CHEMICAL COMPOSITION AND ANTIOXIDANT PROPERTIES OF MAYA NUT (*Brosimum alicastrum)*

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ABSTRACT

Maya nut was investigated in term of its moisture, ash content, antioxidant activities, phenolic components by high pressure liquid chromatography-ultraviolet detector (HPLC-UV) and fatty acid composition by gas chromatography-flame ionization detector (GC-FID). The results showed that Maya nut was mainly composed of 55.06% linoleic acid (C18:2 n6), 27.2% palmitic acid (C16:0), 6.26% stearic acid (C18:0), 9.9% linolenic acid (C18:3 n3), and 3.36% Cis-11-Eicosenoic (C20:1). Maya nut possesses a higher content of polyunsaturated fatty acids (PUFAs) (linoleic and linolenic acid), but lower content of monounsaturated fatty acids (MUFAs) than canola oil and olive oil. The antioxidant capacities and total phenolic contents of the methanolic Maya nut extract and other commercially available nuts including walnut, almond, and peanut were determined and compared by three different *in vitro* antioxidative assays, i.e., DPPH* free radical scavenging assay, ferric reducing antioxidant potential (FRAP) assay, and ABTS free radical scavenging assay. The DPPH radical scavenging activity and $ABTS^{\cdot+}$ radical quenching activity of the Maya nut extract were 83.35% and 92.55%, respectively, which were higher than walnut, almond, peanut and commercial antioxidant BHT. In addition, FRAP value of the Maya nut extract was 8.08 mmol Fe^{2+}/100 g sample, which was higher than that of peanut and almond, but lower than that of walnut. The TPC of Maya nut was 24.67 mg of gallic acid equivalents per gram of sample, which were 3, 6, and 28 times higher than that of walnut, peanut, almond. Furthermore, free phenolic acids in the Maya nut were analyzed by HPLC. 3, 4-Hydroxybenzoic acid (45 mg/kg), gallic acid (27.06 mg/kg), vanillic acid (21.15 mg/kg), and caffeic acid (6.5 mg/kg) were found after

the acidic hydrolysis. After the alkaline treatment and the second acidic hydrolysis, 3,4 hydroxybenzoic acid (342.2 mg/kg), vanillic acid (103.9 mg/kg), caffeic acid (17.1 mg/kg), *p*-coumaric acid (13.5 mg/kg), epicatechin (53 mg/kg), and sinapic acid (8.16 mg/kg) were detected. These results demonstrated that Maya nut is rich in bioactive phytophenolics that results in high antioxidant activities; therefore, Maya nut is suitable to be made into health-benefiting nutraceuticals.

DEDICATION

I would like to dedicate this work to my husband, Ufuk Ozer, my parents, Hacer Tokpunar, Mevlut Tokpunar, my lovely sisters, Betul Tokpunar and Sumeyra Zeynep Tokpunar, my brother, Seyit Ali Tokpunar, with great thanks, love, and pride.

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CHAPTER 1

LITERATURE REVIEW

1.1 History of Maya nut

Brosimum alicastrum, Maya nut, a fast growing and huge tropical rainforest tree that is indigenous in Central and Northern South America can grow up to 40 m in height and 150 cm d.b.h as seen in Figure 1.1 (The Equilibrium Fund 2008). It was once plentiful throughout Central America (see its geographical distribution shown in Figure 1.2), but faces extinction in parts of its range because of overcut of trees for firewood and corn planting (Global Facilitation Unit (GFU) for Underutilized Species, 2007). This species is under the Moraceae family whose other genuses consist of mulberries and fig. The taxonomy of Brosimum alicastrum is determined as follows (Integrated Taxonomic Information System, 2000).

Kingdom Plantae

Subkingdom Tracheobionta

Division Magnoliophyta

Order Urticales

Family Moraceae

Genus Brosimum

Species Brosimum alicastrum

According to the studies (Puleston, 1968; Puleston, 1972), the seed of B. alicastrum, or called Maya nut (shown in Figure 1.1) that has other names such as Ramon nut, Breadnut, Ojoche, Ox, Ash, Ujuxte, Ojite, Ojushte, Ujushte, Capomo, Pisba waihka and Masica, was one of the main staple foods for ancient Mayas and other Neotropical pre-Columbians. It was also used as an alternative food when the production of traditional crop was low (Puleston, 1968; Puleston, 1972). Thousands of villages have survived for years against drought and famine by consumption of Maya nut. In addition, there is evidence that Ancient Mayas intentionally cultivated or managed the tree for harvesting Maya nut because of the edulis fruits and nutritional seeds of this large rainforest tree. Moreover, its leaves provide pleasing forage, the wood is outstanding for construction, and various parts of the tree have been used as regional medicines (Peters, 1989; Peters, 1983).

1.2 Nutritional Properties of Maya nut

Seeds of Maya nut are rich in starch, calcium, potassium, iron, folate, fiber, and vitamins A, E, C and B. The nutritional value of Maya nut is similar to soybean and comparable to some other nutritional foods (Figure 1.3 and Figure 1.4) (Vohman & Sanchez-Garduño, 2008). It is an organic food without pesticides, fertilizers, or other chemical contamination. One of the reasons why the ancient people could survive was possibly partially due to the consumption of Maya nut as food. In addition, the tree can conserve soil, biodiversity and environment. For example, the tree can produce 2 tons of processed foods per year and simultaneously support the populations of birds and wildlife. It is believed that the Maya nut forest is important to maintain the habitat of

wildlife, healthy environment and local ecosystem. However, in last decades, because of the effect of large destructive plantation of crops that has caused significant biological erosion of the survival of Maya Nut forest, the life quality of the people has been affected resulting in less job opportunities, lower income and shortage of food supply (Vohman & Sanchez-Garduño, 2008). In order to fight those difficulties, the Equilibrium Fund was set up and has trained over 7000 women from 348 villages in Honduras, Nicaragua, Guatemala, El Salvador and Mexico, to participate in the Maya nut Program to earn their own money by producing and selling Maya nut for increased income, better health and nutrition (GFU for Underutilized Species, 2007).

1.3 Comparison of Chemical Composition of Nuts

1.3.1 Moisture

According to USDA Nutrient Database, the moisture content of walnut *(Juglands regia)* is about 4.07%, in comparison with that of almond (*Prunus dulcis*) 4.70%, peanut (*Arachis hypogaea*) 6.50% (USDA, 2009).

1.3.2 Ash

The ash compositions of walnut (*Juglands regia*), almond (*Prunus dulcis*), and peanut (*Arachis hypogaea*) are as follows: 1.78%, 2.99%, and 2.33%, respectivley (USDA, 2009).

1.3.3 Protein

The protein content of walnut *(Juglands regia)* is 15.23%, while almond (*Prunus dulcis*) has a protein content of 21.22%, and peanut (*Arachis hypogaea*) has 25.80% (USDA, 2009).

1.3.4 Lipid

Lipid is the major constituents of some nuts, i.e., walnut (*Juglands regia*), almond (*Prunus dulcis*), and peanut (*Arachis hypogaea*). Their total lipid contents are as follows: 65.21%, 49.42%, and 49.24% (USDA, 2009), respectively.

1.3.5 Fatty Acid Composition

Fatty acids (FA) are main components of lipids. Most part of these carboxylic acids is found esterified as triacylglycerides. Recently, determination of the FA composition has become a subject of increased interest for both routine food analysis and lipid research, because of the nutritional importance and beneficial effects on human health. For example, total fat intake, the type of fat or fatty acid composition may affect several physiological and biochemical processes, including blood pressure regulation, glucose-lipid metabolism, and platelet aggregation (Amaral, Casal, Pereira, Seabra & Oliveira, 2003). Some tree nuts are considered healthy foods because of rich in health benefiting FAs. For example, walnuts contain rich omega-6 and omega-3 polyunsaturated fatty acids (PUFA), and some other nuts have more monounsaturated fatty acids (MUFA) (Amaral, Casal, Pereira, Seabra & Oliveira, 2003). According to the USDA National Nutrient Database (USDA, 2009) which lists the components of walnuts, peanuts, and almonds, the English walnut (*Juglans regia)* contains 38% linoleic acid, 9% gamma linolenic acid (PUFAs), 8.8% oleic acid (MUFA), 4.4% palmitic acid, and 1.6% stearic acid (saturated fatty acids), respectively. Peanut (*Arachis hypogaea)* has 15.5% linoleic acid, 23.7% oleic acid, 5.1% palmitic acid, and 1.1% stearic acid. Almond (*Prunus dulcis)* involves 3% palmitic acid, 30.6% oleic acid, 12% linoleic acid (Table 1.1).

For general FA analysis, the lipids are at first extracted with appropriate solvents, e.g., non-polar solvents such as hexane, ethyl ether, etc., then the fatty acids are converted into fatty acid methyl esters (FAMEs) and analyzed by gas chromatography / flame ionization detector (GC/FID) (Christie, 1998; Shantha & Napolitano, 1992) with the aid of comparison of retention times (or more accurately, the retention index, RI) of authentic standards and sample (Wetter & Thurnhofer, 2005; Spitzer, 1997).

1.4 Mechanism of Antioxidants and Oxidative stress

Organisms are able to develop various defensive mechanisms to prevent excessive exposure to free radicals from a variety of sources (Serafini, Villano, Spera & Pellegrini, 2006). Defensive systems against free radical-induced oxidative stress include several means through preventative mechanism, repair mechanism, physical defense and antioxidant defense (Labuckas, Maestri, Perelló, Martínez, & Lamarque, 2008). The latter in the body consists of enzymatic and molecular players to form an antioxidant network which varies in components and concentration in distinct environmental conditions (Serafini, Villano, Spera & Pellegrini, 2006). Enzymatic antioxidant defenses are composed of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), etc. In contrast, some well known non-enzymatic antioxidants include ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants that can play an important role in plasma (Labuckas, Maestri, Perelló, Martínez, & Lamarque, 2008). Many of the biological effects of antioxidants are based on their ability to scavenge deleterious free radicals, which include reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that are products of normal

cellular metabolism. Free radicals are described as molecules which have an unpaired electron in the outer orbit. These molecules are usually unstable and very reactive and can be transformed to other non-radical reactive species. These radicals act as a double bladed sword since they can be either harmful or beneficial to living systems. Beneficial effects of ROS include their physiological roles in cellular responses to infectious agents and various numbers of cellular signaling systems. Oxygen radicals carry out significant biochemical activities such as signal transduction, gene transcription, and regulation of soluble guanylate cyclase activity in cells (Zhang, Liao, Moore, Wu & Wang, 2009; Uttara, Singh, Zamboni & Mahajan, 2009). At low concentrations, ROS can cause mitogenic response in the cells. NO (Nitric oxide) serves as a significant signaling molecule and is involved in the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics (Uttara, Singh, Zamboni & Mahajan, 2009). However, at high concentrations, ROS and RNS can be severely harmful to cell structures composed of proteins, lipids, membranes, and nucleic acids (Valko, Rhodes, Moncol, Izakovic & Mazur, 2006). Therefore, if overproduction of free radicals cannot be balanced by enzymatic and non-enzymatic antioxidants, oxidative and nitrosative stresses will damage the biological systems and the redundant free radicals can alter cellular lipids, proteins, or DNA and suppress their normal function (Barreira, Ferreira, Oliveira, & Pereira, 2008). Oxidative stress is responsible for several pathological conditions including cardiovascular disease, cancer, neurological disorders, diabetes and ageing. On the other hand, in the case of overproduction of RNS, nitrosylation reactions (protein modification)

can alter the structure of proteins and hinder their regular functions. In addition, these agents could induce DNA fragmentation and lipid oxidation in human body (Barreira, Ferreira, Oliveira, & Pereira, 2008). Although antioxidant defensive system of cells opposes oxidative damage from ROS, presence of oxidative damage accumulating during the life cycle and free radical-induced damage to DNA, to lipids, to proteins has been suggested to development of important cell disorders such as cancer, neurodegenerative diseases, cardiovascular diseases, and chronic inflammation (Valko, Rhodes, Moncol, Izakovic & Mazur, 2006).

The capability of antioxidants is not only limited to scavenge hazardous free radicals, but also to modulate cell-signaling pathways. One of the impacts of ROS materials is their role in signal transduction. Signal transduction is a process which a cell transform outer signals or stimulus to components inside the cell. This process is initiated by extracellular signals such as neurotransmitters, cytokines, and hormones and can inhibit various biological activities including gene expression, cell growth, muscle contraction, and nerve transmission. The modulation of cell signaling pathways by antioxidants might help preclude cancer by protecting normal cell cycle regulation, delaying proliferation and stimulating apoptosis, inhibiting tumor occupation, restraining inflammation and other effects. In addition, genetic material modification due to oxidative stress circumstances can be the first step in carcinogenesis, mutagenesis, and ageing. Increasing levels of oxidative DNA lesions and DNA mutation have been implicated in several tumors and damages in the etiology of cancer (Prior, Wu & Schaich, 2005). ROS-induced DNA damage resulting in single or double-stranded DNA breaks,

modifications of purine, pyrimidine, and deoxyribose, as well as DNA cross-links can cause inhibition of the transcription, delay of signal transduction, increase of replication errors, and genomic imbalance, all of which are involved in carcinogenesis. Besides of DNA damage, the lipid peroxidation process resulting from reactive nitrogen species (RNS) has been shown in the carcinogenesis mechanism (Lee & Lee, 2006).

In the past years, because of the concern of toxicity and carcinogenic claims of synthetic antioxidants, finding new natural antioxidants without undesirable side effects have become more popular. As a result, studies have centered on antioxidants, such as phenolics, from natural sources, for instance, naturally present in fruits, vegetables, nuts and herbs (Frankel & Finley, 2008). Moreover, these naturally occurring antioxidants might be developed as nutraceuticals helping to inhibit oxidative damage occurring in the body (Dudonné, Vitrac, Coutiére, Woillez & Mérillon, 2009). In this context, phenolic acids, due to their variety and immense distribution, have been generally studied as likely models for the development of new basic antioxidants.

1.5 Phenolics and Antioxidants

Phytochemicals are defined as non-nutritive bioactive plant chemicals in fruits, vegetables, grains, and other plant foods having protective or disease preventive properties and are considered to reduce the risk of chronic diseases (Dong, He & Liu, 2007). Many individual phytochemicals have been identified and classified in fruits, vegetables, and grains. However, still large amount of phytochemicals need to be characterized to comprehend their health benefits. Some well-known phytochemicals include carotenoids, phenolics or polyphenols, alkaloids, nitrogen-including compounds,

and organosulfur compounds (Dong, He & Liu, 2007). Phenolic compounds constituting one of the most widespread groups of substances are the products of secondary metabolism in plants. More than 8000 phenolic structures have been characterized, which involve essential activities in the reproduction and growth of the plants, play important role as defense agents against pathogens, parasites, and predators, as well as promotion to the color of plants (Dong, He & Liu, 2007). Natural phenolics can vary from simple (such as phenolic acids with a C6 ring structure), low molecular weight, and single aromatic-ring compounds to the large, tannins (highly polymerized compounds) and derived polyphenols (Crozier, Jaganath & Clifford, 2009). They can be classified by the number and arrangement of their carbon atoms, and are generally conjugated to sugars and organic acids. These phenolics naturally occurring in plant tissue can be divided into two groups, the flavonoids, and non-flavonoids. The term “phenolic acids”, in general, defines phenols possessing one carboxylic acid functional group. However, phenolic acids naturally occurring in plant tissue implies to a different group of organic acids. These naturally occurring phenolic acids can be subdivided into two major groups including two discrete carbon frame-works, hydroxycinnamic and hydroxybenzoic acids (Figure 1.5 and Figure 1.6). The numbers and positions of the hydroxyl groups on the aromatic ring make them different and designate the variety of phenolic acids (Table 1.2 and Table 1.3) (Stalikas, 2007). *P*-Hydroxybenzoic, protocatechuic, vanilic, syringic, and gallic acids constitute hydroxybenzoic acid derivatives and are generally found in the bond form and part of a complex structure like lignins and hydrolysable tannins. In addition, they can be present in the form of sugar derivatives and organic acids in plant

foods. The most common hydroxycinnamates include *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid, with caffeic acid dominating (Figure 1.7). These acids occur as conjugated form, for example with tartaric acid or quinic acid, collectively referred to as chlorogenic acids (Crozier, Jaganath & Clifford, 2009). Gallic acid is the most widespread phenolic acid of the biosynthetic precursor of hydrolysable tannins, the C6–C3 hydroxycinnamates and their conjugated derivatives, and the polyphenolic C6–C2–C6 stilbenes and occurs largely as complex sugar esters in gallotannins. However, these are only present in limited extent in dietary components. Non-sugar galloyl esters in grapes, wine, mangoes, green tea and black tea compose the considerable source of gallic acid in the human diet. Moreover, ellagic acid and ellagitannins found in raspberries and strawberries are also present largely in fruits including pomegranate, blackberries, persimmon, walnuts, hazelnuts, as well as oak-aged wines (Crozier, Jaganath & Clifford, 2009).

Although phenolic acids have been known involving various functions, including nutrient uptake, protein synthesis, structural components, enzyme activity, phytosynthesis, and allelopathy, there is still much unknown functions regarding their roles in plants (Robbins, 2003). Benzoic and cinnamic acid derivatives occur in nearly all plant foods (e.g., fruits, vegetables, and grains) and are substantially distributed throughout the plant in seeds, leaves, roots, and stems. Only a small fraction is present as “free acids”, while the majority are bonded through ester, ether, or acetal bonds either to structural components of the plant such as cellulose, proteins, lignin or to larger polyphenols (flavonoids), or smaller organic molecules (e.g., glucose, quinic, malic, or

tartaric acids) or other natural products (e.g., terpenes). These linkages lead to a huge amount of derivatives. This diversity is also a crucial factor in the complexity of the analysis of phenolic acids (Robbins, 2003). Investigating their roles in plant life represents only one aspect of phenolic acids, there has been huge interests from point of view of food industry. Phenolic acids have been related to color, sensory qualities, nutritional and antioxidant properties of foods. The reason for analytical investigations of phenolics has been their contribution in organoleptic properties (flavor, astringency, and hardness) of foods. In addition, the food industry has interest of exploration of the nature and profile of phenolic acids, their influence on fruit maturation, prevention of enzymatic browning, and their roles as food preservatives. Phenolics act as antioxidants, due to the reactivity of the phenol group (hydroxyl substituent on the aromatic ring). It is known that there are several mechanisms. However, the dominant model of antioxidant activity is believed to be radical scavenging by means of hydrogen atom donation. In addition, electron donation and singlet oxygen quenching are defined as other antioxidant - radical quenching mechanisms. The stabilization of phenolic acids is influenced by substituents on its aromatic ring, which, on the other hand, affects its radical-quenching ability. Therefore, distinct acids can have distinct antioxidant activities, for example, the antioxidant behavior of the free, esterified, glycosylated, and nonglycosylated phenolics has been reviewed (Robbins, 2003).

1.6 Sample Preparation and Extraction of Phenolic Compounds

Sample preparation is critical to any reliable analysis, without exception to analyzing polyphenolics and simple phenolics in plants, foods, and liquid samples

(including biological fluids and beverages) that contain chemical functional groups with a great diversity regarding polarity, acidity, number of hydroxyl groups and aromatic rings, concentration levels, and complexity of the matrix. The analyses of phenolic acids and flavonoids can be different substantially, and require detailed elaboration of sample preparation, from use of simple filters to more complicated work-up routines, such as hydrolysis of glycosides, extraction or clean-up steps prior to analysis. For this reason, no single pretreatment procedure can be applied to all samples. Choosing the optimal pretreatment method with respect to the chemical structures and characteristics of the analyzed substances has been the common sense in real practice (Stalikas, 2007). Broadly, solid samples are usually exposed to milling, grinding, and homogenization, which may be processed by air-drying or freeze-drying. On the other hand, liquid samples are first filtered or centrifuged before they are either directly injected into the separation system or the compounds are separated by means of additional steps using corresponding techniques (Stalikas, 2007).

1.6.1 Hydrolysis of Phenolic Acids

Acidic hydrolysis and saponification are the most common means of releasing the acids, although they may decompose under these conditions. Enzymatic release is an alternative but less prevalent technique. The acidic hydrolytic method involves treating the plant extract or the food sample itself with a strong inorganic acid (e. g. HCl) at reflux or above reflux temperatures in aqueous or alcoholic solvents (methanol being the most common). Acid ranges from 1 to 2 N HCl and the reaction times range from 30 min to 1 h. Aqueous HCl was reported to be able to destroy the hydroxycinnamic acids. Krygier et

al. (Krygier, Sosulski & Hogge, 1982) reported that losses under acidic conditions varied with the form of phenolic acid, ranging from 15 to 95% for p-coumaric acid and sinapic acid, respectively. Saponification entails treating the sample with a solution of NaOH at concentrations from 1 to 4 M. Most of the reactions are left to proceed at room temperature for 15 min up to overnight (Rommel & Wrolstad, 1993). Some investigations report that the reactions are carried out in the dark, as well as under an inert atmosphere such as argon or nitrogen gas (Stalikas, 2007; Robbins, 2003).

1.6.2 Extraction

Extraction techniques need to take into account the location of phenolic acids in the plant. Most phenolic acid derivatives present in the plant matrix are stored in vacuoles and are commonly extracted by alcoholic or organic solvents. The exceptions are those bound to insoluble carbohydrates and proteins within the plant matrix (Robbins, 2003). There are various factors influencing the extraction of phenolic acids. Solubility of phenolics is managed by their chemical nature in the plant depending on simple, highly polymerized and very highly polymerized substances. Plant materials may be composed of variable amounts of phenolic acids, phenylpropanoids, anthocyanins and tannins, among others. The interaction of phenolics with other plant substances such as carbohydrates and proteins may be seen in plants. This interaction might cause the formation of considerably insoluble complexes, which has an effect on the extraction procedure and efficiency. As a result, it is not simple to develop a universal desirable method for all plant phenolics. Because of diverse form of plant phenolics, supplemental steps might be necessary to remove the unwanted phenolics and non-phenolic substances

such as waxes, terpenes, fats and chlorophylls (Naczk & Shahidi, 2006). Another factor influencing the solubility of phenolics is the polarity of solvents. Different solvents such as methanol, ethanol, propanol, acetone, ethyl acetate, dimethylformamide and their combinations can be used for extraction of phenolics with distinct proportions of water (Antolovich, Prenzler, Robards & Ryan, 2000; Zadernowski, Naczk & Nesterowicz, 2005). However, very polar phenolic acids (benzoic, cinnamic acids) might not be able to be extracted properly with pure organic solvents. Less polar solvents (dichloromethane, chloroform, hexane, benzene) are more convenient for the extraction of nonpolar extraneous compounds such as waxes, oils, sterols, chlorophyll from the plant matrix. In addition, there are also various significant factors influencing the extraction of phenolics such as pH, temperature, sample-to-solvent volume ratio, and time intervals of each of extraction steps in the extraction process (Stalikas, 2007). The extraction time is thought as an efficient parameter for extraction of polyphenols. Desirable extraction times for phenolic acids can alter from 1 min to 6 h using Soxhlet extraction (Sun, Sun & Zhang, 2001) and change from 1 min to 24 hour using different techniques (Sun, Sun & Zhang, 2001; Price & Butler, 1977; Maxson & Rooney, 1972). It has been demonstrated that longer extraction times induce the oxidation of phenolics in the absence of reagents in the solvent system (Khanna, Viswanathan, Krishnan & Sanwal, 1968). Also, in order to improve the analytical efficiency, vortex followed by centrifuge, sonication, mechanical stirring, and continuous rotary extraction have been often adopted (Montedoro, Servili, Baldioli, & Miniati, 1992; Guillén, Barroso, & Pérez-Bustamante, 1996a; Guillén, Barroso, & Pérez-Bustamante, 1996b). The extraction of polyphenols from plant material

might also be affected by the ratio of solvent-to-sample (Naczk & Shahidi, 2006). Escarpa et al. (Escarpa & González, 2001) demonstrated that good yields were obtained when using 100% methanol for apples and pears, and 80% aqueous methanol for green beans, lentil and pomace, in the absence of light and addition of 1% BHT (2,6-di-*tert*-butyl-4 methylphenol). In some studies, successful extraction of phenolic acids and their methyl esters could be implemented by chloroform (Smolarz, 2001).

