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POLYPHENOLIC ANTIOXIDANTS FROM SAPOTACEAE FRUITS

By

Jun Ma

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

2004

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Abstract

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By

Jun Ma

Advisor: Dr. Edward J. Kennelly

Tropical edible plants are rich sources of phytochemical antioxidants. In this study, forty-nine tropical edible plants were selected to undergo screening for antioxidant activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay. Among them, thirty-six displayed antioxidant activity. Of these thirty-six plants with antioxidant activity, four were from the Sapotaceae family, namely, *Manilkara zapota*, *Pouteria campechiana*, *Pouteria sapota*, and *Pouteria viridis*, these plants were selected to undergo activity-guided fractionation and isolation to study their antioxidant constituents.

Activity-guided fractionation of a methanol (MeOH) extract from the fruit of *Manilkara zapota* cv. Tikal resulted in the isolation of two new antioxidants, methyl 4-*O*-galloylchlorogenate and 4-*O*-galloylchlorogenic acid, along with eight known polyphenolic antioxidants, namely, methyl chlorogenate, dihydromyricetin, quercitrin, myricitrin, (+)-catechin, (–)-epicatechin, (+)-gallocatechin, and gallic acid. Of the ten polyphenols, methyl 4-*O*-galloylchlorogenate showed the highest antioxidant activity in the DPPH assay with IC_{50} = 12.9 μM, and displayed cytotoxicity to the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 190 and 160 μM, respectively.

With an IC_{50} of 23.5 μM, 4-*O*-galloylchlorogenic acid showed a high antioxidant activity in the DPPH assay, and displayed cytotoxicity to the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 154 and 134 μM, respectively.

Fresh fruits of *Pouteria campechiana*, *Pouteria sapota*, and *Pouteria viridis*, were extracted and activity-guided fractionations of the corresponding extracts were performed to identify the antioxidant constituents. Seven polyphenolic antioxidants, gallic acid, (+)-gallocatechin, (+)-catechin, (-)-epicatechin, dihydromyricetin, (+)-catechin-3-*O*-gallate, and myricitrin, were isolated and identified. Extracts of these three *Pouteria* fruits were analyzed by a selected ion monitoring liquid chromatography–mass spectrometry method to quantify their polyphenolic antioxidants. The highest level of the seven measured polyphenols was found in *P. sapota*, the second highest in *P. viridis*, and the lowest in *P. campechiana*. The levels of the seven polyphenols corresponded with the results of the DPPH assay, where *P. sapota* had the highest antioxidant activity, *P. viridis* the second highest, and *P. campechiana* the lowest.

The results of this study may provide additional nutrition information for little used tropical fruits grown commercially in the US, as well as in other subtropical and tropical areas. These fruits may be a good source of potential chemopreventive agents.

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Chapter 1

Introduction

Antioxidants and Human Health

Humans have used plants to cure and prevent diseases for thousands of years. Written records about medicinal plants date back at least 5000 years, and archaeological records suggest an even earlier use of medicinal plants (Swerdlow, 2000). Many societies, such as Egyptian, Assyrian, Chinese, and Indian, have long histories of using medicinal plants (Evans, 1998). Many modern drugs are plant-derived, such as aspirin, paclitaxel, and vinblastine (Raskin et al., 2002). Through the ages, people have used edible plants not only for foods, but also for health as plants used for food have bioactive constituents, and are known as functional food. Much interest in the area of functional foods concerns about prevention of coronary heart disease (CHD) and cancer (Pezzuto, 1995; Renaud and de Lorgeril, 1992).

Free radicals are any chemical species that have one or more unpaired electrons, such as hydroxyl radical, nitric oxide, and superoxide, and they are formed constantly in living organisms, including humans (Halliwell, 1994). The free radicals can cause oxidative damage to DNA, lipids, and proteins, and lead to certain diseases such as CHD and cancer. A leading current hypothesis of the pathogenesis of atherosclerosis is that free radicals can oxidize low-density lipoprotein (LDL). Oxidized LDL is taken up by the scavenger receptors on macrophages, leading to the formation of foam cells. The accumulation of oxidized LDL in foam cells results in the formation of fatty streaks. The fatty streak itself is benign but leads to the formation of atherosclerotic plaques and CHD (Figure 1.1. A) (Jacob and Burri, 1996). The current hypothesis of the pathogenesis of cancer is that free radicals can cause oxidative damage to DNA. The damaged DNA produces a somatic mutation in a normal cell, and the replication and clonal expansion of

this genetically altered cell forms a benign tumor. Further initiation and mutation can result in cancer (Figure 1.1. B) (Jacob and Burri, 1996). Besides these two common diseases, the oxidation of biomolecules induced by free radicals has been implicated in the pathogenesis of other age-related diseases, such as Alzheimer's disease (Smith et al., 1991), Parkinson's disease (Luft, 1994), and diabetes (Rabini et al., 1994).

Much evidence shows the oxidation of LDL by free radicals as the key step of pathogenesis of atherosclerosis (Steinbrecher, 1997). For example, oxidized LDL has been found in atherosclerotic lesions from both rabbits and humans (Steinberg, 1997). In both humans and animals, antibodies prepared against oxidized LDL react with materials in atherosclerotic lesions. Autoantibodies against oxidized LDL have been obtained from both rabbits and humans, indicating the presence of oxidized LDL or a closely related antigen (Steinbrecher, 1997). Moreover, elevated amounts of oxidized LDL are present in blood of patients with CHD disease (Holvoet et al., 1995).

A large body of evidence also shows that the oxidative damage of DNA by free radicals is also a key step in the pathogenesis of cancer (Wiseman and Halliwell, 1996). It is well known that excess radiation can induce cancers such as leukemia and skin cancers. Ionizing radiations invariably generate free radicals. Therefore, the interrelationship between free radicals and cancer is axiomatic (Simic, 1988). Lung cancer is mostly related to smoking. Smoking cessation leads to a decreased risk of lung cancer. Cigarette smoke is rich in carcinogens such as nitrosamine, acrolein, and carcinogenic hydrocarbons, but free radicals present in cigarette smoke may also contribute to cancer development, since smoke is rich in free radicals (Pryor and Stone, 1993). Higher levels of oxidative damage of DNA have been reported in lung cancer

tissue (Olinski et al., 1992) compared with surrounding normal tissue and in cells exposed to smoke (Kiyosawa et al., 1990). In addition, a 4-10 fold elevation of products of DNA oxidative damage have been found in urine of smokers (Loft et al., 1992). Oxidative damage of DNA may be also involved in the development of breast cancer. More than 9-fold elevation of oxidative damaged DNA can be seen in breast cancer compared with control tissue (Malins and Haimanot, 1991). Usually, liver cancer may develop from chronic hepatitis (Blumberg et al., 1975), which in turn is associated with the presence of inflammatory cells, presumably generating free radicals (Stein, 1991). Increased levels of oxidative damaged DNA have been detected from livers with chronic hepatitis (Shimoda et al., 1994).

Antioxidants, which scavenge free radicals, can prevent the oxidation of LDL and DNA, thus breaking the chain reaction of pathogenesis of CHD and cancer, and thereby preventing the pathogenesis of CHD and cancer (Scarfiotti et al., 1997).

The oxidation of LDL can be inhibited by a wide range of antioxidants polyphenols and polyphenol-rich extracts, as shown in studies in vitro (Aviram and Fuhrman, 1998; Frankel et al., 1993). Such effects may be due to direct scavenging by the polyphenols of the free radicals. In addition to studies in vitro, some animal models and studies with human subjects indicate that ingestion of polyphenols or polyphenol-rich extracts increase the resistance of LDL to oxidation in vivo (Ishikawa et al., 1997; Nigdikar et al., 1998). Epidemiological studies show that intake of high amounts of the antioxidants vitamin E or flavonoids was associated with a decreased risk of CHD, possibly as a result of inhibition of oxidation of LDL (Hertog et al., 1993; Rimm et al., 1993; Stampfer et al., 1993). But recently, large-scale clinical trials have failed to

demonstrate that vitamin E can decrease the risk of CHD (Dutta and Dutta, 2003). This may be due to inappropriate dose and duration of the vitamin E treatment.

Many studies have reported flavonoid-mediated antiproliferative effects against both human and rodent ovarian, leukemic, intestinal, lung, breast, and cancer cells (Duthie et al., 2000). Genistein, kaempferol, and quercetin inhibit the proliferation of human colon cancer cells (Agullo et al., 1994; Kuo, 1996). Epigallocatechin gallate (EGCG) can inhibit cell growth and the activation of the epidermal growth factor receptor (EGFR) (Liang et al., 1997), causes G1 arrest of the cell cycle, and induces apoptosis in human prostate, lung, colon, gastric carcinoma, leukemia, squamous cell carcinoma, and head and neck cancer cell lines (Masuda et al., 2001). There are also animal models for anticancer activity of antioxidants. For example, quercetin and luteolin decrease the incidence of fibrosarcoma and tumor size in male Swiss albino mice following treatment with the model chemical carcinogen 20-methylcholanthrene (Elangovan et al., 1994). The results of epidemiological studies are contradictory. Epidemiological studies indicate that high intake of antioxidant vitamin C is associated with reduced risk of developing cancer, particularly in the stomach, esophagus, or oral cavity (Enstrom, 2002). Epidemiological studies also suggest that tea consumption lowers the risk of developing cancer whereas other investigators failed to find such association or have even indicated procarcinogenic effects (Blot et al., 1996; Borrelli et al., 2004).

Phytochemical Antioxidants from Fruits and Vegetables

Through evolution, plants have developed extremely efficient defense systems that are critical to protect their intracellular environment against the oxidative stress caused by free radicals; such systems include enzymatic inactivation and nonenzymatic

protection (Dugan, 1980). Research has indicated that endogenous antioxidants play an important role in the antioxidant defense mechanism in biological systems (Osawa et al., 1985). Antioxidants have been found in many plant materials such as seeds, crops, vegetables, fruits, leaves, barks, and roots (Osawa et al., 1990). They mainly function as free radical scavengers, but they can also be peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, and synergists (Pziezak, 1986). By means of these functions, antioxidants can protect plants from various oxidative damage.

The high consumption of fruits and vegetables has been associated with lower incidence and mortality rates of CHD and cancer (Omenn, 1995). This phenomenon has been attributed to the various antioxidants contained in fruits and vegetables (Omenn, 1995), which are a rich source of natural antioxidants.

Wine and tea are two botanical-based beverages that contain phytochemical antioxidants that may prevent CHD and cancer. It was noticed that mortality rates from CHD are much lower in France than in other industrialized countries (Renaud and de Lorgeril, 1992). Epidemiological studies found that although many French people have a diet high in fat, and smoke tobacco, there is a lower incidence of CHD in France than in many other industrial countries. This phenomenon is known as the "French paradox." Since the French paradox was first reported, there have been many hypotheses to explain it. Most frequently, the French paradox has been attributed to the regular consumption of red wine. Recent studies have suggested that the polyphenolic antioxidants in red wine, such as the flavonoid quercetin and the stilbene resveratrol, inhibit the oxidation of human LDL in vitro and may prevent the pathogenesis of CHD (Constant, 1997; Frankel et al., 1993; Schneider et al., 1996).

Both green and black tea (*Camellia sinensis*) also have been extensively studied for cardioprotective and anticancer activity. The major flavonoid of tea, (–)-epigallocatechin (EGC) was found to inhibit LDL oxidation (Yokozawa and Dong, 1997), and EGCG was found to have anticancer activity (Ahmad and Mukhtar, 1999; Mukhtar and Ahmad, 1999).

There is a large variety of phytochemical classes of antioxidants (Halliwell et al., 1995). Table 1.1 shows twenty-eight selected phytochemical antioxidants from eleven compound classes. The monoterpenes carvacrol and thymol inhibit peroxidation of phospholipids liposomes in the presence of Fe^{3+} and ascorbate (Aeschbach et al., 1994). The monoterpene *p*-cymen-7-ol, one of the major volatile constituent of *Eucalyptus camaldulensis*, has antioxidant activity (Fadel et al., 1999). The diterpenes carnosol and carnosic acid both are potent inhibitors of endothelial cell-mediated oxidation of LDL in a dose-dependent manner (Pearson et al., 1997). The triterpene esters, 3β,23,28-trihydroxy-12-oleanene 23-caffeate and 3β,23,28-trihydroxy-12-oleanene 3β-caffeate display lipid peroxidation inhibitory activity (Yun et al., 1999). The triterpene acids, oleanolic and ursolic acid have protective effects against free radical-induced damage (Balanehru and Nagarajan, 1991). The saponins glycyrrhizin from licorice, and soyasaponin I and soyasaponin Ab all inhibit hydrogen peroxide damage to mouse fibroblast cells (Yoshikoshi et al., 1996).

Flavonoids are a class of polyphenolic phytochemicals that have been widely studied for their antioxidant activity. Besides (–)-epigallocatechin and EGCG mentioned in the previous paragraph, (+)-catechin and (–)-epicatechin can inhibit LDL oxidation (Mangiapane et al., 1992; Zhang et al., 1997). Quercetin, found in many plants including

apples, can inhibit the oxidation of LDL, and thus may act as a CHD chemopreventive agent (Chopra et al., 2000); it is also a potent anticancer a gent (Lamson and Brignall, 2000).

Resveratrol, a stilbene found in grapes, may play an important role in the prevention of CHD by inhibiting the oxidation of LDL (Ferroni et al., 2004). It is also a cancer chemopreventive agent because it acts as a reactive free radical scavenger (Khanduja et al., 2004). Mahanimbine, a carbazole alkaloid from *Murray koenigii*, displays antioxidant activity (Ramsewak et al., 1999).

Certain aromatic acids such as chlorogenic and gallic acid are antioxidants (Jung et al., 1999; Luo et al., 2002). Chlorogenic acid is a well-known antioxidant, it and its derivatives have been identified from many plants (Kweon et al., 2001; Nakatani et al., 2000). Gallic acid is often esterified to a central glucose and comprises hydrolyzable tannins, or gallitannins. It may also be esterified to flavonoids, such as EGCG. The coumarins esculentin, 5,7-dihydroxy-4-methylcoumarin, and 6,7-dihydroxy-4-methylcoumarin are antioxidants. They inhibit lipid peroxidation, and scavenge superoxide and alkylperoxyl radicals (Hoult and Paya, 1996).

The best-known phytochemical antioxidants are also nutrients. These include the carotenoids, especially β-carotene (Krinsky, 1998) and lycopene (Kelloff et al., 1999); the monosaccharide derivative ascorbic acid (Diplock, 1993); and the benzoquinone α-tocopherol (Diplock, 1993). There is growing evidence that the major antioxidant activity of many edible plants is likely due to a combination of vitamins and non-nutritional antioxidants (Cao et al., 1996).

Besides known antioxidants, many additional antioxidants that have not been isolated, may be present in edible plants, and may play an important role in the overall antioxidant activity (Cao et al., 1996). For example, in blueberries, less than 10% of the total antioxidant capacity of the fruits can be attributed to known antioxidants (Prior et al., 1998). Therefore, more research on antioxidant constituents from the edible fruits and vegetables need to be conducted to understand their entire antioxidant profile.

Selection of Plant Material

It is well-known that plant biodiversity is greatest in tropical flora. Over two thirds of the approximately 270,000 known species of higher plants live in the tropics. Therefore, tropical plants may provide a rich source of phytochemicals. In my research, tropical fruits and vegetables were collected and extracted for examination from southern Florida, because there are a number of small-scale growers there, and the climate is favorable (Anonymous, 1989). Many of the plants studied in this research were food plants, so the toxicity of the phytochemicals contained in these plants is likely to be very low. Plants were selected based on literature searches to ensure that no related research had been conducted on a given species. Database searches included Medline, Chemical Abstracts, and Natural Products Alert.

In this study, I selected plants from several species in the Sapotaceae family, one from the *Manilkara* genus, three from *Pouteria* genus. Sapotaceae is a large, usually latex-producing and mostly tropical plant family with 107 genera and more than 1,000 species (Mabberley, 1993). There are many Sapotaceae genera that produce edible fruits, such as *Chrysophyllum*, *Manilkara*, *Mimusops*, and *Pouteria* (Mabberley, 1993). Sapotaceae species grow well in tropical and subtropical areas. In the United States, some

Sapotaceae species are grown in California, southern Florida, and Hawaii and some are sold commercially. But in general, Sapotaceae plants are not widely used in the United States. Not many phytochemical studies have been conducted in the Sapotaceae family. A detailed study of phytochemical antioxidants from these four Sapotaceae plants is the main objective of this project.

Antioxidant Assays

A number of assays have been developed to evaluate antioxidant activity (Khal and Hilderbrant, 1986). Table 1.2 shows some of the available antioxidant assays. Generally, they are classified into two classes according to their mechanisms. One class of antioxidant assay measures the free radical scavenging activity of antioxidants by using some known free radicals, for example, the chemiluminescence assay (Whitehead et al., 1992), the crocin based assay (Tubaro et al., 1998), the dichlorofluorescin-diacetate (DCFH-DA) based assay (Valkonen and Kuusi, 1997), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Blois, 1958), the oxygen radical absorbance capacity (ORAC) assay (Cao et al., 1993), the phycoerythrin (PE) based assay (Ghiselli et al., 1994), the Trolox equivalent antioxidant capacity (TEAC) assay (Miller et al., 1993), the total oxyradical scavenging capacity (TOSC) (Winston et al., 1998), and the total radical trapping parameter (TRAP) assay (Wayner et al., 1985). These assays use different free radicals, or free radical producing agents, such as luminal radicals in the chemiluminescence assay, DPPH in the DPPH assay, and 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) in the TEAC assay. The amounts of free radicals scavenged are determined by different methods, such as spectroscopy, to determine the antioxidant activity of samples.

The other class of antioxidant assay involves the measurement of inhibition of lipid peroxidation by antioxidants, and includes the conjugated dienes products assay (Esterbauer et al., 1991), the fluorescent products assay (Esterbauer et al., 1991), the headspace gas chromatography (GC) assay (Frankel et al., 1992), the peroxide value assay (Jessup et al., 1990), the thiobarbituric acid reacting substance (TBARS) assay (Auddy et al., 2003). In these assays, antioxidant activity is calculated by measuring the inhibition of lipid peroxidation by antioxidants via techniques such as headspace GC.

Among these techniques, the DPPH assay is widely used to measure antioxidant activity. The free radical DPPH (Figure 1.2) has a maximum absorbance at 517 nm in ethanol, and therefore can be detected by visible spectroscopy. As its odd electron becomes paired off by a hydrogen donor, a free radical-scavenging antioxidant, DPPH is reduced to 1,1-diphenyl-2-picrylhydrazine, the absorbance decreases, and the resulting change in color (purple to yellow) is stoichiometric with respect to the number of electrons captured (Figure 1.3) (Blois, 1958). In this method, the Lambert-Beer Law is obeyed over the useful range of absorption (Segel, 1976). This technique can measure the sample's scavenging ability of DPPH free radicals through monitoring the change of absorbance of DPPH free radicals. The DPPH assay is sensitive and reproducible because of DPPH's strong absorption at 517 nm. Additionally, the DPPH assay is simple because no expensive reagents are required. It is also rapid since the reaction time is only 30 min. This assay is very convenient for the screening of large numbers of samples. Therefore, in this research, DPPH assay was used for screening plant samples for antioxidant activity and further activity-guided fractionation and isolation of the antioxidant constituents. However, like other chemical assays, it can not be used to predict a direct

correlation between the determined antioxidant activity and potential protective properties in biological systems. Therefore, after the novel antioxidants are isolated and identified, some specific bioassays such as LDL oxidation and cell-based assays are needed to test their protective properties.

