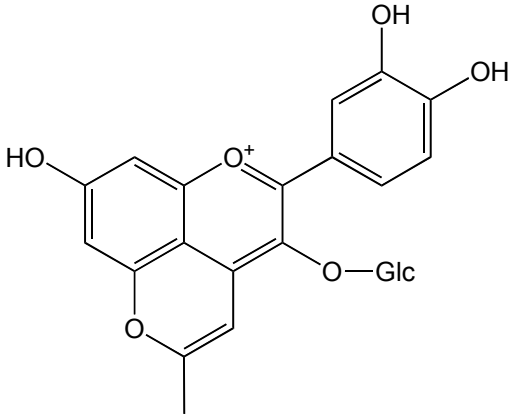

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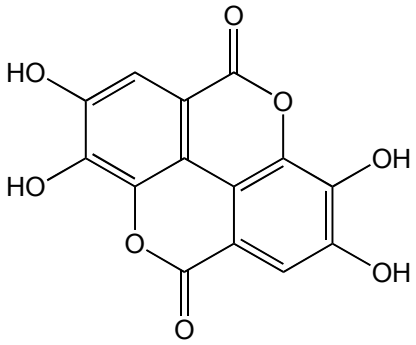

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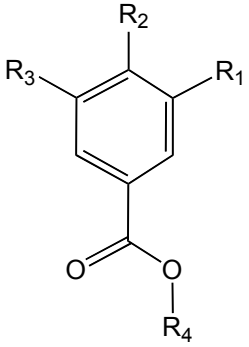
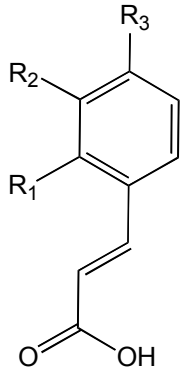


14 - 16



17 - 18

Figure A1. Primary chemical structures in the text

	Compound	R
1	Jaboticabin	Me
2	2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid	H

	Compound	R
3	Cyanidin 3-glucoside	H
4	Delphinidin 3-glucoside	OH

	Compound	R_1	R_2	R_3	R_4
5	Quercetin	OH	H	H	H
6	Quercitrin	OH	H	rhamnose	H
7	Isoquercitrin	OH	OH	glucose	H
8	Rutin	OH	H	rutinose	H
9	Myricitrin	OH	OH	rhamnose	H
10	Quercimeritrin	OH	H	H	Glucose
11	Kaempferol	H	H	H	H

12 Pyranocyanin B

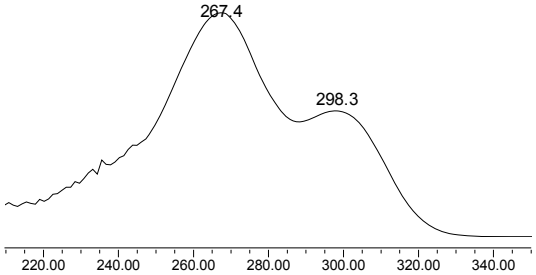
13 Ellagic acid

	Compound	R_1	R_2	R_3	R_4
14	Gallic acid	OH	OH	OH	H
15	Protocatechuic acid	OH	OH	H	H
16	Methyl protocatechuate	OH	OH	H	Me

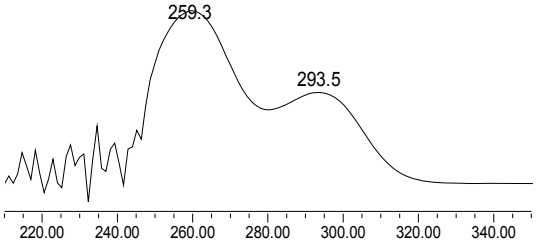
	Compound	R_1	R_2	R_3
17	Cinnamic acid	H	H	H
18	*o*-Coumaric acid	OH	H	H

Figure A2. UV-Vis spectra of some common phenolics

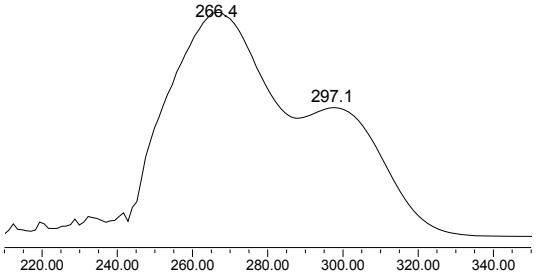
Jaboticabin



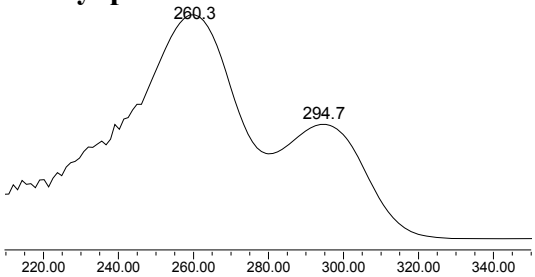
Protocatechuic acid



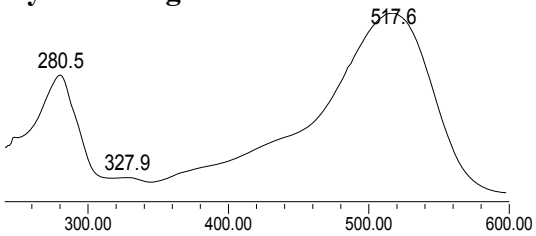
2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid



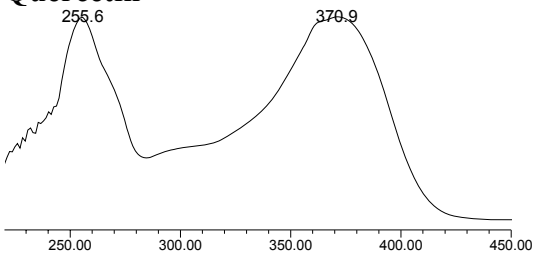
Methyl protocatechuate



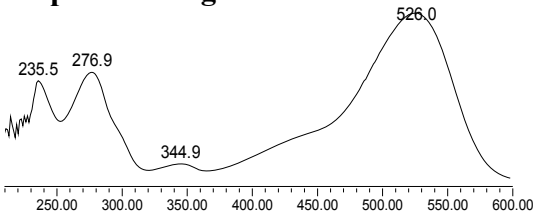
Cyanidin 3-glucoside



Quercetin



Delphinidin 3-glucoside



Quercitrin

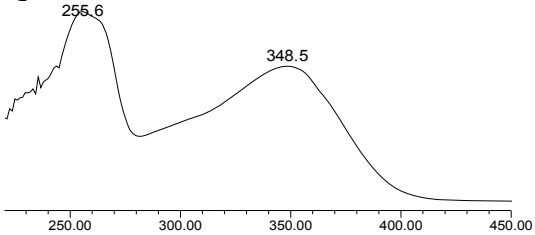
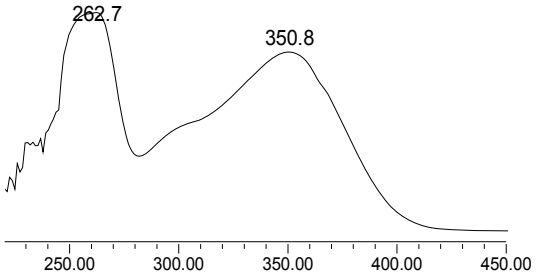
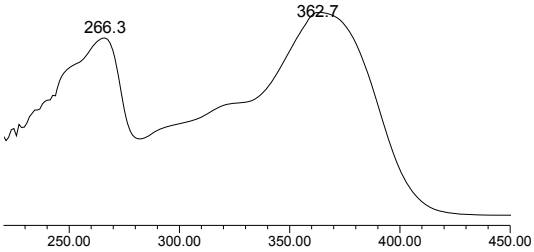