1.6.2.1.Liquid-Liquid Extraction Method

Liquid-liquid and solid-liquid extraction (LLE) are the most mainly preferred techniques before analysis of polyphenolics and simple phenolics in plants due to their convenience of use, efficiency, and wide-ranging applicability (Stalikas, 2007). Liquid-liquid extraction is one of the most prevalent sample pre-treatments in an analytical process to increase selectivity by separating the analyte from matrix interfering species, or to raise selectivity by concentrating the analyte from a large sample volume (Silvestre, Santos, Lima & Zagatto, 2009). In this method, hydrophobic sample constituents are extracted from aqueous samples with a water-immiscible organic phase. Different volatile organic solvents can be utilized, including pentane, hexane, diethyl ether, ethyl acetate, chloroform and methylene chloride (Pederson-Bjergaard, Rasmussen, Halvorsen, 2000). The need for the separation usually derives from the fact that other solutes or original solvent(s) interfere in some way with the chosen analytic technique. It is also called selective dissolution method in which the analyte is removed from the original solvent and subsequently dissolved in a different solvent (extracted). At the same time, most of the remainder of the sample remains unextracted in the original solution. The

technique obviously includes two liquid phases, original solution and extracting solvent. The important criteria for a successful separation of the analyte are that these liquids should be immiscible and that the analyte should be more soluble in the extracting solvent (Kenkel, 1994). For successful LLE, the analyte should be extracted quantitatively from the sample and into the organic solvent (Pederson-Bjergaard, Rasmussen, Halvorsen, 2000).

1.6.2.2 Solid-Phase Extraction Method (SPE)

Solid-phase extraction (SPE) is a simple preparation technique based on the principles used in liquid chromatography, in which the solubility and functional group interactions of sample, solvent, and adsorbent are optimized to effect sample fractionation and/or concentration. A wide range of chemically modified adsorbent materials (silica gel or synthetic resins) enable precise group separation on the basis of different types of physicochemical interaction, i.e. reversed phase (C2, C8, C18), cation- and anion-exchange, etc. It should be noted that SPE is well suited to the treatment of sample matrices with high water content, e.g. extracts of herbal materials (Huie, 2002). Solid-phase extraction (SPE) is a generally utilized method to fractionate and to remove undesirable components from the sample and useful for the clean-up method of crude plant extracts or biological samples (Stalikas, 2007). Larger phenolics and sugars can be distinguished from the smaller phenolic components by eluting with solvents of varying pH (Robbins, 2003). SPE offers several advantages, including better selectivity, faster speed, and convenience to automate (Guillén, Barroso, & Pérez-Bustamante, 1996). A repetition of acidification, treatment of alkaline solutions and extraction steps are

generally required to separate the phenolic acids. A basic SPE required for all acidic and basic analyte isolations from the plant extract is an ordinary for this method. It has seen that there is coherence in the choice of absorbents for isolating the phenolic acids and flavonoids. Predominantly, the absorbent is C18 bonded silica while the sample solution or solvents are generally acidified to prohibit ionisation of the phenolics. It could be said that another big advantage of SPE is that the crude extract of plant material is injected directly into this SPE–HPLC system (Guillén, Barroso, & Pérez-Bustamante, 1996a). Its use in flavonoid analysis is, however, relatively new. In most cases like the extraction and separation of phenolics, the sorbent is a C18-bonded silica while the sample solution and solvents are usually slightly acidified to prevent ionization of the flavonoids in order to reduce their retention (Rijke, Out, Niessen, Ariese, Gooijer & Brinkman, 2006). The advantages of SPE include providing higher precision and throughput, lower solvent consumption, and prevention of the formation of emulsions, which are often time-consuming. Additionally, SPE can be easily incorporated into automated analytical procedures with relatively simple and inexpensive equipment, which can lead to greater accuracy and precision and higher laboratory throughput. The main issue in the SPE method development could be the choice of sorbent material and the elution protocol (Gilbert-López, García-Reyes & Molina-Díaz, 2009).

1.6.2.3 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) has been used for many years for the extraction of volatile components, essential oils and aroma compounds from plant materials, on an industrial scale. Recently, the application of this technique on an

analytical scale has started to attract wide interest for sample preparation before chromatographic analysis. The potential advantages include the ability to perform rapid (often less than 30 min) extractions, to reduce the use of hazardous solvents replaced by carbon dioxide, and to combine the extraction steps with gas, liquid, or supercritical-fluid chromatography. Another important advantage of applying SFE to the extraction of active compounds from medicinal plants is that degradation as a result of the lengthy exposure to elevated temperatures and atmospheric oxygen can be avoided and/or minimized. Carbon dioxide has been by far the most used supercritical fluid for extracting a variety of pesticides from solid matrices, such as soil, sediments, vegetables, animal tissues and foodstuffs. The main advantages of using fluid CO_2 rely on its non-toxic, non-flammable, inexpensive properties, and the automation due to the instruments available for that purpose and also the ability of being coupled to chromatographic systems. Finally, relatively selective and fast extraction procedures can be performed by modifying the density of the supercritical fluid. Increasing the density of the fluid increases the extraction yield of high molecular weight compounds. The density of the fluid can be varied by varying its temperature and pressure. The SFE is conceptually not difficult to perform. The extraction vessel is filled with a sample and placed in a heated extraction chamber. A pump is used to supply a known pressure of the supercritical fluid to an extraction vessel which is thermostated at a temperature above the critical temperature of the supercritical fluid. During the extraction, the analytes are removed from the bulk sample matrix into the fluid and swept into a decompressing region. Herein, the supercritical fluid becomes a gas and is vented, while analytes abandoning the

gas are collected in a vial containing a small volume of a suitable solvent such as methanol (so-called solvent mode). A variation of this scheme is that of substituting the collecting liquid at the outlet of the extractor with a sorbent cartridge such as C18 (so-called sorbent mode). The extraction of hydrophobic compounds from complex matrices containing sugar, proteins, and fat can be achieved almost quantitatively, but polar molecules usually give poor recovery rates. The recovery of these compounds can be improved significantly by adding of a modifying solvent, such as methanol or acetonitrile. Ono et al., (2006) developed a multi-residue method for the determination of over 300 pesticides in soybeans using SFE and GC–MS, obtaining recoveries between 70% and 120% for 245 out of the 303 compounds tested. Carbon dioxide was used as a supercritical fluid and acetone as collection solvent. An additional clean-up step was required for soybean samples using a C18 cartridge and after that an Envicarb-NH_2 cartridge column. Despite its selectivity and automation capabilities, nowadays the use of SFE has decreased dramatically during the last couple of years, due to the requirement of green chemistry of extraction and separation, high cost of the instrumentation maintenance, and the difficulties in method development. For instance, optimization of the extraction conditions on the sample can be time-consuming and complex optimization procedures (Gilbert-López, García-Reyes & Molina-Díaz, 2009).

1.7 Prevention of Cancer

Cells in humans and other organisms are permanently exposed to various oxidizing agents, which may be produced by metabolic activity within cells. The important fact to maintain an optimal physiological and healthy condition to human

beings is to obtain a balance between oxidants and antioxidants. Oxidative stress, especially in chronic diseases (infections) may result from the overproduction of oxidants leading to an imbalance. In addition, large biomolecules such as lipids, proteins, and DNA can be affected by this damage resulting in an increased risk for cancer and CVD. It is very well known that adequate amounts of antioxidant compounds (phytochemicals), such as phenolics and carotenoids can inhibit the oxidative stress induced by free radicals (Liu, 2004). Antioxidant phytochemicals have supplementary mechanisms, e.g., stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, and antibacterial and antiviral effects (Sun, Chu, Wu & Liu, 2002). One-third of all cancers are considered to be related to unhealthy diet and be preventable. However, identifying relevant dietary components to prevention of specific cancers is a great challenge.

Many phytochemicals possessing different chemical structures have anticarcinogenic properties. They are able to activate cytoprotective enzymes and inhibit DNA damage to inhibit initiation in healthy cells, or modulate cell signalling to eliminate unhealthy cells at later stages in the carcinogenic process. It has been demonstrated that many well known phytochemicals can influence many aspects of cell biochemistry *in vitro* test, but different agents have many similar activities. The crucial *in vivo* targets for particular dietary molecules are unknown, making it difficult to predict which cancer phenotypes are most likely to be affected. Generally, many phytochemicals are insufficient bioavailable and studies recommend that combinations may be more effective than single agents. There may also be advantages in combining them with chemo- or

radio-therapies (Manson, Foreman, Howells & Moiseeva, 2007). In addition, phytochemicals can act as blocking agents preventing the initiation phase of carcinogenesis or suppressing agents retarding the promotion and progression phases of carcinogenesis (Kwon, Barve, Yu, Huang & Kong, 2007). The use of natural product as anticancer agents was approved in the 1950s by the U.S. National Cancer Institute (NCI) under the leadership of the Dr. Jonathan Hartwell. Later, NCI has been important institution through its contribution as a grant to discover new naturally occurring anticancer agents (Gragg, Kingston & Newman, 2005 (missing in the ref list). In this context, Maya nut might be a promising healthy food because of existence of bioactive phytochemical substances.

1.8 Methods for Measuring Antioxidant Capacity

Antioxidant capacity assays are classified into two groups in terms of principal of chemical reactions. These are hydrogen atom transfer (HAT) reaction based assays and single electron transfer (ET) reaction based assays (Huang, Ou & Prior, 2005), which are shown in Table 1.4. The HAT-based assays including competitive reaction scheme, usually are consisted of a free radical generator, an oxidizable molecular probe, and an antioxidant. The ET-based assays measure the reduction capacity of an antioxidant and when oxidant is reduced, color changes occur (Apak et.al., 2007). The HAT- and ET-based assays determine the radical (or oxidant) scavenging capacity, rather than the suppressive antioxidant effect of a sample (Huang, Ou & Prior, 2005). In the chemical industry, antioxidants are defined as compounds that postpone autoxidation of a chemical product. The autoxidation process results from radical chain reactions between oxygen

and the substrates. Powerful antioxidants, radical scavengers can prevent radical chain reactions. In food science, dietary antioxidants markedly diminish the effects of reactive species, such as reactive oxygen and nitrogen species. Radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors, and antioxidant enzyme cofactors constitute dietary antioxidants (Huang, Ou & Prior, 2005). In this research, the DPPH assay, ferric reducing antioxidant power assay (FRAP), and ABTS radical scavenging assay were utilized to evaluate the antioxidant capacity of Maya nut. Moreover, total phenolic content was analyzed by the Folin-Ciocalteu reducing capacity assay (Singleton & Rossi, 1965).

1.8.1 Diphenylpicryl-hydrazyl (DPPH) Free Radical Scavenging Assay

DPPH is a stable and commercially available organic radical and has an absorbance maximum centered at about 515 nm (Huang, Ou & Prior, 2005). The radical DPPH is generally used to evaluate the antiradical/antioxidant properties of synthetic and natural phenols using methanol or ethanol as the most appropriate solvents (Foti, Daquino & Geraci, 2004) because it is more stable than superoxide and hydroxyl radicals (Alasalvar & Shahidi, 2008). In this assay, the purple chromogen radical 2,2-diphenyl- 1-picrylhydrazyl (DPPH•) is reduced by antioxidant/reducing compounds to pale yellow hydrazine. The reaction progress and the scavenging capacity are generally evaluated in organic media by monitoring the absorbance decrease at 515–528 nm with a spectrophotometer until the absorbance remains stable (Magalhães, Segundo, Reis & Lima, 2008). The reaction mechanism depends on an electron transfer (ET) reaction because it happens slowly in strong hydrogen-bond accepting solvents, such as methanol

and ethanol (Foti, Daquino & Geraci, 2004). The scheme for scavenging the DPPH radicals by an antioxidant RH is shown in Figure 1.8.

1.8.2 Free Radical Scavenging by the Use of the ABTS Radical

Generation of the ABTS [2, 29-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] radical cation constitutes the basis of one of the spectrophotometric methods that have been carried out to the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixtures and beverages. The pre-formed radical monocation of 2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and reaction time on the inhibition of the radical cation absorption are considered when evaluating the antioxidant activity. The method possesses maxima reaction absorption at wavelengths 645 nm, 734 nm and 815 nm (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). Because of its operational convenience, the ABTS assay has been used in many research laboratories for studying antioxidant capacity (Huang, Ou & Prior, 2005). This method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999).

1.8.2 Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP assay also benefits from electron-transfer reactions. A ferric salt, $Fe(III)(TPTZ)_2CI_3$ (TPTZ= 2,4,6-tripyridyls- triazine) (Figure 1.9) is utilized as an oxidant (Huang, Ou & Prior, 2005). The ferric reducing antioxidant power (FRAP) assay

is based on a SET (Single electron transfer) reaction and quantifies the ability of the antioxidant to reduce a ferric 2, 4, 6-tripyridyl-s-triazine salt (Fe^{3+}-TPTZ) to the blue-colored ferrous complex (Fe^{2+}- TPTZ) at low pH (Jimenez-Alvarez et.al, 2008). In the FRAP assay, excess Fe3+ is used, and the rate-limiting factor of Fe^{2+}-TPTZ, and color formation show the reducing ability of the sample (Benzie & Strain, 1996). FRAP values are estimated by measuring the absorbance increase at 593 nm of the sample and an antioxidant standard solution (ascorbic acid or trolox solution, for instance). In addition, this method is also used with 96-well microplate reader which provides preferable reproducibility and more sample throughput (Tsao, Yang, & Young, 2003). In spite of the low pH value, the ferric-reducing ability might indirectly demonstrate the antioxidant capacity. For example, Cao et al. showed a weak but significant linear correlation between serum FRAP and serum ORAC (Cao & Prior, 1998).

1.8.4 Total Phenolic Content with Folin- Ciocalteu Reducing Capacity Assay (FC).

Although the certain chemical nature of the Folin–Ciocalteu reagent is not known, it is considered that it includes phosphomolybdic/ phosphotungstic acid complexes (Singleton & Rossi, 1965). The principle of FC assay depends on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes. Therefore these complexes can be measured spectrophotometrically at 750–765nm (Magalhães, Segundo, Reis & Lima, 2008). Gallic acid is preferred as the reference standard compound and results are stated as gallic acid equivalents (GAE mg L^{-1}). The weakness of this assay is that FC reagent is not specific to phenolic compounds. It may be reduced by many non-phenolic compounds, such as

aromatic amines, sulfur dioxide, ascorbic acid, etc. Because of that reason, the FC assay was lately suggested for the measurement of total reducing capacity of samples (Huang, Ou & Prior, 2005). Some studies demonstrate that there are good linear correlations between FC assay and other ET-based assays, such as TEAC and DPPH. (Roginsky, Lissi, 2005). Even though the FC assay has disadvantages, it is reproducible, convenient, and simple. Moreover, at a long-wavelength, the product absorption reduces interferences from the sample (Magalhães, Segundo, Reis & Lima, 2008).

1.9 Significance of the Project

Different parts of Maya nut tree have been used as regional medicines for a long time and seeds of Maya nut are rich in nutritional values. The objectives of this research were to explore other potential health beneficial characteristics of Maya nut (*Brosimum alicastrum*). Thus, the specific objectives of this study were:

1) To evaluate physical properties and to compare the fatty acid composition of Maya nut with other commercial oils,
2) To determine the radical scavenging capacities using ABTS, DPPH, and FRAP assays and to uncover the total phenolic content, and
3) To separate and identify the individual bioactive compounds.

1.10 Figures and Tables

A. Full size of Maya nut tree

Figure 1.1-A was authorized to be added in this thesis (from Vohman, 2008)

B. Powder of Maya nut seeds

Figure 1.1. The appearance of *Brosimum alicastrum*

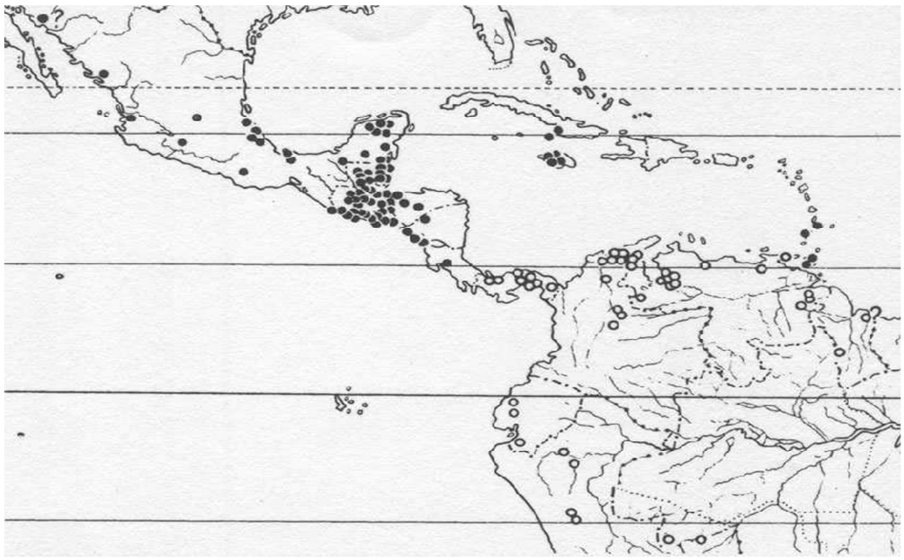


Figure 1.2 Distribution of *Brosimum alicastrum, sub species alicastrum;*

This figure was authorized to be added in this thesis (from Vohman & Sanchez Garduño, 2008)

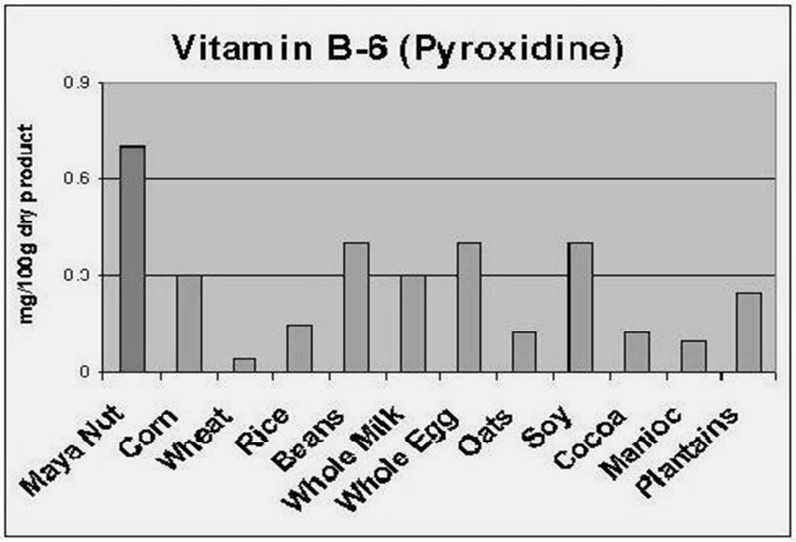


Figure 1.3 The vitamin B-6 values of Maya nut and other foods

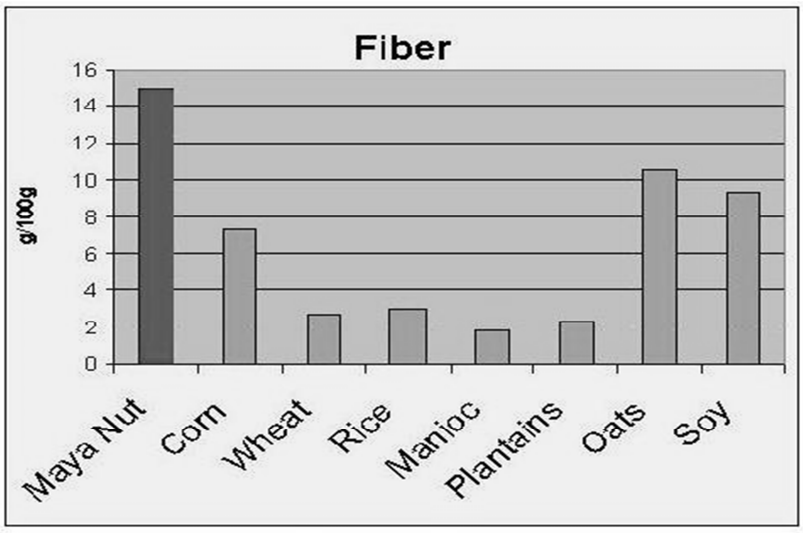


Figure 1.4 The fiber values of Maya nut and other foods

Figure 1.3 and **Figure 1.4** are authorized to be added in this thesis (from the Equilibrium Fund 2008)

Nutrient (Value per 100 grams)	Walnut (*Juglands regia*)	Almond (*Prunus dulcis*)	Peanut (*Arachis hypogaea*)
Moisture (g)	4.07	4.70	6.50
Protein (g)	15.23	21.22	25.80
Ash (g)	1.78	2.99	2.33
Total lipid (fat) (g)	65.21	49.42	49.24
Fatty acids (g)	62.233	46.69	46.82
Linoleic acid (g)	38	12	15.5
Gamma linolenic acid (g)	9.08	0.006	0
Oleic acid (g)	8.8	30.6	23.7
Palmitic acid (g)	4.4	3	5.1
Stearic acid (g)	1.6	0.658	1.1

Table 1.1 The comparison of nutrients of *Juglands regia*, *Prunus dulcis*, and *Arachis hypogaea* (USDA, 2009)

Hydroxybenzoic acids

Name	**R_1**	**R_2**	**R_3**	**R_4**
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H

Table 1.2 The frameworks of the hydroxybenzoic acids

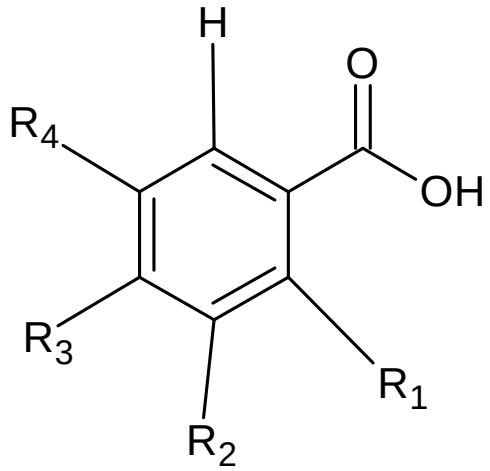


Figure 1.5 The structures of the hydroxybenzoic acids

Hydroxycinnamic acids

Name	**R_1**	**R_2**	**R_3**	**R_4**
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p- Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

Table 1.3 The frameworks of the hydroxycinnamic acids

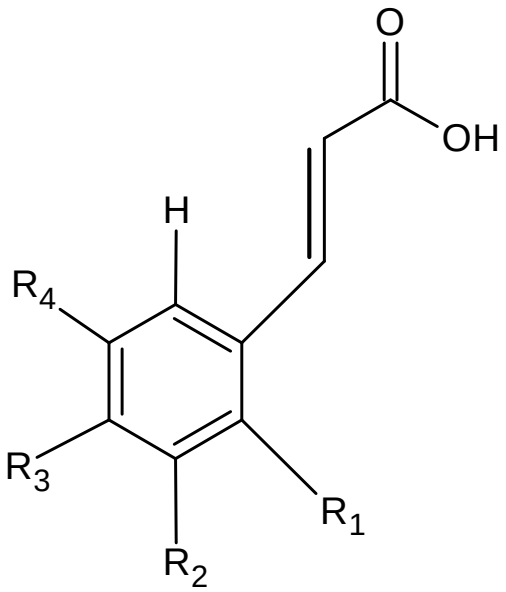


Figure 1.6 The structures of the hydroxycinnamic acids

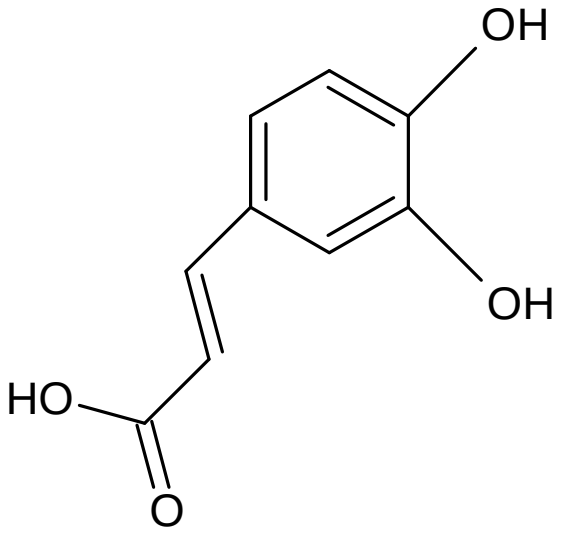
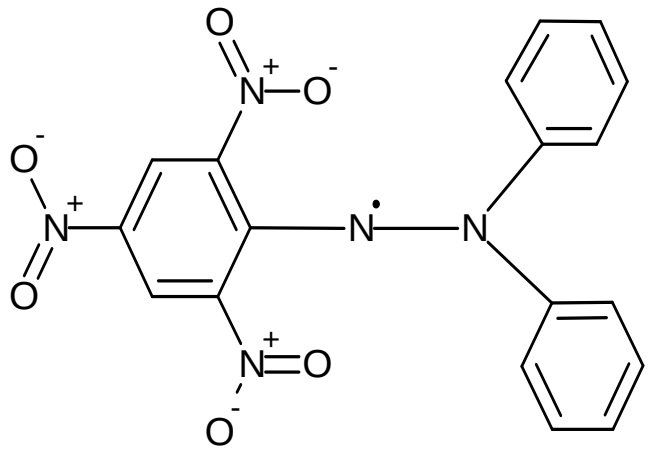