Activity-guided Fractionation

For many years, many active compounds, including cancer chemopreventive agents, have been isolated using activity-guided fractionation (Kinghorn et al., 1998; Kinghorn et al., 2003). Crude non-polar, mid-polar, and polar extracts prepared from each plant sample are evaluated for their potential antioxidant activity using DPPH assay, and based on the results, active extracts undergo further activity-guided fractionation until active compounds are isolated. No further work is performed on inactive extracts. Since antioxidative compounds belong to different phytochemical classes, they are structurally diverse and may have different polarities when extracted with or dissolved in organic solvents. All active extracts and fractions were subjected to further activity-guided fractionation without missing any active extract. Activity-guided fractionation is an efficient and timesaving technique that was used through this project.

Conclusions

Free radical apecies are formed constantly in living organisms, cause oxidative damage to biomolecules, and lead to a myriad of diseases. Antioxidants can scavenge free radicals and may prevent the pathogenesis of these diseases. A large variety of phytochemical classes of antioxidants were found from edible plants. Besides known antioxidants, many unknown antioxidants may be present in edible plants and may play

an important role in the overall antioxidant activity. Over two/thirds of higher plant species are tropical and provide a rich source of phytochemicals.

In this project, four edible plants from the Sapotaceae family underwent activity-guided fractionation to isolate and identify their antioxidant constituents.

Table 1.1. Selected phytochemical antioxidants from different compound classes

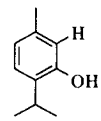
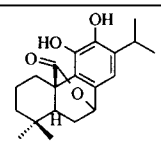
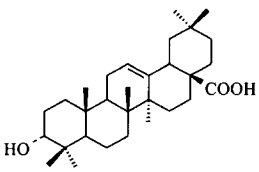
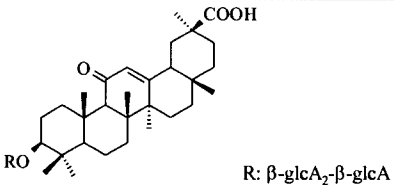
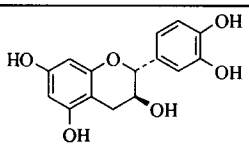
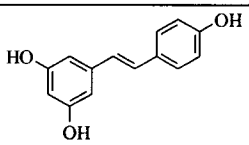
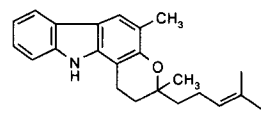
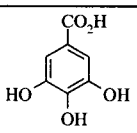
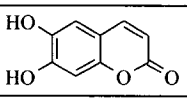
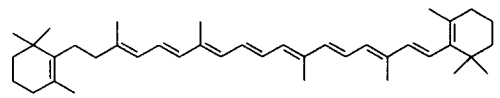
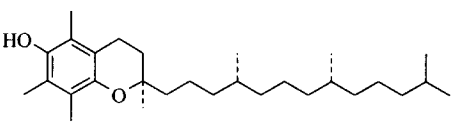
Compound Class	Compound Name	Structure	Reference
Monoterpene	Thymol		Aeschbach et al., 1994
Diterpene	Carnosol		Pearson et al., 1997
Triterpene	Oleanolic acid		Balanehru and Nagarajan, 1991
Saponin	Glycyrrhizin	 R: β-glcA$_2$-β-glcA	Yoshikoshi et al., 1996
Flavonoid	(+)-Catechin		Mangiapane et al., 1992
Stilbene	Resveratrol		Fremont, 2000; Jang et al., 1997
Alkaloid	Mahanimbine		Ramsewak et al., 1999
Aromatic acids	Gallic acid		Luo et al., 2002
Coumarins	Esculentin		Hoult and Paya, 1996
Carotenoids	β-Carotene		Krinsky, 1998
Vitamins	α-Tocopherol		Diplock, 1993

Table 1.2. Some antioxidant assays

Mechanism	**Assay Name**	**Reference**
Scavenging activity of free radicals	Chemiluminescence	Whitehead et al., 1992
	Crocin based	Tubaro et al., 1998
	DCFH-DA based	Valkonen and Kuusi, 1997
	DPPH	Blois, 1958
	OARC	Cao et al., 1993
	PE based	Ghiselli et al., 1994
	TEAC	Miller et al., 1993
	TOSC	Winston et al., 1998
	TRAP	Wayner et al., 1985
Inhibition of lipid peroxidation	Conjugated dienes products	Esterbauer et al., 1991
	Fluorescent products	Esterbauer et al., 1991
	Headspace GC	Frankel et al., 1992
	Peroxide value	Jessup et al., 1990
	TBARS	Auddy et al., 2003

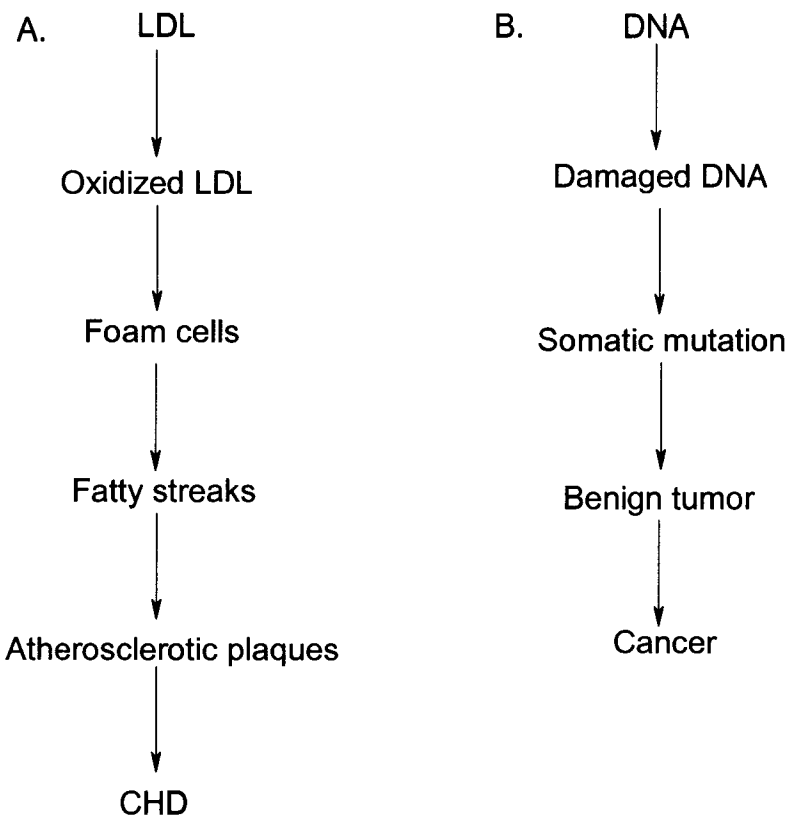


Figure 1.1. The pathogenesis of CHD (A) and cancer (B)

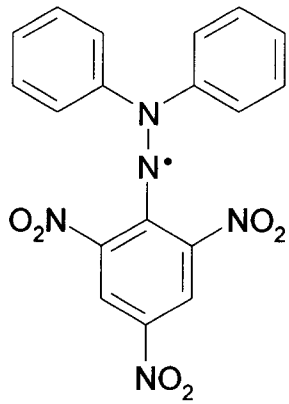


Figure 1.2. The structure of DPPH

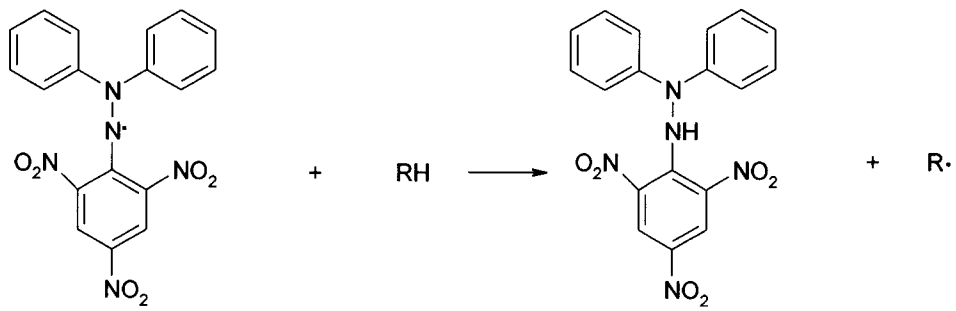


Figure 1.3. Scheme for scavenging of DPPH free radical by an antioxidant (RH)

Chapter 2

Screening of Forty-nine Less-used Edible Fruits and Vegetables for Antioxidant Activity

Introduction

Edible plants are regarded as excellent resources of natural antioxidants. Besides the traditional nutrient antioxidants such as α-tocopheral (vitamin E), β-carotene, and ascorbic acid (vitamin C), edible plants contain various other antioxidants, such as polyphenols, which may play an important role in their overall antioxidant activity (Cao et al., 1996). Epidemiological studies indicate that a high consumption of fruits and vegetables is associated with lower incidence and mortality rates of CHD and cancer, and some researchers have suggested that this phenomenon is not solely due to increased levels of vitamins and fibers, but may be attributed to polyphenolic antioxidants (Hertog et al., 1995; Parodi, 1997).

Some edible fruits and vegetables have been studied for their natural antioxidants beyond the traditional nutrient antioxidants. Resveratrol, found in grapes, may play an important role in the prevention of CHD by inhibiting the oxidation of low-density lipoprotein (LDL) (Fremont, 2000). Resveratrol is also a cancer chemopreventive agent because it acts as a reactive free radical scavenger (Jang et al., 1997; Lin and Tsai, 1999). Lycopene, abundant in tomatoes, may prevent CHD and cancer by inhibiting the oxidation of LDL and DNA (Balestrieri et al., 2004; Tapiero et al., 2004). Quercetin, found in apples and many other plants, can inhibit the oxidation of LDL, and thus may act as a CHD chemopreventive agent (Hou et al., 2004). Quercetin is also a potent anti-cancer agent (Wenzel et al., 2004). Many additional antioxidants may be present in fruits, but have not been isolated. For example, in blueberries, less than 10% of the total antioxidant capacity of the fruits can be attributed to known antioxidants (Prior et al.,

1998). Therefore, further research on understanding the compounds responsible for antioxidant activity from edible fruits and vegetables is necessary.

In this study, a number of edible fruits and vegetables were screened for their antioxidant activity. Many of them are not widely used in the United States, and are therefore regarded as less-used. Most of them grow in tropical and subtropical areas. The plants were selected based on studies of their ethnobotanical use and as reported in the scientific literature. The fresh plant material was extracted with methanol (MeOH), and partitioned with hexane and ethyl acetate (EtOAc). The EtOAc-soluble fractions were tested by the DPPH free radical assay to quantify the antioxidant capacity of the plant material.

Results and Discussion

After solvent-solvent partitioning, the EtOAc-soluble fractions were screened for their antioxidant activity in the DPPH assay. The known antioxidant vitamins C and E and the flavonoid kaempferol were used as positive controls. Fractions with an IC_{50} of greater than or equal to 200 μg/mL are considered inactive in this study. Of the forty-nine EtOAc-soluble fractions tested, thirty-six displayed antioxidant activity in the DPPH assay. The IC_{50} values for active plant extracts varied from 11.7 μg/mL in *Monstera deliciosa* Liebm. (Araceae) to 169.1 μg/mL in *Colocasia esculenta* (L.) Schott (Convolvulaceae). The IC_{50} values for standard antioxidants were 9.4 μg/mL for kaempferol, 18.3 μg/mL for ascorbic acid, and 53.3 μg/mL for α-tocopherol. The results for the EtOAc-soluble fractions tested in the DPPH assay are presented in Table 2.1.

Out of forty-nine plants studied, thirty-six displayed antioxidant activity in the DPPH assay. High antioxidant activity ($IC_{50} < 50$ μg/mL) was observed in fractions of

eighteen plants, namely, *Chrysophyllum cainito*, *Garcinia gardneriana*, *G. livingstonei*, *G. spicata*, *Harrisia taylorii*, *Kigelia pinnata*, *Lecythis usitata*, *Manilkara zapota*, *Monstera deliciosa*, *Pachira aquatica*, *Pouteria caimito*, *P. hypoglauca*, *P. lucuma*, *P. sapota*, *Psidium friedrichsthalianum*, *Quararibea cordata*, *Rheedia edulis*, and *Theobroma grandiflorum*. Moderate antioxidant activity (50 μg/mL $\leq IC_{50} <$ 100 μg/mL) was observed in fractions of the plants *Antidesma bunius*, *Mammea americana*, *Mangifera indica*, *Manihot esculenta*, *Parmentiera cereifera*, *Pouteria viridis*, and *Spondias tuberosa*. Low antioxidant activity (100 μg/mL $\leq IC_{50} <$ 200 μg/mL) was observed in fractions of eleven plants, *Antidesma montanum*, *Averrhoa carambola*, *Colocasia esculenta*, *Crescentia cujete*, *Dovyalis hebecarpa*, *Garcinia xanthochymus*, *Melicoccus bijugatus*, *Opuntia dilleni*, *Pouteria campechiana*, *Quararibea funebris*, and *Spondias purpurea*. The remaining thirteen plant extracts were inactive ($IC_{50} \geq$ 200 μg/mL) in the DPPH assay. The antioxidant activity of these thirty-six plant EtOAc extractions is not attributable to ascorbic acid, because the water-soluble vitamin is present in the water, not in the EtOAc fraction.

Of the thirty-six active plants, eight plants are from the Sapotaceae family, six from the Clusiaceae family, three from the Anacardiaceae family, three from the Bignoniaceae family, three from the Bombaceae family, three from the Euphorbiaceae family, two from the Cactaceae family, and the remaining eight active plants are from other eight families. A total of fourteen (38.9%) active plants are from only two plant families, Sapotaceae and Clusiaceae.

(+)-Catechin and (–)-epicatechin are well-known antioxidants and are present in many fruits. For the purpose of dereplication, if the plant extract had high levels of (+)-

catechin and (–)-epicatechin, and not many other compounds, it was given a low priority score. For example, *Pachira aquatica* appears to be a promising lead plant (IC_{50} = 15.0 μg/mL), but its EtOAc fraction display high level of (+)-catechin and (–)-epicatechin but not many other compounds (Figure 2.1); therefore, it was given a low priority score. The active fractions were further analyzed for (+)-catechin and (–)-epicatechin, first by TLC and by comparison of R_f values to standards, then the fractions with positive results were confirmed by HPLC through their UV spectra and retention times. Both catechin and epicatechin were detected in the fractions of *Bunchosia argentea, Colocasia esculenta, Lecythis usitata, Mammea Americana, Manihot esculenta, Manilkara zapota, Pachira aquatica, Pouteria lucuma, P. sapota, P. viridis, Quararibea cordata,* and *Theobroma grandiflorum.* Catechin was detected in the EtOAc fraction of *Pouteria campechiana* (Table 2.1). (+)-catechin and (–)-epicatechin contribute, at least in part, to the antioxidant activity of these twelve plants. Of these plants with (+)-catechin and (–)-epicatechin detected, five are from the Sapotaceae family. (+)-Catechin and (–)-epicatechin appear be the major antioxidant constituents in Sapotaceae plants, and may contribute significantly to the antioxidant activity observed in this family.

Further activity-guided fractionation work has been performed on eight active plant extracts in our laboratory. Two new antioxidants, methyl 4-*O*-galloylchlorogenate and 4-*O*-galloylchlorogenic acid, along with eight known polyphenolic antioxidants, gallic acid, (+)-gallocatechin, (+)-catechin, (–)-epicatechin, dihydromyricetin, quercitrin, myricitrin, and methyl chlorogenate, were identified from *Manilkara zapota* cv. Tikal. These two new antioxidants also displayed cytotoxicity (Ma et al., 2003). Nine antioxidant compounds gallic acid, (+)-gallocatechin, (–)-epicagallotechin, (+)-catechin,

(–)-epicatechin, quercetin, quercitrin, isoquercitrin, and myricitrin were identified from *Chrysophyllum cainito* (Luo et al., 2002). Seven antioxidant compounds (+)-catechin, (–)-epicatechin, isoscutellarein 8-*O*-*β*-D-glucuronide, hypolaetin 8-*O*-*β*-D-glucuronide, quercetin 3-*O*-*β*-D-glucuronide, quercetin 3-*O*-*β*-D-glucuronide 6″-methyl ester, and quercetin were identified from *Theobroma grandiflorum* (Yang et al., 2003). Four antioxidants gallic acid, (+)-gallocatechin, (+)-catechin, and myricitrin were identified from *Pouteria campechiana* (Ma et al., in press). Seven antioxidant compounds, gallic acid, (+)-gallocatechin, (+)-catechin, (–)-epicatechin, dihydromyricetin, (+)-catechin-3-*O*-gallate, and myricitrin, were identified from both *P. sapota* and *P. viridis* (Ma et al., in press). Two new benzophenone antioxidants with eleven known antioxidants were identified from *Garcinia xanthochymus*. These two new antioxidants are cytotoxic (Baggett et al., submitted). Two new coumarin antioxidants with seven known coumarin antioxidants were identified from *Mammea americana*. These two new coumarin antioxidants are also cytotoxic (Yang et al., in preparation) and may contribute significantly to the total antioxidant activity of the EtOAc fractions of these eight active plants.

From the results of activity-guided fractionation of the plants under study, most antioxidants identified from five Sapotaceae plants were catechins in the flavonoid phytochemical class, e.g. catechin, epicatechin, and gallocatechin. Other classes of antioxidants were still identified from other families, such as benzophenones from *Garcinia xanthochymus* (Clusiaceae), and coumarins from *Mammea americana* (Clusiaceae). A plausible explanation for this phenomenon is that different families of

plants have developed divergent biosynthetic pathways, and therefore, different plant families contain various classes of antioxidants.

Fruits and vegetables are a rich source of natural antioxidants. The antioxidant activity of many fruits and vegetables extends beyond the traditional vitamin antioxidants. Other classes of phytochemical antioxidants, such as benzophenones, coumarins, and flavonoids, are important natural antioxidants, and the extent of their impact on human health is beginning to be understood. In this study, forty-nine less-used edible fruits and vegetables were screened for their antioxidant activity using the DPPH assay. Thirty-six plants (73.5%) showed antioxidant activity in the DPPH assay. This is not surprising since antioxidants are very important for the plants to protect the intracellular environment against the oxidative stress caused by free radicals formed via biochemical reactions such as photosynthesis and oxidative phosphorylation. They mainly function as free radical scavengers, but they can also be peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, and synergists (Osawa, 1994).