Figure A2. UV-Vis spectra of some common phenolics (continued)

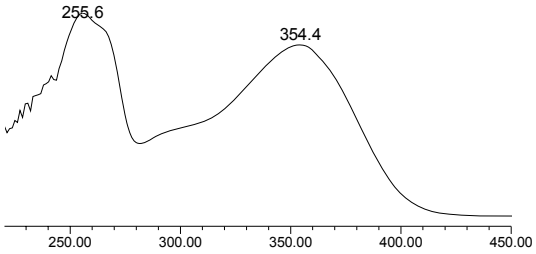
Isoquercitrin



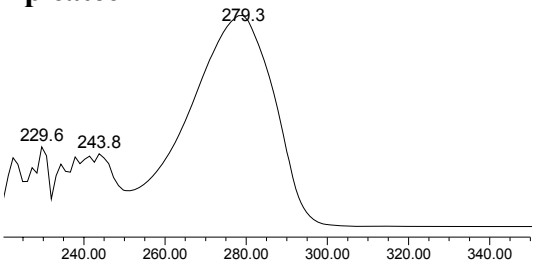
Kaempferol



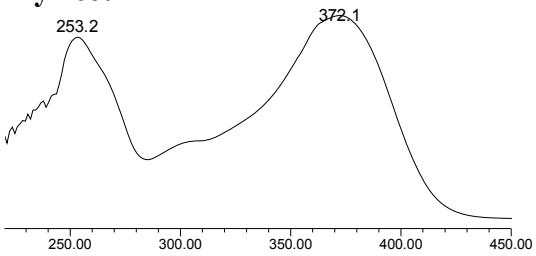
Rutin



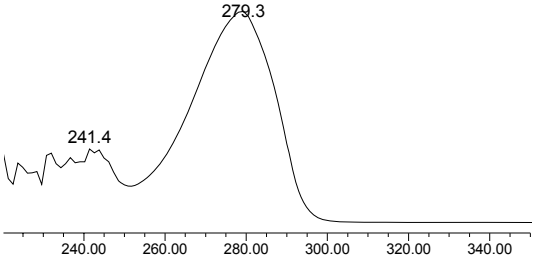
Epicatechin



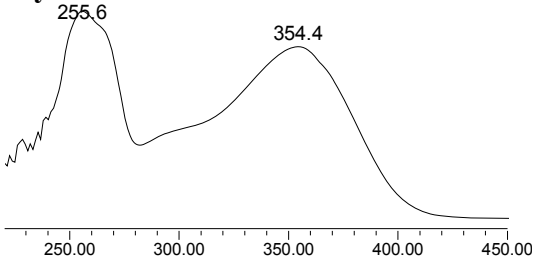
Myricetin



Catechin



Myricitrin



EGCG

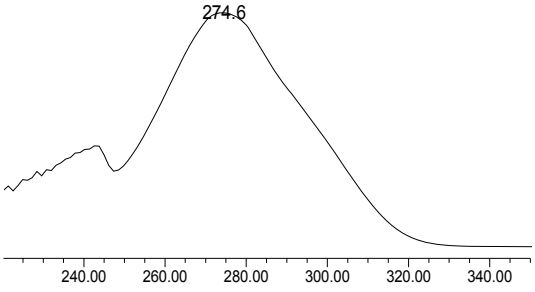
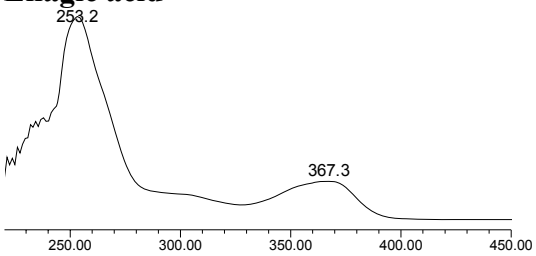
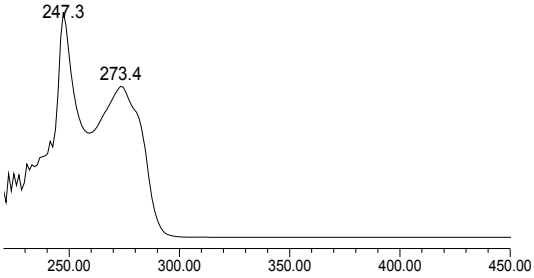


Figure A2. UV-Vis spectra of some common phenolics (continued)