Figure 1.7 The structure of caffeic acid

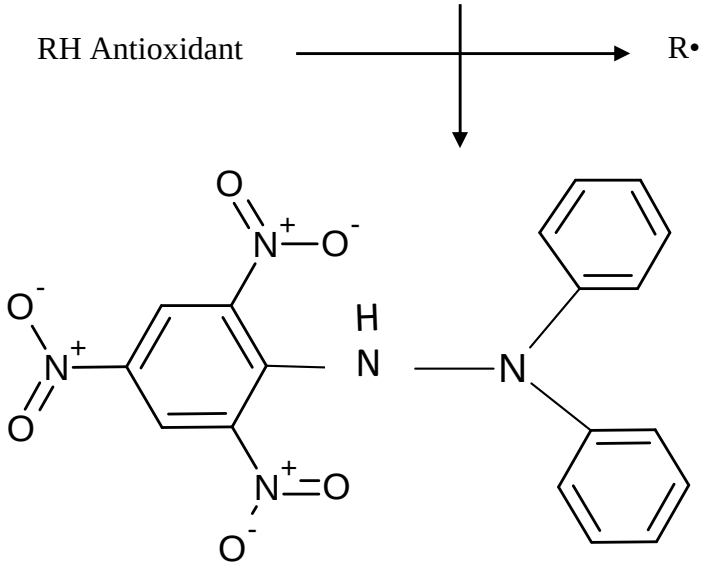
Antioxidant Capacity Assays (in vitro)

Single electron transfer-based assays	DPPH (diphenyl-1-picrylhydrazyl)
$M(n) + e \text{ (from AH)} \longrightarrow AH^{\cdot +} + M(n-1)$	FRAP (ferric ion reducing antioxidant parameter)
	TEAC(Trolox equivalent antioxidant capacity)
	Copper(II) reduction capacity
	Total phenols assay by Folin-Ciocalteu reagent
Hydrogen atom transfer-based assays	TRAP (Total radical trapping antioxidant parameter)
Reaction: $X^{\bullet} + AH \longrightarrow XH + A^{\bullet}$	Crocin bleaching assay
	Oxygen radical absorbance capacity(ORAC)
	IOU (inhibited oxygen uptake)
	Inhibition of linoleic acid oxidation
	Inhibition of LDL oxidation
Other assays	TOSC (total oxidant scavenging capacity)
	Chemiluminescence
	Electrochemiluminescence

Table 1.4 *In vitro* Antioxidant capacity measurement assays



2, 2-Diphenyl-1-picrylhydrazyl (DPPH•)



2, 2-Diphenyl-1-picrylhydrazyl (DPPH)

Figure 1.8 The scheme for scavenging the DPPH radicals by an antioxidant RH

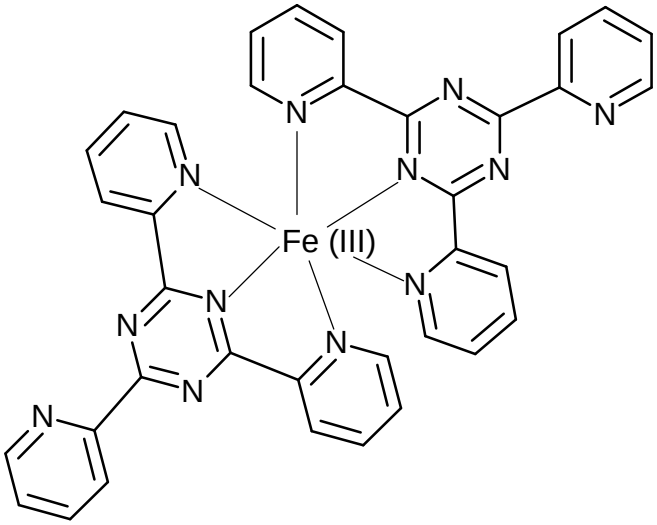


Figure 1.9 The structure of [Fe (III) (TPTZ) $_2$]$^{3+}$

1.11 References:

Alasalvar, C., & Shahidi, F. (2008). Tree nuts: composition, phytochemicals, and health effects. pp (226). Boca Raton, Florida: CRC Press.

Amaral, J.S., Casal, S., Pereira, J.A., Seabra, R.M., & Oliveira, B.P.P. (2003). Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (*Juglans regia* L.) cultivars grown in Portugal. Journal of Agricultural and Food Chemistry, 51, 7698- 7702.

Antalovich, M., Prenzler, P., Robards, K., & Ryan, D. (2000). Sample preparation in the determination of phenolic compounds in fruits. Analyst, 125, 989- 1009.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S.E., Bektaşoğlu, B., & Berker, K.I., & Özyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC Assay. Molecules, *12*, 1496-1547.

Barreira, J.C.M., Ferreira, I.C.F.R.M., Oliveira, M.B.P.P., & Pereira, J.A., (2008). Antioxidant activities of the extracts from chestnut flower, leaf, skins and fruit. Food Chemistry, 107(3), 1106-1113.

Benzie, I.F.F., & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of Antioxidant Power: The FRAP Assay. Analytical Biochemistry, 239, 70-79.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Reviews, 56(11), 317-333.

Cao, G., & Prior, R.L. (1998). Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clinical Chemistry, 44 (6), 1309-1315. Christie, W.W. (1998). Gas chromatography–Mass spectrometry methods for structural analysis of fatty acids. Lipids, 33, 343-353.

Crozier, A., & Jaganath, I.B., & Clifford, M.N. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. Natural Products Reports, 26, 1001-1043.

Dong, M., He, X., & Liu, R.H. (2007). Phytochemicals of black bean seed coats: isolation, structure elucidation, and their antiproliferative and antioxidative activities. Journal of Agricultural and Food Chemistry, 55, 6044- 6051.

Dudonné S., Vitrac X., Coutiére P., Woillez M., & Mérillon J.M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. Journal of Agricultural and Food Chemistry, 57, 1768-1774.

The Equilibrium Fund. [Cited 2009 July 06].Maya nut Program. [Internet]. Available from http://www.theequilibriumfund.org/.

Escarpa, A., & González, M. C. (2001). Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. Analytica Chimica Acta, 427, 119-127.

Frankel E. N., & Finley J. W. 2008. How to standardize the multiplicity of methods to evaluate natural antioxidants. Journal of Agricultural and Food Chemistry, 56(13), 4901-4908.

Foti, M.C., Daquino, C., & Geraci, C. (2004). Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH• radical in alcoholic solutions. Journal of Organic Chemistry, 69, 2309-2314.

Gilbert-López, B., García-Reyes, J.F., & Molina-Díaz, A. (2009). Sample treatment and determination of pesticide residues in fatty vegetable matrices: A review. Talanta 79, 109–128.

Global Facilitation Unit (GFU) for Underutilized Species. 2007 [Cited 2009 July 06]. Maya nut (Brosimum alicastrum). [Internet]. Available from: http://www.underutilized-species.org/species/brochures/Maya%20Nut.pdf.

Gragg, G.M., Kingston, D.G.I., & Newman, D.J. (2005). Introduction. In Gragg, G.M., Kingston, D.G.I., Newman, D.J. Anticancer agents from natural products (pp.1-3). Boca Ration: CRC Press.

Guillén, D.A., Barroso, C.G., & Perez-Bustamante, J.A. (1996a). Selection column and gradient for the separation of polyphenols in sherry wine by high-performance liquid chromatography incorporating internal standards. Journal of Chromatography A, 724, 117-124.

Guillén, D.A., Barroso, C.G., & Perez-Bustamante, J.A. (1996b). Automation of sample preparation as a preliminary stage in the high performance liquid chromatographic determination of polyphenolic compounds in sherry wines. Journal of Chromatography A, 730, 39-46.

Huang, D., Ou, B., & Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry, 53, 1841- 1856.

Huie, C.W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. Analytical and Bioanalytical Chemistry, 373, 23-30.

Jimenez-Alvarez, D., Giuffrida, F., Vanrobaeys, F., Golay, P.A., Cotting, C., Lardeu, A., & Keely, B.J. (2008). High-throughput methods to assess lipophilic and hydrophilic antioxidant capacity of food extracts in *vitro*. Journal of Agricultural and Food Chemistry, 56, 3470–3477.

Kenkel, J. (1994). Analytical chemistry for technicians. pp (324). Boca Raton, Florida: CRC Press.

Khanna, S.K., Viswanathan, P.N., Krishnan, P.S., & Sanwal, G.G. (1968). Extraction of total phenolics in the presence of reducing agents. Phytochemistry, 7(9), 1513-1517.

Krygier, K., Sosulski, F., & Hogge, L. (1982). Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. Journal of Agricultural and Food Chemistry, 30, 330-334.

Kwon, K.H., Barve, A., Yu, S., Huang, M.T., & Kong, A.T. (2007). Cancer chemoprevention by phytochemicals: potential molecular targets, biomarkers and animal models. Acta Pharmacologica Sinica, 28 (9): 1409–1421.

Labuckas, D. O., Maestri, D.M., Perelló, M., Martínez, M.L., & Lamarque, A.L. (2008). Phenolics from walnut (*Juglans regia L.*) kernels: Antioxidant activity and interactions with proteins. Food Chemistry, 107, 607-612.

Lee, K.W., & Lee, H.J. (2006). The roles of polyphenols in cancer chemoprevention. Biofactors, 26, 105-121.

Liu, R.H. (2004). Potential synergy of phytochemicals in cancer prevention: Mechanism of action. The Journal of Nutrition, 134 (12), 3479- 3485.

Magalhães L.M., Segundo, M.A., Reis, S., & Lima, J.L.F.C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. Analytica Chimica Acta, 613, 1-19.

Manson, M.M., Foreman, B.E., Howells, L.M., & Moiseeva, E.P. (2007). Determining the efficacy of dietary phytochemicals in cancer prevention. Biochemical Society Transactions, 35, 1358-1363.

Maxson, E.D., & Rooney, L.W. (1972). Evaluation of methods for tannin analysis in sorghum grain. Cereal Chemistry, 49, 719 – 728.

Montedoro, G., Servili, M., Baldioli, M., & Miniati, E. (1992). Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. Journal of Agricultural and Food Chemistry, 40, 1571-1576.

Naczk, M., & Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. Journal of Pharmaceutical and Biomedical Analysis, 41, 1523- 1542.

Ono Y., Yamagami T., Nishina T., &Tobino T. (2006). Pesticide multiresidue analysis of 303 compounds using supercritical fluid extraction. Analytical Sciences, 22 (11), 1473.

Pederson-Bjergaard, S., Rasmussen, K.E., & Halvorsen, T.G. (2000). Liquid–liquid extraction procedures for sample enrichment in capillary zone electrophoresis. Journal of Chromatography A, 902, 91–105.

Peters, C.M. (1989). Reproduction, growth and the population dynamics of *Brosimum alicastrum* in a moist tropical forest of Central Veracruz, Mexico. PhD Dissertation. School of Forestry and Environmental Studies, Yale Univ., New Heaven, Connecticut, U.S.A.

Peters, C.M. (1983). Observations on Maya subsistence and the ecology of a tropical tree. The American Antiquities Journal, 48, 610-614.

Price, M.L., & Butler, L.G. (1977). Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. Journal of Agricultural and Food Chemistry, 25(6), 1268-1273.

Prior, R.L., Wu, X., & Schaich K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry, 53, 4290-4302.

Puleston, D.E. (1968). Brosimum alicastrum as a subsistence alternative for the classic maya of the central southern lowlands. M.A. Thesis. Univ. Pennsylvania, Philadelphia.

Puleston, D.E. (1972). Ancient Maya settlement patterns and environment at Tikal, Guatemala: implications for subsistence models. Ph.D. Dissertation, Univ. of Pennsylvania, Philadelphia.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine, 26, (9-10), 1231–1237.

Rijke, E., Out, P., Niessen, W.M.A., Ariese, F., Gooijer, J. & Brinkman, U.A.T. (2006). Analytical separation and detection methods for flavonoids. Journal of Chromatography A, 1112, 31–63.

Robbins, R.J. (2003). Phenolic acids in foods: An overview of analytical methodology. Journal of Agricultural and Food Chemistry, 51, 2866-2887.

Roginsky, V., Lissi, E.A. (2005). Review of methods to determine chain-breaking antioxidant activity in food. Food Chemistry, 92, 235-254.

Rommel, A., & Wrolstad, R.E. (1993). Influence of acid and base hydrolysis on the phenolic composition of red raspberry juice. Journal of Agricultural and Food Chemistry, 41, 1237- 1241.

Serafini, M., Villano, D., Spera, G., & Pellegrini, N. (2006). Redox molecules and cancer prevention: The importance of understanding the role of the antioxidant network. Nutrition and Cancer, 56(2), 232-240.

Shantha, N.C., & Napolitano, G.E. (1992). Gas chromatography of fatty acids. Journal of Chromatography A, 624(1-2), 37-51.

Silvestre, C.I.C., Santos, J.L.M., Lima, J.L.F.C., & Zagatto, E.A.G. (2009). Liquid–liquid extraction in flow analysis: A critical review. Analytica Chimica Acta, 652, 54-65.

Singleton, V.L., & Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16,144.

Smolarz, H. D. (2001). Application of GC-MS method for analysis of phenolic acids and their esters in chloroformic extracts from some taxons of Polygonum L. genus. Chemia Analityczna, 46, 439-444.

Spitzer, V. (1997). Structure analysis of fatty acids by gas chromatography- Low resolution electron impact mass spectrometry of their 4, 4-Dimethyloxazoline derivatives- A Review. Progress in Lipid Research, 35(4), 387-408.

Stalikas, C.D. (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. Journal of Seperation Science, 30, 3268-3295.

Sun, J., Chu, Y.F., Wu, X., & Liu, R.H. (2002). Antioxidant and antiproliferative activities of common fruits. Journal of Agricultural and Food Chemistry, 50, 7449-7454.

Sun, R.C., Sun, X.F., & Zhang, S.H. (2001). Quantitative determination of hydroxycinnamic acids in wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood. Journal of Agricultural and Food Chemistry, 49, 5122- 5129.

Tsao, R., Yang, R., & Young, C. (2003). Antioxidant isoflavones in osage orange, maclura pomifera (Raf.) Schneid. Journal of Agricultural and Food Chemistry, 51, 6445-6451.

USDA. 2009 [cited 2010 April 19]. USDA National nutrient database for standard reference, Release 22, Nuts, almonds; walnuts, english; and peanuts, all types, raw [Internet]. Available from: http://www.nal.usda.gov/fnic/foodcomp/cgi-bin/list_nut_edit.pl.

Uttara, B., Singh, A. V., Zamboni, P., & Mahajan, R.T. (2009). Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. Current Neuropharmacology, 7, 65-74.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interactions, 160, 1-40.

Vohman E. Traditional rainforest food for healthy forests and families. International symposium of underutilized plants for food, nutrition, income and sustainable development; 2008 March 3-8; Arusha, Tanzania. Mexico: The Equilibrium Fund.

Vohman E.C., & Sanchez-Garduño C. (2008). Maya Nut (*Brosimum alicastrum*): Traditional rainforest food for healthy forests and families in Central America. International symposium "Underutilized plants for food, nutrition, income and sustainable development".

Wetter, W., & Thurnhofer, S. (2005). A Gas chromatography/electron ionization-Mass spectrometry- selected ion monitoring method for determining the fatty acid pattern in food after formation of fatty acid methyl esters. Journal of Agricultural and Food Chemistry, 53, 8896- 8903.

Zadernowski, R., Naczk, M., & Nesterowicz, J. (2005). Phenolic acid profiles in some small berries. Journal of Agricultural and Food Chemistry, 53, 2118- 2124.

Zhang, Z., Liao, L., Moore, J., Wu, T., & Wang, Z. (2009). Antioxidant phenolic compound from walnut kernels (*Juglans regia L.).* Food Chemistry, 113, 160-165.

CHAPTER 2

ANTIOXIDANT POTENTIAL AND NUTRITIONAL VALUES OF MAYA NUT, WALNUT, PEANUT AND ALMOND

Abstract:

Different parts of the Maya tree (*Brosimum alicastrum*) have been used for different applications, including forage, construction, and medicine. Recent studies have also shown that the seeds of Maya nut possess various nutritional values. In this study, Maya nut oil was compared with other two commercially edible oils: olive oil and canola oil, in terms of their fatty acid composition and quantity. In addition, proximate analyses, antioxidant capacities, and total phenolic content of the Maya nut and other commercially available nuts including walnut, almond, and peanut were measured and compared. Antioxidant capacities of Maya nut, walnut, almond, and peanut were evaluated by three different *in vitro* antioxidative assays (i.e., DPPH* free radical scavenging assay, ferric reducing antioxidant potential (FRAP) assay, and ABTS free radical scavenging assay). The Maya nut seed (dry) powder contain 1.31% oil that is composed of 27.20% palmitic acid (C16:0), 6.26% stearic acid (C18:0), 9.90% linolenic acid (C18:3 n3), 55.06% linoleic acid (C18:2 n6), and 3.36% Cis-11-Eicosenoic (C20:1). It has a higher content of linoleic and linolenic acid (PUFAs), but lower content of oleic acid than canola oil and olive oil. The TPC of Maya nut was 24.67 mg of gallic acid equivalents per gram of sample, which were 3, 6, and 28 times higher than that of walnut, peanut, almond. The FRAP value of Maya nut was 8.08 mmol Fe^{2+}/100 g sample, which is lower than that of walnut but higher than that of peanut and almond. Maya nut exhibited a stronger DPPH

(83.35%) free radical scavenging activity than walnut, peanut, and almond at concentration of 1 mg/mL. The level of DPPH scavenging activity of Maya nut was higher than that of BHT (76.46%) at 1000 ppm concentration. Furthermore, Maya nut showed powerful $ABTS^{\cdot+}$ radical quenching activity than walnut, peanut, and almond. The Maya nut extract possessed the highest antioxidant capacity (92.55% of ABTS inhibition) followed by the walnut extract (92.17%), peanut extract (91.12%), and almond extract (58.96%), respectively.

2.1 Introduction

Maya tree (*Brosimum alicastrum*) is widely distributed in Mexico and its leaves are usually used for forage, and/or as medicines. Its nut seed is immensely high in fiber, calcium, potassium, folate, iron, zinc, proteins and vitamins A, B, C and E (Vohman & Sanchez-Garduño, 2008), and has been used as an alternative food by the ancient Mayas, who traditionally either ate the raw nut seeds or made them into juice or marmalades (Roys, 1931). Though some studies on nutritional values of the Maya nut were conducted, its fatty acid profile and antioxidant potential have not been studied yet.

Fatty acid composition may affect lipid oxidation and final quality of food, as well as human health. For example, omega-6 and omega-3 polyunsaturated fatty acids (PUFA) are implicated to have anti-inflammatory effects in the body, and are used in the nutritional dietary supplements for treatments of arthritis, asthma, allergies, and skin conditions (Amaral, Casal, Pereira, Seabra & Oliveira, 2003).

Since oxygen-derived free radicals were found to be closely relevant to various diseases such as cancer, inflammation, aging, Alzheimer's disease, cardiovascular diseases, diabetes. etc (Yeh & Yen, 2003; Yu, Perret, Harris, & Haley, 2003), antioxidants, particularly natural antioxidants, which can quench the free radicals and cease their propagation to prevent the biological damages, have attracted much attention (Parry et. al., 2006; Parry, Cheng, Moore, & Yu 2008). Although the development of free radicals is a natural procession, when defected, free radicals may become biologically dangerous. The steps of the chain reaction responsible for the actions of free radicals include initiation, propagation, and termination steps. The final step, termination, is

considered the cause of free radical onset of cancer because this step might bring about DNA damage, mutations and other hazardous effects. For instance, their involvement in the onset and development of colorectal cancer is widely accepted.

In addition, many foods are subject to different factors for the quality deterioration, e.g., lipid autoxidation. Consequently, natural and/or synthetic antioxidants are frequently added as food additives to secure food quality in terms of flavor, color, texture and nutritional values, as well as the food shelf life. Though some synthetic antioxidants such as butyl hydroxyanisole (BHA) and butyl hydroxytoluene (BHT) are very effective, their use is limited in commercial applications because they might be hazardous to human health (Ito, Fukushima, Tsuda, Shirai, Hagiwara, & Imaida, 1985). Therefore, it is crucial to discover some other naturally occurring non- or less-toxic antioxidants which could be used to preclude food oxidative deterioration. Natural antioxidants also have been used as nutraceutical or cosmetic ingredients since they constitute multiple functions. In this study, we analyzed the antioxidant capacity of Maya nut using three complimentary *in vitro* assays: 2, 2-diphenyl-1-picrylhydrazyl (DPPH*) free radical scavenging assay, ferric reducing antioxidant potential (FRAP) assay, and ABTS free radical scavenging assay. The antioxidant activity, total phenolic content, and proximate values of Maya nut, were also compared with those of the commercial nuts and standard antioxidant, butylated hydroxytoluene (BHT). Moreover, the fatty acid composition of Maya nut was compared with that of the olive oil and canola oil which are both considered health benefiting oils and used commonly in the United States.

The goals of this study were: (i) to evaluate antioxidant capacity and total phenolic content of Maya nut and to compare these values with the commercial nuts including walnut, peanut, and almond; and (ii) to determine proximate analyses and fatty acid composition of Maya nut and compare these values with olive oil and canola oil.

2.2 Materials and Methods:

2.2.1 Materials and Chemicals

The Maya nut (*Brosimum alicastrum*) sample was obtained from The Equilibrium Fund (TheEquilibriumFund.org, Colorado, CO, USA). Unsalted dry roasted peanuts (Wal-Mart Stores, Inc, Bentonville, AR, USA), pecans, walnuts, almonds (Hines Nut Company, Dallas, Texas, USA), extra virgin olive oil (Bertolli Company, New Jersey, USA) and canola oil (Pure Wesson, ConAgra Foods Company, Memphis, Tennessee, USA) were purchased from a local market in Clemson, SC, USA. Fatty acid methyl ester (FAME) standards were purchased from Supelco Co. (Bellefonte, PA, USA). Heptadecanoic acid was obtained from MP Biomedicals (Ohio, OH, USA). Sodium methoxide was purchased from Acros Organics (New Jersey, NJ, USA). Potassium carbonate was from the Alfa Aesar, 99% min (Massachusetts, MA, USA). The Folin-Ciocalteu reagent, gallic acid, 2, 4, 6-tri (2-pyridyl)-s-triazine (TPTZ), 2, 2-diphenyl-1-picryl-hydrazyl radical (DPPH) and ferric chloride were purchased from Sigma-Aldrich Co, MO, USA. Sodium carbonate and sodium acetate were from Fisher Scientific. Ferrous sulfate was from J.T. Baker Chemical Company. Hydrochloric acid and glacial

acetic acid were obtained from EM Science. HPLC grade and laboratory grade solvents were purchased from Fisher Scientific (Suwanee, GA, USA)

2.2.2 Preparation of Defatted Samples:

The oil extraction method was modified from previously reported method (Shahidi, Alasalvar & Liyana-Pathirana, 2007). All samples were ground separately in a spice and nut grinder (Model SG 10C, Blank and Cuisinart Company, Stamford, CT) for 5 min and then defatted by sonication with hexane (1:5, w/v, 3×10 min) with the Branson sonicator (Model 5510, Bransonic Tabletop Ultrasonic Cleaners, Branson Ultrasonics Corporation, Danbury, CT) at an ambient temperature. The samples were centrifuged at 3024*g* (Model Eppendorf Centrifuge 5810 R, Eppendorf North America Inc., Westbury, NY) for 10 min before the upper layer was removed. Defatted samples were subsequently air-dried for 1 hour for further analysis.