From these thirty-six active plants, twelve plants (33.3%) were found to contain (+)-catechin and (–)-epicatechin, compounds that may contribute partly to the antioxidant activity of these plants.

Eight plants were selected for further study, and activity-guided fractionation was performed on their EtOAc-soluble fractions to isolate their antioxidant constituents. Many polyphenolic antioxidants including six new antioxidants from different compound classes and six cytotoxic agents were isolated and identified. Therefore, we can conclude that the DPPH assay is a simple, quick, and sensitive method to screen plant samples for

antioxidant activity. These results set the stage for my examination of polyphenolic antioxidants from four species of plants in the Sapotaceae family.

Experimental Section

Plant Material. Most fruits and vegetables were collected from southern Florida and bought in the New York City area. The fruits of *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum. (Sterculiaceae) were collected from French Guiana. Plants were stored at –20°C until extracted. Plants were identified by the authors, with the help of the Institute of Economic Botany, The New York Botanical Garden (NYBG), and voucher specimens were deposited at NYBG.

Preparation of Extracts. A standard technique of extracting antioxidants from fresh plant material was developed from a scheme successfully utilizeded by researchers in the antioxidant field (Gamez et al., 1998). Fresh plant material (100 g) was extracted with MeOH (100 mL) for 1 hour. The extract was concentrated under reduced pressure at a temperature not exceeding 40 °C. A portion of each MeOH/water extract was sequentially partitioned with hexane (3 x 50 mL) and EtOAc (3 x 50 mL) (Figure 2.2). The hexane-soluble fraction was dried in vacuo, and stored at –20 °C. The EtOAc-soluble fraction was dried over sodium sulfate, then brought to dryness in vacuo, and stored at –20 °C. The water extract was dried and stored at –20 °C. The EtOAc-soluble fractions were screened for antioxidant activity in the DPPH free radical assay.

DPPH Assay. EtOAc-soluble fractions were subjected to the DPPH assay as previously described (Blois, 1958; Smith et al., 1987). Briefly, EtOAc-soluble fractions were dissolved in dimethyl sulfoxide (DMSO) and further diluted into four 2-fold serial dilutions. DPPH was dissolved in 95% ethanol (EtOH) to prepare a 400 μM solution.

Reaction mixtures containing test samples (dissolved in DMSO) and the DPPH ethanolic solution in 96-well microtiter plates were incubated at 37 °C for 30 min. Each well contained 150 μL of 400 μM DPPH solution and 50 μL of sample solution, final concentrations of test samples were typically in the 1-50 μg/mL range. Absorbances were measured at 515 nm using a *VERSA*$_{max}$ tunable microplate reader (Molecular Devices, Sunnyvale, CA). Percent inhibition by sample treatment was determined by comparison with a DMSO-treated control group: % Inhibition = [(C-S)/C] x 100, where C is the absorbance of the DMSO-treated control at 515 nm, and S is the absorbance of the sample at 515 nm. A plot of the percent inhibition versus the concentration of sample at 515 nm was constructed, and IC_{50} values were defined as the concentration of the sample required to scavenge 50% of DPPH free radicals. The lower the IC_{50} value, the higher the antioxidant activity. The known antioxidants ascorbic acid, α-tocopherol, and kaempferol were used as positive controls. For each plant tested, mean IC_{50} values were obtained from duplicate experiments with less than 5% difference between duplications. For the purposes of these experiments, IC_{50} values less than 50 μg/mL are considered to be highly active. IC_{50} values greater than or equal to 50 μg/mL and less than 100 μg/mL are moderately active. IC_{50} values greater than or equal to 100 μg/mL and less than 200 μg/mL are weakly active. IC_{50} values greater than or equal to 200 μg/mL are considered inactive.

Identification of Polyphenolic Antioxidants. Polyphenolic antioxidants (+)-catechin and (–)-epicatechin in plant extracts were identified using thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) comparing with standard compounds. TLC analysis was performed on RP-18 F_{254} plates (Merck,

Darmstadt, Germany), with a mobile phase composed of 3:7 MeOH/H_2O. The compounds were visualized by spraying with 1% vanillin in 10% (v/v) H_2SO_4 in EtOH followed by heating. The R_f values were 0.53 and 0.42 for catechin and epicatechin, respectively. HPLC analysis was carried out on a Waters 2690 separations module equipped with a Waters 996 photodiode array detector and Millenium[32] software using a Phenomenex Nucleosil C18 column (250 x 4.6 mm, 5 μm) and a solvent system of 1:19 to 1:1 MeCN/H_2O linear gradient, with a flow rate of 1 mL/min, and a 20 min run time. The HPLC profile was monitored at 280 nm. (+)-Catechin and (–)-epicatechin were identified via their UV spectra (maximum absorbance at 214 nm and 279 nm) and retention times in the solvent system and conditions described (8.3 and 9.5 min, respectively).

Table 2.1. Results of DPPH assay for the EtOAC extracts of selected edible plants and standard antioxidants

Name	Family	Vernacular name	Plant part	EtOAc extract IC_{50} (μg/mL)	Detection of catechin and epicatechin
Antidesma bunius (L.) Spreng.	Euphorbiaceae	Bignay	Fruit	92.5	
Antidesma montanum Blume	Euphorbiaceae	Gunchak	Fruit	102.8	
Averrhoa carambola L.	Oxalidaceae	Star fruit	Fruit	150.5	
Blighia sapida K.D. Koenig	Sapindaceae	Akee	Fruit	>200	
Brassica chinensis L. var. parchinensis	Cruciferae	Flowering cabbage	Flowers, stems	>200	
Bunchosia argentea (Jacq.) DC.	Malphighiaceae	Bunchosia	Fruit	>200	
Chrysophyllum cainito L.	Sapotaceae	Star apple	Fruit	22	+
Cnidoscolus chayamansa McVaugh	Euphorbiaceae	Chaya	Leaves	>200	
Colocasia esculenta (L.) Schott	Convolvulaceae	Taro	Corm	169.1	+
Crescentia cujete L.	Bignoniaceae	Calabash tree	Fruit	123.5	
Diospyros digyna Jacq.	Ebenaceae	Black sapote	Fruit	>200	
Diospyros kaki L. f.	Ebenaceae	Persimmon	Fruit	>200	
Dovyalis hebecarpa (Gardner) Warb.	Flaucortiaceae	Ceylon gooseberry	Fruit	141.0	
Garcinia gardneriana (Planch. & Triana) Zappi	Clusiaceae		Fruit	16.9	
Garcinia livingstonei T. Anderson	Clusiaceae	Imbé	Fruit	27.4	
Garcinia spicata Hook.	Clusiaceae	Spicata	Fruit	16.6	
Garcinia xanthochymus Hook.f. ex T. Anderson	Clusiaceae	Gamboge	Fruit	113.3	
Harrisia taylorii Britton	Cactaceae		Fruit	19.4	
Kigelia pinnata (Jacq.) DC.	Bignoniaceae	Sausage tree	Fruit	34.9	
Lagenaria siceraria (Molina) Standl.	Curcurbitaceae	Bottle gourd	Fruit	>200	
Lecythis usitata Miers	Lecythidaceae	Paradise nut	Fruit	14.2	+
Luffa acutangula (L.) Roxb.	Curcurbitaceae	Angled gourd	Fruit	>200	
Mammea americana L.	Clusiaceae	Mammee apple	Fruit	55.9	+
Mangifera indica L.	Anacardiaceae	Mango	Fruit	84.0	
Manihot esculenta Crantz	Euphorbiaceae	Cassava	Fruit	72.1	+
Manilkara zapota (L.) P. Royen cv. Tikal	Sapotaceae	Sapodilla plum	Fruit	33.2	+
Melicoccus bijugatus Jacq.	Sapondaceae	Mamoncillo	Fruit	110.3	
Mimusops balata (Aubl.) C.F. Gaertn.	Sapotaceae	Balata	Fruit	>200	

Momordica charantia L.	Curcurbitaceae	Bitter cucumber	Fruit	>200	
Monstera deliciosa Liebm.	Araceae	Ceriman	Fruit	11.7	
Moringa pterygosperma Gaertn.	Moringaceae	Horseradish tree	Fruit	>200	
Opuntia dilleni (Ker Gawl.) Haw.	Cactaceae	Drillen prickly-pear	Fruit	136.8	
Pachira aquatica Aubl.	Bombaceae	Guiana chestnut	Seeds	15.0	+
Parmentiera cereifera Seem.	Bignoniaceae	Candle tree	Fruit	93.7	
Persea americana Mill.	Lauraceae	Avocado	Fruit	>200	
Pouteria caimito (Ruiz & Pav.) Radlk.	Sapotacea	Abiu	Fruit	23.1	
Pouteria campechiana (Kunth) Baehni	Sapotacea	Canistel	Fruit	142.1	c
Pouteria hypoglauca (Standl.) Baehni	Sapotacea	Cinnamon apple	Fruit	17.5	
Pouteria lucuma (Ruiz & Pav.) Kuntze	Sapotaceae	Lucmo	Fruit	33.8	+
Pouteria sapota (Jacq.) H.E. Moore & Stearn.	Sapotacea	Sapote	Fruit	12.8	+
Pouteria viridis (Pittier) Cronquist	Sapotacea	Green sapote	Fruit	52.6	+
Psidium friedrichsthalianum (O. Berg) Nied.	Myrtaceae	Cas	Fruit	42.1	
Quararibea cordata (Bonpl.) Vischer	Bombaceae	Chupa-chupa	Fruit	20.9	+
Quararibea funebris (La Llave) Vischer	Bombaceae	Llave	Fruit	102.5	
Rheedia edulis (Seem.) Planch. & Triana	Clusiaceae	Mameyito	Fruit	27.8	
Rollinia pulchrinervis A. DC.	Annonaceae	Abriba	Fruit	>200	
Spondias purpurea L. var. lutea	Anacardiaceae	Yellow mombin	Fruit	158.6	
Spondias tuberosa Arruda.	Anacardiaceae	Imbu	Fruit	84.6	
Theobroma grandiflorum (Willd. ex Spreng.) K. Schum.	Sterculiaceae	Cupuaçu	Seeds	40.9	+
L-Ascorbic acid		Vitamin C		18.3	
α-Tocopherol		Vitamin E		53.3	
Kaempferol				9.4	

+, both catechin and epicatechin were detected.
c, only catechin was detected.

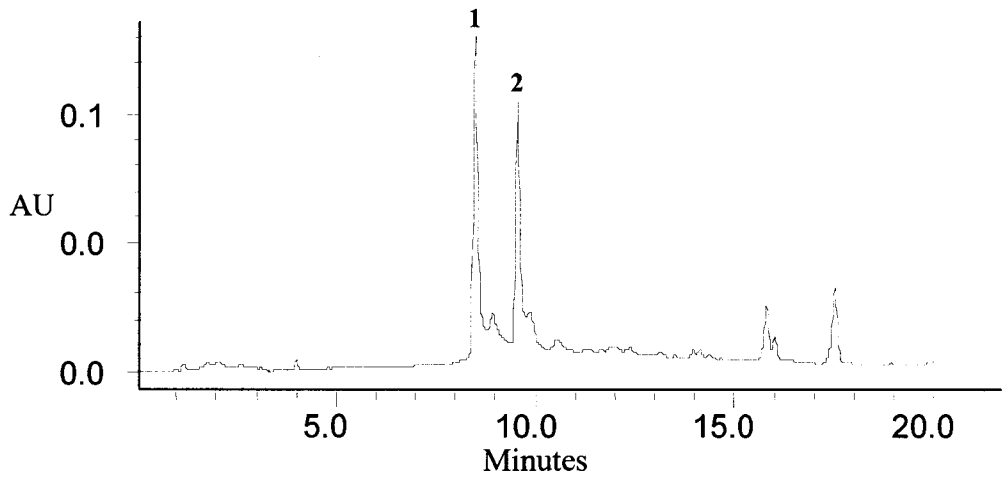


Figure 2.1. HPLC chromatogram at 280 nm of the EtOAc extract of *Pachira aquatica*: **1**, (+)-catechin; **2**, (–)-epicatechin

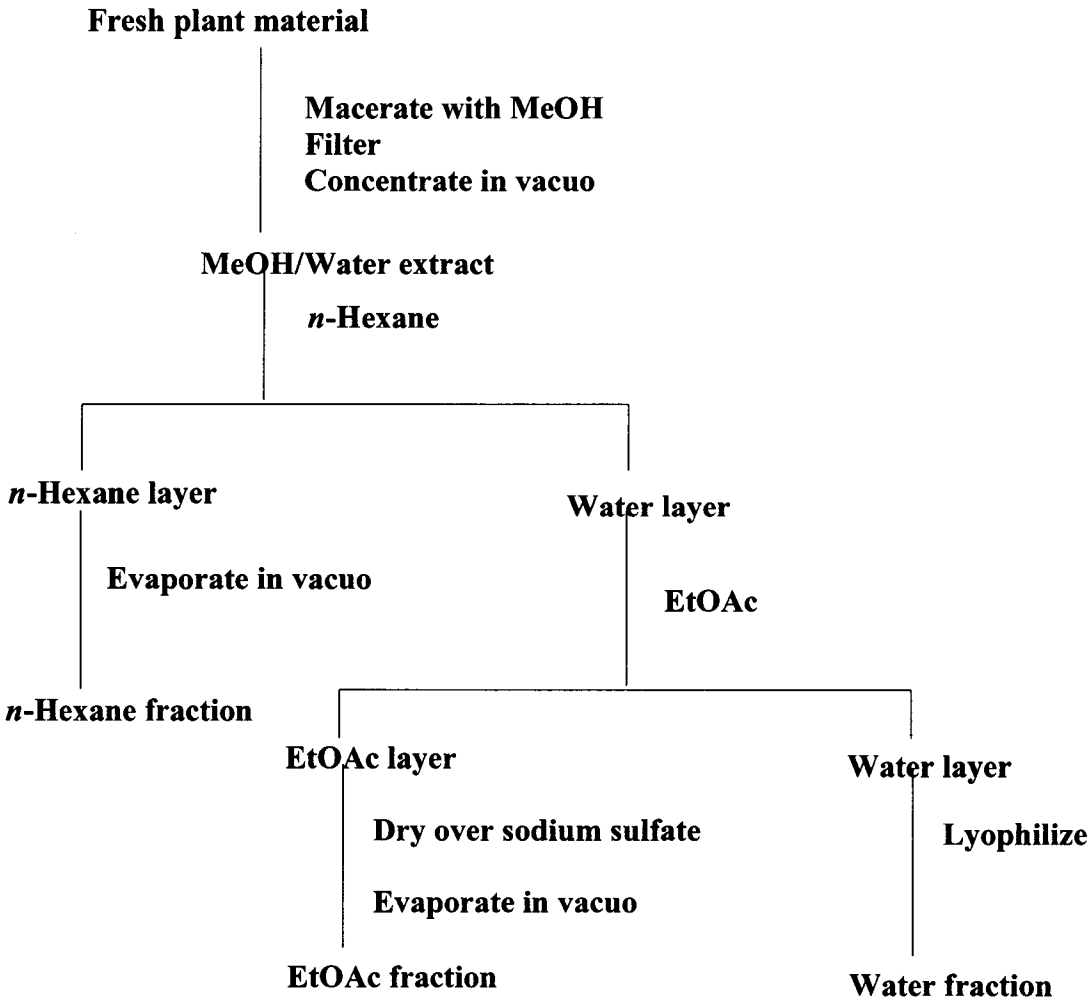


Figure 2.2. Extraction procedure for plant material

Chapter 3

Bioactive Novel Polyphenols from the Fruit of *Manilkara zapota* (Sapodilla)

Introduction

In living systems, free radicals are formed constantly and can cause oxidative damage to DNA, lipids, and proteins, and may lead to certain diseases such as CHD and cancer (Jacob and Burri, 1996). Antioxidants, which scavenge free radicals, can prevent the oxidation of biomolecules, such as low-density lipoprotein (LDL) and DNA, and may thereby break the reaction chains of pathogenesis of CHD and cancer (Scarfiotti et al., 1997). High fruit consumption has been associated with lower incidence and mortality rates of CHD and cancer (Omenn, 1995), and dietary antioxidants have been shown to be beneficial in reducing the risk of cognitive dysfunction in aged humans (Engelhart et al., 2002). This phenomenon has been attributed to the various antioxidants contained in fruit, which are a rich source of natural antioxidants (Omenn, 1995). In addition to the classical nutrient antioxidants (e.g. vitamins C and E, and β-carotene), fruits contain polyphenolic antioxidants, which may play an important role in the overall antioxidant activity of fruits (Cao et al., 1996).

As well as antioxidant activity, polyphenols have other mechanisms of action that may be important in disease prevention. EGCG, for example, inhibits cell growth and the activation of the epidermal growth factor receptor (EGFR) (Liang et al., 1997), causes G1 arrest of the cell cycle, and induces apoptosis in human prostate, lung, colon, gastric carcinoma, leukemia, squamous cell carcinoma, and head and neck cancer cell lines (Masuda et al., 2001). EGCG inhibits activation of growth factor receptors and extracellular signal-regulated protein kinase (ERK) proteins and also inhibits activator protein-1 (AP-1) activity and c-*fos* promoter activity (Masuda et al., 2001). We are

interested in identifying cytotoxic polyphenols from edible plants that may be important in cancer prevention by inhibiting the activation of the EGFR.

The tropical fruit tree *Manilkara zapota* (L.) P. Royen (Sapotaceae) (Figure 3.1), known commonly as sapodilla or naseberry, is native to Mexico (Yucatan Peninsula) and Central America. The sapodilla is a slow-growing, long-lived tree and can reach a height of 12 to 18 m (Balerdi and Crane, 2000). The ripe fruit range from nearly round to spindle-shaped, 5 to 10 cm in diameter, edible though grainy, and has a sweet pleasant flavor resembling that of a pear (Morton, 1987). Cultivated varieties, such as *M. zapota* cv. Tikal, have been developed for enhanced flavor and edible skin (Morton, 1987). Sapodilla fruit is often eaten fresh, but the pulp is also incorporated into sherbets, milkshakes, and ice cream (Balerdi and Crane, 2000). The nutrient value of sapodilla fruit (100 g) includes 0.4 g protein, 1.1 g fat, 20 g carbohydrate, 5.3 g total dietary fiber, 210 mg calcium, 0.8 mg iron, 12.0 mg magnesium, 12.0 mg phosphorus, 193.0 mg potassium, 12.0 mg sodium, and 14.7 mg vitamin C, and a total calorie count of 83 kcal (Balerdi and Crane, 2000). The gummy latex of sapodilla, called chicle, is used to make chewing gums and the fruit is used to treat diarrhea and pulmonary diseases (bin Haji Mohiddin et al., 1992; Morton, 1987). The leaves have been used to treat cough, cold, and diarrhea, and the bark to treat diarrhea and dysentery (Morton, 1987).