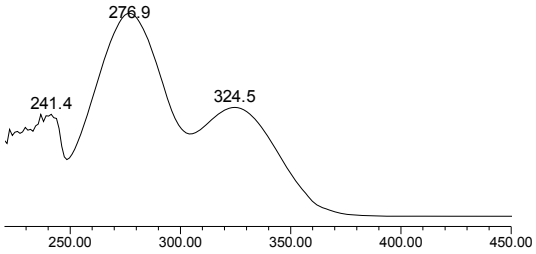
Ellagic acid



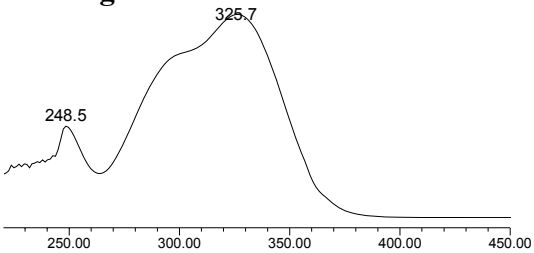
Benzoic acid



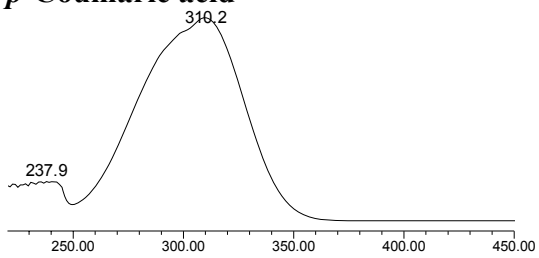
***o*-Coumaric acid**



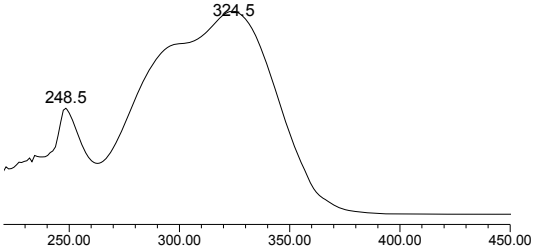
Chlorogenic acid



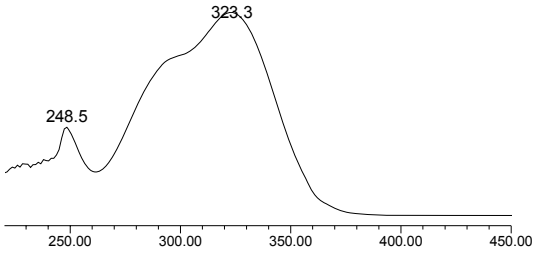
***p*-Coumaric acid**



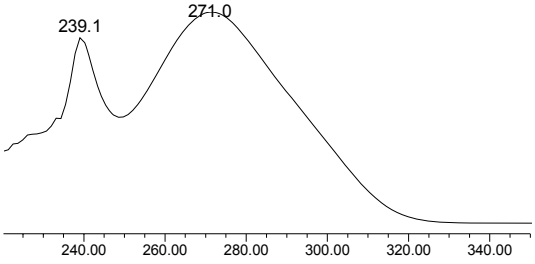
Caffeic acid



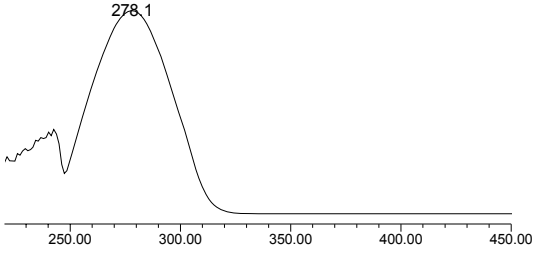
Ferulic acid



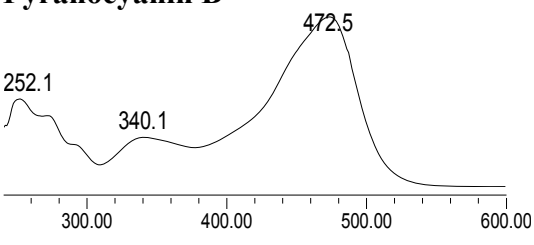
Gallic acid



***t*-Cinnamic acid**



Pyranocyanin B



Appendix B:
1,1-Diphenyl-2-picrylhydrazyl (DPPH) Assay

Basic Method

The 1,1-diphenyl-2-picrylehydrazyl (DPPH) assay is a rapid and effective colometric method for estimating antiradical activity. This chemical assay is widely used in natural products research to isolate phytochemical antioxidants and to test general radical absorbing capacity of extracts and pure compounds. The DPPH radical is a stable nitrogen-containing organic compound with a strong absorbance at λ_{max} 517 nm and a dark purple color. After reacting with antioxidant compounds, it is reduced, and the color changes to yellow. The change can be measured by a spectrophotometer, and plotted against concentration.

The procedure is an adaptation of Yamaguchi [*159*]. Extracts are dried completely and resuspended in dimethylsulfoxide (DMSO). DPPH in ethanol (400 μM) is used as the reaction mixture. Serial dilutions of the extracts are combined with the DPPH solution in a 96-well microtiter plate. DMSO is used as a negative control. Gallic acid, ascorbic acid, or α-tocopherol (Trolox) can be used as positive controls. The plate is incubated for thirty min at 37 ºC, and the unreacted DPPH is measured with microplate spectrophotometer. Mean values are obtained from duplicate experiments. Inhibition percent is calculated using the equation:

$$\% \text{ Inhibition} = [(C-S)/C] \times 100$$

where C is the net absorbance of the control and S is the net absorbance of the sample. Percent inhibition is plotted against concentration, and the equation for the line is used to obtain the IC_{50} value. A lower IC_{50} value indicates greater activity. IC_{50} < 50 μg/mL is very active; 50 μg/mL < IC_{50} < 100 μg/mL is active; 100 μg/mL < IC_{50} < 200 μg/mL is moderately active; IC_{50} > 200 μg/mL is not active.

DPPH has some problems, however, which should be considered when using this assay to estimate antioxidant activity [*145*]. Small molecules have more access to the DPPH radical site, and therefore have higher a antiradical activity. Gallic acid acts fairly quickly in this assay as can be seen in Figure B2. Many active antiradical phenolics show this same exponential curve, which can be sigmoidal on close examination of the lower concentrations. For this reason, it is important to use the linear portion of the curve to estimate the antiradical activity (Figure B3), which is sometimes a very limited range (2 to 3-fold) (Figure B4).

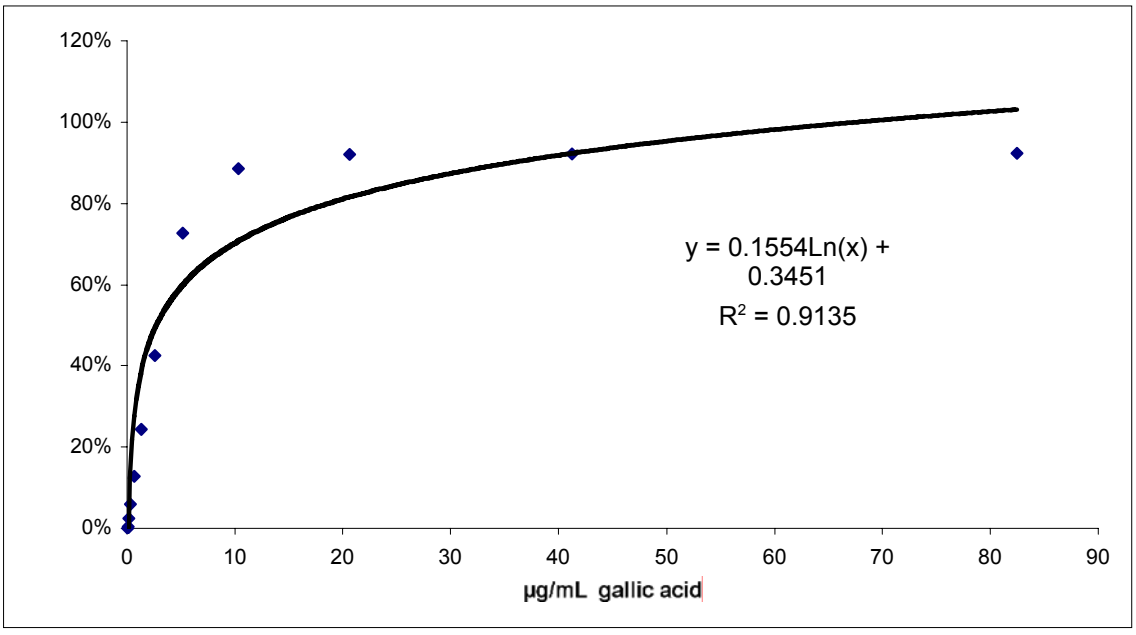


Figure B2. Antiradical activity of gallic acid in the DPPH assay.

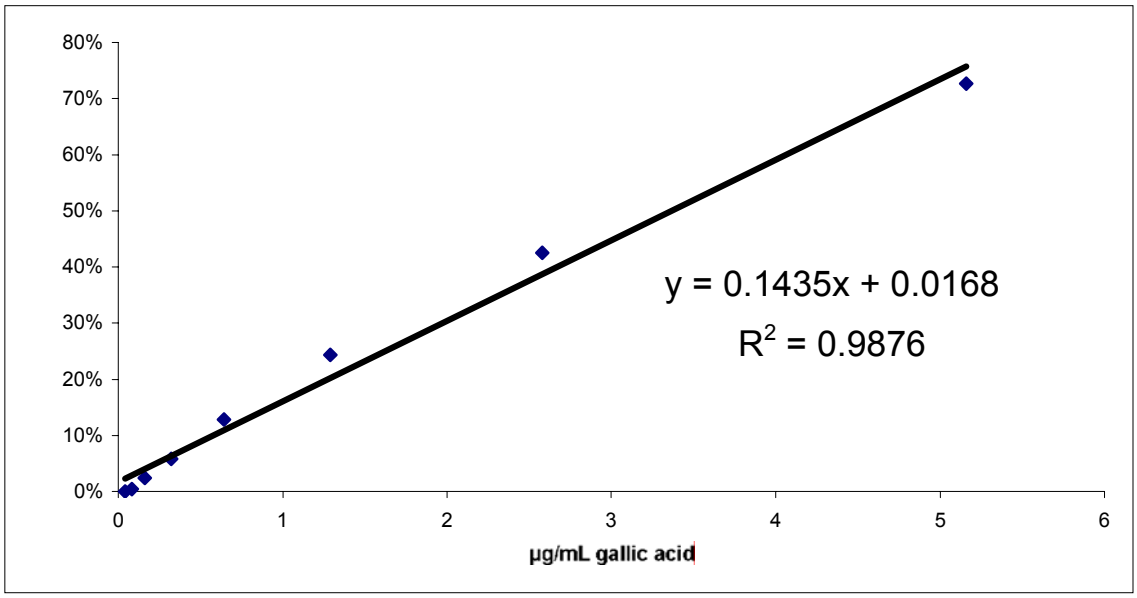


Figure B3. Linear range of DPPH assay with gallic acid.