2.2.3 Extraction of Crude Phenolics:

The preparation of antioxidant extracts was modified from the method reported by Siriwardhana and Shahidi (2002). Phenolic compounds present in the defatted samples were extracted by 80:20 (v/v) ethanol/water mixture (6 g of sample /100 mL of solvent) at 80°C for 30 min by a Soxhlet extractor. The resulting slurries in the flask were collected. The residue was re-extracted twice under the same condition, the remaining slurries were collected. The solvent in the slurries was removed by a rotary evaporator at 40 °C (Model R-200; Buchi, Switzerland). The prepared crude extracts were re-dissolved in methanol, and filtrated by Whatman filter paper (No. 5). The final concentration was

expressed as mg of sample equivalent per mL of methanol. They were stored at -20 °C in the dark until they were used.

2.2.4 Determination of Total Phenolic Content

Content of phenolics in the extracts was determined according to a modified procedure described by Singleton and Rossi (Singleton & Rossi, 1965) using the Folin-Ciocalteu phenol reagent. The Folin-Ciocalteu phenol reagent (1:10 diluted with distilled water) (1 mL) was added to each centrifuge tube containing 200 µL of the extract, and 800 µL saturated sodium carbonate (75.05 g/L). The tubes were thoroughly mixed by vortexing before the tubes were allowed to stand at ambient temperature for 2 hours until the characteristic blue color developed. Absorbance of the clear supernatants was measured at 750 nm using a UV spectrophotometer (Genesys 20 Model 4001/4; ThermoSpectronic, Rochester, NY, USA). The blank devoid of any extract was used for background subtraction. The content of total phenolics in each extract was determined from a series of standard solutions in concentrations of 25, 50, 100, 200, and 400 µg/mL of gallic acid as a standard and expressed as µg gallic acid equivalents (GAE) per gram of extract.

2.2.5 Determination of DPPH Radical Scavenging Activity and Antioxidant Capacity

The method described by Kitts et al. (Kitts, Wijewickreme & Hu, 2000) was used with some modifications to evaluate the DPPH radical scavenging activity of the extracts. A volume of 0.5 mL of antioxidant extract solution (1, 2, 3, 4, 5 mg/mL in methanol) was mixed with 0.5 mL of 0.125 mM DPPH solution dissolved in methanol. Methanol was

used as a blank solution. The absorbance of the reaction mixture was measured at 515 nm after 1 hour and compared with the standard antioxidant BHT. Lower absorbance of reaction mixture demonstrated higher free radical scavenging activity. Finally, the absorbance of the resulting solution was read spectrophotometrically at 515 nm. Results were expressed as the content of extract (mg per assay) versus absorbance at 515 nm. The scavenging of DPPH radical of the extracts was calculated according to the equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{Control} - A_{sample}}{A_{Control}} \times 100$$

2.2.6 Ferric Reducing Antioxidant Potential (FRAP) Assay

The ferric reducing power of extracts was determined by a modified version of the FRAP assay described by Benzie and Strain (Benzie and Strain, 1996). This method was based on the reduction, at low pH, of a colorless ferric complex (Fe^{3+}-tripyridyltriazine) to a blue colored ferrous complex (Fe^{2+}-tripyridyltriazine) by the action of electron-donating antioxidants. The reduction is carried out by measuring the change of absorbance at 593 nm.

The working FRAP reagent was freshly prepared daily by mixing 10 volumes of 300 mM acetate buffer at pH 3.6, with 1 volume of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-*s*-triazine) in 40 mM hydrochloric acid and with 1 volume of 20 mM ferric chloride. A standard curve was prepared using various concentrations (i.e., 0.5, 1, 2, 4, 5 mM) of $FeSO_4 \cdot 7H_2O$. All solutions were used on the day of preparation. One hundred microliters of sample solutions and 300 μL of deionized water were added to 3 mL of freshly prepared FRAP reagent. The reaction mixture was incubated at 37 ºC for 30 min in a

water bath. Then, the absorbance of the samples was measured at 593 nm. A sample blank reading using acetate buffer was also taken. The difference between sample absorbance and blank absorbance was calculated and used to calculate the FRAP value. In this assay, the reducing capacity of the extracts tested was calculated with reference to the reaction signal given by a Fe^{2+} solution. FRAP values were expressed as mmol Fe^{2+}/g of sample. All measurements were done in triplicate.

2.2.7 Free Radical Scavenging by the Use of the ABTS Radical

The free radical scavenging capacity of extracts was also studied using the ABTS radical cation decolorization assay (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999), which is based on the reduction of $ABTS^{+\bullet}$ radicals by antioxidants of the plant extracts tested. ABTS was dissolved in deionized water to a 7 mM concentration. ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting ABTS solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. For the study, the $ABTS^{+\bullet}$ solution was diluted in deionized water to an absorbance of 0.7 (±0.02) at 734 nm. An appropriate solvent blank reading was taken (*A*B). After the addition of 100 μL of aqueous extract solutions to 3 mL of $ABTS^{+\bullet}$ solution, the absorbance reading was taken at 30ºC for 20 min after initial mixing (*A*E). All solutions were used in the same day of preparation, and all determinations were carried out in triplicate.

The percentage of inhibition of $ABTS^{+\bullet}$ was calculated using the following formula. % inhibition = $[A_B - A_E / A_B] \times 100$

where A_B = absorbance of the blank sample, and A_E = absorbance of the extract

2.2.8 Proximate Analyses

Moisture content was determined by placing 2 grams of ground Maya nut in disposable aluminum pans. The samples were then placed in a Thelco Laboratory Oven (Model 130DM by Precision Scientific Inc., Maharashtra, India) set to100 ºC for 5 hours. The samples were then immediately re-weighed. The samples were then held in a desiccator prior to fat analysis.

The samples were placed in porcelain crucibles and put into a Thermolyne Type 6000 programmable furnace to determine ash content. Furnace temperature was started at room temperature and increased 5ºC per minute until 250 ºC was reached. The temperature then was increased 10ºC per minute until 525ºC was reached. The temperature was held at 525 ºC for 5 hours. After cooling, samples were weighed.

The dried samples from the moisture analysis were put into dried thimbles, stopperred with cotton, and held in a Soxhlet unit for 16 hours. Hexane was used as the extraction solvent. After extraction, the thimbles were dried in the oven for overnight at 100 ºC. The thimbles were then weighed to determine the fat content.

Maya nut was analyzed for protein content using slightly modified bicinchoninic acid assay (BCA assay) method described by Smith et al (Smith et al, 1985). The BCA reagent was prepared by combining 50 mL of reagent A containing sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1 N sodium hydroxide with 1 mL of reagent B consisting of 4% copper sulfate pentahydrate solution. Five mg of maya nut samples (ground in a mill) were weighed into the centrifuge tubes and suspended in 1.0 mL of the BCA reagent. The samples were then incubated at 37 °C

for 30 min in a water bath with vortex mixing in every 10 minutes. The samples were then cooled in an ice bath for 1 min and centrifuged at 3000*g* for 10 min at room temperature. An aliquot of 0.2 mL supernatant was removed and diluted with 0.8 mL of BCA reagent. The solution was thoroughly mixed by vortex and its absorbance was measured at 562 nm. A standard curve was constructed using a series of standard bovine serum albumin solutions in concentrations of 50, 100, 200, 400 µg/mL. Protein content was expressed as mg protein/mL.

2.2.9 Preparation of Fatty Acid Methyl Esters (FAMEs)

The preparation of fatty acid methyl esters (FAMEs) was modified from the procedure described by Surh, Ryu & Kwon (Surh, Ryu & Kwon, 2003). Sixty milligrams of the sample were put into a screw capped culture tube with 1 mL internal standard of heptadecanoic acid (2mg/mL in hexane) as an internal standard. Then, 2 mL of 0.5 N sodium methoxide was slowly added during stirring. The sample tubes were tightly closed with Teflon-lined caps, placed in a water bath at 50°C for 10 min and cooled down within 5 minutes. Then 3 mL of 5% methanolic HCI was added and the mixture was incubated in water bath at 80 °C for another 10 minutes and cooled down for 5 minutes. Eventually, 1 mL hexane and 7.5 mL of 6% aqueous K_2CO_3 was slowly added to stop the reaction and neutralize the mixture. After centrifugation at 1200 rpm for 5 minutes, an aliquot of hexane upper phase was transferred into a vial and subjected to gas chromatography. All of these reactions were performed in triplicate for the extract.

2.2.10 Analysis of Methyl Esters with GC

The fatty acid composition of Maya nut oil was evaluated from the FAMEs by GC-FID (Model GC-17A, Shimadzu Scientific Instruments Inc., MD, and USA). The results were expressed as % individual fatty acid of total fatty acids. A fused silica capillary column DB-Wax was used (I.D. 0.25 mm, length 60 m, thickness 0.25 µm, J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas in a 1:15 split mode at a flow rate of 1.2 mL/min. Injection volume was 5 µL. Column was operated at 70 °C for 4 min, then temperature-programmed at 13 °C/min to 175 °C, held there for 27 min, programmed at 4 °C/min to 215 °C, and finally held there for 31 min; Total run was 80 min. It is worthy of note that the start-up temperature at 70 °C allowed the reasonable resolution of the short-chain FAME, while maintaining the temperature at 215 °C for a longer period of time permitted appearance of the very long-chain FAME. Fatty acids were identified by comparing the retention time of the peaks in the sample with those of the standard mixtures. Determination of the retention time and area of peaks were carried out by the software "Shimadzu ClassVP 7.0" (Shimadzu Scientific Instruments Inc., MD, USA).

2.2.11 Statistical Analyses

Data were reported as the mean ± standard deviation. All statistical analysis was performed on the SAS V9.2 software for Windows (SAS Institute Inc., Cary, NC, USA). Differences among sample means were determined by analysis of variance (one-way analysis of variance, ANOVA) at $p<0.05$.

2.3 Results and Discussion

This is the first research about Maya nut and there is no proximate analysis values provided by The USDA National Nutrient Database. In this research, Maya nut was compared with different nuts such as walnut, almond, and peanut. The proximate compositions of walnut, almond, peanut, and Maya nut are summarized in Table 2.1.

Moisture content of the Maya nut powder was 5.53%, which had a low moisture value compared with other nuts. Low moisture content is critical for keeping quality and shelf life of seeds as low moisture (low *A*w) reduces the probability of microbial growth, unwarranted fermentation, premature seed germination, and many undesirable biochemical changes normally associated with these processes. When expressed on a dry weight basis, the lipid content of the samples ranges from 1.31 % for Maya nut to 64.50 % for walnut. Maya nut has the lowest lipid content among the nuts. When expressed on a dry weight basis, the values for ash content are almost close to each other. However, Maya nut has the highest ash content as compared to 1.60- 2.48% ash in peanut, walnut, and almond (Table 2.1). As we know, ash is the remaining inorganic residue after the water and organic matter have been removed by heating in the presence of oxidizing agents. Ash content represents a measure of the total amount of minerals present within a food (Nielsen, 2003). Among the nuts, Maya nut has the largest amount of inorganic components such as Ca, Na, K and Cl. When expressed on a dry weight basis, the value for protein is 62.6 g/100g. Maya nut has a higher protein content compared with walnut (15.23 g/100g), almond (21.22g/100g), and peanut (23.68g/100g).

Fatty acids are recognized as essential nutrients in both human and animal diets, and are implicated to possess numerous health benefits. Their use in the pharmaceutical and food industries has also been well documented. Saturated fatty acids are considered to contribute to cardiovascular diseases, whereas the unsaturated fatty acids are shown to help cellular function and promote a healthy heart. The essential fatty acids, omega-6 and omega-3 polyunsaturated fatty acids (PUFA), are also implicated to have anti-inflammatory effects in the body, and are used in the nutritional treatment of arthritis, asthma, allergies, and skin conditions (Amaral, Casal, Pereira, Seabra & Oliveira, 2003). These essential fatty acids are considered to lower the incidence of immune system disorders such as cancer, multiple sclerosis, and lupus (Henry, Momin, Nair & Dewitt, 2002).

In this research, fatty acid composition of olive oil, canola oil and Maya nut was compared. Edible vegetable oils such as olive oil and canola oil contain high amounts of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) which are considered to promote hearth health, to lower LDL cholesterol. Olive oil is attributed to a high proportion of monounsaturated fatty acids (MUFAs), namely oleic acid, which represents 70–80% of the fatty acids present in virgin olive oil (Cicerale, Conlan, Sinclar & Keast, 2009). The fatty acid profiles of Maya nut, olive oil, and canola oil were determined by GC-FID, and the results are shown in Table 2.2. The peaks of major fatty acids of Maya nut and the peaks of standard fatty acids mixture were shown with respect to their retention times in the GC-FID chromatograms (Figure 2.1 and 2.3). In addition, As the result shown in Figure 2.2, the major fatty acid of olive oil and canola oil is oleic

acid (C18:1), a monosaturated fatty acid (MUFA), but the major fatty acid of Maya nut is linoleic acid (C18:2 n6), a polyunsaturated fatty acid (PUFA). In our study, the mean values of the oleic acid (C18:1) in olive oil and canola oil were 67.40 and 64.2 (% FAME), respectively, while the mean value of the linoleic acid (C18:2 n6) in Maya nut was 55.06 (% FAME). All samples contained predominantly monounsaturated fatty acids (MUFAs) (e.g. 67.40% for olive oil) or polyunsaturated fatty acids (PUFAs) (e.g. 3.36% for Maya nut). It was reported that the oleic acid (C18:1) content of the olive oil ranged from 62.66 to 76.84 % in European olive cultivars (Dabbou, Rjiba, Nakbi, Gazzah, Issaoui, Hammami, 2010) and ranged from 64.73 to 77.04 % in Spanish olive cultivars (Allohuche, Jiménez, Gaforio, Uceda & Beltran, 2007). It was noted that and the oleic acid content of canola oil ranged from 58.0 to 80.5 % (Gunstone, Harwood & Dijsktra, 2004).

In our study, olive oil and canola oil have similar amounts of oleic acid, but surprisingly, there was no oleic acid detected in the Maya nut. However, depending on the sample, there was a significant variation in total PUFAs. Linoleic acid (C18:2) and linolenic acid (C18:3) were the predominant contributors toward the makeup of the PUFAs. Linoleic acid (LA) and alpha linolenic acid (ALA) belong to the n-6 (omega-6) and n-3 (omega-3) series of polyunsaturated fatty acids (PUFA), respectively. They are defined as the "essential" fatty acids since they cannot be synthesized in the human body and are mostly obtained from the diet (Russo, 2009). In the current study we found Maya nut containing 55.06 % linoleic acid, whereas olive oil and canola oil have 11.84% and 22.52%, respectively. Wijendran and Hayes reported that the linoleic acid **(**LA) was the

major dietary fatty acid regulating low-density lipoprotein (LDL)-C metabolism by downregulating LDL-C production and protect against coronary heart disease (Wijendran and Hayes, 2004). In addition, we found that Maya nut had a higher amount of linolenic acid than olive oil and canola oil. The mean values of linolenic acid of the Maya nut, canola oil and olive oil were 9.9, 7.38, and 1.26 (% FAME), respectively.

Results from the present study revealed that Maya nut had higher PUFAs than olive oil and canola oil. Both n-6 (linoleic acid, C18:2 n6) and n-3 (linolenic acid, C18:3 n3) polyunsaturated fatty acids have been associated with lower cardiovascular risk. Within the n-6 series, linoleic acid seems to decrease cardiovascular risk. A high intake of long-chain n-3 fatty acids has consistently been associated with a lower incidence of CHD in prospective cohort studies. The shorter chain n-3 fatty acid, such as the a-linolenic acid, has been considered to have anti-arrhythmic effect in experimental models, and seem to decrease primarily fatal coronary events (Erkkila, Mello, Risérus & Laaksonen, 2008). Therefore, presence of the linolenic acid (C18:3 n3) and other highly unsaturated fatty acids in the Maya nut provide potentials of lowing free radical production in human beings. The highly unsaturated fatty acid composition of Maya nut makes it well-suited for improving nutritional benefits of foods (Savoini, Agazzi, Invernizzi, Cattaneo, Pinotti & Baldi, 2010). Figure 2.4 shows the comparison of the polyunsaturated fatty acid (PUFA), monounsaturated fatty acid (MUFA) and total unsaturated fatty acid (TUFA) in Maya nut, olive oil, and canola oil. As seen in the figure, PUFA value of Maya nut was 64.96%. Polyunsaturated fatty acids (PUFAs) are increasingly in demand due to the beneficial effects on human health. Comprehensive

studies have shown that PUFAs have two main functions: (1) regulating membrane fluidity and membrane proteins; and (2) serving as metabolite precursors, such as prostaglandins and Leukotrienes (Ho, Jiang & Chen, 2007; Certik & Shimizu, 1999). As PUFAs have their unique structures and functions, deficiencies of PUFAs would cause various abnormalities in humans (Gill & Valivety, 1997). Dupont et al. reported that PUFAs and MUFAs of corn oil were 59 and 24 % (% FAME) (Dupont et.al., 1989). The PUFA is linoleic acid (C18:2n-6) primarily, with a small amount of linolenic acid (C18:3n-3) giving an n-6/n-3 ratio of 83 (Dupont et.al., 1990). In another study, Li, Cherian, Ahn, Hardin and Sim noted that PUFAs in other edible oils such as palm oil, sunflower oil were 10.3 and 66.4% (FAME%), respectively (Li, Cherian, Ahn, Hardin & Sim, 1996). Compared with the olive, canola, corn, and palm oils, the Maya nut oil possesses a higher amount of PUFA. Despite the high PUFAs, Maya nut was low in MUFA, especially in oleic acid.

Many sample preparation methods have been developed to evaluate polyphenolics and simple phenolics in a wide range of sample types. Most importantly, extraction is the crucial step for the recovery and isolation of bioactive phytochemicals from plant materials before analysis. In addition, it is worth noting that the removal of unwanted phenolics and non-phenolic substances such as waxes, fats is also critical. Solid-liquid extraction (Soxhlet extraction) is the most commonly used method prior to analysis of polyphenolics and simple phenolics. Methanol, ethanol, acetone, diethyl ether, and ethyl acetate are the commonly used extraction solvents (Stalikas, 2007). It was reported that among the five organic solvent extracts made by ethanol, methanol, acetone, ethyl

acetate, and hexane, the ethanolic extracts of peanut seed produced higher yields and stronger antioxidant activity than other organic solvent extracts (Huang, Yen, Chang, Yen & Duh, 2003). Also, Wijeratne, Abou-Zaid, and Shahidi indicated that extraction conditions were the best when carried out in 80% ethanol at 80 °C for 30 min to obtain the highest amount of phenolic extracts from almond (Wijeratne, Abou-Zaid & Shahidi, 2006).

Many plants and nuts contain several therapeutically active constituents, especially polyphenols. Plant phenolics constitute one of the main classes of natural antioxidants (Dimitrios, 2006). Phenolic compounds could react with the molybdenum-containing Folin-Ciocalteu reagent and be induced by an electron transfer. With the electron transfer, the deep yellow color was converted to a blue color that can be measured spectroscopically (Singleton & Rossi, 1965). As shown in Table 2.3, total phenolic contents (TPC) of the samples were illustrated as gallic acid equivalents (GAE) per gram of sample of the extract. The TPC of the Maya nut, walnut, peanut and almond were 24.67±0.85, 8.12±0.51, 4.3±1.78, and 0.875±0.02 mg of GAE per gram of sample (mg GAE/ g sample), respectively. It is seen that there are significant differences in the phenolic contents between the Maya nut and other nut extracts. Maya nut had the considerably higher TPC in ethanol solvent system. Almond has the lowest TPC in the solvent system. A higher concentration of total phenolics in almond seed using 80% (v/v) acetone extract (16.1 mg of CE/g of extract) was obtained by Amarowicz et al. (Amarowichz, Troszyńska & Shahidi, 2005). Our result suggests that Maya nut can potentially be a good natural source of dietary phenolic compounds.

In the present study, the FRAP assay was developed to determine the ferric reducing ability of the samples. The antioxidant capacity of the samples was estimated from their ability to reduce the TPTZ-Fe^{3+} complex to the TPTZ-Fe^{2+} complex (Benzie & Strain, 1996). Calculation of the FRAP values was based on iron (II) sulfate (Table 2.3). Walnut had the greatest ability to induce the TPTZ-Fe^{3+} complex to the TPTZ-Fe^{2+} complex, followed by the Maya nut, peanut, and almond. The mean values of the walnut, Maya nut, peanut, and almond were 22.64±0.009, 8.08±.0.35, 1.61±0.00032, and 1.52±0.0009 mmol Fe^{2+}/100 g sample, respectively. Maya nut was comparable to the values of different kinds of nuts. The mmol Fe^{2+}/100g sample equivalent of Maya nut was higher than that of different species of chestnuts (4.7 mmol/100g), roasted peanuts (2.0 mmol/100g), and pistachios (1.7mmol/100g) (FRAP-nuts). Moreover, ferric reducing antioxidant power of Maya nut was preferable to various kinds of beverages such as orange juice (0.64 mmol/100g), grape juice (1.2 mmol/100g) (Carlsen et.al, 2010).

The DPPH radical scavenging assay is commonly employed in evaluating the ability of antioxidants to scavenge free radicals. The change in absorbance at 515 nm is used as a measure of the scavenging effect of a particular extract for DPPH radicals (Farhoosh, Kenari & Poorazrang, 2009). The absorbance at 515 nm decreases as the reaction between antioxidant molecules and DPPH radical progresses. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract in terms of its hydrogen atom-donating capacity (Alasalvar, Karamać, Amarowichz & Shahidi, 2006). As shown in Figure 2.5, after 60 minutes of the reaction, the DPPH

remaining % of the Maya nut, walnut, almond, peanut, and standard antioxidant BHT at concentration of 400 ppm (µg/mL) were 78.07%, 84.85%, 45.50, 45.47%, and 83.27%, respectively. After 60 minutes, their DPPH remaining % approached stable. In this study, Maya nut showed a higher level of DPPH scavenging activity than almond and peanut, but lower level of DPPH scavenging activity than walnut and almond at 400 ppm. The phytochemical extracts of the samples exhibited potent antioxidant activities. Variety, process methods, in-shell or without shell, cultivation conditions, and storage conditions markedly affect the content of total antioxidants (Yang, Liu & Halim, 2009). Based on the FRAP, TRAP, and TEAC assays, the contribution of bound phytochemicals from six nuts (almonds, hazelnuts, peanuts, pine nuts, pistachios, and walnuts) extracted by methanol and alkaline, to the total antioxidant capacity (TAC) was also evaluated by Pellegrini et al. (Pellegrini, Serafini, Salvatore, Del Rio, Bianchi & Brighenti, 2006). In all three assays, walnuts had the highest TAC values (Pellegrini, Serafini, Salvatore, Del Rio, Bianchi & Brighenti, 2006). In our model system, free radical scavenging activity was in the following order: Maya nut > BHT > walnut > almond > peanut at 1mg/ml concentration (Figure 2.5). In addition, the comparison of % DPPH scavenging values of the Maya nut extract and BHT is presented in Figure 2.6. Maya nut showed a higher % DPPH scavenging value than that of BHT antioxidant at 1000 µg/mL (1000 ppm) concentration.

Numerous methods are used to evaluate antioxidant activities of natural compounds in foods or biological systems with varying results. Two free radicals that are commonly used to assess antioxidant activity in vitro are 2, 2 azinobis (3-

ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) and 2, 2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$). The $ABTS^{\cdot+}$ is generated by reacting a strong oxidizing agent (potassium persulfate) with the ABTS salt. The reduction of the blue-green $ABTS^{\cdot+}$ radical by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum. The method is rapid and can be used over a wide range of pH values (Arnao, Cano & Acosta, 1999; Lemanska et. al., 2001) in both aqueous and organic solvent systems. It also has good repeatability and is simple to perform; hence, it is widely reported (Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003). In the ABTS assay, the values ranged from 58.96 to 92.55%. Maya nut extract possessed the highest antioxidant capacity (92.55% of ABTS inhibition) followed by the walnut extract (92.17%), peanut extract (91.12%), and almond extract (58.96%), respectively. The methanolic extracts of Maya nut and walnut with stronger DPPH radical scavenging activities also exhibited higher ABTS values (Figure 2.7). Moreover, high correlations have been found between the DPPH scavenging activities and ABTS radical scavenging capacities of the antioxidant extracts ($R^2 = 0.9139$, Figure 2.8).