The polyphenols (+)-catechin, (–)-epicatechin, leucocyanidin, leucodelphinidin, leucopelargonidin, chlorogenic acid, and gallic acid, have been reported previously from unripe sapodilla fruit (Lakshminarayana and Mathew, 1967; Mathew and Lakshminarayana, 1969; Prabha and Patwardhan, 1986). From ripe fruit, the level of 5-caffeoyl quinic acid was measured recently in the peel (94.6 ± 9.50 mg/kg) and the pulp

(32.1 ± 18.7 mg/kg) (Pontes et al., 2002). The antioxidant activity of sapodilla fruit has been reported to be very high in the 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay (AEAC = 3396 ± 387.9 mg/kg) (Leong and Shui, 2002). This paper describes the isolation and structural elucidation of two new chlorogenic acid derivatives from *M. zapota* with antioxidant and cytotoxicity activities.

Results and Discussion

The fresh frozen *M. zapota* cv. Tikal fruit was extracted with MeOH and partitioned with hexane and EtOAc sequentially. The EtOAc-soluble fraction displayed high antioxidant activity in the DPPH assay and cytotoxicity against the HCT-116 and SW-480 human colon cancer cell lines. Activity-guided fractionation of the EtOAc-soluble fraction was performed to isolate the antioxidant constituents. The EtOAc-soluble fraction was separated by Sephadex LH-20 column chromatography (CC). Fractions were recombined according to their reversed-phase C18 (RP18) TLC profiles to give eleven combined fractions in total (A-K). All fractions were tested in the DPPH assay. Active fractions A, B, and K were further separated by RP18 CC. Two new antioxidants, **1** and **2**, were isolated from fraction B.

Compound **1** was obtained as a yellow amorphous powder. In the positive ion HR-FABMS, a peak corresponding to $[M + H]^+$ was observed at *m/z* 521.1294, showing the molecular formula of **1** to be $C_{24}H_{24}O_{13}$. The ^1H NMR spectrum of **1** displayed an AMX spin system consisting of δ 7.01 (1H, d, J = 2.2 Hz), 6.77 (1H, d, J = 8.2 Hz), and 6.91 (1H, dd, J = 8.2, 2.2 Hz), thereby indicating a 1′,3′,4′-trisubstituted benzene ring (Kweon et al., 2001). The presence of a *trans* double bond was noted in the ^1H NMR spectrum of **1**, δ 6.16 (1H, d, J = 16.0 Hz) and 7.50 (1H, d, J = 16.0 Hz), suggesting the

presence of a *trans* caffeoyl moiety in **1**. A gallic acid moiety in **1** was determined by ^{1}H NMR δ7.12 (2H, s, H-2″ and 6″) and ^{13}C NMR, δ166.5 (C-7″), 145.5 (C-3″ and 5″), 138.8 (C-4″), 119.9 (C-1″), 109.2 (C-2″). The ^{1}H and ^{13}C NMR data of **1** are consistent with previously published data for methyl chlorogenate and gallic acid (Gottlieb et al., 1991; Miyazaki et al., 1991; Wu et al., 1995). HMBC correlations (Table 3.1) clearly revealed H-4 (δ5.18) in the quinic acid moiety of chlorogenic acid is long range coupled to the C-7″ carbonyl group (δ166.5) of gallic acid (Figure 3.2). Therefore, the gallic acid moiety is attached at the 4 position of the quinic acid moiety. In addition, the *O*-methyl protons (δ3.72) are long range coupled to the C-7 carbonyl group (δ174.0) in the quinic acid moiety of chlorogenic acid (Figure 3.2). This indicates the methyl group is attached at the 7 position of the quinic acid moiety.

Analysis of ^{1}H NMR experiments was used to determine the stereochemistry of **1**. The ^{1}H NMR spectrum of **1** displayed H-4 as a dd at δ5.18 with *J* values of 2.9 and 8.1 Hz, indicating *axial-axial* (J = 8.1 Hz) and *axial-equatorial* (J = 2.9 Hz) couplings. Homodecoupling experiments were carried out on **1** by irradiating H-3 (δ4.41) and H-5 (δ5.59). The large coupling of H-4 and H-5 (J = 8.1 Hz) (Figure 3.3) demonstrates *axial-axial* coupling and the small coupling of H-3 and H-4 (J = 2.9 Hz) (Figure 3.4) shows *axial-equatorial* coupling. These results confirm **1** has the same stereochemistry in its quinic acid moiety as chlorogenic acid (Pauli et al., 1999). Therefore, **1** was identified as methyl 4-*O*-galloylchlorogenate. The configuration of chlorogenic acid derivatives is of biological significance, as in the protection against lipid peroxidation (Muller et al., 1998) and as inhibitors of hepatic glucose-6-phosphate translocase (Hemmerle et al., 1997), but there is confusion in the literature on the nomenclature of these compounds

(Haribal et al., 1998). Previous studies have found the quinic acid moiety of chlorogenic acid to be in the chair conformation (Haribal et al., 1998), and this conclusion is further supported by the NMR data of **1**.

Compound **2,** a yellow amorphous powder, displayed a peak at *m/z* 507.1135 in the positive ion HR-FABMS, corresponding to $[M + H]^+$, indicating a molecular formula of $C_{23}H_{22}O_{13}$. In a manner similar to **1**, homodecoupling experiments were carried out on **2**. The results showed that **2** has the same stereochemistry in the quinic acid moiety as chlorogenic acid (Pauli et al., 1999). Compound **2** has similar 1D and 2D NMR data as **1,** except **2** does not display chemical shifts for an *O*-methyl group (Table 1). Therefore, **2** was identified as 4-*O*-galloylchlorogenic acid.

The possibility that **1** is a MeOH extraction artifact of **2** was studied by extracting *M. zapota* cv. Tikal fruit with 95% EtOH and partitioning with hexane and EtOAc sequentially. The EtOAc-soluble fraction was separated by Sephadex LH-20 CC with 95% EtOH. By TLC, fraction I from the Sephadex separation contained a compound with the same R_f value as **1**. Fraction I was further analyzed by liquid chromatography–electrospray ionization mass spectrometry (LC-ESIMS) in the negative mode, and a peak with retention time 11.7 min displayed an ion with *m/z* 519 $[M - H]^-$. Under the same LC-MS conditions, standard compound **1** also had a retention time 11.7 min and displayed an ion with *m/z* 519 $[M - H]^-$. These results confirmed the presence of **1** in the EtOH extract of sapodilla fruit, and therefore we conclude that **1** is not a MeOH extraction artifact of **2**.

In the DPPH assay, **1** and **2** showed high antioxidant activity, IC_{50} = 12.9 and 23.5 μM, respectively (Table 3.2). The IC_{50} value of commercially available chlorogenic acid is 39.5 μM. Thus, the addition of a gallic acid moiety to chlorogenic acid improves the

latter's antioxidant activity. In the microtetrazolium (MTT) assay, **1** displayed cytotoxicity against the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 190 and 160 μM, respectively. Compound **2** displayed cytotoxicity in the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 154 and 134 μM, respectively. EGCG, known to inhibit the activation of the EGFR (Masuda et al., 2001), displayed IC_{50} values of 161 and 195 μM in the HCT-116 and SW-480 human colon cancer cell lines, respectively. Chlorogenic acid displayed cytotoxicity against the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 367 and 353 μM, respectively. Thus the addition of a gallic acid moiety to chlorogenic acid results in a more cytotoxic compound.

Eight additional known antioxidants, namely methyl chlorogenate (**3**), dihydromyricetin (**4**), quercitrin (**5**), myricitrin (**6**), (+)-catechin (**7**), (–)-epicatechin (**8**), (+)-gallocatechin (**9**), and gallic acid (**10**) (Figure 3.5), were isolated from fractions A, B, and K, and identified by spectroscopic methods.

Experimental Section

General Experimental Procedures. Melting points were determined on a Mel-Temp II melting point apparatus (Laboratory Devices, Placerville, CA) and are uncorrected. Optical rotations were measured on an Autopol II Automatic Polarimeter (Rudolph Research Analytical, Flanders, NJ). ^{1}H NMR and ^{13}C NMR spectra were recorded using a JOEL GX-400 MHz, operating at 400 and 100 MHz, respectively. 2D-NMR experiments were run on a Varian Unity Plus 500 MHz. All compounds were measured in CD_3OD (Pauli et al., 1999). ESIMS was performed on a Finnigan LCQ Deca instrument (Thermo Finnigan, San Jose, CA) equipped with Xcalibur software. Samples

were dissolved in MeOH and introduced by direct injection. The capillary voltage was 10 V, the spray voltage was 4.5 kV, and the tube lens was offset at 0 V. The capillary temperature was 230 °C. HR-FABMS was performed on a 70-SE-4F mass spectrometer (Micromass). Samples were dissolved in MeOH. HPLC analysis was carried out on a Waters 2690 Separations Module equipped with a Waters 996 Photodiode Array (PDA) Detector and Millenium[32] software using a Phenomenex Nucleosil C18 column (4.6 x 250 mm, 5 μm) and a solvent system of 1:19 to 1:1 MeCN/H_2O linear gradient, and a flow rate of 1 mL/min, 20 min running time. TLC analysis was performed on RP-18 F_{254} plates (Merck, Darmstadt, Germany), with compounds visualized by spraying with 1% vanillin in 10% (v/v) H_2SO_4 in EtOH. Sephadex LH-20 (25–100 μm; Pharmacia Fine Chemicals, Piscataway, NJ) and C18 reversed-phase silica gel (40 μm; J. T. Baker, Phillipsburg, NJ) were used for column chromatography.

Plant Material. Fruit of *M. zapota* cv. Tikal was collected from the Fruit and Spice Park (Homestead, FL) and Dr. Richard J. Campbell's tropical fruit collection at the Montgomery Botanical Center (Coral Gables, FL). Frozen fruit was shipped to New York City by overnight courier and stored at –20 °C until extracted. A voucher specimen of *M. zapota* was prepared, identified, and deposited at the herbarium of The New York Botanical Garden (Bronx, NY).

Extraction and Isolation. The fresh frozen fruit (20.2 kg) of *M. zapota* was twice extracted with MeOH at room temperature for 1 h per extraction. After the MeOH was removed in vacuo, the resulting aqueous extract was partitioned with hexane and EtOAc, respectively. The EtOAc fraction (IC_{50} = 10.7 μg/mL in the DPPH assay, IC_{50} = 95 μg/mL for HCT-116, and IC_{50} = 100 μg/mL for SW-480) was concentrated in vacuo to

give 14.6 g of a residue, of which 13.8 g was separated by Sephadex LH-20 CC (from 100% H_2O to 100% MeOH in 10% steps). The resulting fractions were combined according to their RP18 TLC profiles to give 11 combined fractions in total (A-K). All fractions were tested in the DPPH assay, and fractions A (2.2 g), B (1.1 g), and K (0.6 g) showed high activity, 5.2, 7.1, and 19.2 μg/mL, respectively.

Fraction A was separated over RP18 CC (from 1:9 to 3:2 MeOH/H_2O). Subfraction A-1 was purified by RP18 CC eluting with a gradient of 100% H_2O to 1:9 MeOH/H_2O to yield gallocatechin (**9**) (114.6 mg) and gallic acid (**10**) (95.6 mg). Subfraction A-2 was purified by RP18 CC eluting with a gradient of 1:9 to 4:21 MeOH/H_2O to obtain catechin (**7**) (14.9 mg). Subfraction A-3 was purified by RP18 CC eluting with a gradient of 1:4 to 3:7 MeOH/H_2O to obtain epicatechin (**8**) (30.9 mg).

Fraction B was separated over RP18 CC (from 1:9 to 3:2 MeOH/H_2O) to yield methyl 4-*O*-galloylchlorogenate (**1**) (45.6 mg) and dihydromyricetin (**4**). Subfraction B-7 was purified by RP18 CC eluting with a gradient of 1:4 to 2:3 MeOH/H_2O to obtain B-7-2. B-7-2 was further purified by continual RP18 CC eluting with a gradient of 1:9 to 1:4 MeCN/H_2O to yield 4-*O*-galloyl chlorogenic acid (**2**) (15.9 mg). Subfraction B-8 was purified by RP18 CC eluting with a gradient of 3:7 to 1:1 MeOH/H_2O to obtain B-8-4. B-8-4 was further purified by continual RP18 CC eluting with an isocratic solvent system of 1:4 MeCN/H_2O to yield myricitrin (**6**) (14.3 mg). Subfraction B-10 was purified by RP18 CC eluting with a gradient of 3:7 to 2:3 MeOH/H_2O to obtain B-10-4. B-10-4 was further purified by continual RP18 CC eluting with an isocratic solvent system of 1:4 MeCN/H_2O to yield quercitrin (**5**) (4.0 mg). Fraction K was separated over RP18 CC (from 1:4 to 2:3 MeOH/H_2O) to yield methyl chlorogenate (**3**) (145.2 mg).

Methyl 4-*O*-galloylchlorogenate (1): white powder, mp 183-185 °C; $[\alpha]^{25}_{D}$ -137.4° (*c* 0.0048, MeOH); negative ESIMS *m/z* 519 $[M - H]^-$ (negative ESIMS/MS *m/z* 357 $[M - H - 162]^-$; negative ESIMS/MS *m/z* 367 $[M - H - 152]^-$); positive HR-FABMS *m/z* 521.1294 $[M + H]^+$ (calculated for $C_{24}H_{25}O_{13}$, 521.1295); ^1H NMR (CD_3OD, 400 MH_Z) δ 7.50 (1H, d, *J* = 16.0 H_Z, H-7′), 7.12 (2H, s, H-2″ and 6″), 7.01 (1H, d, *J* = 2.2 H_Z, H-2′), 6.91 (1H, dd, *J* = 8.2, 2.2 H_Z, H-6′), 6.77 (1H, d, *J* = 8.2 H_Z, H-5′), 6.16 (1H, d, *J* = 16.0 H_Z, H-8′), 5.59 (1H, m, H-3), 5.18 (1H, dd, *J* = 8.1, 2.9 H_Z, H-4), 4.41 (1H, m, H-5), 3.72 (3H, s, OCH_3), 2.12-2.30 (4H, m, H-2, 6); ^{13}C NMR (CD_3OD, 100 MH_Z) δ174.0 (C-7), 166.7 (C-9′), 166.5 (C-7″), 148.4 (C-4′), 146.4 (C-7′), 145.5 (C-3″, 5″), 145.2 (C-3′), 138.8 (C-4″), 126.3 (C-1′), 121.9 (C-6′), 119.9 (C-1″), 115.3 (C-5′), 113.9 (C-2′), 113.3 (C-8′), 109.2 (C-2″), 74.5 (C-1), 73.8 (C-4), 67.8 (C-3), 67.2 (C-5), 51.9 (OCH_3), 37.2 (C-2, 6).

4-*O*-Galloylchlorogenic acid (2): white powder, mp >300 °C (began decomposing at 180 °C); $[\alpha]^{25}_{D}$ -65.3° (*c* 0.00075, MeOH); negative ESIMS *m/z* 505 $[M - H]^-$ (negative ESIMS/MS *m/z* 343 $[M - H - 162]^-$); positive HR-FABMS *m/z* 507.1135 $[M + H]^+$ (calcd for $C_{23}H_{23}O_{13}$, 507.1139); ^1H NMR (CD_3OD, 400 MH_Z) δ 7.49 (1H, d, *J* = 15.8 H_Z, H-7′), 7.10 (2H, s, H-2″ and 6″), 6.98 (1H, d, *J* = 2.2 H_Z, H-2′), 6.88 (1H, dd, *J* = 8.2, 2.2 H_Z, H-6′), 6.74 (1H, d, *J* = 8.2 H_Z, H-5′), 6.17 (1H, d, *J* = 15.8 H_Z, H-8′), 5.75 (1H, m, H-3), 5.14 (1H, dd, *J* = 9.9, 2.9 H_Z, H-4), 4.39 (1H, m, H-5), 2.05-2.25 (4H, m, H-2, 6); ^{13}C NMR (CD_3OD, 100 MH_Z) δ178.6 (C-7), 167.3 (C-9′), 166.7 (C-7″), 148.2 (C-4′), 146.0 (C-7′), 145.4 (C-3″, 5″), 145.1 (C-3′), 138.6 (C-4″), 126.4 (C-1′), 121.8 (C-6′), 120.1 (C-1″), 115.2 (C-5′), 113.8 (C-2′), 113.6 (C-8′), 109.1 (C-2″), 76.1 (C-1, 4), 69.2 (C-5), 68.2 (C-3), 39.3 (C-2), 37.6 (C-6).

Methyl chlorogenate (3): white powder; negative ESIMS *m/z* 367 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 7.53 (1H, d, J = 15.8 H_Z, H-7′), 7.05 (1H, d, J = 2.0 H_Z, H-2′), 6.95 (1H, dd, J = 8.4, 2.2 H_Z, H-6′), 6.79 (1H, d, J = 8.1 H_Z, H-5′), 6.23 (1H, d, J = 15.8 H_Z, H-8′), 5.29 (1H, m, H-3), 4.15 (1H, m, H-5), 3.74 (1H, dd, J = 7.3, 2.9 H_Z, H-4), 3.70 (3H, s, OCH_3), 2.00-2.23 (4H, m, H-2, 6); ^{13}C NMR (CD_3OD, 100 MH_Z) δ174.2 (C-7), 167.1 (C-9′), 148.4 (C-4′), 145.9 (C-7′), 145.5 (C-3′), 126.4 (C-1′), 121.7 (C-6′), 115.3 (C-5′), 113.9 (C-2′), 113.8 (C-8′), 74.6 (C-1), 71.3 (C-4), 70.8 (C-3), 69.1 (C-5), 51.7 (OCH_3), 36.7 (C-2), 36.5 (C-6). ^1H and ^{13}C NMR data are consistent with published data (Miyazaki et al., 1991; Wu et al., 1995).

Dihydromyricetin (4): yellow powder; negative ESIMS *m/z* 319 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 6.55 (2H, s, H-2′ and 6′), 5.93 (1H, d, J = 2.2 H_Z, H-6), 5.88 (1H, d, J = 1.8 H_Z, H-8), 4.85 (1H, d, J = 11.4 H_Z, H-2), 4.48 (1H, d, J = 11.4 H_Z, H-3); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 197.0 (C-4), 167.5 (C-5), 164.0 (C-9), 163.2 (C-7), 145.6 (C-3′, 5′), 133.6 (C-4′), 127.8 (C-1′), 106.8 (C-2′, 6′), 100.5 (C-10), 96.1 (C-6), 95.0 (C-8), 84.0 (C-2), 72.4 (C-3). ^1H and ^{13}C NMR data are consistent with published data (Chung et al., 1996).