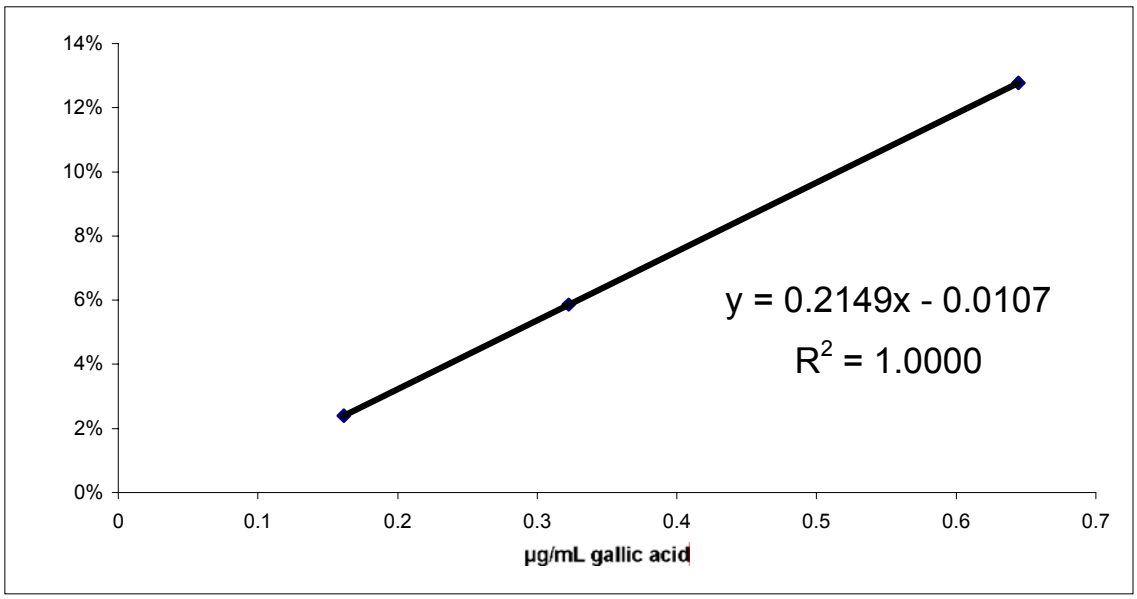


Figure B4. The limited linear reaction range of the DPPH assay is approximately 2 to 3-fold.

Preparation

1. Prepare a 400 μM solution of DPPH in etOH. To do so, place 17.6 mg of DPPH in a 100 mL volumetric flask and add approximately 80 mL etOH. Place on a magnetic stirrer or in a sonicator to dissolve the DPPH completely, and then bring up to volume (100mL).
2. Gallic acid should be prepared in a concentration of approximately 0.25 mg/mL as a positive control. Make several serial dilutions of this stock solution for the assay.
3. Prepare the test sample in the range of 1.0 to 0.5 mg/mL and make several serial dilutions. Often, it is best to make as many serial dilutions as can be placed on the plate (11 total) when running the assay on a new sample, as the concentration of sample necessary to achieve results in the linear range is not known.

Procedure

See Figure B5 for an example of the microplate layout.

1. Place 50 μL of etOH in wells A1, B1, D1, and E1 as the blank.
2. Place 50 μL of the sample serial dilutions (S1-S11) or gallic acid serial dilutions (G1-G11) in wells 2-12, beginning with the most dilute (well 2) to the most concentrated (well 12).
3. Place 150 μL of DPPH solution in each well with a sample.
4. Cover the well with tin foil and place in an incubator at 37 ◦C for 30 min.
5. Remove from incubator, remove cover and place in microplate reader. Read results at 517 nm.
6. Transfer results to a spreadsheet program for further processing.

7. Use the equation

$$\% \text{ Inhibition} = [(C-S)/C] \times 100$$

to process the results, and plot the % Inhibition against the concentration. Choose the points that give the best linear curve in a range below 60% inhibition. Using the equation for the line, calculate the IC_{50}.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
B	blank	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
C												
D	blank	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
E	blank	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
F												
G												
H												

Figure B5. Example layout of microtiter plate for DPPH assay.

Appendix C:
Total Phenolic Content (TPC) Analysis Using the Folin-Ciocalteu Method

Basic Method

This method of TPC measurement is based on studying the F-C methods published in the peer-reviewed literature. The published methods vary greatly from lab to lab, and are generally different from the original assay developed by Singleton and Rossi [*151*]. This Appendix is meant to create a standard method of TPC analysis for use in our lab.

Folin-Ciocalteu (F-C) reagent is a molybdotungstophosphoric heteropolyanion acid with a λ_{max} of 765 nm [*145*]

$$3H_2O - P_2O_5 - 13WO_3 - 5MoO_3 - 10H_2O$$

and

$$3H_2O - P_2O_5 - 14WO_3 - 4MoO_3 - 10H_2O$$

Preparation

Perform an extraction of plant material as appropriate to the study species. For quantitative purposes, a highly efficient extraction method should be developed prior to this experiment. On the other hand, this method can be used to evaluate the phenolic-extracting power of a method. The F-C method is widely used in natural products because it is simple, sensitive, and precise. One limitation of this method is that with complex samples, there may be interfering compounds such as sugars and ascorbic acid [*145*].

A sample may be dried and resuspended in the appropriate dilution range for the detector or the extraction itself can be brought up to a known volume for analysis. Determine an appropriate dilution factor by diluting an aliquot with water; the amount will vary depending on the amount of phenolics in the plant and the concentration of the extract in solution. The concentration should fall within the linear range for the UV spectrophotometer being used for the analysis. If the concentration absorbs is outside the linear range of the standard curve (either too concentrated or too dilute), then the sample must be diluted or concentrated and retested.

Apparatus

Molecular Devices VersaMax Tuneable Absorbance detector; clear 96-well microtiter plates; 25 mL volumetric flask; 100 mL volumetric flask; 1000 mL volumetric flask; test tubes; test tube rack; hot plate; and volumetric pipettes.

Reagents

2 N Folin-Ciocalteu reagent (FCR); sodium carbonate (Na_2CO_3); and gallic acid

Prepare the following solutions

1) Folin-Ciocalteu reagent (FCR): dilute stock 2 N FCR with distilled water to make a 10% (v/v) solution. Pipette 10 mL in to a 100 mL volumetric flask and bring up to volume with water.
2) Sodium carbonate solution (10%): Add 100 g Na_2CO_3 to approximately 800 mL distilled water and bring to a boil. Cool, add a crystal of Na_2CO_3 and place in refrigerator overnight. Filter and bring volume up to 1000 mL.
3) Gallic acid standard solution: Prepare a 0.5 mg/mL stock solution of gallic acid by accurately weighing 12.5 mg gallic acid into a 25 mL volumetric flask. Dissolve in a small amount of MeOH, and bring volume up to 25 mL with water. Using water, create four serial dilutions for a standard curve based on 0.5, 0.25, 0.125, 0.0625, 0.03125, and a blank is distilled water. The standard curve will have six points. These concentrations will keep the gallic acid concentration within the linear range of the detector (Molecular Devices VersaMax Tuneable Absorbance detector). If you use a different detector, you may need to alter these concentrations.

Procedure

1) Add 100 μL of sample or standard and 1 mL diluted FCR to a test tube and incubate for 5 min at room temperature (22 °C).

2) Add 1 mL of the 10% Na_2CO_3 solution and incubate for 90 min at room temperature.
3) Pipette 200 µL of each sample and standard solution into separate wells of a microtiter plate and read absorbance at 765 nm.
4) Plot gallic acid absorbance vs. concentration to create standard curve. Subtract blank from each measurement, including sample.
5) Using the equation for the line, calculate concentration of gallic acid equivalents (GAE) in sample solution, and then calculate mg GAE per g (or 100 g) plant material.