2.4 Conclusions

In summary, Maya nut has the high amount of ash content and the low amount of lipid content among the nuts. The experiments showed that Maya nut contained 27.2% palmitic acid (16:0), 6.26% stearic acid (18:0), 9.9% linolenic acid (18:3), 55% linoleic acid (18:2), 3.36% Cis-11-Eicosenoic acid (20:1), or 64.96% PUFA, and 3.36% MUFA. In contrast, olive oil and canola oil have similar amounts of oleic acid compositions and

there was no oleic acid found in Maya nut. However, we demonstrated that Maya nut had higher amount of linolenic acid than olive oil and canola oil. The mean values of linolenic acid of the Maya nut, canola oil and olive oil were 9.9, 7.38, and 1.26 (% FAME), respectively. Results from this study showed that Maya nut had higher PUFA and lower MUFA than olive oil and canola oil. The TPC of the Maya nut is 24.67±0.85 mg of gallic acid equivalents per gram of sample (mg GAE/ g sample). These values are considerably higher than that of walnut, almond, and peanut. In FRAP assay, walnut had the greatest ability to induce the TPTZ-Fe^{3+} complex to the TPTZ-Fe^{2+} complex, followed by the Maya nut, peanut, and almond. Besides, at the concentration of 1 mg/mL, the DPPH remaining % of the Maya nut, walnut, almond, peanut, and standard antioxidant BHT were 83.35%, 71.58%, 18.1%, 9%, and 82.97%, respectively. The level of DPPH scavenging activity of Maya nut was close to that of BHT at concentrations of 400 and 500 ppm. ABTS free radical scavenging values ranged from 58.96 to 92.55% among nuts and Maya nut extract possessed the highest antioxidant capacity (92.55% of ABTS inhibition). Moreover, we have found a high correlation between the DPPH scavenging activity and the ABTS radical scavenging capacity of the antioxidant extracts (R^2 = 0.9139). These results demonstrated that Maya nut had suitable nutritional properties (higher linoleic, medium linolenic, low oleic acid, and high ash contents), which could make it a health-benefiting food. Also, Maya nut had potent antioxidant activities that resulted from its intrinsic contents of bioactive compounds. Although the chemical profiles in terms of the fatty acid composition and phenolic acids in the Maya

nut have been characterized, further research is required to promote its usage as nutraceuticals and functional foods.

2.5 Figures and Tables

Table 2.1 The proximate compositions of walnut, almond, peanut, and Maya nut

	Percentage on Dry Weight Basis[1]			
	Maya nut	Peanut	Walnut	Almond
Moisture	5.53±0.11	6.50±0.093	4.07±0.15	4.70±0.046
Ash	3.27±0.015	2.33±0.064	1.78±0.019	2.99±0.015
Protein	62.6±0.85	25.80±0.24	15.23±0.23	21.22±0.044
Lipid	1.31±0.32	49.24±0.29	65.21±0.49	49.42±0.18

Values are means of triplicates and shown with standard error

Values for peanut, walnut, and almond are from the USDA National Nutrient Database

Table 2.2 Fatty acid composition of Maya nut, olive oil and canola oil

Fatty Acid Composition (% of methyl esters)			
Fatty acid	Maya nut	Olive oil	Canola oil
C16:0 (Palmitic)	27.22±2.05	12.1±0.76	3.52±0.6
C 16:1 n9 (Palmitoleic acid)	Nd	Nd	0.5±0.18
C 18:0 (Stearic)	6.26±0.88	2.72±0.16	1.62±0.2
C18:3 n3 (Linolenic)	9.9±3.3	1.26±0.07	7.38±0.6
C18:1 n9 (Oleic)	Nd	67.40±2.72	64.2±4.82
C18:2 n6 (Linoleic)	55.06±4.92	11.84±0.42	22.52±1.73
C20:1 (Cis-11-Eicosenoic)	3.36±1.36	4.3±1.74	1.14±0.08

Values are expressed as means with (standard error)

None of the values are different as determined by one-way analysis of variance, ANOVA at $p<0.05$

Table 2.3 Total Phenolic Content (TPC) and Ferric Reducing Antioxidant Power (FRAP) of Maya nut, walnut, almond and peanut extracts

Sample	TPC (mg GAE/g)	FRAP (mmol Fe^{2+}/100 g)
Maya nut	24.67±0.85	8.08±.0.35
Walnut	8.12±0.51	22.64±0.009
Peanut	4.3±1.78	1.61±0.00032
Almond	0.875±0.02	1.52±0.0009

TPC: Total phenolic content and expressed as gallic acid equivalent (GAE)

TPC and FRAP measurements were done in triplicate.

Values are expressed as means with (standard error)

None of the values are different as determined by one-way analysis of variance, ANOVA at $p<0.05$

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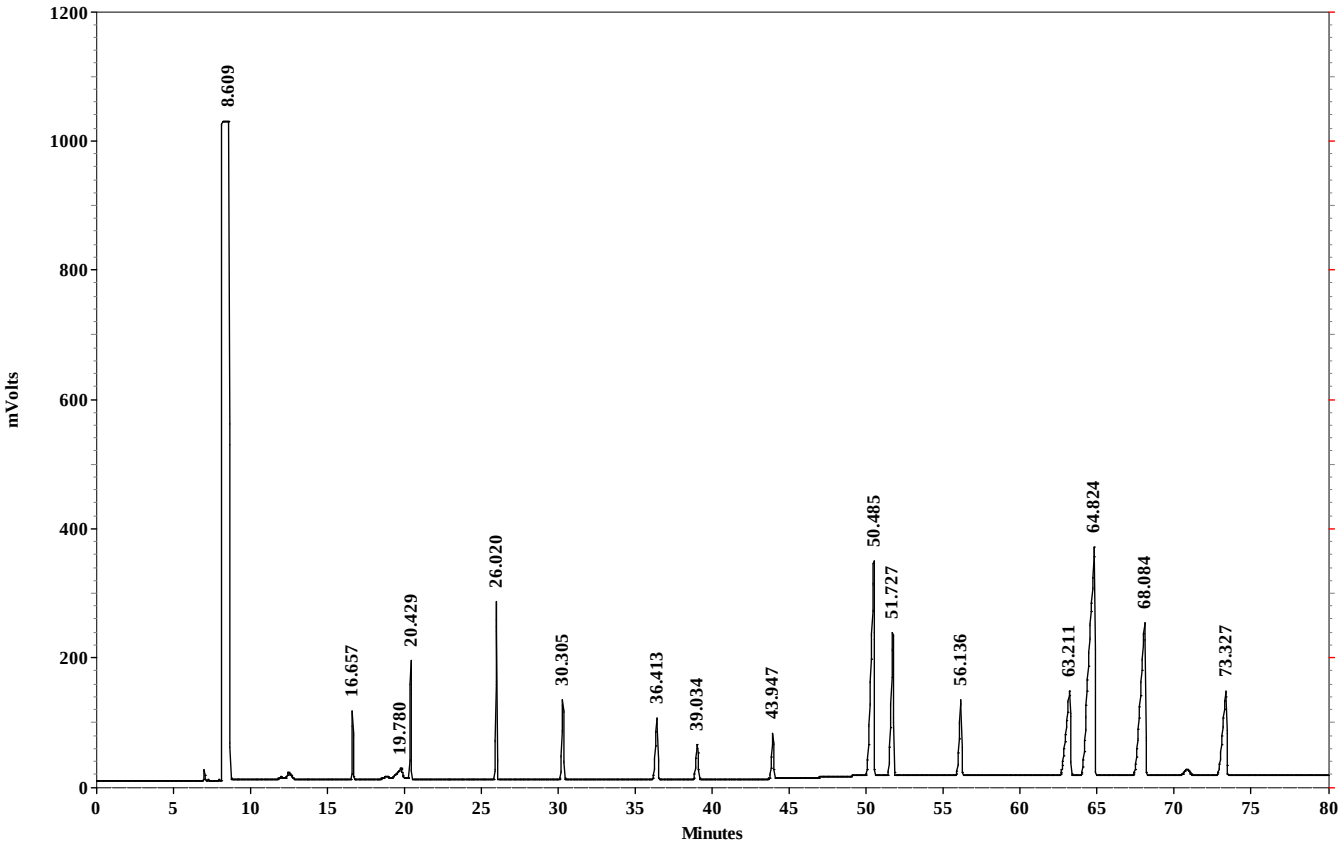


Figure 2.1 GC Chromatogram of Standard Fatty Acids Mixture

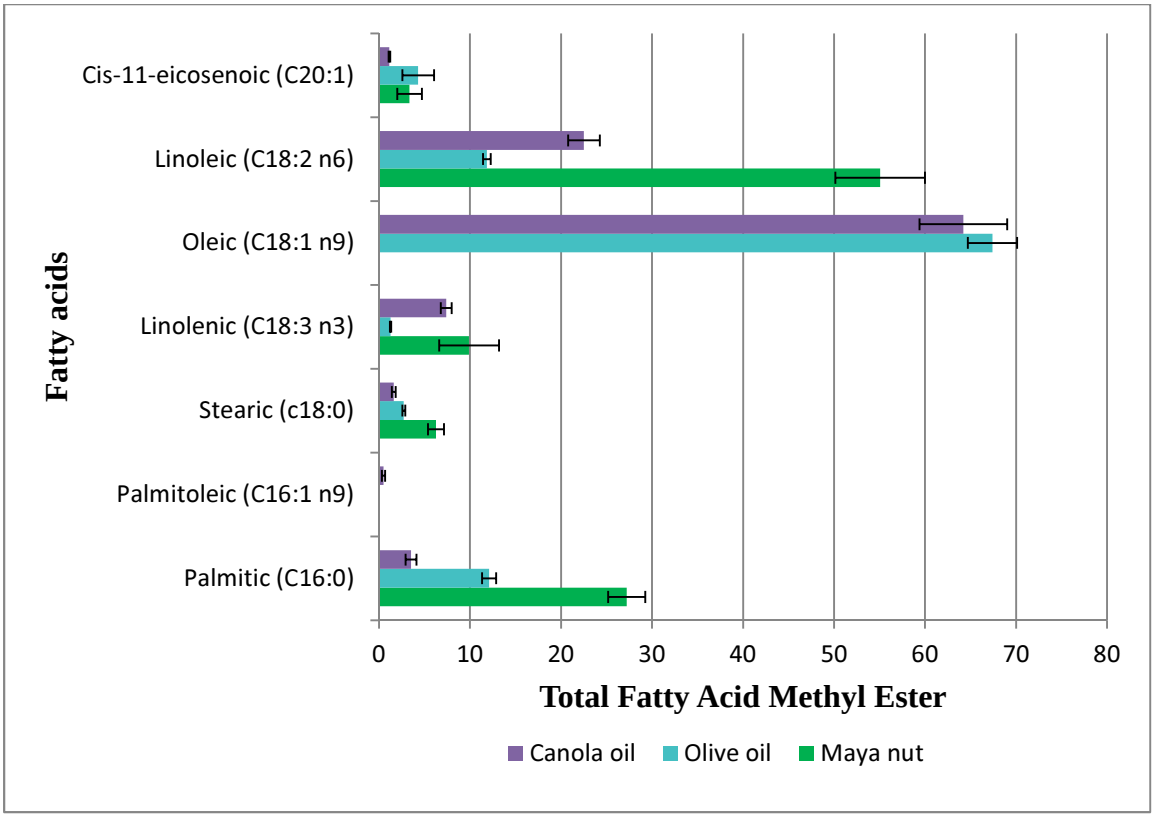


Figure 2.2 Comparison of fatty acid content within Maya nut, olive oil, and canola oil

Values are expressed as means with (standard error)

None of the values are different as determined by one-way analysis of variance, ANOVA at $p<0.05$

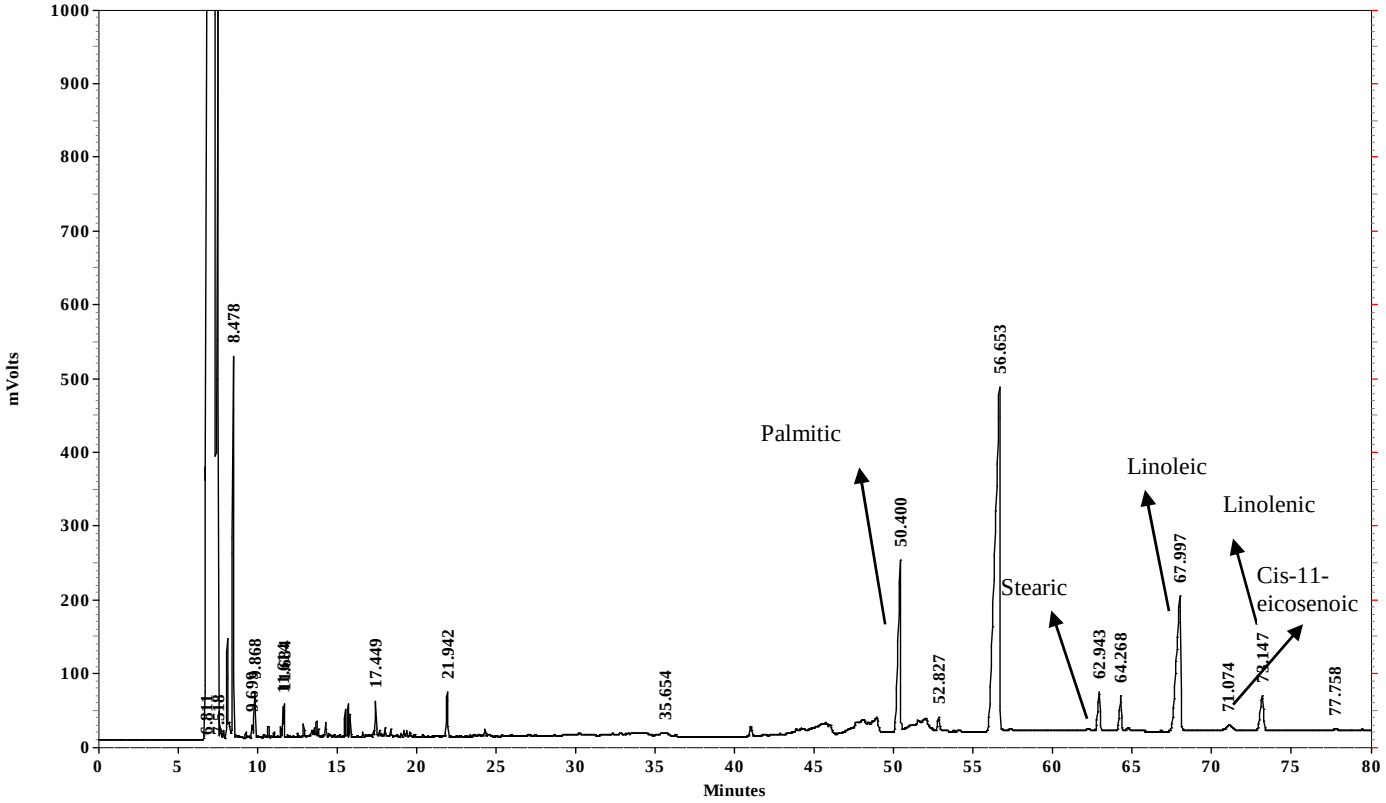


Figure 2.3 GC Chromatogram of Maya nut fatty acid composition

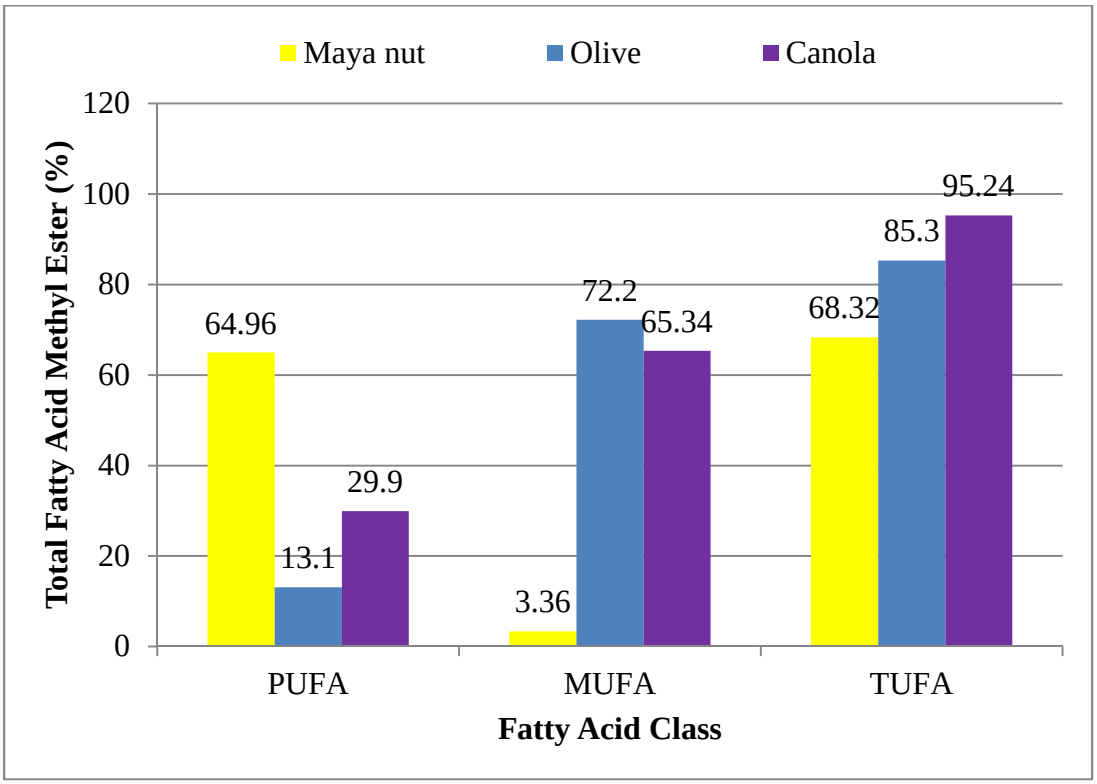


Figure 2.4 Comparison of Polyunsaturated Fatty acid (PUFA), Monounsaturated Fatty Acid (MUFA) and Total Unsaturated Fatty acid (TUFA) within Maya nut, olive oil and canola oil

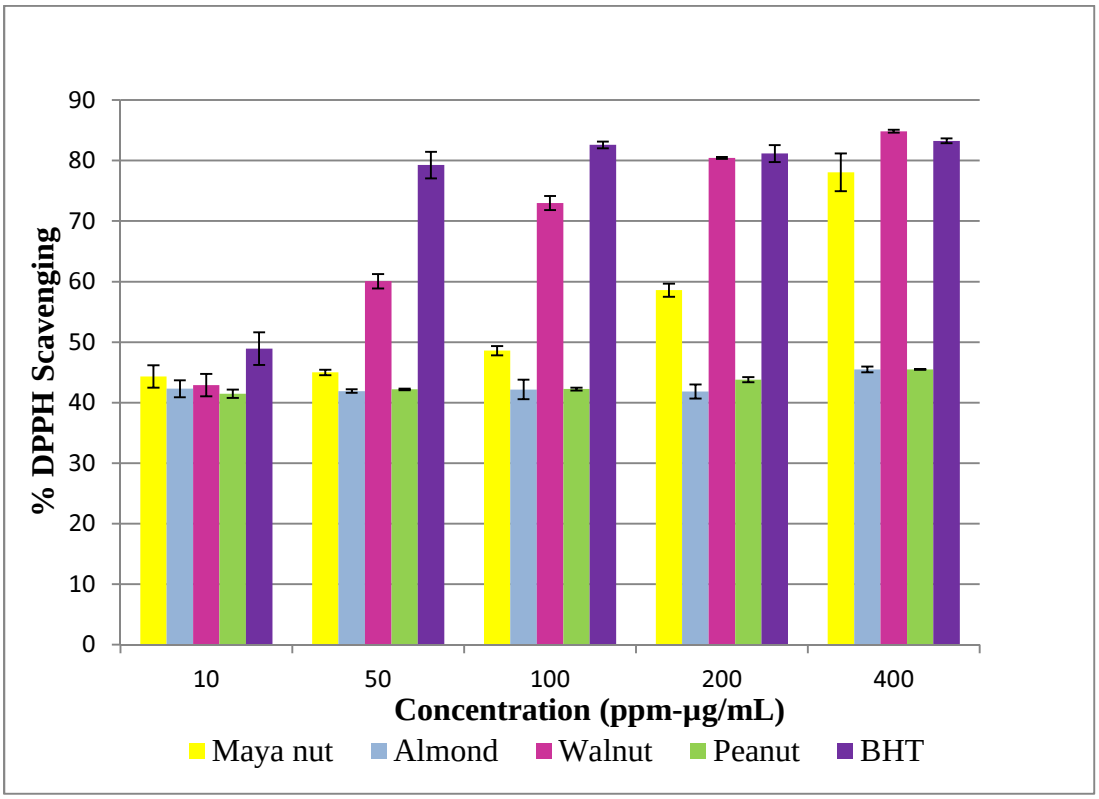
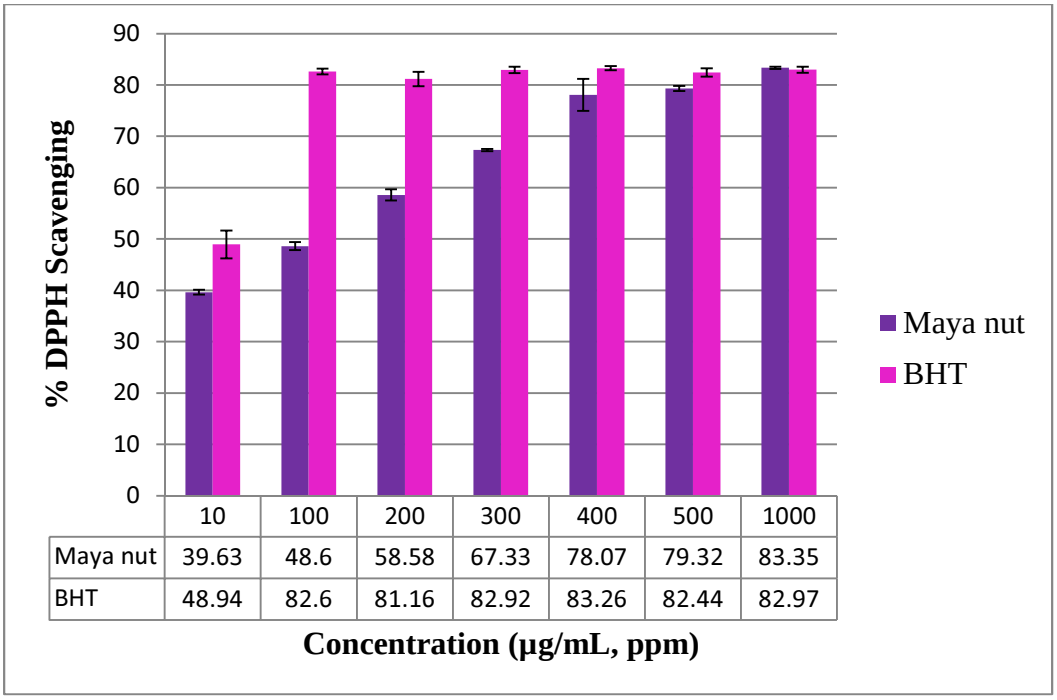


Figure 2.5 Comparison of free radical scavenging activity of Maya nut, walnut, almond, peanut, and BHT- DPPH assay

Values are expressed as means with (standard error)

None of the values are different as determined by one-way analysis of variance, ANOVA at $p<0.05$



	10	100	200	300	400	500	1000
Maya nut	39.63	48.6	58.58	67.33	78.07	79.32	83.35
BHT	48.94	82.6	81.16	82.92	83.26	82.44	82.97

Figure 2.6 The comparison of % DPPH scavenging capacity of Maya nut and standard antioxidant BHT