Quercitrin (5): yellow powder; negative ESIMS *m/z* 447 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 7.34 (1H, brs, H-2′), 7.31 (1H, brd, J = 8.4 H_Z, H-6′), 6.92 (1H, d, J = 8.0 H_Z, H-5′), 6.35 (1H, brs, H-6), 6.18 (1H, brs, H-8), 5.35 (1H, brs, H-1″), 4.22 (1H, brs, H-2″), 3.76 (1H, dd, J = 9.5, 3.7 H_Z, H-3″), 3.45 (1H, m, H-5″), 3.35 (1H, m, H-4″), 0.95 (3H, d, J = 5.8 H_Z, H-6″); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 177.6 (C-4), 164.6 (C-7), 161.9 (C-5), 158.0 (C-9), 157.2 (C-2), 148.5 (C-4′), 145.1 (C-3′), 134.9 (C-3), 121.7 (C-1′), 121.6 (C-6′), 115.7 (C-5′), 115.1 (C-2′), 104.6 (C-10), 98.5 (C-6), 93.4 (C-8), 102.2

(C-1″), 71.9 (C-4″), 70.9 (C-3″), 70.7 (C-2″), 70.6 (C-5″), 16.3 (C-6″). ^{1}H and ^{13}C NMR data are consistent with published data (Chung et al., 1996; Markham et al., 1978).

Myricitrin (6): yellow powder; negative ESIMS *m/z* 463 $[M - H]^-$; ^{1}H NMR (CD_3OD, 400 MH_Z) δ 6.96 (2H, s, H-2′ and 6′), 6.37 (1H, d, J = 2.2 H_Z, H-6), 6.20 (1H, d, J = 1.8 H_Z, H-8), 5.32 (1H, brs, H-1″), 4.24 (1H, brs, H-2″), 3.80 (1H, dd, J = 9.4, 2.9 H_Z, H-3″), 3.53 (1H, m, H-5″), 3.35 (1H, m, H-4″), 0.97 (3H, d, J = 6.2 H_Z, H-6″); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 178.4 (C-4), 164.8 (C-7), 161.9 (C-5), 158.1 (C-9), 157.2 (C-2), 145.6 (C-3′, 5′), 136.6 (C-4′), 135.0 (C-3), 120.6 (C-1′), 108.3 (C-2′, 6′), 104.5 (C-10), 98.6 (C-6), 93.4 (C-8), 102.3 (C-1″), 71.9 (C-4″), 70.8 (C-3″), 70.7 (C-5″), 70.6 (C-2″), 16.4 (C-6″). ^{1}H and ^{13}C NMR data are consistent with published data (Chung et al., 1996).

(+)-Catechin (7): white powder; negative ESIMS *m/z* 289 $[M - H]^-$; ^{1}H NMR (CD_3OD, 400 MH_Z) δ 6.85 (1H, d, J = 1.8 H_Z, H-2′), 6.78 (1H, d, J = 8.1 H_Z, H-5′), 6.73 (1H, dd, J = 8.1, 1.8 H_Z, H-6′), 5.94 (1H, d, J = 2.2 H_Z, H-6), 5.87 (1H, d, J = 2.2 H_Z, H-8), 4.58 (1H, d, J = 7.7 H_Z, H-2), 3.98 (1H, ddd, J = 7.5, 8.0, 5.5 H_Z, H-3), 2.86 (1H, dd, J = 16.1, 5.5 H_Z, H-4a), 2.52 (1H, dd, J = 16.1, 7.7 H_Z, H-4b); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 156.5 (C-9), 156.3 (C-7), 155.6 (C-5), 144.9 (C-3′, 4′), 130.9 (C-1′), 118.8 (C-6′), 114.8 (C-5′), 114.0 (C-2′), 99.6 (C-10), 95.1 (C-6), 94.3 (C-8), 81.5 (C-2), 67.5 (C-3), 27.2 (C-4). ^{1}H and ^{13}C NMR data are consistent with published data (Chung et al., 1996; Foo et al., 1996). The identification was further supported by TLC comparison with an authentic standard (Sigma, St. Louis, MO).

(–)-Epicatechin (8): white powder; positive ESIMS *m/z* 291 $[M + H]^+$; ^{1}H NMR (CD_3OD, 400 MH_Z) δ6.99 (1H, d, J = 1.8 H_Z, H-2′), 6.81 (1H, dd, J = 8.4, 1.8 H_Z, H-6′),

6.77 (1H, d, J = 8.4 H_Z, H-5′), 5.95 (1H, d, J = 2.2 H_Z, H-6), 5.93 (1H, d, J = 2.2 H_Z, H-8), 4.82 (1H, brs, H-2), 4.19 (1H, q, J = 2.9 H_Z, H-3), 2.87 (1H, dd, J = 16.8, 4.6 H_Z, H-4a), 2.75 (1H, dd, J = 16.8, 2.9 H_Z, H-4b); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 156.7 (C-9), 156.4 (C-7), 156.1 (C-5), 144.6 (C-4′), 144.5 (C-3′), 131.0 (C-1′), 118.1 (C-6′), 114.6 (C-5′), 114.0 (C-2′), 98.8 (C-10), 95.2 (C-6), 94.6 (C-8), 78.6 (C-2,), 66.2 (C-3), 27.9 (C-4). ^{1}H and ^{13}C NMR are consistent with published data (Foo et al., 1996; Watanabe, 1998). The identification was further supported by TLC comparison with authentic compound (Sigma, St. Louis, MO).

(+)-Gallocatechin (9): white powder; negative ESIMS *m/z* 305 $[M - H]^-$; ^{1}H NMR (CD_3OD, 400 MH_Z) δ 6.42 (2H, s, H-2′ and 6′), 5.94 (1H, d, J = 2.2 H_Z, H-6), 5.88 (1H, d, J = 2.2 H_Z, H-8), 4.55 (1H, d, J = 7.0 H_Z, H-2), 3.98 (1H, ddd, J = 7.7, 8.1, 5.1 H_Z, H-3), 2.82 (1H, dd, J = 16.1, 5.1 H_Z, H-4a), 2.52 (1H, dd, J = 16.1, 7.7 H_Z, H-4b); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 156.5 (C-9), 156.3 (C-7), 155.5 (C-5), 145.6 (C-3′, 5′), 132.7 (C-1′), 130.3 (C-4′), 105.6 (C-2′, 6′), 99.5 (C-10), 95.0 (C-6), 81.6 (C-2), 67.5 (C-3), 26.8 (C-4). ^{1}H and ^{13}C NMR data are consistent with published data (Foo et al., 1996).

Gallic acid (10): white powder; negative ESIMS *m/z* 169 $[M - H]^-$; ^{1}H NMR (CD_3OD, 400 MH_Z) δ 7.08 (2H, s, H-2 and 6); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 171.3 (C-7), 144.9 (C-3, 5), 137.2 (C-4), 123.0 (C-1), 109.0 (C-2, 6). ^{1}H and ^{13}C NMR data are consistent with published data (Gottlieb et al., 1991).

LC-ESIMS Analyses. The fresh frozen fruit (500 g) of *M. zapota* cv. Tikal was twice extracted with 95% EtOH at room temperature for 1 h per extraction. After the EtOH was removed in vacuo, the resulting aqueous extract was partitioned with hexane and EtOAc, respectively. The EtOAc fraction was concentrated in vacuo, and the

resulting residue (527 mg) was separated by Sephadex LH-20 CC using an isocratic solvent system of 95% EtOH to yield 20 fractions A-T. These fractions, along with **1**, were analyzed by RP18 TLC using a solvent system of 7:3 MeCN/H_2O, and fraction I contained a compound with the same R_f value as **1**. Fraction I was further analyzed by LC-ESIMS in the negative mode using a Finnigan LCQ mass spectrometer with a Waters 2690 Separations Module and a Waters 2487 Dual λ Absorbance Detector. The instrument was equipped with an electrospray ionization source, and controlled by Xcalibur software. The capillary temperature was 230 °C. Nitrogen was used as the sheath gas and the auxiliary gas at flow rates of 80 and 30 units, respectively. The capillary voltage was 10 V, the spray needle voltage was 4.5 kV, and the tube lens offset was 0 V. A mass range of 75 to 1500 was scanned in the negative mode. Compounds were also monitored using the absorbance detector at 280 nm. Compounds were separated using a Phenomenex Nucleosil C18 column (4.6 x 250 mm, 5 μm) and a solvent system of 1:19 to 1:1 MeCN/H_2O linear gradient, and a flow rate of 1 mL/min, 20 min running time. Compound **1** was run under the same LC-MS conditions.

DPPH Free-Radical Scavenging Assay. The DPPH assay was performed on fractions and purified isolates as previously described in Chapter 2.

Cell Culture. HCT-116 and SW-480 human colon cancer cells were maintained in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (Gibco-BRL) in a normal atmosphere with 5% CO_2 at 37 °C. Cells were grown to 40% confluence prior to treatment with polyphenols. All cultures were passaged weekly and the media was changed three times a week. No antibiotics were added at any

time during the experiments. In all experiments, compounds to be studied were dissolved in DMSO and added to the medium at the start of the incubation.

Microtetrazolium (MTT) Assay. This assay was carried out according to manufacturer's instructions (Boeringher-Mannheim, Indianapolis, IN). Briefly, about 30,000 cells were plated in 96-well flat-bottom plates in 100 μL of medium. When cells reached 40% confluence, the medium was changed and cells were exposed to the plant extracts or isolates. After 72 h, cells were washed three times with PBS followed by the addition of 100 μL of Dulbecco's modified Eagle medium with 10 μL of 5 mg/mL MTT solution in PBS was added to each well for 4 h. Finally, 100 μL of MTT solubilization solution included in the assay was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was determined using a Biokinetics plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Octuplicate wells were assayed for each condition, and mean as well as standard deviations were determined. The IC_{50} values were determined by linear regression analysis.

Table 3.1. ^{1}H and ^{13}C NMR data for compounds **1**and **2** (100 and 400 MHz, CD_3OD)

	1			**2**		
C/H	δ_C	δ_H (int., mult., *J* in Hz)	HMBC (^{13}C No.)	δ_C	δ_H (int., mult., *J* in Hz)	HMBC (^{13}C No.)
1	74.5			76.1		
2	37.2	2.12-2.23 (2H, m)	4, 5, 7	37.6	2.05-2.25 (2H, m)	4
3	67.2	4.41 (1H, m)		69.2	4.39 (1H, m)	4
4	73.8	5.18 (1H, dd, 8.1, 2.9)	2, 3, 6, 7″	76.1	5.14 (1H, dd, 9.9, 2.9)	3, 7″
5	67.8	5.59 (1H, m)		68.2	5.75 (1H, m)	4, 9′
6	37.2	2.12-2.23 (2H, m)	4, 7	39.3	2.05-2.25 (2H, m)	3, 4
7	174.0			179.1		
1′	126.3			126.4		
2′	113.9	7.01 (1H, d, 2.2)	4′, 6′, 7′	113.8	6.98 (1H, d, 2.2)	4′, 6′, 7′
3′	145.2			145.1		
4′	148.4			148.2		
5′	115.3	6.77 (1H, d, 8.2)	1′, 3′, 4′, 6′	115.2	6.74 (1H, d, 8.2)	1′, 3′, 4′
6′	121.9	6.91 (1H, dd, 8.2, 2.2)	2′, 4′, 5′, 7′	121.8	6.88 (1H, dd, 8.2, 2.2)	2′, 4′, 7′
7′	146.4	7.50 (1H, d, 16.0)	1′, 2′, 6′, 9′	146.0	7.49 (1H, d, 15.8)	2′, 6′, 9′
8′	113.3	6.16 (1H, d, 16.0)	1′, 9′	113.6	6.17 (1H, d, 15.8)	1′, 9′
9′	166.7			167.3		
1″	119.9			120.1		
2″	109.2	7.12 (1H, s)	1″, 3″, 4″, 7″	109.1	7.10 (1H, s)	1″, 3″, 4″, 7″
3″	145.5			145.4		
4″	138.8			138.6		
5″	145.5			145.4		
6″	109.2	7.12 (1H, s)	1″, 4″, 5″, 7″	109.1	7.10 (1H, s)	1″, 4″, 5″, 7″
7″	166.5			166.7		
OCH_3	51.9	3.72 (3H, s)	7			

Table 3.2. DPPH scavenging activity and yields of antioxidant constituents of *M. zapota* cv. Tikal

Compound	DPPH Activity IC_{50} (μM)	Yield (mg/kg fresh weight)
1	12.9	2.4
2	23.5	0.9
3	39.8	7.7
4	30.8	10.8
5	77.7	0.2
6	26.4	0.8
7	34.7	0.8
8	38.3	1.7
9	20.7	3.9
10	20.9	5.1

Figure 3.1. Photograph of *M. zapota* fruit

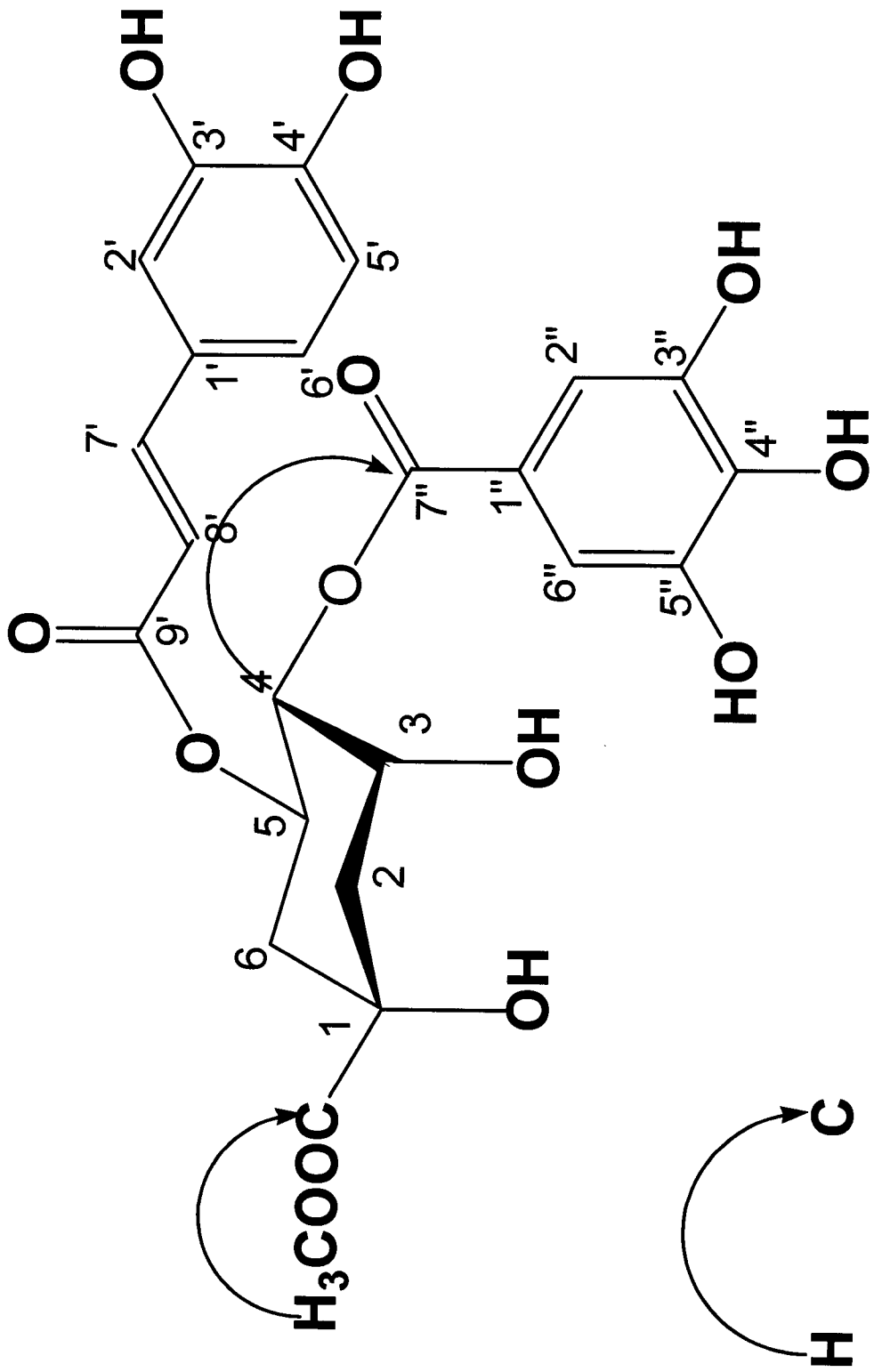


Figure 3.2. Key HMBC correlations of compound 1

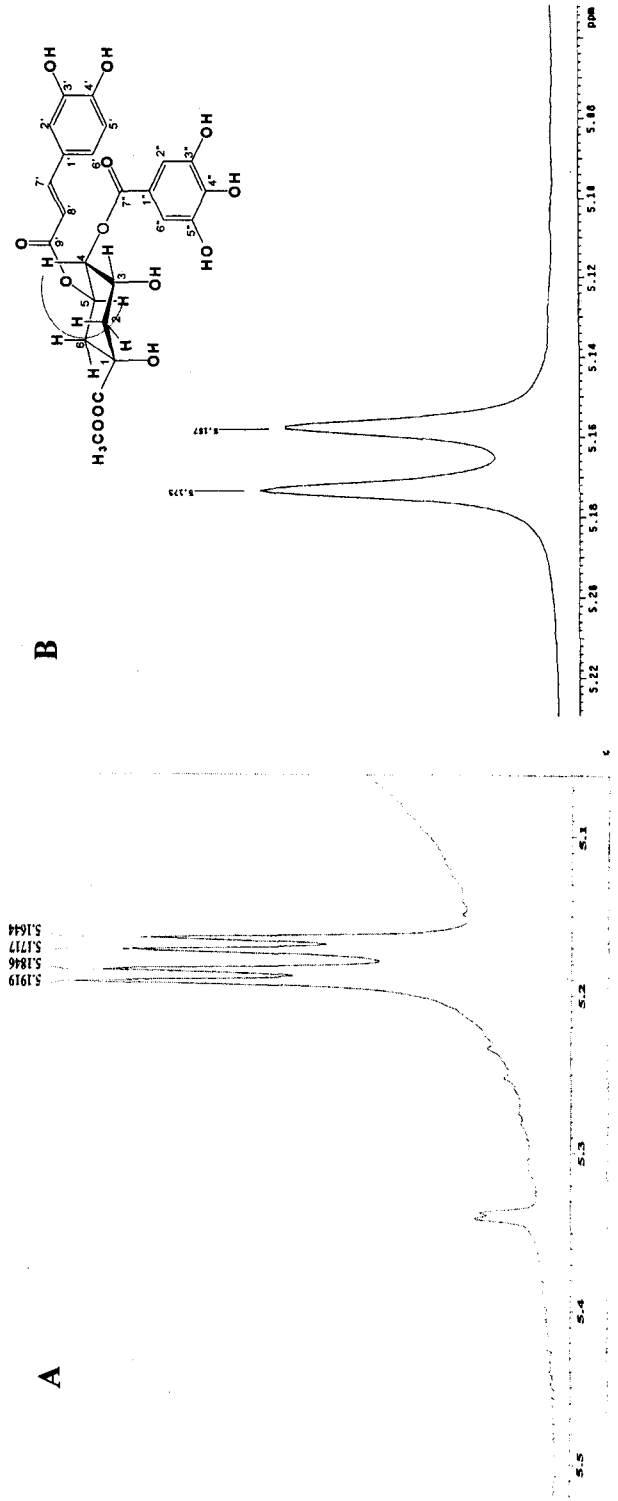


Figure 3.3. Homonuclear decoupling experiment on compound **1** (500 MHz, CD_3OD, decoupling at H-3, δ4.41): **A**, Before decoupling; **B**, After decoupling