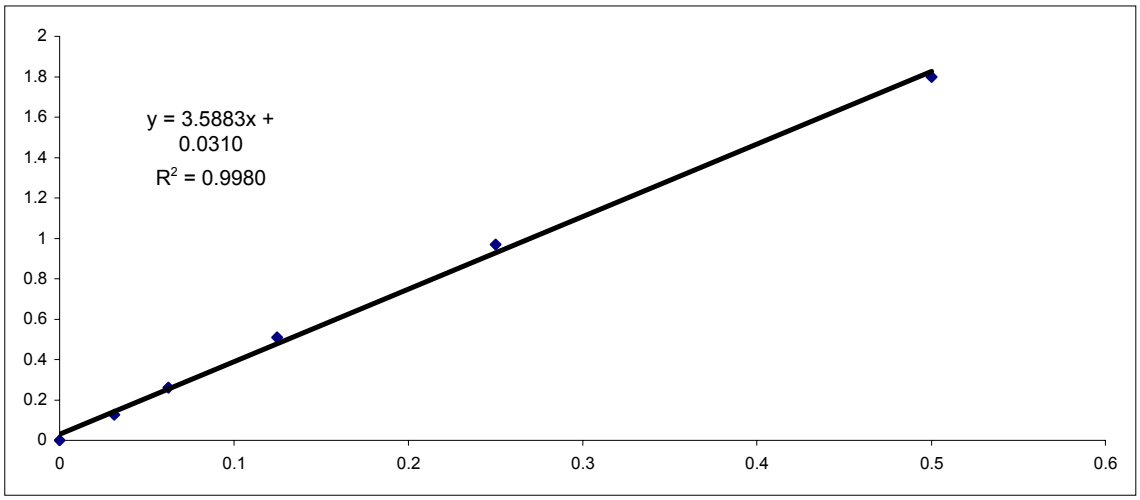


Figure C1. Example of a gallic acid standard curve.

Further Notes for Consideration

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	S 1.1	S 2.1	S 3.1	S 4.1	S 5.1		0.5	0.25	0.125	0.0625	0.03125	blank
D	S 1.2	S 2.2	S 3.2	S 4.2	S 5.2		0.5	0.25	0.125	0.0625	0.03125	blank
E	S 1.3	S 2.3	S 3.3	S 4.3	S 5.3		0.5	0.25	0.125	0.0625	0.03125	blank
F												
G												
H												

Figure C2. Layout of microtiter plate for F-C TPC analysis.

C 7-12: gallic acid standard curve dilutions

D 7-12: gallic acid standard curve dilutions

E 7-12: gallic acid standard curve dilutions

S = sample. You should test all samples in triplicate.

You should perform a gallic acid standard curve on each plate with samples. The above concentrations will give you a linear curve within the range of our spectrophotometer if doing the TPC measurements using the above solution concentrations.

Calculations:

Calculate the average of each gallic acid dilution and plot it against the concentration. Subtract the average of the blank from each measurement in the standard curve.

Once you determine the concentration of GAE in the test solution, you must use the dilution factor to calculate the amount of GAE in the extract. This amount can then be used to calculate the GAE for the plant material.

Appendix D:
Quantitative Analysis by HPLC-PDA and LC-MS:
Method Development

Introduction

There are many good guidelines for developing validated methods of analysis available through organizations like the AOAC <http://aoac.org>, the United States Pharmacopoeia (USP) <http://www.usp.org>, the United States Food and Drug Administration (FDA) <http://www.fda.gov>, and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) <http://www.ich.org>. Their protocols and guidelines can be found through their respective websites. This Appendix is based largely on the AOAC Single Lab Validation Certificate course [*160*], as well as Chapter 9 of the Book *Modern HPLC for Practicing Scientists* by Michael Dong [*161*].

There are many issues and parameters to consider when developing an analytical method. Some factors will influence others inasmuch as they must be worked out at the same time, in concert, in order to develop a suitable method.

Fitness of Purpose

The first step in the method development process is determining the so-called "Fitness of Purpose." Broadly speaking, the analyst must answer a few questions, namely:

- What is the intended application of the analytical method? For instance, does the method need to be qualitative or quantitative (or both)? Does the method serve any regulatory purpose? Will the method be used repeatedly over time, or for one set of experiments?
- What are the analytes? How many analytes are there? What class of compounds?
- What are the matrices?
- What are the expected concentration ranges?

These questions may seem obvious, but are often overlooked by the analyst who is not carefully planning a thorough study. These factors will determine many aspects of the method development process.

Method Development

Data Acquisition/Background

Literature searches and discussions with lab members and other colleagues are effective means of method development preparation. Oftentimes, this sort of information can be more immediately useful and applicable than literature information. Aside from peer-reviewed journals and edited books, other useful sources of information include the

Merck Index and the *CRC Handbook of Chemistry & Physics* as well as some vendor application guides.

Extraction and Sample Preparation

For quantitative purposes, a highly efficient extraction method should be developed prior to analysis. Determine extraction efficiency and appropriateness of solvent, duration, temperature, and so on. During this time, various sample preparations can be examined to determine what sample preparation (if any) is necessary prior to analysis. Oftentimes, the sample preparation can help remove interfering non-analyte compounds, and concentrate the analytes of interest. It can also help prolong the life of HPLC columns, which can become clogged by "column killers," non-polar and other compounds that bind permanently to reversed-phase packings. Guard columns help, and should always be used, but can also become problematic if they become clogged. Sample preparation can also be used for enrichment of the trace analytes, to desalt and extract, or for solvent exchange or sample storage and transport.

Sample preparation can involve methods such as filtration, solvent-solvent partition, solid phase extraction (SPE), or other column chromatography. A large number of steps and complicated methods are usually to be avoided because accuracy and precision decrease with each step, and more error is introduced.

Chromatographic Separation

Chromatographic principles to consider: capacity factor, selectivity, efficiency, and system suitability. The choice of detector can also have a great impact on results. Peaks being quantified should achieve baseline separation and be well-separated from

any interfering non-analytes. Co-elution of compounds with different UV-Vis λ_{max} may be acceptable, however it should be known that in some cases there could be some interferences.

Developing a suitable HPLC system is the first step. The system (chromatographic conditions and instrument selection) must remain the same for the results to be accurate. The limit of quantification (LOQ) and detection (LOD) determinations, recovery experiments and quantitative analysis experiments are all performed using the same HPLC setup.

Glassware

Simple dilution of liquid sample can usually be performed with a precision of better than 0.5%. Calibrated volumetric glassware (at least 25 mL) and pipettes (at least 10 mL) should be used. Minimize the numbers of transfers and dilutions. Autopipettors of small volumes (0.1 to 5 mL) can degrade overall method precision. Dissolve samples completely in the mobile phase (ideally) or a solvent that is weaker than the mobile phase to maintain good peak shape (especially for early-eluting compounds). Sample size should not exceed 1 to 10 μg of solute per gram of packing.

Quantification and Calibration

The AOAC guidelines give acceptable percent rates of recovery for analytes based on the concentration of analyte in the matrix.

External standards are standard solutions that are prepared at known concentrations and a fixed volume that is injected and analyzed. Peak response vs. concentrations is plotted, and calibration plot should be linear and have a zero intercept (b = 0). Internal standards are added to the matrix before analysis and often used when there is considerable sample preparation. Generally, five to six replicate injections of a standard with 2 to 2.5 % variance is acceptable. At least five concentrations that are approximately equally spaced across the expected concentration range. For a calibration curve covering a large concentration range, a weighted regression may be used.

The LOQ and LOD are usually determined as a measure of the S/N ratio of 10:1 and 3:1, respectively.

Definitions

Accuracy – the closeness in agreement of the accepted true value or a reference value to the actual results obtained. Typically, this is done by recovery experiment of a spiked sample into a matrix or placebo.