Values are expressed as means with (standard error)

None of the values are different as determined by one-way analysis of variance, ANOVA at $p<0.05$

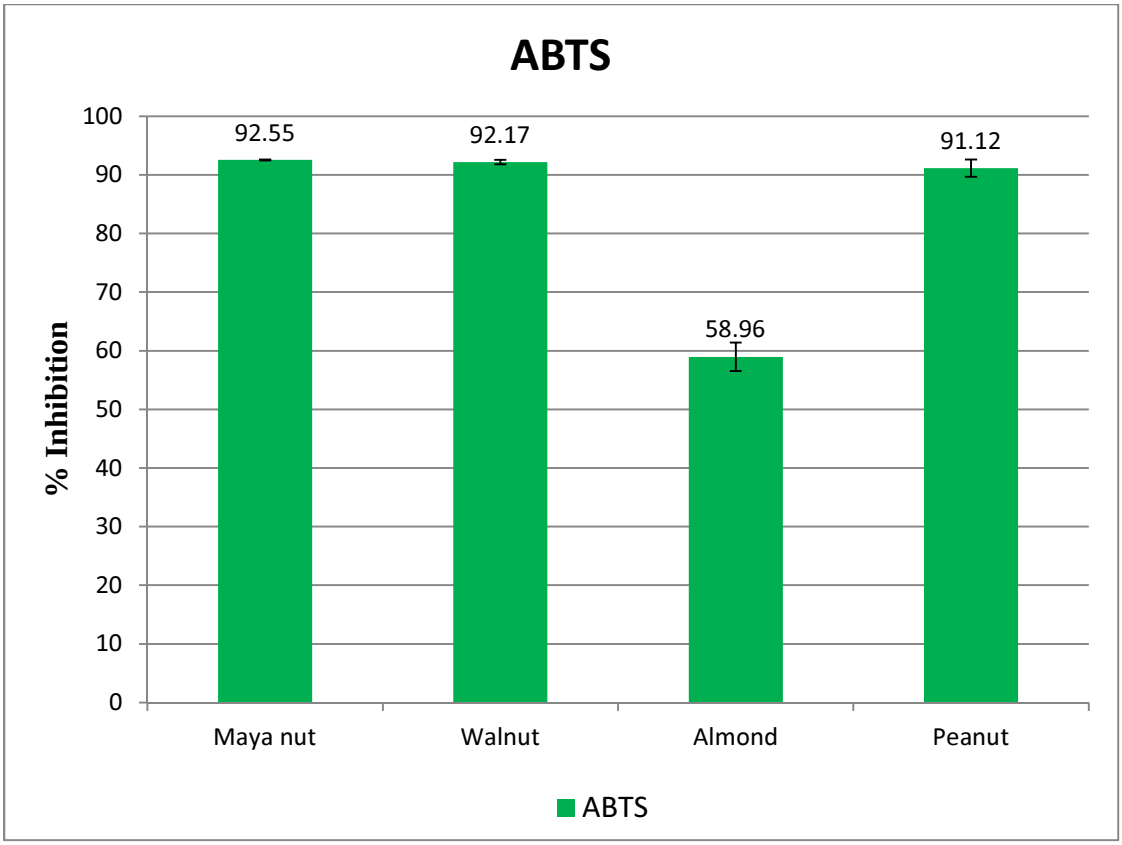


Figure 2.7 The % ABTS inhibition of Maya nut, walnut, peanut and almond at 1mg/mL concentration

Values are expressed as means with standard error

None of the values are different as determined by one-way analysis of variance, ANOVA at $p<0.05$

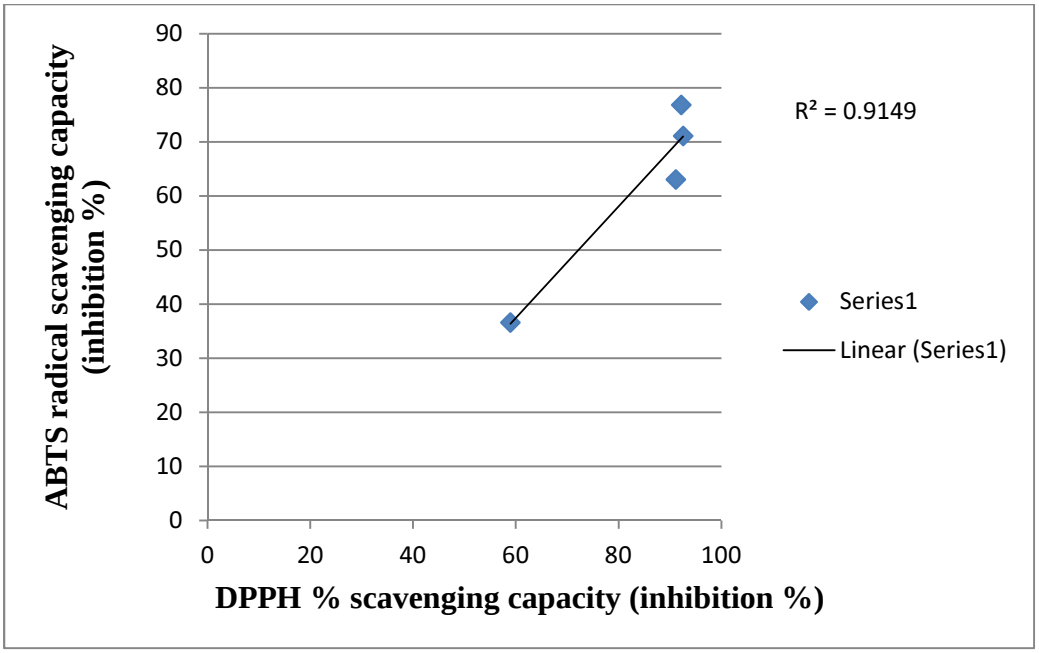


Figure 2.8 Correlation coefficient between DPPH and ABTS assays

Correlation determination R^2=0.914

2.6 References

Alasalvar, C., Karamać, M., Amarowichz, R., & Shahidi, F. (2006). Antioxidant and antiradical activities in extracts of hazelnut kernel (*Corylus avellana* L.) and hazelnut green leafy cover. Journal of Agricultural and Food Chemistry, 54, 4826-4832.

Allouche, Y., Jiménez, A., Gaforio, J.J., Uceda, M., & Beltran, G. (2007). How heating affects extra virgin olive oil quality indexes and chemical composition. Journal of Agricultural and Food Chemistry, 55, 9646–9654.

Amaral, J.S., Casal, S., Pereira, J.A., Seabra, R.M., & Oliveira, B.P.P. (2003). Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (*Juglans regia* L.) Cultivars grown in Portugal. Journal of Agricultural and Food Chemistry, 51, 7698- 7702.

Amarowichz, R., Troszyńska, A., & Shahidi, F. (2005). Antioxidant activity of almond seed extract and its fractions. Journal of Food Lipids, 12, 344–358.

Arnao, M. B., Cano, A., & Acosta, M. (1999). Methods to measure the antioxidant activity in plant material. A comparative discussion. Free Radical Research, 31, 89-96.

Awika, J.M., Rooney, L.W., Wu, X., Prior, R.L., Cisneros-Zevallos, L. (2003). Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. Journal of Agricultural and Food Chemistry, 51, 6657-6662.

Benzie, I.F.F., & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of ‘‘Antioxidant Power’’: The FRAP assay. Analytical Biochemistry, 239, 70-76.

Carlsen, M.H., Halvorsen, B.L., Holte, K., BØhn, S.K., Dragland, S., Sampson, L., Willey, C., Senoo, H., Umezono, Y., Sanada, C., Barikmo, I., Berhe, N., Willett, W.C., Phillips, K.M., Jacobs Jr, D.R., & Blomhoff, R. (2010). The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. Nutrition Journal, 9(3), 1-11.

Certik, M., & Shimizu, S. (1999). Biosynthesis and regulation of microbial polyunsaturated fatty acid production. Journal of Bioscience and Bioengineering, 87(1), 1-14.

Cicerale, S., Conlan, X.A., Sinclair, A.J., & Keast, R.S.J. (2009). Chemistry and health of olive oil phenolics. Critical Reviews in Food Science and Nutrition, 49, 218–236.

Dabbou, S., Rjiba, I., Nakbi, A., Gazzah, N., Issaoui, M., & Hammami, M. (2010). Compositional quality of virgin olive oils from cultivars introduced in Tunisian arid zones in comparison to *Chemlali* cultivars. Scientia Horticulturae, 124, 122–127.

Dimitrios, B. (2006). Sources of natural phenolic antioxidants. Trends in Food Science & Technology, 17, 505–512.

Dupont, J., White, P.J., Johnston, K.M., Heggtveit, H.A., McDonald, B.E., Grundy, S.M., & Bonanome, A. (1989). Food safety and health effects of canola oil. Journal of the American College of Nutrition, 8(5), 360-375.

Dupont, J., White, P.J., Carpenter, M.P., Schaefer, E.J., Meydani, S.N., Elson, C.E., Woods, M., Gorbach, S.L. (1990). Food uses and health effects of corn oil. Journal of the American College of Nutrition, 9(5), 438-470.

Erkkilä A., de Mello, V.D.F., Risérus U., & Laaksonen, D.E. (2008). Dietary fatty acids and cardiovascular disease: An epidemiological approach. Progress in Lipid Research, 47, 172–187.

Farhoosh, R., Kenari, R.E., Poorazrang, R. (2009). Frying stability of canola oil blended with palm olein, olive, and corn oils. Journal of American Oil Chemists' Society, 86, 71-76.

Gill, I., & Valivety, R. (1997). Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications. Tibtech, 15, 401- 409.

Gunstone, F.D., Harwood J.L., & Dijkstra, & A.J. (2004). The Lipid Handbook. pp (54-55). Boca Raton, Florida: CRC Press.

Henry, G.E., Momin, A., Nair, M.G., & Dewitt, D.L. (2002). Antioxidant and cyclooxygenase activities of fatty acids found in foods. Journal of Agricultural and Food Chemistry, 50, 2231-2234.

Ho, S-Y., Jiang, Y., & Chen, F. (2007). Polyunsaturated fatty acids (PUFAs) content of the fungus *Mortierella alpina* isolated from soil. Journal of Agricultural and Food Chemistry, 55, 3960-3966.

Huang, S.C., Yen, G-C., Chang, L-W., Yen, W-J., & Duh, P-D. (2003). Identification of an antioxidant, ethyl protocatechuate, in peanut seed testa. Journal of Agricultural and Food Chemistry, 51, 2380-2383.

Ito, N., Fukushima, S., Tsuda, H., Shirai, T., Hagiwara, A., & Imaida, K. (1985). Antioxidants: carcinogenicity and modifying activity in tumorigenesis. In Food Toxicology: Real or imaginary problems? Gibson, G.G., Walker, R., Eds. pp (181-198). London: Taylor & Francis.

Kitts, D. D., Wijewickreme, A. N., & Hu, C. (2000). Antioxidant properties of a North American ginseng extract. Molecular and Cellular Biochemistry, 203, 1-10.

Li, S.X., Cherian, G., Ahn, D.U., Hardin, R.T., & Sim, J.S. (1996). Storage, heating, and tocopherols affect cholesterol oxide formation in food oils. Journal of Agricultural and Food Chemistry, 44, 3830-3834.

Nielsen, S.S. (2003). Food Analysis. pp (105). New York: Kluwer Academic/ Plenum Publishers.

Parry, J., Cheng, Z., Moore, J., Yu, L. (2008). Fatty acid composition, antioxidant properties, and antiproliferative capacity of selected cold-pressed seed flours. Journal of the American Oil Chemists' Society, 85(5), 457-464.

Parry, J. Su, L., Moore, J., Cheng, Z. Luther, M., Rao, J.N., Wang, J., Yu, L. (2006). Chemical compositions, antioxidant capacities, and antiproliferative activities of selected fruit seed flours. Journal of Agricultural and Food Chemistry, 54(11), 3773- 3778.

Pellegrini, N., Serafini, M., Salvatore, S., Del Rio, D., Bianchi, M., & Brighenti, F. (2006). Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different in vitro assays. Molecular Nutrition & Food Research, 50, 1030-1038.

Peters, C.M., & Pardo-Tejeda, E. (1982). Brosimum alicastrum (Moraceae): Uses and potential in Mexico. Economic Botany, 36(2), 166-175.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine, 26 (9-10), 1231–1237.

Russo, G.L. (2009). Dietary n-6 and n-3 polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention. Biochemical Pharmacology, 77(6), 937-946.

Savoini, G., Agazzi, A., Invernizzi, G., Cattaneo, D., Pinotti, L., & Baldi, A. (2010). Polyunsaturated fatty acids and choline in dairy goats nutrition: Production and health benefits. Small Ruminant Research, 88, 135–144.

Shahidi, F., Alasalvar, C., & Liyana-Pathirana, C.M. (2007). Antioxidant phytochemicals in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproducts. Journal of Agricultural and Food Chemistry, 55, 1212-1220.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic - phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16, 144-158.

Siriwardhana, S.S.K.W., & Shahidi, F. (2002). Antiradical activity of extracts of almond and its by-products. Journal of the American Oil Chemists' Society, 79(9), 903-908.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. E., Fujimoto, E. K.,Golki, N. M., Olson, B. J., & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. Analytical Biochemistry, 150, 76-85.

Stalikas, C.D. (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. Journal of Separation Science, 30, 3268-3295.

Surh, J., Ryu, J-S., & Kwon, H. (2003). Seasonal variations of fatty acid compositions in various Korean shellfish. Journal of Agricultural and Food Chemistry, 51 (6), 1617-1622.

Wijendran, V., & Hayes, K.C. (2004). Dietary n -6 and n -3 fatty acid balance and cardiovascular health. Annual Review of Nutrition, 24, 597-615.

Wijeratne, S.S.K., Abou-Zaid, M.M., & Shahidi, F. (2006). Antioxidant polyphenols in almond and its coproducts. Journal of Agricultural and Food Chemistry, 54, 312-318.

Vohman, E.C., & Sanchez-Garduño C. (2008). Maya Nut (*Brosimum alicastrum*): Traditional rainforest food for healthy forests and families in Central America. International symposium "Underutilized plants for food, nutrition, income and sustainable development".

Yang, J., Liu, R.H., & Halim, L. (2009). Antioxidant and antiproliferative activities of common edible nut seeds. Food Science and Technology, 42, 1–8.

CHAPTER 3

CHEMICAL COMPOSITION AND CHARACTERIZATION OF MAYA NUT

Abstract

Individual phenolic compounds were prepared by both an acidic and an alkaline hydrolysis in order to isolate free phenolic acids from their glycosides. Free phenolics were then identified by high performance liquid chromatography (HPLC-UV), which showed that 3, 4 hydroxybenzoic acid was the major phenolic compound in the Maya nut extract. First of all, acidic hydrolysis was conducted to reveal the presence of 3, 4 hydroxybenzoic acid (45 mg/kg), gallic acid (27.06 mg/kg), vanillic acid (21.15 mg/kg), and caffeic acid (6.5 mg/kg) in Maya nut. Moreover, the bound phenolic acids were identified by HPLC-UV detection at 280 nm. While applying alkaline hydrolysis for the Maya nut extract, more and larger amount of free phenolic acids were detected, including 3, 4 hydroxybenzoic acid (326.2 mg/kg), vanillic acid (103.9 mg/kg), caffeic acid (17.1 mg/kg), *p*-coumaric acid (13.5 mg/kg), and a flavanol, epicatechin (53 mg/kg). In addition, second acidic hydrolysis of the remained bound phenolic acids was performed to obtain more 3, 4 hydroxybenzoic acid and sinapic acid in the Maya nut extract. Results indicate that 3, 4 hydroxybenzoic acid and vanillic acid were detected in high amounts. The presence of rich phenolic compounds is an important character of Maya nut for its various biological and nutritional properties. Moreover, further investigation is suggested to be carried out for the nutraceutical application of these secondary metabolites present in Maya nut.

3.1 Introduction

Maya nut has been used as a staple food for many years by the Ancient Mayas. Its seeds are nutritious, including the rich amount of polyphenols. In our study, we found that the Maya nut powders possessed high antioxidant activities and might contain a number of polyphenols including phenolic acids, flavonoids, flavanols, many of which are strong free radical scavengers (Jacobs, Meyer & Solvoll, 2001), and are considered health benefiting (i.e. anti-atherogenic, anticarcinogenic, anti-ischemic, etc.), which can be prepared as nutraceutical and functional foods through food-grade extraction (Lapornik, Prošek, & Wondra, 2005). In addition, because of the growing interest in natural health benefiting compounds, there is a need to isolate, identify and quantify them from raw materials and evaluate their potential health benefits (Sellappan, Akoh, Krewer, 2002). However, the analyses of those different phytochemicals (e.g., phenolic acids and flavonoids) can be significantly different, and demand exhaustive elaboration of sample preparation, from use of simple filters to more complicated procedures, such as hydrolysis of glycosides, and extraction or clean-up steps prior to analysis. Therefore, no single pretreatment process can be performed to all samples (Stalikas, 2007). Acidic hydrolysis and saponification are among the most commonly used procedures to release the phenolic acids, even though the methods might decompose the target compounds under the conditions. The acidic hydrolytic treatment of the sample usually uses a strong inorganic acid (e.g. HCl) at high temperatures in aqueous solvents (methanol, ethanol or acetone). The concentrations of acids might differ from 1 to 2 N, and the reaction times range from 30 min to 1 h (Krygier, Sosulski & Hogge, 1982). With the usage of increased

temperature and high concentration of the acid solutions, analytes can be extracted quicker and more efficiently. Saponification procedure requires the sample to be treated with a solution of NaOH at concentrations from 1 to 4 M. Most of the reactions occur at room temperature for 15 min up to overnight (Rommel & Wrolstad, 1993).

The goals of applying two different hydrolytic procedures (i.e., acidic vs. alkaline hydrolyses) in this research project were: (i) to compare and optimize the extraction methods to extract phenolic compounds from the Maya nut; and (ii) to quantify the individual phenolic compounds.

3.2 Materials and Methods

3.2.1 Materials and Chemicals

2, 6-Di-tert-butyl-4-methyl phenol and butylated hydroxytoluene (BHT) in 99% purity were purchased from Acros Organics (Newjersey, NJ, USA). Acetic acid was obtained from EM Science. Sodium hydroxide, diethyl ether, ethyl acetate, and methanol were purchased from Fisher Scientific. Gallic acid, vanillic acid (97% purity), caffeic acid (in minimum of 99% purity), 3,4-hydroxybenzoic acid, p-coumaric acid (>98 %), and sinapic acid (>97 purity) were from Sigma-Aldrich. (-)-Epicatechin was from MP Biomedicals.

3.2.2 Acidic Hydrolysis

A homogenized dried (0.2-0.5 g) sample was weighed into a 50-mL graduated plastic test tube and mixed with 7 mL of methanol (containing 2 g/L of 2, (3)-*tert*-butyl-4-hydroxyanisole) and 10% acetic acid (85:15) (7 mL of methanol and 1.05 mL of acetic

acid) using Vortex for 1 minute. A 4-g sample of the mixture was then weighed into a 50-mL test tube and 5 mL of the extraction solution (4.25 mL of methanol and 0.75 mL of acetic acid) was added. After mixing by vortex, sample extract was ultrasonicated for 30 min, made up to 10 mL with distilled water, and mixed. Then, 1 mL of the extract was filtered through a membrane filter (0.45 μm, 25 mm; Fisher Scientific) for the HPLC analysis of free phenolic acids.

3.2.3 Alkaline Hydrolysis

In comparison of the acidic hydrolysis for releasing free phenolic acids, the alkaline hydrolysis methods used 12 mL of distilled water and 5 mL of 10 M NaOH to hydrolyze the sample in a sealed test tube, which was stirred overnight at room temperature (about 16 h) using a magnetic stirrer. The solution was then adjusted to a pH of 2. The liberated phenolic acids were extracted three times with 15 mL of a mixture of cold diethyl ether (DE) and ethyl acetate (EA, 1:1) by manually shaking and centrifuge. DE/EA layers were combined, evaporated to dryness, and dissolved into 1.5 mL of methanol. After the sample was filtered through a membrane filter (0.45 μm, 25 mm; Fisher Scientific), it was analyzed by the HPLC.

3.2.4 The Second Acid Hydrolysis

After the alkaline hydrolysis was carried out, an acidic hydrolysis was completed by adding 2.5 mL of concentrated HCl into the test tube followed by incubating the tube in a water bath (85 °C) for 30 min. After the acidic hydrolysis, the sample was allowed to cool down followed by the adjustment of pH to 2. The DE/EA extraction was collected and treated as that as the alkaline hydrolysis. The solvents were evaporated. The extract

was then dissolved into 1.5 mL of methanol and filtered through a membrane filter (0.45 μm, 25 mm; Fisher Scientific), and analyzed by HPLC.

3.2.5 HPLC Analysis

Phenolic analyses were carried out using a Shimadzu UFLC- 20AT HPLC system (Kyoto, Japan) equipped with a model SPD-20A UV/VIS detector, a model DGU-20A5 degasser, and a model SIL-20 AHT auto sampler, all of which was connected and controlled by the LC Solution software. Wavelengths used for the identification of phenolic acids with UV detector were 280 nm for gallic acid, 3,4-hydroxybenzoic acid, vanillic acid, caffeic acid, p-coumaric acid, and epicatechin; 280 nm and 329 nm for sinapic acid. Separation of phenolic acids was conducted on a Premier C18 column (150 x 4.6 mm; 5 μm particle size) with a C-18 guard column. Gradient elution was employed with a mobile phase consisting of 50 mM H_3PO_4, pH 2.5 (Solution A) and acetonitrile (Solution B) as follows: isocratic elution 95% A/5% B, 0-5 min; linear gradient from 95% A/5% B to 50% A/ 50% B, 5-55 min; isocratic elution 50% A/50% B, 55-65 min; linear gradient from 50%A/50%B to 95% A/5% B, 65-67 min; post-time 6 min before next injection. Flow rate of the mobile phase was maintained at 0.7 mL/min, and the injection volumes of the standards and sample extracts were 10 μL. All phenolic acids were quantified using the external standard method. Quantification was based on the peak area. Calibration curves of the standards were made by diluting stock standards in methanol to yield 10-50 μg/mL.

3.2.6 Statistical Analyses

Samples for HPLC analysis were performed on the SAS V9.2 software for Windows (SAS Institute Inc., Cary, NC, USA). Data were reported as the mean ± standard deviation. Differences among sample means were determined by analysis of variance (one-way analysis of variance, ANOVA) at $p<0.05$.

3.3 Results and Discussion

Phenolic acids are well known for their various biological functions, including nutrient uptake, protein synthesis, regulation of enzyme activity, photosynthesis, serving as structural components, allelopathy, etc, although there are still many unknown regarding their roles in plants. Only a minor fraction of phenolic acids exists as “free acids”. In contrast, the majority exists through ester, ether, or acetal bonds either to the structural components of the plant (cellulose, proteins, lignin), or to polyphenols (flavonoids), or to smaller organic molecules (e.g., glucose, quinic, maleic, or tartaric acids) or other natural products (e.g., terpenes). These linkages elicit to a vast range of derivatives. This diversity is one of the major factors resulting in the analytical complexity of phenolic acids. Moreover, phenolic acids are not evenly distributed throughout plant tissues (Robbins, 2003) and there exists a large variation during various stages of maturation. Growing conditions, such as temperature and soil nutrient, are well known to affect the phenolic acid content as well (Zheng & Wang, 2001).

Phenolic acids have been associated with color, sensory qualities, nutritional values and antioxidant properties of foods. For example, one reason for previous

analytical investigations is that the phenolics have the influential organoleptic properties (e.g., flavor, astringency, and hardness) of foods. In addition, the content and profile of phenolic acids in foods, their effect on fruit maturation, prevention of enzymatic browning, and their roles as food preservatives are frequently examined (Tomás-Barberán & Espín, 2001; Maga, 1978). However, accurate analysis of their contents in foods seems difficult, which, in part, arises from the unsatisfactory extraction of these compounds from food matrixes. Previously, very complicated extraction methods have been employed to determine free, esterified, and glycolysated phenolic acids in plant material. Generally, three different hydrolysis procedures have been used for the determination of free and total phenolic acids. Hydrolysis of the ester to a carboxylic acid has been used to simplify the analysis and obtain a more specific profile of the phenolic acids in foods. There are two known procedures reported in the literature to cleave the ester bond, which are acidic hydrolysis and saponification (or the alkaline hydrolysis) (Robbins, 2003; Escarpa & González, 2001). The third, a less prevalent technique, conducts cleavage through the use of enzymes (esterases). Though reaction times and temperatures for the acidic hydrolysis are highly variable, this method commonly uses strong inorganic acid (e.g., HCl) at reflux or above reflux temperatures in aqueous or alcoholic solvents (methanol being the most common). The saponification method requires a solution of NaOH with specific concentrations ranging from 1 to 4 M to treat a sample. Most of the reactions are allowed to stand at room temperature for time ranging from 15 min to overnight (Escarpa & González, 2001; Leung, Fenton, & Clandinin, 1981).