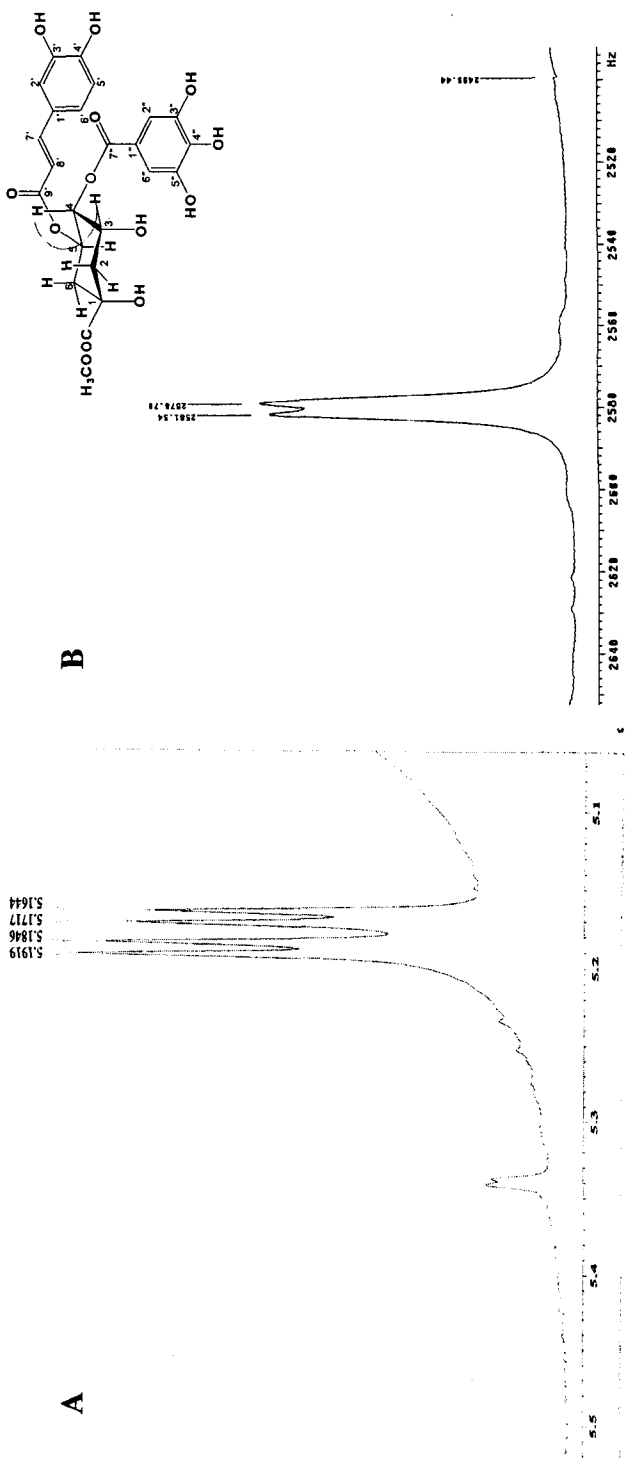


Figure 3.4. Homonuclear decoupling experiment on compound **1** (500 MHz, CD_3OD, decoupling at H-5, δ5.59): **A**, Before decoupling; **B**, After decoupling

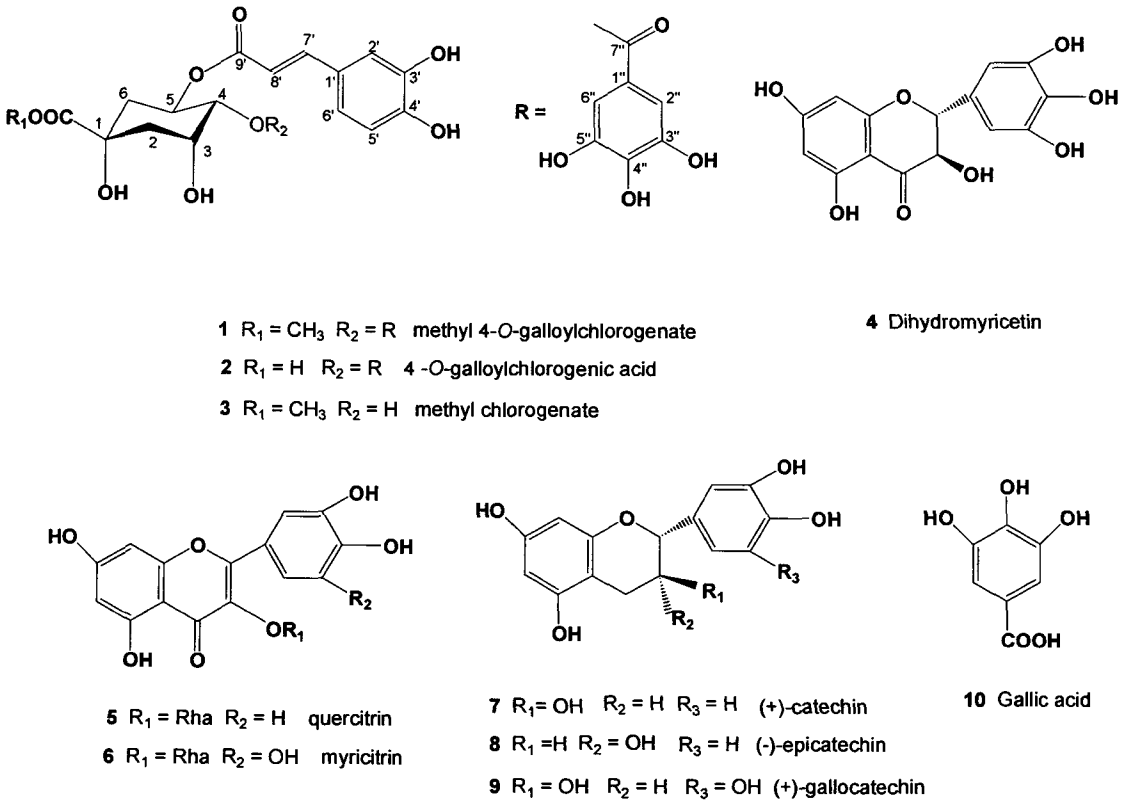


Figure 3.5. Polyphenolic antioxidants from *M. zapota* cv. Tikal

Chapter 4

Analysis of Polyphenolic Antioxidants from the Fruits of Three *Pouteria* Species by SIM LC-MS

Introduction

Sapotaceae is a large, mostly tropical, plant family with 107 genera, and more than 1,000 species (Mabberley, 1993). There are many Sapotaceae genera that produce edible fruits, such as *Chrysophyllum*, *Manilkara*, *Mimusops*, and *Pouteria* (Mabberley, 1993). Sapotaceae species grow well in tropical and subtropical areas. In the United States, Sapotaceae species are grown in California, southern Florida, and Hawaii and some are sold commercially. Sapotaceae species are a rich source of polyphenolic antioxidants. From *Manilkara zapota* (L.) P. Royen cv. Tikal, ten polyphenolic antioxidants were identified, including two new chlorogenic acid derivatives (Ma et al., 2003), and from *Chrysophyllum cainito* L., nine known polyphenolic antioxidants were identified (Luo et al., 2002). These polyphenols may be important in the prevention of diseases in humans such as cancer and CHD (Ma et al., 2003; Tijburg et al., 1997). In the present study, we examine the antioxidant content of three edible fruits in the Sapotaceae *Pouteria campechiana* (Kunth) Baehni, *Pouteria sapota* (Jacq.) H. E. Moore & Stern, and *Pouteria viridis* (Pittier) Cronquist.

P. campechiana (Figure 4.1A), known commonly as canistel, is a native of Central America, and in the United States, it is grown in California, southern Florida, and Hawaii (Balerdi and Shaw, 1998; Facciola, 1998). The fruit is nearly round, oval, ovoid, or spindle-shaped, 7.5–12.5 cm long and 5–7.5 cm wide. When unripe, the skin is green, and upon ripening turns yellow. The pulp is yellow with one to four hard seeds (Balerdi and Shaw, 1998), and the flavor is sweet like that of a baked sweet potato (Morton, 1987). The fruit can be eaten fresh or after baking, and also can be used in making custards, ice creams, milkshakes, jam, and marmalade (Morton, 1987). The bark has been

used to treat fevers and skin eruptions. The seeds have been used to treat ulcers (Morton, 1987). No phytochemical studies have been published on this species.

P. sapota (Figure 4.1B), known commonly as sapote, or mamey sapote, is indigenous to southern Mexico and Central America, and is cultivated in California, southern Florida, and Hawaii in the United States (Balerdi and Shaw, 1998; Facciola, 1998). The fruit is round, ovoid or elliptic, brown and scurfy, 8–23 cm long, 8–12 cm wide (Balerdi and Shaw, 1998), with a thick rind and red, orange, or yellow, soft, sweet, pumpkin-like in flavor pulp, enclosing one to four large, hard seeds (Morton, 1987). The sapote fruits are typically eaten by spooning out the flesh. They are used in milkshakes, and can be found incorporated into ice creams. The fruits and ice creams are both sold commonly in southern Florida supermarkets. Sapote fruit pulp has been used to treat gastric ulcers and dysentery (Morton, 1981), and the seed shell has been used to treat coronary diseases, kidney stones, and rheumatism (Morton, 1981; Morton, 1987). There has been one phytochemical study of *P. sapota*, and three benzenoids, lucumin, lucuminamide, and lucuminic acid, were identified from the seeds (Takeda et al., 1997).

P. viridis (Figure 4.1C), commonly called green sapote, is also originated from Central America, and is cultivated in California, Florida, and Hawaii in the United States (Kennard and Winters, 1960; Thomson, 1973). The fruit is nearly round or ovoid, 9–13 cm long and 6–8 cm wide, with a thin, green skin dotted with red-brown (Morton, 1987). The flesh is pale red-brown, of fine texture, juicy and sweet with one or two seeds (Kennard and Winters, 1960). The fruit is picked while hard and stored until soft. The flesh is generally eaten raw, spooned from the skin. The fruits are sold commonly in Central America for food. No phytochemical studies have been published on this species.

Growers in southern Florida have begun to consider *P. viridis* for further commercial development because it is similar in flavor to the popular *P. sapota*, but has a superior shipping characteristic in terms of size (personal communication, Chris Rollins, Director, Metro-Dade County Fruit and Spice Park, Homestead, FL).

We report the identification of a total of seven polyphenolic antioxidants from these three species. Using a selected ion monitoring liquid chromatography–mass spectrometry (SIM LC-MS) technique, the levels of the seven antioxidants were quantified in these *Pouteria* species.

Results and Discussion

The EtOAc-soluble fractions of *P. sapota* and *P. viridis* displayed antioxidant activity (IC_{50} = 12.8 and 52.6 μg/mL, respectively) in the DPPH assay. Activity-guided fractionation was performed on both plants to isolate the antioxidant constituents. The EtOAc-soluble fractions of *P. sapota* and *P. viridis* were each separated by Sephadex LH-20 CC. Active fractions were further separated by CC (RP18 and LH-20) and preparative RP18 TLC. Six antioxidant compounds, gallic acid (**1**), (+)-gallocatechin (**2**), (+)-catechin (**3**), (–)-epicatechin (**4**), dihydromyricetin (**5**), and (+)-catechin-3-*O*-gallate (**6**), were isolated and identified from the fruits of *P. sapota*. Five antioxidant compounds, gallic acid (**1**), (+)-gallocatechin (**2**), (+)-catechin (**3**), (–)-epicatechin (**4**), and myricitrin (**7**), were isolated and identified from the fruits of *P. viridis*.

Initially, no antioxidant compounds could be isolated from a MeOH extract of *P. campechiana* fruits. Subsequently, the *P. campechiana* fruits were extracted with 80% acetone in an attempt to enhance the extraction of polyphenols and tannins (Escribano-Bailón and Santos-Buelga, 2003). The 80% acetone extract was fractionated by HP-20

CC eluting with H_2O, MeOH/H_2O (1:1), MeOH, and acetone. The MeOH/H_2O (1:1) fraction displayed antioxidant activity (IC_{50} = 35.2 μg/mL) in the DPPH assay. Activity-guided fractionation was performed on this fraction, and two antioxidant compounds, gallic acid (**1**) and (+)-catechin (**3**), were isolated and identified from the fruits of *P. campechiana*.

HPLC-PDA analysis was conducted on the mixture of seven standards and the EtOAc fractions of three *Pouteria* species using a previously published method (Cabrera et al., 2003). Baseline separation was achieved among the seven standard polyphenols (Figure 4.2A) using this method. In the three *Pouteria* extracts, however, the seven polyphenols could not all be resolved using this method (Figure 4.2B–4.2D) because other compounds in the extracts coeluted with these polyphenols and interfered with their separation. As a result, quantitative results of polyphenols using HPLC-PDA would be somewhat inaccurate. In order to achieve more accurate quantitative results, SIM LC-MS instead of HPLC-PDA was used to quantify the polyphenols in the plant extracts.

The antioxidant constituents of the three *Pouteria* species were quantified by SIM LC-MS. Baseline separation of seven polyphenols was achieved by modifying a previously published HPLC-PDA method (Cabrera et al., 2003) for application by LC-MS. Compounds were detected using MS total ion current (TIC) and UV absorbance at 280 nm (Figure 4.3A and 4.3B). Each of the specific $[M - H]^-$ ions was detected by MS (Figure 4.3C).

The retention time of each standard was obtained from the SIM chromatograms of individual compounds. Peak areas for individual standards were measured from the SIM chromatograms and used for quantification. Individual calibration curves for each

standard measured in the SIM mode were linear, with r^2 values of > 0.99 in the range of calibrations of the standards tested. In this SIM LC-MS method, the limits of detection (LOD) were 250 pg for gallic acid and 500 pg for (+)-catechin; the limits of quantification (LOQ) were 1 ng for gallic acid and 2 ng for (+)-catechin.

In a spike–recovery experiment, 50 g of *P. campechiana* fruits spiked with 30 mg of dihydromyricetin was extracted with MeOH twice, at room temperature for 1 h each time. After the MeOH was removed in vacuo, the resulting aqueous extract was partitioned with hexane and EtOAc, respectively. The EtOAc fraction was concentrated in vacuo to dryness and dissolved with HPLC grade MeOH to prepare 1 mg/mL solution. This solution was analyzed for dihydromyricetin using SIM LC-MS, and a percent recovery was calculated. This experiment was repeated, and the average percent recovery was 85%.

By SIM LC-MS, all seven polyphenols (**1**-**7**) were detected from *P. sapota* and *P. viridis*, and four polyphenols (**1**-**3** and **7**) were detected from *P. campechiana*. All of these polyphenols have antioxidant activity (IC_{50} < 40 μM) (Table 4.1). By activity-guided fractionation, we were not able to isolate myricitrin (**7**) from *P. sapota*, dihydromyricetin (**5**) and (+)-catechin-3-*O*-gallate (**6**) from *P. viridis*, nor (+)-gallocatechin (**2**) and myricitrin (**7**) from *P. campechiana*, despite the fact that these compounds were detected by SIM LC-MS. The inability to isolate these compounds by activity-guided fractionation may result from their low content and/or interference of coeluting compounds.

Of these three *Pouteria* species, the fruits of *P. sapota* contain the highest level of total polyphenolic antioxidants; the contents of individual polyphenolic antioxidants

range from 24.42 ± 0.97 ppm for (–)-epicatechin (**4**) to 200.77 ± 11.73 ppm for dihydromyricetin (**5**). *P. sapota* contains high amounts of gallic acid (**1**) (170.91 ± 0.53 ppm), (+)-gallocatechin (**2**) (172.85 ± 2.21 ppm), and dihydromyricetin (**5**) (200.77 ± 11.73 ppm). These three polyphenolic antioxidants likely contribute significantly to the high antioxidant activity of its EtOAc fraction (IC_{50} = 12.8 μg/mL in the DPPH assay), the highest antioxidant activity of the three *Pouteria* species studied. The fruits of *P. viridis* contain the second highest level of total polyphenolic antioxidants in this study. The contents of individual polyphenolic antioxidants range from 2.73 ± 0.21 ppm for (+)-catechin-3-*O*-gallate (**6**) to 47.26 ± 1.46 ppm for (+)-gallocatechin (**2**). Its EtOAc fraction showed the second highest antioxidant activity (IC_{50} = 52.6 μg/mL in the DPPH assay). In this study, the lowest level of total polyphenols measured was found in the fruits of *P. campechiana*, and this corresponds to the lowest antioxidant activity of the three plant extracts (Table 4.1). Only four polyphenols were detected in *P. campechiana*, and the contents of individual polyphenolic antioxidants range from 1.04 ± 0.05 ppm for (+)-catechin (**3**) to 16.85 ± 0.09 ppm for gallic acid (**1**). The EtOAc fraction of *P. campechiana* had an IC_{50} = 146.3 μg/mL in the DPPH assay.

(+)-Gallocatechin (**2**), (+)-catechin (**3**), (–)-epicatechin (**4**), and (+)-catechin-3-*O*-gallate (**6**) belong to the catechin, or flavan-3-ol, class of flavonoids, and they have been studied intensely because they are important components of edible foods, such as tea (Davis et al., 1996). They are all strong antioxidants with IC_{50} values in the DPPH free radical assay ranging from 19.0 to 38.3 μM (Table 4.1). The tea catechins have been shown to prevent the oxidation of plasma low-density lipoprotein (LDL) in vitro (Tijburg et al., 1997). Because LDL oxidation is a key step in the pathogenesis of cardiovascular

disease (Upston et al., 2003), catechins are thought to be important cardioprotective agents. Epidemiological studies have shown that the intake of tea catechins can decrease the risk of cardiovascular disease (Miyazawa, 2000). Gallic acid (**1**), dihydromyricetin (**5**), and myricitrin (**7**) are found in many edible plants (Luo et al., 2002; Ma et al., 2003), and also have strong antioxidant activity (IC_{50} from 20.9 to 30.8 μM) (Table 4.1). The fruits of *P. sapota* and *P. viridis* are rich sources of polyphenolic antioxidants.