Linearity – ability of a method to obtain test results that are directly proportional to the sample concentration over a given range. The relationship between sample concentration and detector response (peak height or area). The equation for a line is y = mx + b; a value for b that approaches or equals zero is best. A method that is linear with b = 0 permits a quick, convenient check with one or two points to confirm accuracy.

LOD – Limit of Detection. The smallest (or highest) amount or concentration that can be detected. A S/N ratio of 3:1 is the most commonly accepted value.

LOQ – Limit of quantification. The lowest (or highest) level that an analyte can be quantified with some degree of certainty (e.g. with a precision of ± 5%) 10:1 S/N

Matrix blank – all substances in a typical test article except the analyte(s) of interest.

Maximum level of quantification – highest concentration that can reliably be determined using the conditions of the method – the limit of the detector

Peak height vs. peak area – Peak area is the most widely used method. Care must be taken to determine the proper baseline and properly integrate the peak from beginning to end. For symmetrical peaks, peak height can be as precise and more accurate than peak area.

Precision – measure of the ability of a method to generate reproducible measurements of a sample. Precision can measured for repeatability, intermediate precision, & reproducibility, on a single day or multiple days, using different instruments, analysts, sample preparations, etc.

Selectivity – the ability to assess unequivocally the analyte in the presence of components that may be expected to be present.

Specificity – the ability of a method to discriminate between the intended analyte(s) and other components in the sample. Baseline separation and peak purity using either the PDA or MS.

Validation – process of demonstrating or confirming the performance characteristics and limitations of a method and identification of the limitations.

Appendix E:
Dioctyl Phthalate
A Common Contaminant of Reagent-Grade Solvents

Dioctyl phthalate (DOP) [syn: di-2-ethyl hexyl phthalate (DEHP); Bis(2-ethylhexyl)phthalate (BEHP); 1,2-Benzenedicarboxylic acid; dioctyl ester]

Trade names: Bisoflex 81, Bisoflex DOP, Compound 889, DAF 68, Dielektrol, Eviplast 80, Eviplast 81, Fleximel, Flexol DOP, Hatcol DOP, Kodaflex DOP, Reomol DOP, Sicol 150, Staflex DOP, Truflex DOP, Union Carbide Flexol 380, Vestinol AH, Viniciser 80, Witcizer 312

CAS 117-84-0; $C_{24}H_{38}O_4$; molecular weight: 390.562; Mp -25 °C

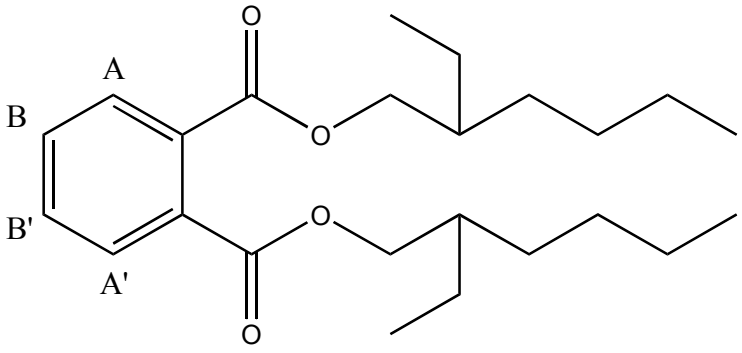


Figure E1. Chemical structure of dioctyl phthalate (DOP).

Dioctyl phthalate (DOP) (Figure E1) is a common plasticizer contaminant in reagent grade solvents. It can also often found in silica gel that is stored in plastic bags and buckets. It is important to recognize this contaminant and remove it from test samples as it has been shown to interfere with cytotoxicity tests and may also affect other bioassays. This spectral information is provided as information in order to help avoid time-consuming isolation and identification.

Properties

Soluble in MeOH. Looks oily, yellowish at room temperature. The UV profile is presented in Figure E2. DOP ionizes well in APCI positive mode $[M + H]^+ = 391$ (Figure E3). Following extraction of plant material, it can often be found in high concentrations (Figure E4). The plane of symmetry creates the characteristic AA'BB' spin system of a 1,2-symmetrically substituted aromatic ring (Figure E1). An easily diagnosable proton multiplet is observable in the ^{1}H NMR spectrum (Figure E5). The plane of symmetry also produces a "misleading" ^{13}C-NMR spectra – only 12 (half) carbon signals are detected (Figure E6).

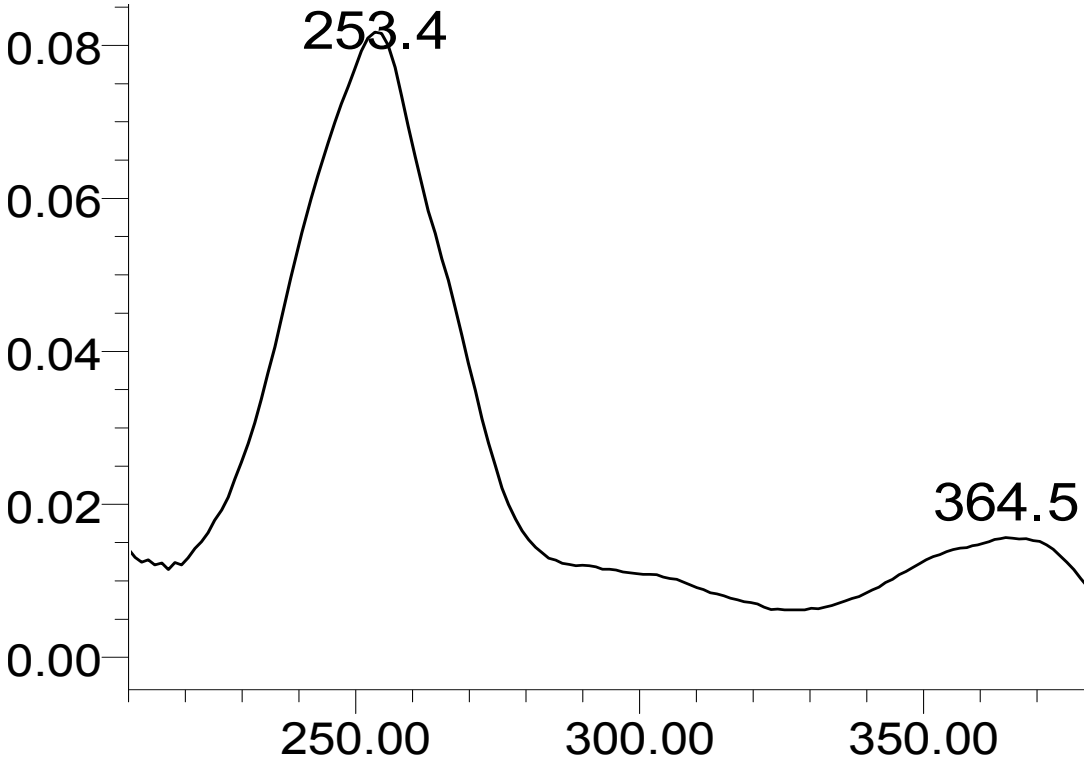


Figure E2. UV-Vis spectrum of DOP.