In this study, an acidic hydrolysis and alkaline hydrolysis were used to allow reasonable estimation of composition and contents of phenolic acids in Maya nut. The extractability, products of acid and alkaline hydrolysis after each extraction step are presented as a scheme in Figure 3.1.

Ultrasonication for 30 min during the hydrolysis of phenolic acids resulted in better extraction values. To optimize the alkaline hydrolysis procedure, the most effective combination for most phenolic acids was 16 h and 5 mL of 10 M NaOH. Generally, alkaline hydrolysis liberated most of the bound phenolic acids. Following the alkaline hydrolysis, the acidic hydrolysis was performed to liberate the rest of the bound phenolic acids. The combination of 2.5 mL of concentrated HCl, 30 min, and 85 °C was decently effective with minimal losses. In the previous researches, extractability of phenolic acids into the DE/EA solution was examined by recovery tests. These recovery tests showed that, generally, free phenolic acids extracted well into DE/EA solution with recoveries ranging from 87 to 112%. In addition to free phenolic acids, some forms of bound phenolic acids could also be extracted to some degree into the DE/ EA solution (Mattila & Kumpulainen, 2002).

High performance liquid chromatography (HPLC) is a pressurized system that separates compounds based on partition between the stationary phase in the column and liquid solvent(s) (Rounds & Gregory, 2003). In normal phase HPLC, the stationary phase is polar and the mobile phase is non-polar. On the contrary, reversed-phase HPLC utilizes a non-polar stationary phase and polar mobile phase such as water, or a polar solvent such as acetonitrile or methanol. Solutes are retained in the column based on

hydrophobicity. Increasing the percentage of organic solvent in the mobile phase will decrease retention time (Rounds & Gregory, 2003). A C18 stationary phase with hydrophilic endcapping was utilized, which has been shown to be highly suitable for the separation of polyphenolics in various matrices. Advantages of HPLC analysis are its speed of analysis, and high sensitivity if the appropriate detector is used. A disadvantage is the requirement for volatile solvents in the mobile phase. Another disadvantage is that samples usually require preparation and filtering prior to analysis (Rounds & Gregory, 2003).

The chromatographic profiles in this section correspond to the acidic hydrolysis, alkaline hydrolysis and the second acid hydrolysis of the nut powders (Maya nut powder), which were collected into the DE/EA solution and detected at 280 nm (Figure 3.2, 3.3, and 3.4). Among the hydroxybenzoates, gallic, *3, 4*-hydroxybenzoic, and vanillic acids were identified by their UV spectra and by comparison of their retention times (RT in minutes) with standard compounds (Figure 3.5). The hydroxycinnamates, such as caffeic, *p*-coumaric, and sinapic acid and one of flavanols, epicatechin were identified accordingly. After the acidic hydrolysis, peaks of 1, 3, 4, and 5 yielded gallic acid (RT = 5.3), 3, 4-hydroxybenzoic acid (RT = 10.1), vanillic acid (RT = 20.2) and caffeic acid (RT =21.3), after the alkaline hydrolysis, peaks of 7, 8, 9, 12, and 13 yielded 3, 4-hydroxybenzoic acid (RT=10.1), vanillic acid (RT= 20.2), caffeic acid (RT=21.3), p-coumaric acid (RT= 26.2), and epicatechin (RT= 28.8). Besides, after the 2^{nd} acidic hydrolysis, the sinapic acid (RT= 28.4) and 3,4-hydroxybenzoic acid were also identified.

Hydroxybenzoic acid derivatives

Each individual phenolics, such as the caffeic or vanillic, was measured in mg and the total phenolics were reported in mg per kg of Maya nut. The total content of hydroxybenzoic acids constituted from 508.91 to 590.75 of the total phenolic acids present in the Maya nut. After the acidic hydrolysis, gallic acid content is 27.06 mg/kg, 3, 4-hydroxybenzoic acid content is 45 mg/kg, and vanillic acid content is 21.15 mg/kg; after the alkaline hydrolysis, the content of the *p*-coumaric acid is 13.5 mg/kg, epicatechin content is 44.74 mg/kg, 3, 4-hydroxybenzoic acid content is 326.2 mg/kg, vanillic acid content is 89.5 mg/kg, and caffeic acid content is 17.1 mg/kg; after the second acidic hydrolysis, 3,4-hydroxybenzoic acid content is 16 mg/kg and sinapic acid content is 8.17 mg/kg (Table 3.1). These phenolic acids may act as natural antioxidants and play important roles in the health benefits. (Jacobs, Meyer & Solvoll, 2001; Jacobs, Meyer, Kushi & Folsom, 1998). Our HPLC analysis showed that the Maya nut seed exhibited a high content of the vanillic acid after the alkaline hydrolysis. This phenolic acid has been reported as an efficient antioxidant compound, scavenging reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and hydroxyl radical (Zhou, Yin & Yu, 2006). Zhou, Yin, and Yu revealed that only ferulic and vanillic acids had significant capacity to form a chelating complex with Cu^{2+} among the tested phenolic acids in the ESR (erythrocyte sedimentation rate) experiment (Zhou, Yin & Yu, 2006). Earlier, in 1999, a study compared the activity of benzoic acid derivatives with cinnamic acid derivatives in prevention of human LDL oxidation (Natella, Nardini, Felice, & Scaccini, 1999). Ferulic, vanillic, syringic, 4-coumaric (*p*-coumaric acid) and 4-OH (3,4-

hydroxybenzoic) benzoic acids all showed significant activity in prevention of human LDL oxidation (Natella, Nardini, Felice, & Scaccini, 1999) which quenched cation radical $ABTS^{\pm}$, and protected protein molecules from radical attacks, as determined by the oxygen radical absorbing capacity (ORAC) assay (Yeh & Yen, 2003). Our study demonstrated that the extract of Maya nut seed was mainly dominated by the 4-OH benzoic acid (371.2 mg/kg). In addition, Maya nut seed was rich in vanillic acid (110.65 mg/kg). The amounts of 4-OH benzoic acid found in apple juice, tomato, carrot, coffee (drink), red raspberry, strawberry, and black currants were 0.66, 0.51, 55, 1.5, 26, 75, and 39.3 mg/kg, respectively, and the amount of vanillic acid found in tomato, carrot, coffee (drink), red raspberry, strawberry, black mulberries, black currants, and blackberries were 0.23, 8.9, 0.7, 5.2, 1.0, 6.5, 48.3, and 45.1 mg/kg, respectively. The results showed that Maya nut extract possesses much higher vanillic acid and 4-OH benzoic acid than apple juice, tomato, carrot, coffee (drink), red raspberry, strawberry, black mulberries, black currants, and blackberries. However, gallic acid was found in a small amount (27.06 mg/kg). Under the alkaline condition, it was noted that the gallic acid was not stable. Hence, the results obtained for this phenolic after alkaline hydrolysis was too low and are not shown in Table 3.1.

Hydroxycinnamic acid derivatives

Lately much attention has focused on the role and mechanism of several flavonoids as inhibitors of oxidative processes (Van Acker et.al., 1996). Minor attention, however, has been directed to the antioxidant activity of the simple phenolic acids, the derivatives of benzoic and cinnamic acids. These compounds are abundant in plant foods

(i.e., fruits, vegetables) (Herrmann and Nagel, 1989), and therefore a certain quantity of them is consumed in our daily diet. A specific absorption of phenolic acids has been demonstrated both in animal (i.e., rat) and in human tests, and specific metabolites have been identified in human and rat urine consequently (Natella, Nardini, Felice & Scaccini, 1999). In addition, a role of caffeic acid in the antioxidant defense in vivo has been demonstrated in rat (Nardini, Natella, Gentili, Felice & Scaccini, 1997). In the past few years, the antioxidant activity of some of these small monomeric phenols was studied in different model systems. Some phenolic derivatives of cinnamic and benzoic acids were studied for their capacity to reduce ferrylmyoglobin which has the potential for oxidising polyunsaturated fatty acid side chains in low density lipoproteins and to inhibit the LDL oxidative modification induced by metal catalysts or by ferrylmyoglobin (Natella, Nardini, Felice & Scaccini, 1999). The wavelength of maximum absorption of hydroxycinnamic acids is near 320 nm (Määttä, Kamal-Eldin, Törrören, 2001), but these compounds were generally absorbed appreciably at 280 nm in our study. Peaks were assigned as *p*-coumaric acid, caffeic acid, and sinapic acid by comparison with the spectra of the respective standards. The retention times of the hydroxycinnamic acid derivatives ranged from 7 to 30 min, indicating that these compounds are present as derivatives of different degrees of polarity (Määttä, Kamal-Eldin, Törrören, 2001). The content of p-coumaric acid after alkaline hydrolysis is 13.5 mg/kg. The content of caffeic acid after acid hydrolysis is 6.5 mg/kg, while its content after the alkaline hydrolysis is 17.1 mg/kg. The contents of free caffeic acid found in blueberries, black mulberries, black currants, skin of white grape pomace, seeds of white grape pomace, apple (Granny

Smith), and apple juice are 17.4, 4.9, 10.6, 1.7, 1.9, 1.4, and 1.92 mg/kg (Mattila & Kumpulainen, 2002; Kammerer, Claus, Carle & Schieber, 2004; Zadernowski, Naczk & Nesterowicz, 2005). When comparing the caffeic acid amount with these foods, the content of free caffeic acid in Maya nut is lower than blueberries and black currants but higher than black mulberries, skin of white grape pomace, seeds of white grape pomace, apple (Granny Smith), and apple juice. The contents of caffeic acid liberated from glycosides found in black mulberries, apple juice, tomato, red raspberry, and strawberry are 2.7, 22.08, 22.5, 5.1, and 1.86 mg/kg. The bound caffeic acid amount found in Maya nut seed is 17.1 mg/kg. Therefore, Maya nut has higher content of caffeic acid than the black mulberries, red raspberry, and strawberry. Beyond the protective antioxidant behavior, other biological activities of phenolic acids have been reported. Caffeic acid, one of the most prominent naturally occurring cinnamic acids, is known to block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma, and allergic reactions (Koshihara, Neichi, Murota, Lao, Fujimoto & Tatsuno, 1984).

Other studies have reported that caffeic acid and some of its esters might possess antitumor activity against colon carcinogenesis (Olthof, Hollman & Katan, 2001). Recent investigations by Maggi-Capyeron et al. have linked a series of phenolic acids with the inhibition of AP-1 transcriptional activity (Maggi- Capeyron et. al., 2001). AP-1 is an activator protein implicated in the processes that controls inflammation, cell differentiation, and proliferation. The caffeic acid derivatives (e.g., dicaffeoylquinic and dicaffeolytartaric acids) have been shown to be potent for the inhibitors of human

immunodeficiency virus type 1 (HIV-1) integrase (An HIV enzyme). This enzyme is used by the virus to integrate its genetic material into the host cell's DNA. Therefore, these hydroxycinnamate derivatives are currently being investigated for their potential antiviral therapy (King et.al., 1999). Moreover, *in vitro* reaction of caffeic with nitrite in human gastric fluid can inhibit nitrosation of dimethylamine and aminopyrine (Torres y Torres & Rosazza, 2001). Another hydroxycinnamic acid, *p*-coumaric acid was detected only after alkaline hydrolysis. Fergusan, Zhu & Harris demonstrated that the *p*-coumaric acid has the ability to protect against oxidative stress and genotoxicity in cultured mammalian cells (Fergusan, Zhu & Harris, 2005). In addition, the p-coumaric acid can act as an antimutagen to protect against degenerative diseases such as cancer (Fergusan, Zhu & Harris, 2005; Ferguson, Fong, Pearson, Ralph, & Harris, 2003). Antioxidants such as *p*-coumaric acid and other hydroxycinnamic acids serve as chemoprotective agents by reacting with reactive nitrogen species (RNSs) such as nitrite or peroxynitrite to suppress *N*-nitrosamine formation (Torres y Torres & Rosazza, 2001). Torres and Rosazza showed that *p*-coumaric acid might behave as an effective chemoprotective agent by quenching nitrosating agents in various biological cells, including salivary and gastric fluids (Torres y Torres & Rosazza, 2001).

In the study, after the acid hydrolysis, *p*-coumaric was not detected. However, after the alkaline hydrolysis, small amount of *p*-coumaric acid was found. The content of *p*-coumaric acid after the alkaline hydrolysis is 13.5 mg/kg. In addition, epicatechin, known as the subgroup of flavanols, was detected in an amount of 44.74 mg/kg after the alkaline hydrolysis when compared with the authentic standard. The monomeric flavanol

(-)-epicatechin is of particular interest because this compound and its metabolites have been identified as bioactive molecules *in vivo* (Schroeter et al., 2006). (-)-Epicatechin can be absorbed into circulation after ingestion of flavanol-containing foods (Schroeter et al., 2006) and may cross the blood–brain barrier (Abd El Mohsen et al., 2002). Studies in humans demonstrated that (-)-epicatechin-rich foods and purified (-)-epicatechin promoted cardiovascular function (Heiss et al., 2005; Schroeter et al., 2006). Praag *et al*. found that spatial memory was enhanced in mice fed a (-)-epicatechin-containing diet compared with controls. Moreover, the study indicated that genes associated with learning and angiogenesis (the development of blood vessels) were upregulated. However, those genes involved with inflammation and cell death were decreased by (-)-epicatechin consumption (Praag et al, 2007). When comparing the (-)-epicatechin content of Maya nut, cocoa and cholocate that have the highest (-)-epicatechin amounts among foods, the content of (-)-epicatechin in Maya nut is not as high as that in cholocate and cocoa, but is considered with a moderate amount. The (-)-epicatechin contents of milk chocolate, dark chocolate and cocoa are 180, 520, 1580 mg/kg (Gu, House, Wu, Ou & Prior, 2006), which is higher than that in various kinds of black tea. The (-)-epicatechin content in black tea ranges from 1.52 to 14.9 mg/kg among different varieties (Rechner, Wagner, Van Buren, Van de Put, Wiseman & Rice-Evans, 2002).

Another hydroxycinnamic acid presenting in Maya nut after the second acidic hydrolysis is sinapic acid, which is a member of the 4-hydroxy-cinnamic acids. The sinapic acid has also been shown to possess strong antioxidative and antibacterial activity in vitro (Nowak, Kujawa, Zadernowski, Roczniak,&Kozlowska, 1992; Tesaki, Tanabe,

Ono, Fukushi, Kawabata, & Watanabe, 1998). In addition, it has been indicated to be a more effective antioxidant than ferulic acid, which in turn is more effective than *p*-coumaric acid (Cos, et. al., 2002). According to Kylli, Nousiainen, Biely, Sipilä, Tenkanen, and Heinonen, sinapic acid and its derivatives were the most effective antioxidants. Moreover, the esterification to glycosides did not decrease the antioxidant activity of ferulic and sinapic acids. Hydroxycinnamic acids esterified to the primary hydroxyls in glucopyranoside and arabinofuranoside are able to move more freely than in other isomers. Therefore, this chemical modification enables them to function as antioxidants more efficiently (Kylli, Nousiainen, Biely, Sipilä, Tenkanen, & Heinonen, 2008). In our study, we identified that the sinapic acid content in Maya nut after the second acid hydrolysis is 8.165 mg/kg. There were some other peaks in the HPLC chromatograph in our study, but they did not match with any of the standards used in the present study.

3.4 Conclusions

The reported high variability of amounts of individual phenolic acids and the total phenolic acids in foods depends on several factors, such as genetic, agronomic, environmental, and extraction procedures (Cai & Arntfield, 2001; Shahidi, & Naczk, 1992). It must be noted that some minor amounts of phenolics may escape from extraction because of interaction with dietary fibers, proteins, and any other polymerized structures. HPLC analysis of Maya nut extract revealed the presence of hydroxycinnamic and hydroxybenzoic acid phenolics. Among the phenolic compounds in the optimal Maya

nut extract, 3, 4-hydroxybenzoic acid was the major compound present. The phenolic compounds consisted of 3, 4 hydroxybenzoic acid, gallic acid, vanillic acid, caffeic acid, p-coumaric acid, epicatechin, and sinapic acid. Some detected phenolic compounds remained unidentified. The results from this study demonstrated that phenolic compounds are diverse and present in considerable amounts. Therefore, Maya nut is suitable as ingredients in functional foods and could serve as an important antioxidant resource. Further investigations are required to evaluate the antiproliferative activities in order to improve its usage in food and dietary supplemental products for health promotion.

3.5 Figures and Tables

Table 3.1 Recoveries of Phenolic Acids after Different Hydrolysis Conditions at 280 nm (mg per kg of Maya nut)

Phenolic acid	Sample (Maya nut)
Gallic acid	
1	27.06 ± 6.7
2	
3	
3,4-hydroxybenzoic acid	
1	45± 1.9
2	326.2± 1.56
3	16±2.043
Vanillic acid	
1	21.15±7.8
2	103.9 ± 6.01
3	
Caffeic acid	
1	6.5± 1.13
2	17.1± 4.28
3	
P-coumaric acid	
1	
2	13.5± 3.7
3	
Epicatechin	
1	
2	53± 0.24
3	
Sinapic acid	
1	
2	
3	8.165±0.205

Procedures; 1: methanol-acetic acid extraction; 2: alkaline hydrolysis; 3: acid hydrolysis

Values are expressed as means with (standard error)

Figure 3.1 Scheme of the extraction procedures for HPLC analysis of phenolic acids

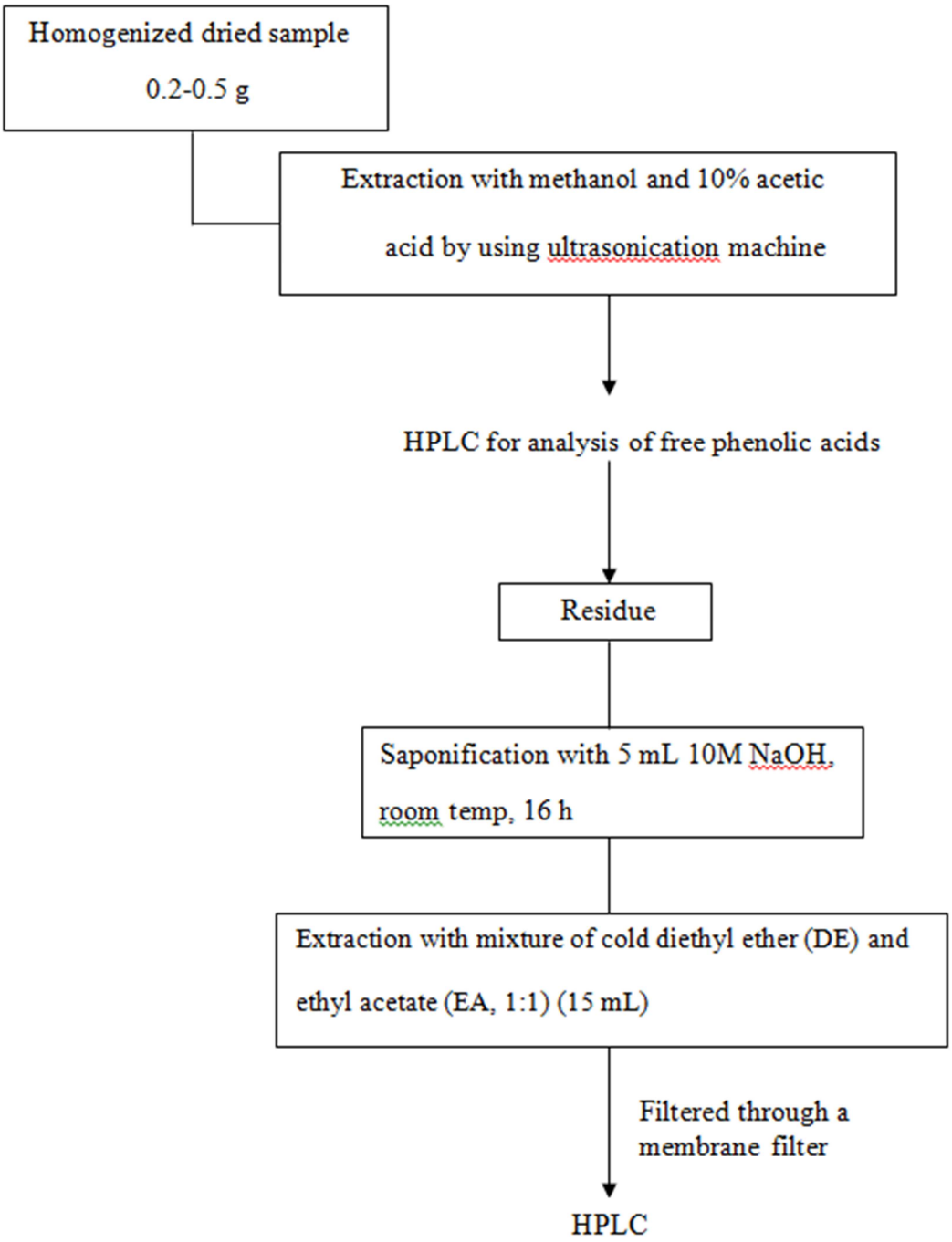


Figure 3.2 Obtained HPLC Chromatogram of phenolic acids after acid hydrolysis

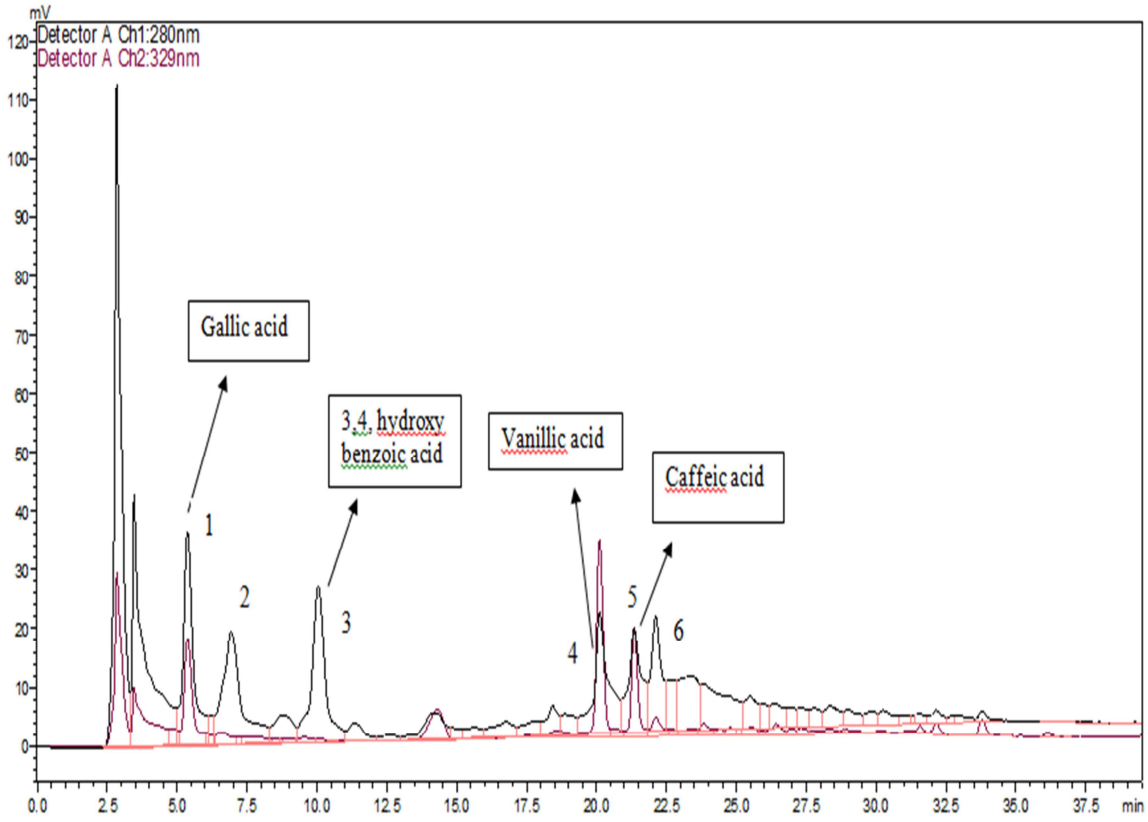


Figure 3.3 Obtained HPLC Chromatogram of phenolic acids after alkaline hydrolysis