In our study, we used a SIM LC-MS method rather than an HPLC-PDA method to quantify polyphenolic antioxidants. SIM LC-MS was useful in this study because LC provided initial separation of the mixture of compounds based on their polarities, and then SIM MS was used to select specific ions corresponding to specific antioxidants. This method allowed the major polyphenolic constituents of the three *Pouteria* species to be distinguished and quantified. The SIM LC-MS method used in this study provided lower LOD and LOQ than HPLC-PDA methods previously published (Donovan et al., 1999). For example, in the HPLC-PDA method reported by Donovan et al. (Donovan et al., 1999), the LOD for (+)-catechin is 22 ng, 44 times higher than in SIM LC-MS method (500 pg). The SIM LC-MS method employed in this study is a quick, sensitive and accurate way to quantify polyphenolic antioxidants in plant samples. In addition to the *Pouteria* species we examined, other plants may be analyzed for polyphenolic antioxidant contents by this method. This method may also be extended to measure other polyphenolic antioxidants.

By SIM LC-MS, we found that different *Pouteria* species have significantly different levels of polyphenolic antioxidants. These results provide additional nutrition

information for three less used tropical fruits that are now being grown commercially in the United States and other subtropical/tropical areas.

Experimental Section

General Experimental Procedure. ^{1}H and ^{13}C NMR spectra were recorded using a JEOL GX-400 MHz, operating at 400 and 100 MHz, respectively. All compounds were measured in CD_3OD. HPLC was carried out on a Waters 2690 separations module equipped with a Waters 996 photodiode array (PDA) detector and Millenium[32] software using a 250 x 4.6 mm i.d., 5 μm, Aqua C18 column (Phenomenex, Torrance, CA). The wavelength was set at 280 nm.

LC-MS was performed on a Thermo Finnigan LCQ mass spectrometer (San Jose, CA) in the negative mode with a Waters 2690 separations module and a Waters 2487 dual wavelength absorbance detector. The instrument was equipped with an electrospray ionization (ESI) source, and controlled by Xcalibur software. The capillary temperature was 230 °C. Nitrogen was used as the sheath gas and the auxiliary gas at flow rates of 80 and 30 units, respectively. The capillary voltage was 10 V, the spray needle voltage was 4.5 kV, and the tube lens offset was 0 V. A mass range of 75–500 was scanned in the negative full or selected ion monitoring (SIM) scan type. Compounds were also monitored simultaneously using the dual-wavelength absorbance detector at 280 nm. Compounds were separated using the Aqua C18 column as for HPLC.

The DPPH free radical assay was conducted on the 96-well microtiter plate (Nalge Nunc International, Rochester, NY). The absorbance of sample in the DPPH assay was detected on a *VERSA*$_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA).

TLC analysis was performed on RP-18 F_{254} plates (Merck, Darmstadt, Germany), with compounds visualized by spraying with vanillin in 10% (v/v) H_2SO_4 in 95% EtOH.

Sephadex LH-20 (25–100 μm) (Pharmacia Fine Chemicals, Piscataway, NJ) and reversed-phase C18 (RP18) silica gel (40 μm) (J. T. Baker, Phillipsburg, NJ) were used for column chromatography (CC). All solvents for chromatographic isolation were of analytical grade. HPLC grade MeCN, MeOH, and H_2O were used for HPLC and LC-MS. Gallic acid, (+)-catechin, (–)-epicatechin, and DPPH were obtained from Sigma Chemical Co. (St. Louis, MO). (+)-Gallocatechin, dihydromyricetin, myricitrin, and (+)-catechin-3-*O*-gallate were isolated and identified by MS and NMR in our laboratory.

Plant Material. Fruits of three *Pouteria* species were collected from the Fruit and Spice Park (Homestead, FL). Frozen fruits were shipped to New York City by overnight courier and stored at –20 °C until extracted. Herbarium voucher specimens of three *Pouteria* species were prepared, identified, and deposited at the Steere Herbarium of The New York Botanical Garden (Bronx, NY).

Extraction and Isolation. The fresh frozen fruits (2.6 kg) of *P. sapota* were macerated with MeOH twice, at room temperature for 1 h each time. After the MeOH was removed in vacuo, the resulting aqueous extract was partitioned with hexane and EtOAc, respectively. The EtOAc fraction displayed antioxidant activity (IC_{50} = 12.8 μg/mL) in the DPPH assay. The EtOAc fraction was concentrated in vacuo to give 13.8 g of a residue, of which 12.9 g was separated by Sephadex LH-20 CC with an isocratic solvent system of 100% MeOH. The resulting fractions were recombined according to their RP18 TLC profiles to give seven fractions in total (SA–SG). All fractions were tested in the DPPH assay, and fractions SC (621.3 mg), SD (296 mg), SE (252.2 mg), SF

(109.4 mg), and SG (954.9 mg) showed antioxidant activity (IC_{50} = 2.8, 4.7, 7.7, 7.3, and 6.8 μg/mL, respectively).

Fraction SC was separated by LH-20 CC with an isocratic solvent system of 100% MeOH to yield gallic acid (**1**) (78.5 mg). Fraction SD was separated by RP18 CC (from 1:4 to 2:3 MeOH/H_2O) to yield (+)-gallocatechin (**2**) (174 mg), (+)-catechin (**3**) (52.7 mg), and (–)-epicatechin (**4**) (5.4 mg). Fraction SE was separated by RP18 CC (from 1:4 to 2:3 MeOH/H_2O) to yield (+)-gallocatechin (**2**) (127.4 mg), (+)-catechin (**3**) (20.8 mg), and dihydromyricetin (**5**) (12.9 mg). Fraction SF was separated by RP18 CC (from 3:7 to 11:14 MeOH/H_2O) to yield dihydromyricetin (**5**) (14.1 mg). Subfraction SF-1 was purified by LH-20 CC eluting with an isocratic solvent system of 100% MeOH to yield (+)-gallocatechin (**2**) (14.5 mg). Fraction SG was separated by RP18 CC (from 3:7 to 1:1 MeOH/H_2O). Subfraction SG-3 was purified by LH-20 CC eluting with an isocratic solvent system of 100% MeOH to yield (+)-catechin-3-*O*-gallate (**6**) (52.3 mg).

The fresh frozen fruits (11.7 kg) of *P. viridis* were extracted with MeOH twice at room temperature for 1 h each time. After the MeOH was removed in vacuo, the resulting aqueous extract was partitioned with hexane and EtOAc, respectively. The EtOAc fraction (IC_{50} = 52.6 μg/mL in the DPPH assay) was concentrated in vacuo to give 9.6 g of a residue, of which 9.5 g was separated by Sephadex LH-20 CC with an isocratic solvent system of 100% MeOH. The resulting fractions were combined according to their RP18 TLC profiles to give six combined fractions in total (VA–VF). All fractions were tested in the DPPH assay, and fractions VD (294.7 mg), VE (134 mg), and VF (546.2 mg) showed antioxidant activity (IC_{50} = 20.1, 17.2, and 22.2 μg/mL, respectively).

Fraction VD was separated by RP18 CC (from 100% H_2O to 13:37 MeOH/H_2O) to yield gallic acid (**1**) (115 mg). Fraction VE was separated by RP18 CC (from 1:19 MeOH/H_2O to 100% MeOH) to yield gallic acid (**1**) (22.6 mg). Fraction VF was separated by RP18 CC (from 1:3 to 11:9 MeOH/H_2O) to yield (+)-gallocatechin (**2**) (31.3 mg). Subfraction VF-3 was purified by RP18 CC eluting with a gradient of 1:4 to 3:7 MeOH/H_2O to yield (+)-gallocatechin (**2**) (32.8 mg), (+)-catechin (**3**) (3.3 mg), and VF-3-4, and then VF-3-4 was purified repeatedly by RP18 CC eluting with a gradient of 1:4 to 2:3 MeOH/H_2O to yield (–)-epicatechin (**4**) (6.3 mg). Subfraction VF-5 was purified by LH-20 CC eluting with an isocratic solvent system of 100% MeOH to obtain VF-5-3, and then VF-5-3 was purified repeatedly by preparative RP18 TLC (3:2 MeOH/H_2O) to yield myricitrin (**7**) (4.8 mg).

The fresh frozen fruits (3.1 kg) of *P. campechiana* were extracted with 80% acetone three times, at room temperature for 1 h each time. After the acetone was removed in vacuo, the resulting aqueous extract was fractionated by HP-20 CC eluting with H_2O, MeOH/H_2O (1:1), MeOH, and acetone. The MeOH/H_2O (1:1) fraction (IC_{50} = 35.2 μg/mL in the DPPH assay) was concentrated in vacuo to give 10.6 g of a residue, of which 10.0 g was separated by Sephadex LH-20 CC with an isocratic solvent system of 100% MeOH. The resulting fractions were combined according to their RP18 TLC profiles to give seven combined fractions in total (CA–CG). All fractions were tested in the DPPH assay, and fractions CA (2002.7 mg) and CC (770.0 mg) showed antioxidant activity (IC_{50} = 90.7 and 11.2 μg/mL, respectively). Fraction CC was separated by RP18 CC (from 1:9 MeOH/H_2O to 100% MeOH) to yield gallic acid (**1**) (12.0 mg) and (+)-catechin (**3**) (16.5 mg).

The fresh frozen fruits (114 g) of *P. campechiana* were extracted at room temperature with MeOH twice, for 1 h each time. After the MeOH was removed in vacuo, the resulting aqueous extract was partitioned with hexane and EtOAc, respectively. The EtOAc fraction was concentrated in vacuo to give 163.6 mg of a residue. It displayed low antioxidant activity (IC_{50} = 146.3 μg/mL) in the DPPH assay, and was kept for SIM LC-MS analysis.

A total of seven polyphenolic antioxidants were isolated from the fruits of these *Pouteria* species (Figure 4.4). The properties of the compounds are presented below.

Gallic acid (1): white powder; negative ESIMS *m/z* 169 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 7.08 (2H, s, H-2 and 6); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 169.1 (C-7), 145.1 (C-3, 5), 138.3 (C-4), 120.8 (C-1), 109.1 (C-2, 6) (1). ^1H and ^{13}C NMR data are consistent with previously published data (Gottlieb et al., 1991).

(+)-Gallocatechin (2): white powder; negative ESIMS *m/z* 305 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 6.42 (2H, s, H-2′ and 6′), 5.93 (1H, d, J = 2.2 H_Z, H-6), 5.87 (1H, d, J = 2.2 H_Z, H-8), 4.54 (1H, d, J = 7.0 H_Z, H-2), 3.98 (1H, ddd, J = 7.7, 8.1, 5.1 H_Z, H-3), 2.82 (1H, dd, J = 16.1, 5.5 H_Z, H-4a), 2.51 (1H, dd, J = 16.1, 7.7 H_Z, H-4b); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 156.4 (C-9), 156.2 (C-7), 155.5 (C-5), 145.5 (C-3′, 5′), 132.8 (C-1′), 130.2 (C-4′), 106.1 (C-2′, 6′), 99.6 (C-10), 95.1 (C-6), 81.5 (C-2), 67.4 (C-3), 26.8 (C-4). ^1H and ^{13}C NMR data are consistent with previously published data (Foo et al., 1996). The identification was further supported by RP18–HPLC comparison (retention time and UV spectrum) with an authentic compound.

(+)-Catechin (3): white powder; negative ESIMS *m/z* 289 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 6.86 (1H, d, J = 1.5 H_Z, H-2′), 6.78 (1H, d, J = 8.0 H_Z, H-5′), 6.73

(1H, dd, J = 8.1, 1.5 H_Z, H-6′), 5.96 (1H, d, J = 2.2 H_Z, H-6), 5.88 (1H, d, J = 1.8 H_Z, H-8), 4.59 (1H, d, J = 7.3 H_Z, H-2), 4.00 (1H, ddd, J = 7.5, 8.0, 5.5 H_Z, H-3), 2.86 (1H, dd, J = 16.1, 5.5 H_Z, H-4a), 2.53 (1H, dd, J = 16.1, 8.0 H_Z, H-4b); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 156.5 (C-9), 156.3 (C-7), 155.6 (C-5), 144.9 (C-3′, 4′), 130.9 (C-1′), 118.8 (C-6′), 114.9 (C-5′), 114.0 (C-2′), 99.6 (C-10), 95.1 (C-6), 94.3 (C-8), 81.5 (C-2), 67.5 (C-3), 27.2 (C-4). ^1H and ^{13}C NMR data are consistent with previously published data (Chung et al., 1996; Foo et al., 1996). The identification was further supported by RP18-HPLC comparison (retention time and UV spectrum) with an authentic compound (Sigma, St. Louis, MO).

(–)-Epicatechin (4): white powder; negative ESIMS *m/z* 289 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ6.98 (1H, d, J = 1.8 H_Z, H-2′), 6.81 (1H, dd, J = 8.0, 1.8 H_Z, H-6′), 6.77 (1H, d, J = 8.0 H_Z, H-5′), 5.95 (1H, d, J = 2.6 H_Z, H-6), 5.92 (1H, d, J = 2.6 H_Z, H-8), 4.83 (1H, brs, H-2), 4.19 (1H, q, J = 2.9 H_Z, H-3), 2.87 (1H, dd, J = 16.8, 4.4 H_Z, H-4a), 2.75 (1H, dd, J = 16.8, 2.9 H_Z, H-4b); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 156.7 (C-9), 156.4 (C-7), 156.1 (C-5), 144.6 (C-4′), 144.5 (C-3′), 131.0 (C-1′), 118.1 (C-6′), 114.6 (C-5′), 114.0 (C-2′), 98.8 (C-10), 95.2 (C-6), 94.6 (C-8), 78.6 (C-2,), 66.2 (C-3), 27.9 (C-4). ^1H and ^{13}C NMR data are consistent with previously published data (Gottlieb et al., 1991; Watanabe, 1998). The identification was further supported by RP18-HPLC comparison (retention time and UV spectrum) with an authentic compound (Sigma, St. Louis, MO).

Dihydromyricetin (5); yellow powder; negative ESIMS *m/z* 319 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 6.54 (2H, s, H-2′ and 6′), 5.93 (1H, d, J = 1.8 H_Z, H-6), 5.88 (1H, d, J = 1.8 H_Z, H-8), 4.84 (1H, d, J = 11.4 H_Z, H-2), 4.47 (1H, d, J = 11.4 H_Z, H-3); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 197.0 (C-4), 167.5 (C-5), 164.0 (C-9), 163.2 (C-7),

145.6 (C-3′, 5′), 133.6 (C-4′), 127.8 (C-1′), 106.8 (C-2′, 6′), 100.5 (C-10), 96.1 (C-6), 95.0 (C-8), 84.0 (C-2), 72.4 (C-3). ^{1}H and ^{13}C NMR data are consistent with previously published data (Chung et al., 1996). The identification was further supported by RP18–HPLC comparison (retention time and UV spectrum) with authentic compound.

(+)-Catechin-3-*O*-gallate (6): white powder; negative ESIMS *m/z* 441 $[M - H]^-$; ^{1}H NMR (acetone-d_6, 400 MH_Z) δ 7.04 (2H, s, H-2″ and 6″), 6.95 (1H, brs, H-2′), 6.81 (1H, brs, H-5′), 6.80 (1H, brs, H-6′), 6.08 (1H, d, J = 2.6 H_Z, H-6), 6.00 (1H, d, J = 2.2 H_Z, H-8), 5.39 (1H, ddd, J = 6.2, 5.5, 6.2 H_Z, H-3), 5.15 (1H, d, J = 6.2 H_Z, H-2), 2.91 (1H, dd, J = 16.5, 5.1 H_Z, H-4a), 2.76 (1H, dd, J = 16.5, 6.2 H_Z, H-4b); ^{13}C NMR (acetone-d_6, 100 MH_Z) δ 165.3 (C-7″), 157.3 (C-7), 156.5 (C-5), 155.5 (C-9), 145.3 (C-3″), 145.1 (C-4′), 145.0 (C-3′), 138.3 (C-4″), 130.6 (C-1′), 120.8 (C-1″), 118.3 (C-6′), 115.2 (C-5′), 113.6 (C-2′), 109.2 (C-2″), 98.4 (C-10), 95.7 (C-6), 94.8 (C-8), 78.0 (C-2), 69.7 (C-3), 23.5 (C-4). ^{1}H and ^{13}C NMR data are consistent with previously published data (Davis et al., 1996).

Myricitrin (7): yellow powder; negative ESIMS *m/z* 463 $[M - H]^-$; ^{1}H NMR (CD_3OD, 400 MH_Z) δ 6.96 (2H, s, H-2′ and 6′), 6.37 (1H, d, J = 2.2 H_Z, H-6), 6.20 (1H, d, J = 1.8 H_Z, H-8), 5.32 (1H, brs, H-1″), 4.24 (1H, brs, H-2″), 3.80 (1H, dd, J = 9.4, 2.9 H_Z, H-3″), 3.53 (1H, m, H-5″), 3.35 (1H, m, H-4″), 0.97 (3H, d, J = 6.2 H_Z, H-6″); ^{13}C NMR (CD_3OD, 100 MH_Z) δ 178.4 (C-4), 164.8 (C-7), 161.9 (C-5), 158.1 (C-9), 157.2 (C-2), 145.6 (C-3′, 5′), 136.6 (C-4′), 135.0 (C-3), 120.6 (C-1′), 108.3 (C-2′, 6′), 104.5 (C-10), 98.6 (C-6), 93.4 (C-8), 102.3 (C-1″), 71.9 (C-4″), 70.8 (C-3″), 70.7 (C-5″), 70.6 (C-2″), 16.4 (C-6″). ^{1}H and ^{13}C NMR data are consistent with previously published data

(Chung et al., 1996). The identification was further supported by RP18–HPLC comparison (retention time and UV spectrum) with an authentic compound.

TLC Analysis. RP18 TLC was performed on all LH-20 fractions with a mobile phase composed of 3:7 MeOH/H_2O. Fractions were pooled according to their TLC profiles to give combined fractions for further activity-guided fractionation.

DPPH Assay. The DPPH assay was preformed on the extracts as previously described in Chapter 2.

Preparation of Samples for HPLC and LC-MS Analyses. The EtOAc fractions of *P. sapota*, *P. viridis*, and *P. campechiana* were redissolved in HPLC grade MeOH to make 10 mg/mL sample solutions. All samples were filtered with a 0.45 μm nylon microfilter (Phenomenex, Torrance, CA) before HPLC and LC-MS analyses.

Preparation of Standards for HPLC Analysis. Stock solutions (1 mg/mL) for the seven standards were prepared by dissolving individual standards in HPLC grade MeOH. The seven standard stock solutions were mixed in equal volumns to make a standard mixture, in which the concentrations of individual polyphenolic standards were 0.1 mg/mL.