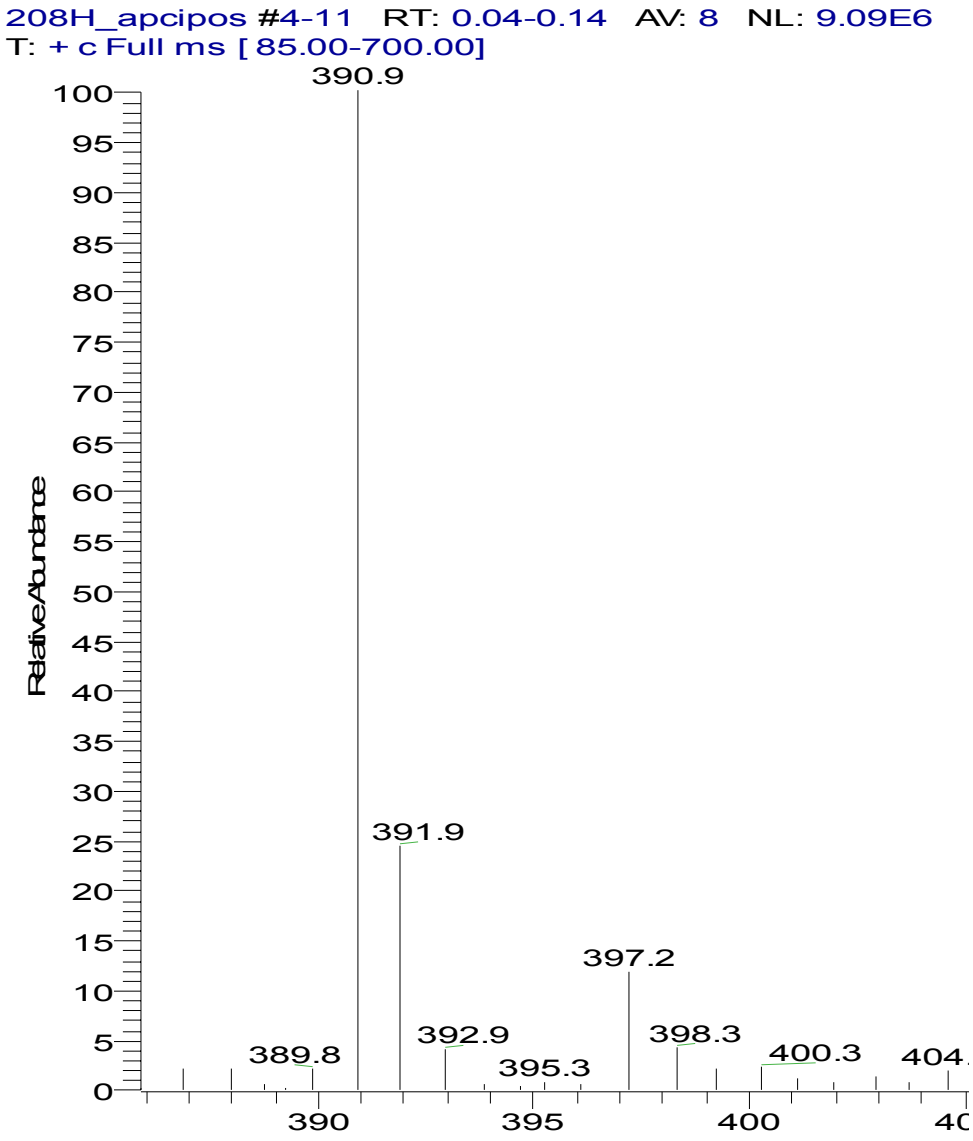


Figure E3. APCI Mass spectral profile of DOP in the postive mode.

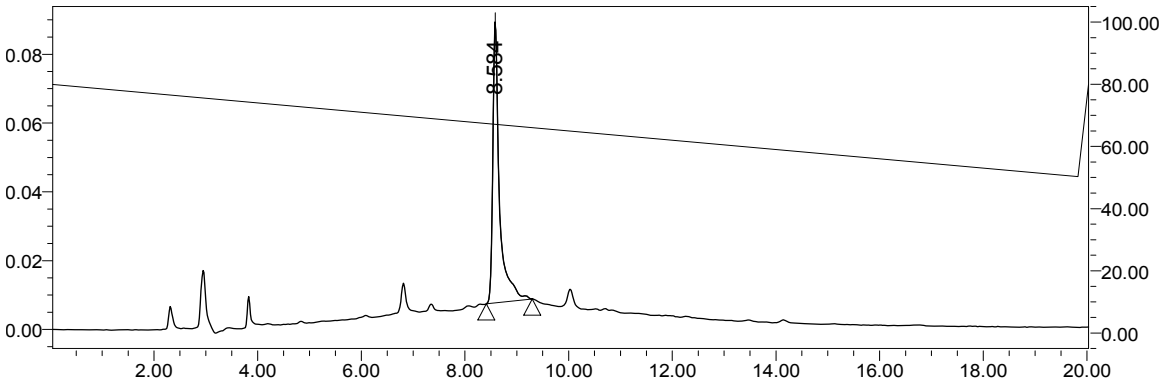


Figure E4. Reversed-phased HPLC chromatogram showing a fraction enriched with DOP after only two open column separations of a crude fruit extract.

Procedure: Waters 2695 Separations module; Phenomenex Aqua 250 x 4.6 mm (4 μm) column; Waters 996 PDA detector monitoring at 254 m; flow rate 1 mL/min; A: 1% Formic acid; B = ACN; 80% A to 50% over 20 min.