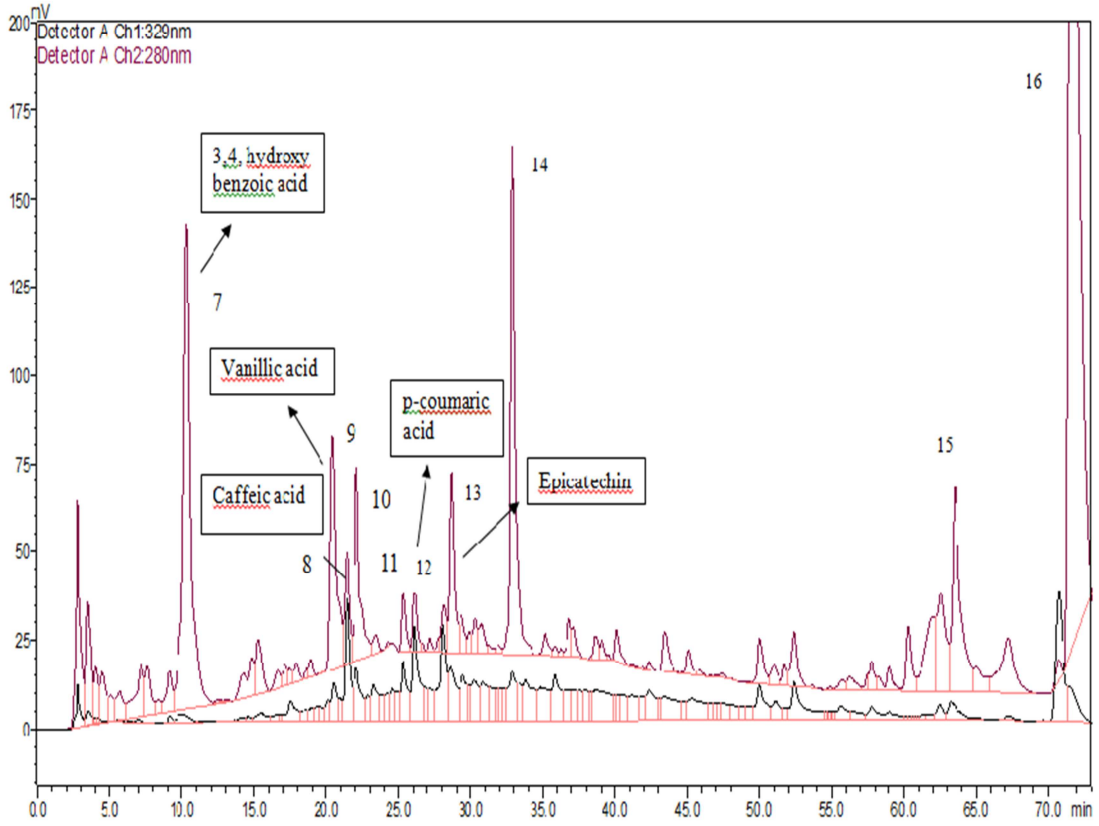


Figure 3.4 Obtained HPLC Chromatogram of phenolic acids after the second acidic hydrolysis

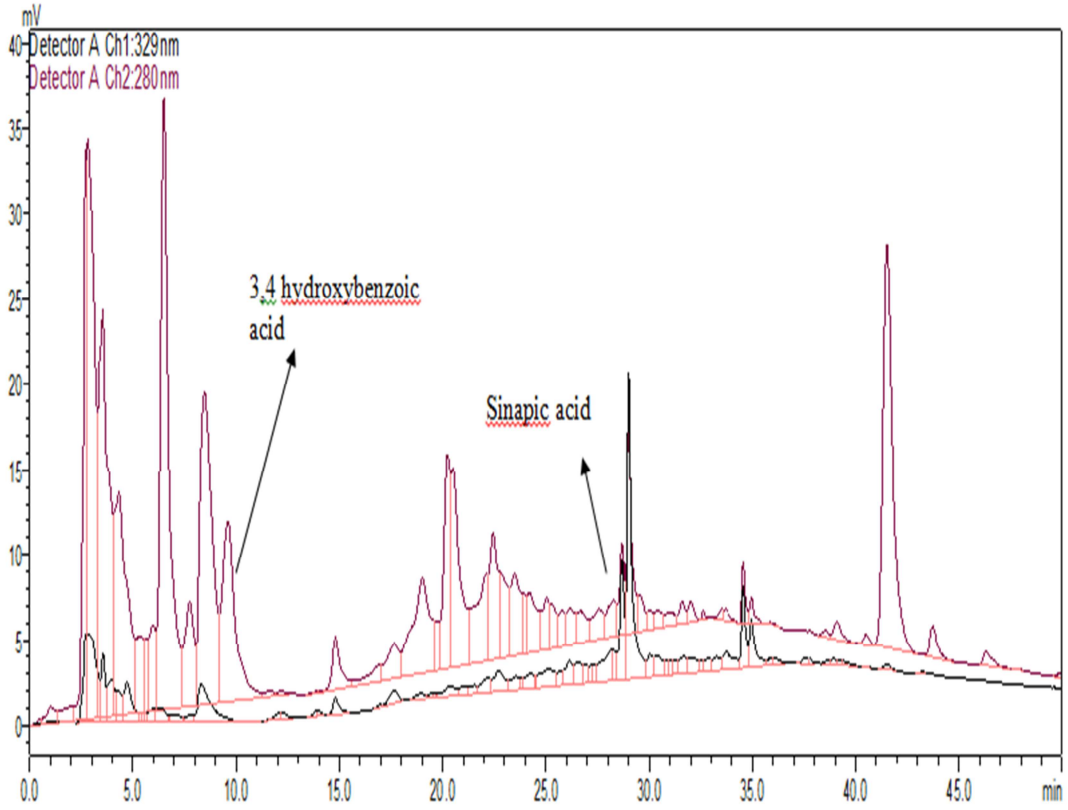
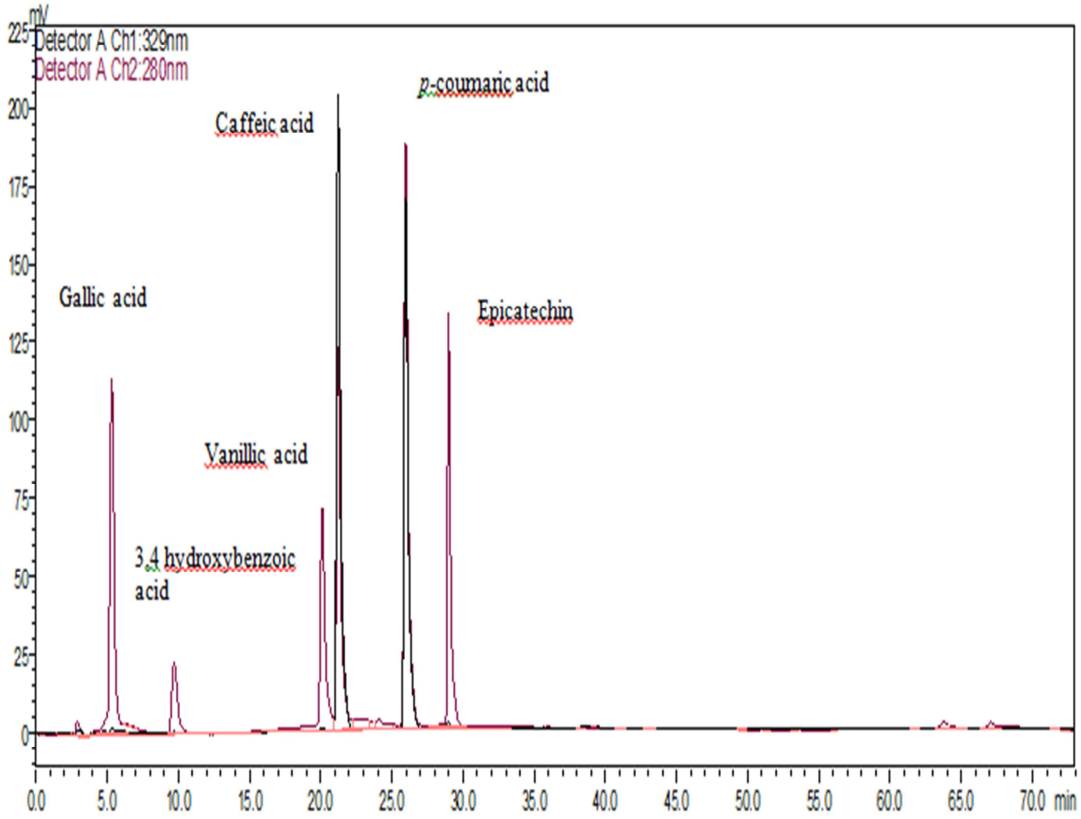


Figure 3.5 Obtained HPLC Chromatogram of mixture of standard phenolic acids



3.6 References

Abd El Mohsen, M.M., Kuhnle, G., Rechner, A.R., Schroeter, H., Rose, S., Jenner, P., Rice-Evans, C.A. (2002). Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. Free Radical Biology Medicine, 33, 1693–1702.

Cai, R., Arntfield, S. D. (2001). A rapid high-performance liquid chromatographic method for the determination of sinapine and sinapic acid in canola seed and meal. Journal of the American Oil Chemists' Society, 78, 903–910.

Cos, P., Rajan, P., Vedernikova, I., Calomme, M., Pieters, L., & Vlietinck, A. J., Augustyns, K., Haemers, A., & Vanden Berghe, D. (2002). In vitro antioxidant profile of phenolic acid derivatives. Free Radical Research, 36, 711–716.

Escarpa, A., & González, M.C. (2001). Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. Analytica *Chimica Acta*, 427, 119-127.

Ferguson, L. R., Fong, I. L., Pearson, A. E., Ralph, J., & Harris, P. J. (2003). Bacterial antimutagenesis by hydroxycinnamic acids from plant cellwalls. Mutation Research, 542, 49–58.

Ferguson, L.R., Zhu, S.-T., & Harris, P.J. (2005). Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29 cells. Molecular Nutrition & Food Research, 49, 585 – 693.

Gu, L., House, S.E., Wu, X., Ou, B., & Prior, R.L. (2006). Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. Journal of Agricultural and Food Chemistry, 54, 4057-4061.

Heiss, C., Kleinbongard, P., Dejam, A., Perre, S., Schroeter, H., Sies, H., Kelm, M. (2005). Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. Journal of the American College of Cardiology, 46, 1276-1283.

Herrmann, K., & Nagel, C.W. (1989). Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. Critical Reviews in Food Science and Nutrition, 28(4), 315-347.

Jacobs, D. R., Meyer, K. A., Kushi, L. H., & Folsom, A. R. (1998). Whole grain intake may reduce risk of coronary heart disease death in postmenopausal women: the Iowa Women's Health Study. American Journal of Clinical Nutrition, 68, 248–257.

Jacobs, D. R., Meyer, H. E., & Solvoll, K. (2001). Reduced mortality among whole grain bread eaters in men and women in the Norwegian Country Study. European Journal of Clinical Nutrition, 55, 137–143.

Kammerer, D., Claus, A., Carle, R., & Schieber, A. (2004). Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera* L.) by HPLC-DAD-MS/MS. Journal of Agricultural and Food Chemistry, 52, 4360-4367.

King, P. J., Ma, G., Miao, W., Jia, Q., McDoughall, B. R., Reinecke, M. G., Cornell, C., Kuan, J., Kim, T. R., & Robinson, Jr. W. E. (1999). Structure-activity relationships: analogues of the dicaffeoylquinic and dicaffeoyltartaric acids as potent inhibitors of human immunodeficiency virus type 1 integrase and replication. Journal of Medicinal Chemistry, 42, 497-509.

Koshihara, Y., Neichi, T., Murota, S.-I., Lao, A.-N., Fujimoto, Y., Tatsuno, T. (1984). Caffeic acid is a selective inhibitor for leukotriene biosynthesis. Biochimica et Biophysica Acta, 792, 92-97.

Krygier, K., Sosulski, F., & Hogge, L. (1982). Free, esterified, and insoluble-bound phenolic Acids. 1. Extraction and purification procedure. Journal of Agricultural and Food Chemistry, 30, 330-334

Kylli, P., Nousiainen, P., Biely, P., Sipilä, J., Tenkanen, M., & Heinonen, M. (2008). Antioxidant potential of hydroxycinnamic acid glycoside esters. Journal of Agricultural and Food Chemistry, 56, 4797- 4805.

Lapornik, B., Prošek, M., & Wondra, A. G. (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. Journal of Food Engineering, 71, 214-222.

Leung, J., & Fenton, T.W., & Clandinin, D.R. (1981). Phenolic components of sunflower flour. Journal of Food Science, 46(5), 1386-1388.

Määttä, K., Kamal-Eldin, A., & Törrören, R. (2001). Phenolic compounds in berries of black, red, green, and white currants (*Ribes* sp.). Antioxidants & Redox Signaling, 3(6), 981-993.

Maga, J. A. (1978). Simple phenol and phenolic compounds in food flavor. Critical Reviews in Food Science and Nutrition, 10, 323-372.

Maggi-Capeyron, M.-F., Ceballos, P., Cristol, J.-P., Delbosc, S., Le Doucen, C., Pons, M., Leger, C. L., & Descomps, B. (2001). Wine phenolic antioxidants inhibit ap-1 transcriptional activity. Journal of Agricultural and Food Chemistry, 49, 5646-5652.

Mattila, P., & Kumpulainen, J. (2002). Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. Journal of Agricultural and Food Chemistry, 50, 3660-3667.

Nardini, M., Natella, F., Gentili, V., Di Felice, M., Scaccini, J. (1997). Effect of caffeic acid dietary supplementation on the antioxidant defense system in rat: An *in vivo* study. Archives of Biochemistry and Biophysics, 342(1), 157-160.

Natella, F., Nardini, M., Felice, M.D., & Scaccini, C. (1999). Benzoic and cinnamic acid derivatives as antioxidants: structure–activity relation. Journal of Agricultural and Food Chemistry, 47, 1453–1459.

Nowak, H., Kujawa, K., Zadernowski, R., Roczniak, B.,& Kozlowska, H. (1992). Antioxidative and bactericidal properties of phenolic compounds in rapeseeds, Fat Science Technology, 94, 149–152.

Olthof, M. R., Hollman, P. C. H., Katan, M. (2001). Chlorogenic acid and caffeic acid are absorbed in humans. Human Nutrition and Metabolism, 131, 66-71.

Praag, H.V., Lucero, M.J., Yeo, G.W., Stecker, K., Heivand, N., Zhao, C., Yip, E., Afanador, M., Schroeter, H., Hammerstone, J., Gage, F.H. (2007). Plant-derived flavanol (–)epicatechin enhances angiogenesis and retention of spatial memory in mice. The Journal of Neuroscience, 27, 5869-5878.

Rechner, A.R., Wagner, E., Van Buren, L., Van de Put, F., Wiseman, S., & Rice Evans, C.A. (2002). Black tea represents a major source of dietary phenolics among regular tea drinkers. Free Radical Research, 36(10), 1127-1135.

Robbins, R.J. (2003). Phenolic acids in foods: An overview of analytical methodology. Journal of Agricultural and Food Chemistry, 51, 2866-2887.

Rommel, A. & Wrolstad, R.E. (1993). Influence of acid and base hydrolysis on the phenolic composition of red raspberry juice. Journal of Agricultural and Food Chemistry, 41, 1237- 1241.

Rounds, M.A., & Gregory, J.F. (2003). High performance liquid chromatography. In Neilsen, S.S. Food Analysis, 3rd Edition. pp. 461-478. Newyork: Plenum Publishers.

Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C.L., Hollenberg, N.K., Sies,H., Kwik-Uribe, C., Schmitz, H.H., Kelm, M. (2006). (-) Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proceedings of the National Academy of Sciences, 103, 1024 –1029.

Sellappan, S., Akoh, C.C., & Krewer, G. (2002). Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. Journal of Agricultural and Food Chemistry, 50, 2432-2438.

Shahidi, F., & Naczk, M. (1992). An overview of the phenolics of canola and rapeseed: chemical, sensory and nutritional significance. Journal of the American Oil Chemists' Society, 69, 917–924.

Stalikas, C.D. (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. Journal of Seperation Science, 30, 3268-3295.

Tesaki, S., Tanabe, S., Ono, H., Fukushi, E., Kawabata, J., & Watanabe, M. (1998). 4-Hydroxy-3-nitrophenylacetic and sinapic acids as antibacterial compounds from mustard seeds. Bioscience, Biotechnology, and Biochemistry. 62, 998–1000.

Tomás-Barberán, F.A., & Espín, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. Journal of Agricultural and Food Chemistry, 81, 853-876.

Torres y Torres, J.L., & Rosazza, J.P.N. (2001). Reactions of *p*-coumaric acid with nitrite: product isolation and mechanism studies. Journal of Agricultural and Food Chemistry, 49, 1486-1492.

Van Acker, S.A.B.E., Dirk-jan Van Den Berg, Tromp, M.N.J.L., Griffioen, D.H., Bennekom, W.P.V., Van Der Vijgh, W.J.F., & Bast, A. (1996). Structural aspects of antioxidant activity of flavonoids. Free Radical Biology & Medicine, 20(3), 331-342.

Yeh, C.-T., & Yen, G.-C. (2003). Effects of phenolic acids on human phenolsulfotransferas in relation to their antioxidant activity. Journal of Agricultural and Food Chemistry, 51, 1474–1479.

Zadernowski, R., Naczk, M., & Nesterowicz, J. (2005). Phenolic acid profiles in some small berries. Journal of Agricultural and Food Chemistry, 53, 2118-2124.

Zheng, W., & Wang, S.Y. (2001). Effect of plant growth temperature on antioxidant capacity in strawberry. Journal of Agricultural and Food Chemistry, 49, 4977-4982.

Zhou, K., Yin, J. J., & Yu, L. L. (2006). ESR determination of the reactions between selected phenolic acids and free radicals or transition metals. Food Chemistry, 95, 446-457.

SUMMARY

Maya nut (*Brosimum alicastrum*) contains some nutritional components which were investigated in this study. Its fatty acid composition was compared with that of other two commercially edible oils: i.e., olive oil and canola oil. The results demonstrated that Maya nut contained 27.2% palmitic acid (16:0), 6.26% stearic acid (18:0), 9.9% linolenic acid (18:3), 55% linoleic acid (18:2), 3.36% Cis-11-Eicosenoic acid (20:1), or 64.96% PUFA and 3.36% MUFA. Results from this study showed that Maya nut had higher PUFA but lower MUFA than olive and canola oil. In addition, proximate analyses, antioxidant capacities, and total phenolic content of Maya nut and other commercially available nuts such as walnut, almond, and peanut were measured. Maya nut has the highest ash content and the lowest lipid content among the nuts. In the FRAP assay, walnut showed the greatest ability to induce the TPTZ-Fe^{3+} complex to the TPTZ-Fe^{2+} complex, followed by the Maya nut, peanut, and almond. Besides, at concentration of 1 mg/mL, the DPPH remaining % of the Maya nut, walnut, almond, peanut, and standard antioxidant BHT were 83.35%, 71.58%, 18.1%, 9%, and 76.46%, respectively. The level of DPPH scavenging activity of Maya nut was close to that of BHT at 400 and 500 ppm concentration. The ABTS free radical scavenging values ranged from 58.96 to 92.55% among the nuts, but the Maya nut extract possessed the highest antioxidant capacity (92.55% of ABTS inhibition). Moreover, we have found high correlations between the DPPH scavenging activities and ABTS radical scavenging capacities of the antioxidant extracts (R^2 = 0.9139). HPLC analysis of the Maya nut extract revealed the presence of hydroxycinnamic and hydroxybenzoic acid phenolics. The phenolic compounds in the

Maya nut extract include 3, 4-hydroxybenzoic acid, gallic acid, vanillic acid, caffeic acid, p-coumaric acid, epicatechin, and sinapic acid, among which 3, 4-hydroxybenzoic acid was the major compound. The result demonstrated that the phenolic compounds are diverse and present in considerable amounts. In summary, Maya nut contains valuable nutritional chemicals and is suitable to be made into functional foods and/or serves as an antioxidant resource for nutraceuticals. Regarding the progress of this research, it is recommended to take further investigations to evaluate more bioassays such as antiproliferative activities in order to improve its usage in food and dietary supplemental products for health promotion.

APPENDICES

APPENDIX A

A Letter of Authority

From: Erica Vohman, the Founder of Equilibrium Fund. (mayanut@gmail.com)

To: Hatice Kubra Tokpunar, Master student, Department of Food Science and Human Nutrition, Clemson University, USA. (htokpun@clemson.edu)

Original e-mail:

From:erika vohman (mayanut@gmail.com)

Date: 27 October 2010 Wednesday

To: hatice tokpunar (htokpunar@hotmail.com); mayanut@gmail.com

Dear Hatice,

Please use this email for all correspondence, thank you.

You may use the photo and the distribution map if you like.

Can you tell me how Maya Nut compares to green tea in antioxidant content?

thank you and good luck! I am very excited about your thesis.

Erika

From: **hatice tokpunar** (htokpunar@hotmail.com)

Date: 27 October,2010 Wednesday

To: info@theequilibriumfund.org

Hi Ms. Vohman,

My name is Hatice Kubra Tokpunar and I am a master student in Food Science Program in Clemson University, USA.

Can I obtain permission from you to add some figures to my thesis?

One of is the picture of the full size of Maya nut tree from Maya nut brochure in Equilibrium Fund web site and the other is figures of nutrition values of Maya nut Brochure in Equilibrium Fund web site. Also, There is picture which shows the distribution of *Brosimum alicastrum, sub species alicastrum* and it is from Maya Nut (*Brosimum alicastrum*) International symposium notes and its name is "Underutilized plants for food, nutrition, income and sustainable development".

I really appreciate your time and help,

Have a good day,

Sincerely,

Hatice Kubra Tokpunar
Master candidate
Department of Food Science and Human Nutrition
P&A Building A201
Clemson University, SC, 29634
htokpun@clemson.edu
htokpunar@hotmail.com

APPENDIX B

A Letter of Authority

Original e-mail:

From: Cecilia Sanchez Garduño, Directora Mexico, www.mayanutinstitute.com. (sanchez_garduno@yahoo.com)

To: Hatice Kubra Tokpunar, Master student, Department of Food Science and Human Nutrition, Clemson University, USA. (htokpun@clemson.edu)

Date: 10/29/2010

Dear Hatice

I'm very sorry for not replying earlier. The figure you say, I imagine, is original to Berg's (Moraceae specialist) work on the distribution of Brosimum. I can send you the bibliographic reference if you don't have it. I think you can use it as long as you put the original source. Erika has told me about your interesting thesis. I can't wait to read it when its published. My biggest congratulations.

best wishes

cecilia

Dra. Cecilia Sanchez Garduño
Directora Mexico
www.mayanutinstitute.com
Buscamos el balance entre las comunidades, la alimentación y los bosques
Matanzas 659, Lindavista
Mexico D.F. 07300
tel (55) 5754 1212
cel 04455 1798 6205
skype ceciliasanchezgarduno

From: Hatice Kubra Tokpunar

To: Cecilia Sanchez-Garduno

Date: 10/27/2010

Hi Ms. Dr.Cecilia,

My name is Hatice Kubra Tokpunar and I am a master student in Food Science Program in Clemson University, USA. Can I obtain permission from you to add this figure to my thesis? It represents Distribution of *Brosimum alicastrum, sub species alicastrum* and it is from Maya Nut (*Brosimum alicastrum*). Traditional rainforest food for healthy forests and families in Central America. International symposium "Underutilized plants for food, nutrition, income and sustainable development".

I really appreciate your time and help,

Have a good day,

Sincerely,

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Master candidate
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