Preparation of Standards for LC-MS Analysis. Stock solutions (2 mg/mL) for the seven standards were prepared by dissolving the individual standards in HPLC grade MeOH. To decrease the number of runs required to generate the calibration curves, four group stock solutions were prepared, group 1: gallic acid (**1**), (+)-gallocatechin (**2**), and (+)-catechin-3-*O*-gallate (**6**); group 2: (+)-catechin (**3**) and (–)-epicatechin (**4**); group 3: dihydromyricetin (**5**); group 4: myricitrin (**7**). Concentrations of individual standards in the group stock solutions were 0.5 mg/mL. The group stock solutions were diluted with

HPLC grade MeOH into six two-fold serial solutions respectively. The concentrations of individual standards in the six two-fold serial solutions were 0.016, 0.031, 0.063, 0.125, 0.25, and 0.5 mg/mL. $[M - H]^-$ ions for the seven standards were monitored in SIM mode and the peak areas calibrated versus the concentrations of individual standards. Standard curves were generated for individual standards after runs of the six two-fold serial solutions.

HPLC Analysis. For HPLC analysis, a sample or standard (10 μL) was separated over a Phenomenex Aqua C18 column, using elution conditions according to a previously published method (Cabrera et al., 2003). The mobile phase consisted of 3% aqueous acetic acid (A) and MeOH (B) with a flow rate of 1 mL/min. The mobile phase composition began with 100% A, which was maintained for 1 min, followed by a linear increase to 63% B in 27 min, and then returned to the initial condition in 5 min for the next run.

Quantitative LC-MS Analysis. For quantitative analysis, a sample or standard (10 μL) was separated over a Phenomenex Aqua C18 column with a flow rate of 1 mL/min. The LC conditions were modified on the basis of a previously published HPLC method (Cabrera et al., 2003). The mobile phase consisted of 0.1% aqueous acetic acid (A) and 0.1% acetic acid in MeOH (B) with a flow rate of 1 mL/min. The mobile phase composition began with 100% A, which was maintained for 1 min, followed by a linear increase to 56% B in 24 min, and then went back to the initial condition in 1 min for the next run. Column elutant was monitored by an ESI probe in the negative ion SIM mode for the seven standard compounds.

Table 4.1. Contents of polyphenolic antioxidants by SIM LC-MS in the fruits of three *Pouteria* species and their DPPH scavenging activity

Compound	*P. sapota* (ppm)	*P. viridis* (ppm)	*P. campechiana* (ppm)	DPPH Activity IC_{50}
Gallic acid (**1**)	170.91 ± 0.53	2.82 ± 0.17	16.85 ± 0.09	20.9 μM
(+)-Gallocatechin (**2**)	172.85 ± 2.21	47.26 ± 1.46	5.62 ± 0.13	20.7 μM
(+)-Catechin (**3**)	75.01 ± 2.67	27.32 ± 0.93	1.04 ± 0.05	34.7 μM
(–)-Epicatechin (**4**)	24.42 ± 0.97	5.91 ± 0.13	ND	38.3 μM
Dihydromyricetin (**5**)	200.77 ± 11.73	4.68 ± 0.29	ND	30.8 μM
(+)-Catechin-3-*O*-gallate (**6**)	80.50 ± 0.81	2.73 ± 0.21	ND	19.0 μM
Myricitrin (**7**)	25.48 ± 3.70	7.34 ± 0.80	2.82 ± 0.02	26.4 μM
P. sapota EtOAc fraction				12.8 μg/mL
P. viridis EtOAc fraction				52.6 μg/mL
P. campechiana EtOAc fraction				146.3 μg/mL

Replication: 4; ND: Not detected by SIM LC-MS

A

B

C

Figure 4.1. Photographs of fruit of **A.** *P. campechiana*; **B.** *P. sapota*; and **C.** *P. viridis*

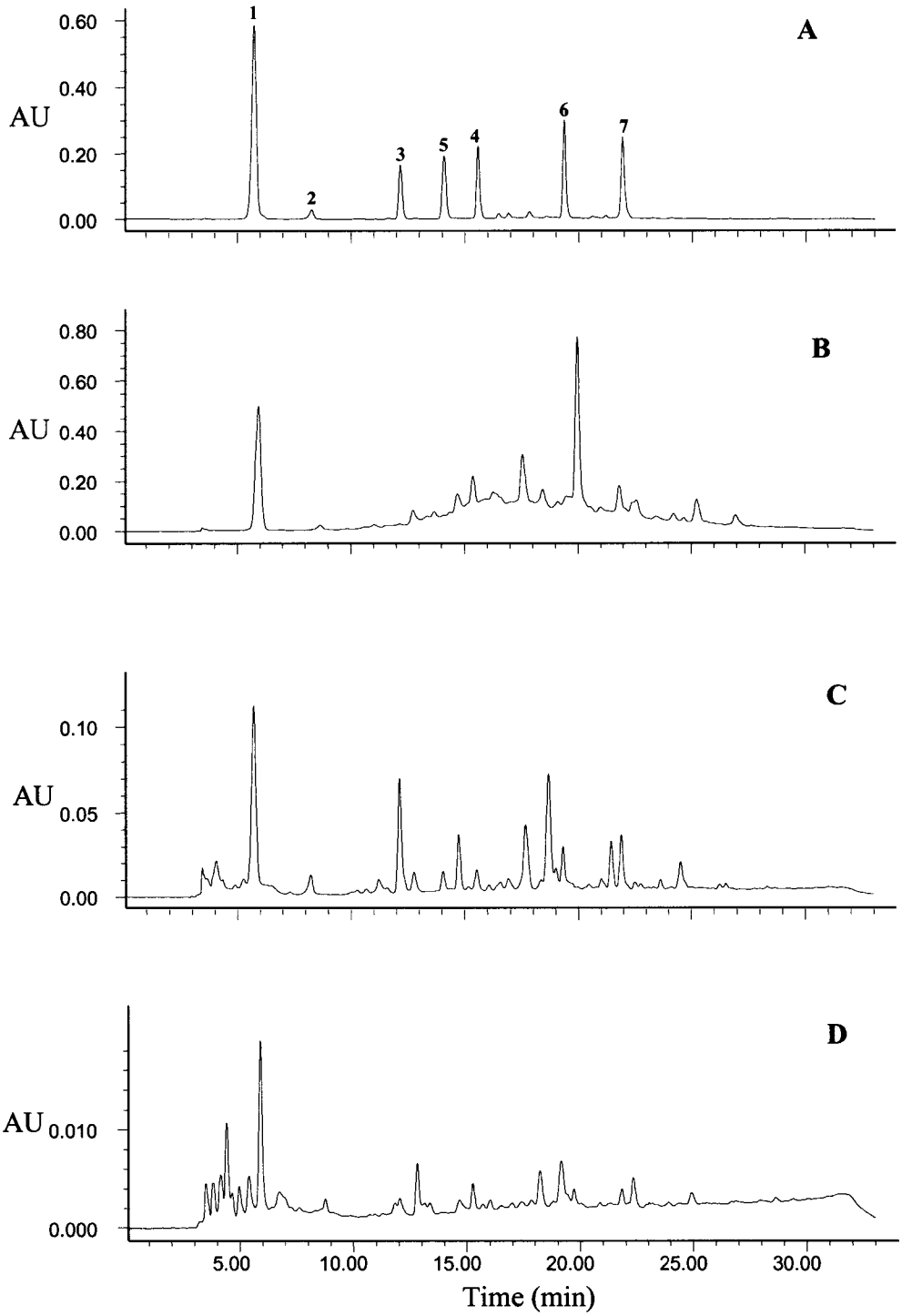


Figure 4.2. HPLC chromatograms at 280 nm of the mixture of seven standards (A), EtOAc extracts of *P. sapota* (B), *P. viridis* (C), and *P. campechiana* (D): **1**, gallic acid; **2**, (+)-gallocatechin; **3**, (+)-catechin; **4**, (–)-epicatechin; **5**, dihydromyricetin; **6**, (+)-catechin-3-*O*-gallate; **7**, myricitrin

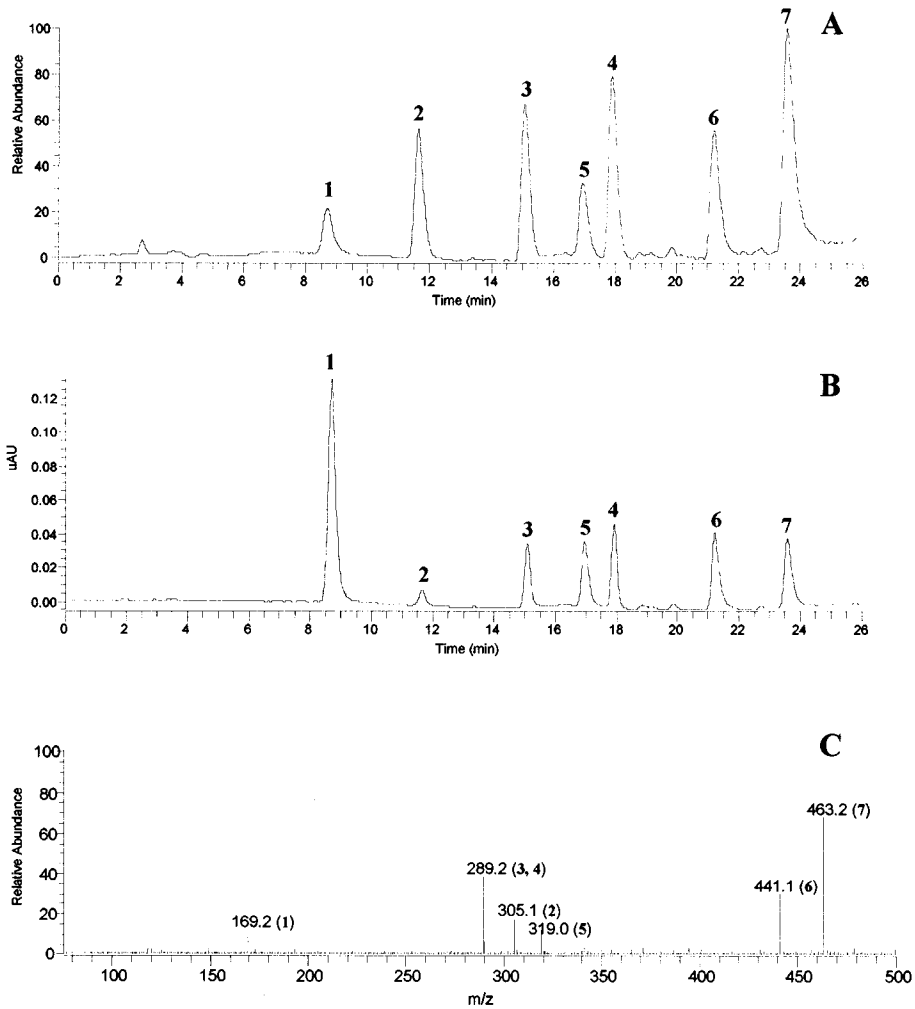
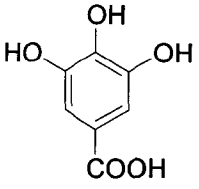
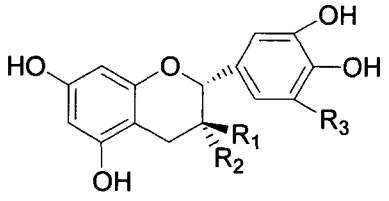


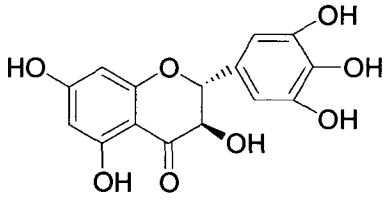
Figure 4.3. (A) Total ion current (TIC) chromatogram from the HPLC separation of the mixture of seven standards: **1**, gallic acid; **2**, (+)-gallocatechin; **3**, (+)-catechin; **4**, (–)-epicatechin; **5**, dihydromyricetin; **6**, (+)-catechin-3-*O*-gallate; **7**, myricitrin; (B) HPLC-UV/Vis chromatogram at 280 nm of the mixture of seven standards; (C) mass spectrometric profile of the mixture of seven standards



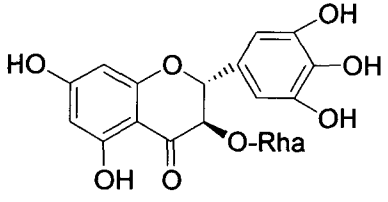
1 Gallic acid



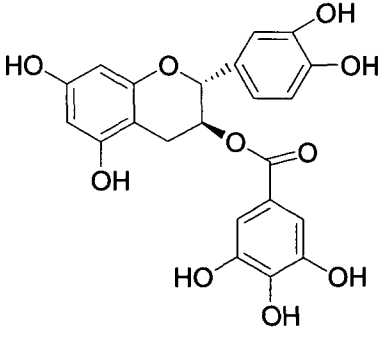
2 (+)-Gallocatechin (R_1 = OH, R_2 = H, R_3 = OH)
3 (+)-Catechin (R_1 = OH, R_2 = H, R_3 = H)
4 (-)-Epicatechin (R_1 = H, R_2 = OH, R_3 = H)



5 Dihydromyricetin



6 Myricitrin



7 (+)-Catechin-3-*O*-gallate

Figure 4.4. Polyphenolic antioxidants from *P. sapota, P. viridis,* and *P. campechiana*

Chapter 5

Summary and Perspectives

The primary purpose of this research was to find novel antioxidants in several edible tropical plants, and quantify some of their specific antioxidant constituents. For this purpose, as described in Chapter 2, I screened for antioxidant activity in forty-nine edible tropical fruits and vegetables using the from these DPPH assay, thirty-six plants displayed antioxidant activity (IC_{50} < 200 μg/mL), and thirteen did not display antioxidant activity (IC_{50} ≥ 200 μg/mL). In these thirty-six active plants, eighteen displayed high antioxidant activity (IC_{50} < 50 μg/mL), seven displayed moderate antioxidant activity (50 μg/mL ≤ IC_{50} < 100 μg/mL), and eleven had low antioxidant activity (100 μg/mL ≤ IC_{50} < 200 μg/mL). Known polyphenolic antioxidants (+)-catechin and (–)-epicatechins were detected from some active plants by TLC and HPLC. These two antioxidants may contribute significantly to the total antioxidant activity of these plants.

Further activity-guided fractionation work has been performed in our laboratory on eight active plants: *Chrysophyllum cainito*, *Garcinia xanthochymus*, *Mammea americana*, *Manilkara zapota*, *Pouteria campechiana, Pouteria sapota*, *Pouteria viridis*, and *Theobroma grandiflorum*, a number of polyphenolic antioxidants from different compound classes, such as benzophenones, coumarins, and flavonoids, were identified from these plants. In addition, six new antioxidants and six potential anticancer agents were identified from these plants (Luo et al., 2002; Ma et al., 2003; Yang et al., 2003; Ma et al., in press; Baggett et al., submitted ;Yang et al., in preparation). From these results, we concluded that the DPPH assay is a simple, rapid, sensitive and reliable method for the preliminary screen of antioxidant activity compared with other antioxidant assays such as LDL oxidation assay.

The work of activity-guided fractionation, isolation of antioxidant constituents, and structural elucidation performed on the fruit of *Manilkara zapota* cv. Tikal described in Chapter 3 focused on the two new polyphenolic antioxidants methyl 4-*O*-galloylchlorogenate and 4-*O*-galloylchlorogenic acid. The active EtOAc fraction was separated by Sephadex LH-20 CC. Active fractions were further separated by RP18 CC. Two new and eight known polyphenolic antioxidants were isolated and identified from the active fractions.

The structural elucidation of the new compounds was mainly conducted by mass spectrometry (MS) and NMR. I used ^{1}H and ^{13}C NMR data with the modern NMR technique HMBC to figure out the basic structure of methyl 4-*O*-galloylchlorogenate. But I also used older NMR techniques, such as homonuclear decoupling, to determine the stereochemistry of the quinic acid moiety. Therefore, in my phytochemical analyses, I used a variety of techniques to elucidate the structure of a new compound. In a similar way, the other new compound was identified as 4-*O*-galloylchlorogenic acid. Both of these two new antioxidants had high antioxidant activity in the DPPH assay, and showed cytotoxity in the MTT assay. Comparing data for these compounds in the DPPH and MTT assays with these data for chlorogenic acid, I concluded that the addition of a gallic acid moiety to chlorogenic acid improves the latter's antioxidant and potential anticancer activity, by adding three more hydroxyl groups to the molecule.

In addition, eight known compounds from *M. zapota*, methyl chlorogenate, dihydromyricetin, quercitrin, myricitrin, (+)-catechin, (–)-epicatechin, (+)-gallocatechin, and gallic acid, were identified by MS and their NMR data was compared with previously published data.

In Chapter 4, I described the work of activity-guided fractionation, isolation, and structural elucidation of antioxidant constituents from the fruits of *Pouteria campechiana*, *P. sapota*, and *P. viridis*, seven polyphenolic antioxidants, namely gallic acid, (+)-gallocatechin, (+)-catechin, (–)-epicatechin, dihydromyricetin, (+)-catechin-3-*O*-gallate, and myricitrin, were isolated and identified by MS and comparing their NMR data with the previously published data. SIM LC-MS was performed on the EtOAc fractions of plants from these three *Pouteria* species. The highest level of the seven polyphenols measured with this technique was found in *P. sapota*, the second highest in *P. viridis*, and the lowest in *P. campechiana*. The levels of the seven polyphenols corresponded to the results of the DPPH assay, where *P. sapota* had the highest antioxidant activity, *P. viridis* second highest, and *P. campechiana* lowest.

I attempted to quantify the seven polyphenols in plants from these three *Pouteria* species using HPLC-PDA, a baseline separation was achieved among the seven standard polyphenols. However, this method was not useful in this case, because other compounds in the extracts coeluted with these polyphenols and interfered with their separation. Thus, the results would have some inaccuracies. In order to get more accurate quantitative results, SIM LC-MS was used to quantify the seven polyphenols. By using SIM LC-MS, LC provided initial separation of the mixture of compounds based on their polarities, and then SIM MS was used to select specific ions corresponding to specific compounds. This method allowed to distinguish and quantify the major polyphenolic constituents of plants from the three *Pouteria* species. The SIM LC-MS is a very sensitive method because only a few ions are monitored in this technique. It provides lower LOD and LOQ than HPLC-PDA methods. In this study, even if some polyphenols were not isolated from

activity-guided fractionation, they were still detected and quantified by SIM LC-MS. SIM LC-MS is a state of art technique for quantifying antioxidants.

In conclusion, in this research, a screen for antioxidant activity using the DPPH assay was conducted on forty-nine edible tropical fruits and vegetables, and thirty-six plants displayed antioxidant activity. Four plants of the Sapotaceae family were selected from these thirty-six active plants to undergo activity-guided fractionation. Two new and eight known polyphenolic antioxidants were isolated and identified from *Manilkara zapota* cv. Tikal. Seven known polyphenolic antioxidants were isolated and identified from *Pouteria campechiana*, *P. sapota*, and *P. viridis*. A quick, sensitive and accurate SIM LC-MS method was developed to quantify these seven polyphenols, the highest level of the seven measured polyphenols was found in *P. sapota*, the second highest in *P. viridis*, and the lowest in *P. campechiana*. In this work, most of the antioxidants identified from the Sapotaceae plants were flavonoids, and these polyphenols are important antioxidant constituents in the Sapotaceae plants.

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