^{1}H NMR and ^{13}C NMR spectra for DOP in CD_3OD

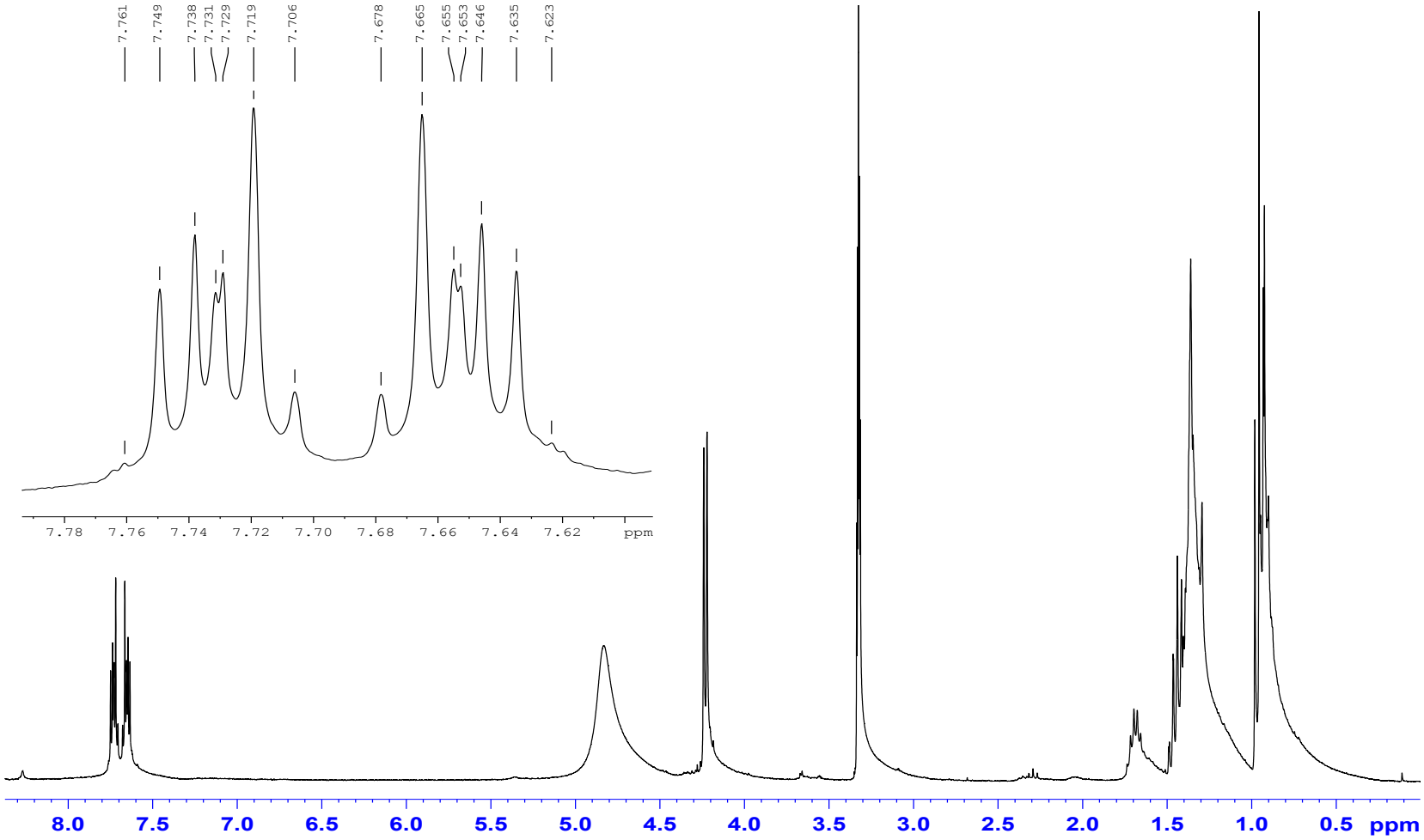


Figure E5. ^{1}H NMR spectrum (300.13 Mhz) for DOP in CD_3OD

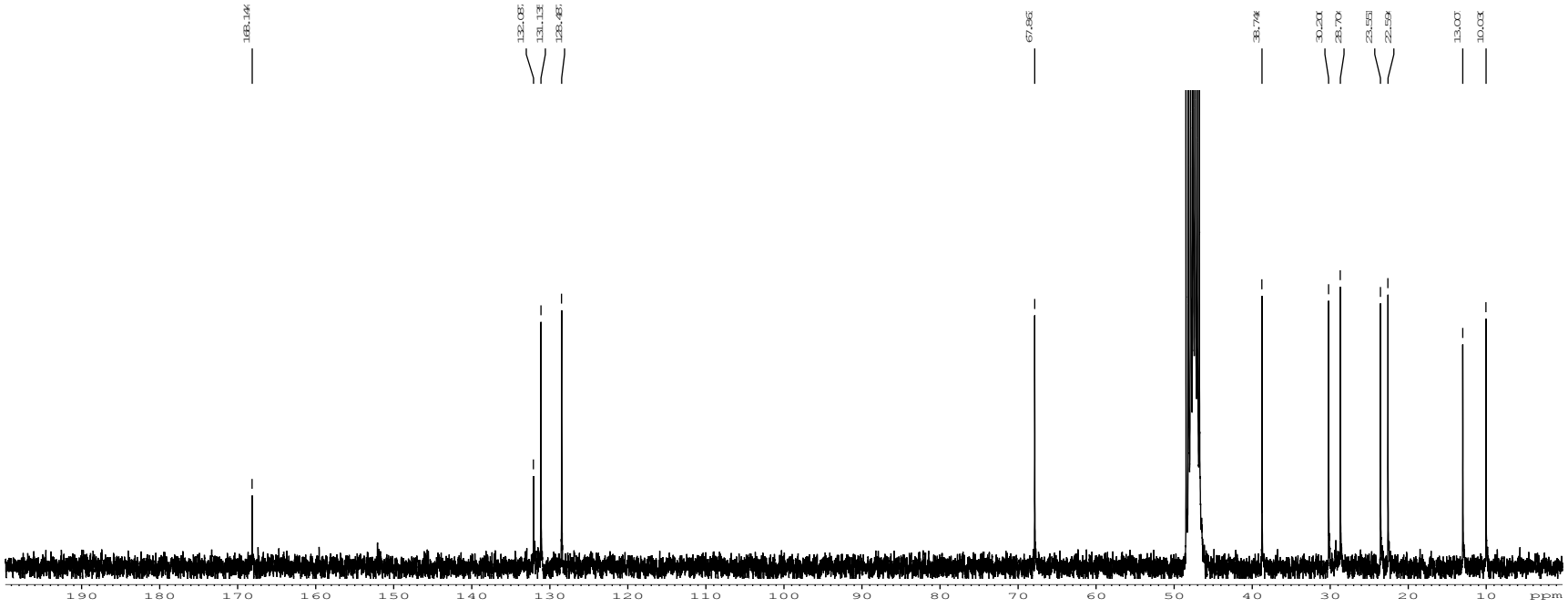


Figure E6. ^{13}C NMR spectrum (75.48 MHz) for DOP in CD_3OD

Appendix F:
Plant Collection and Voucher Information

Several collections were made of some of the study fruits. Each collection was entered into the Phytochemistry lab database and assigned a unique code that was used in tracking the sample. Whenever possible, a voucher specimen was prepared and deposited in the Steere Herbarium of the New York Botanical Garden. With the exception of *Myrciaria dubia*, each species was collected either by myself or collaborator Margaret Basile. In those cases where a voucher was not collected for the specific test sample, a subsequent collection voucher specimen has been made from the same tree or shrub. Each collection comes from an established and documented accession by the collaborating institutions.

For the analyses described in Chapters Two and Three, the following collections utilized are vouchered by the specimens listed in Table F1.

Table F1. Species voucher information

Species	Phytochem DB ID#	Collection location	Collection date	Voucher info
Eugenia aggregata	1178	FSP	5/2001	KAR 7
Eugenia brasiliensis	1175	GGR	5/2001	KAR 9
Eugenia luschnathiana	1210, 1241	FSP	7/2002	KAR 36
Eugenia reinwardtiana	1174	GGR	5/2001	
Myrciaria cauliflora	1207	FSP	6/2001	KAR 20
Myrciaria dubia		ELF		
Myrciaria vexator	1212	FSP	2/2002	
Syzygium cumini	1285	MBC	7/2002	KAR 68
Syzygium curranii	1273	FSP	7/2002	
Syzygium jambos	1169	GGR	4/2001	KAR 16
Syzygium javanicum	1233	FSP	7/2002	
Syzygium malaccense	1190	Kampong	6/2000	KAR 65
Syzygium samarangense	1150	FSP	5/2001	KAR 17
Syzygium samarangense var *Taiwan Pink*	1245	FSP	7/2002	KAR 33

Abbreviations in the table: GGR, Green Grove Ranch; FSP, Fruit and Spice Park; MBC, Montgomery Botanical Center; ELF, Essential Living Foods. Locations and addresses can be found in Appendix G.

Appendix G:
Collaborators and Contributions

Fruits were collected at the following locations:

Green Grove Ranch, Southwest Ranches

4961 SW 193 Lane, Ft. Lauderdale, FL 33332-1230

Margaret Basile

Fruit and Spice Park

24801 SW 187th Avenue, Homestead, FL 33031

(305) 247 5727

Chris Rollins, Director

http://www.fruitandspicepark.org/

The Kampong

4013 Douglas Road, Coconut Grove, FL 33133

(305) 442 7169

Larry Schokman, Director

http://www.ntbg.org/gardens/kampong.php

Tropical Research and Education Center

Institute of Food and Agricultural Sciences

18905 SW 280 Street, Homestead, FL 33031-3314

(305) 246 7001 x290

fax 305 246 7003

Jonathan H Crane, PhD, Topical Fruit Crop Extension Specialist/Horticulture

http://trec.ifas.ufl.edu/

Rare Fruit and Vegetable Council of Broward County

5105 SW 208th Lane

Southwest Ranches, FL 33332

http://www.rfvcbroward.org/

Additional bioassay testing was done in collaboration with the following labs and researchers:

1) IL-8 immunoassay experiments were performed by Alison Wallace, Ph.D. in the lab of Jeanine D'Armiento, M.D., Ph.D.

Department of Medicine

Division of Molecular Medicine

College of Physicians and Surgeons

Columbia University

New York, NY 10032

2) Colon cancer cytotoxicity assays were performed by Seiji Adachi, M.D., Ph.D. in the lab of I. Bernard Weinstein, M.D.

The Herbert Irving Comprehensive Cancer Center

College of Physicians and Surgeons

Columbia University

HHSC-1509, 701 W. 168th Street

New York, NY 10032

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Index