A STUDY OF THE NUTRITIONAL AND MEDICINAL VALUES OF *MORINGA OLEIFERA* LEAVES FROM SUB-SAHARAN AFRICA: GHANA, RWANDA SENEGAL AND ZAMBIA

by

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ABSTRACT OF THE THESIS

A Study of the Nutritional and Medicinal Values of *Moringa oleifera* Leaves from Sub-Saharan Africa: Ghana, Rwanda, Senegal and Zambia

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Moringa oleifera is an important multipurpose tropical tree under-recognized for its nutritional and medicinal properties. Leaves of *M. oleifera* collected from the sub-Saharan African countries of Ghana, Rwanda, Senegal and Zambia were analyzed for their nutritional value and bioactivity by UV-Vis spectrophotometry and HPLC coupled with UV and MS detectors. Moringa leaves were found to provide low amounts of vitamin C (0.351 ± 0.046 to 0.749 ± 0.014 mg/100g dry weight (DW) as determined using UV spectrophotometry. Using LC/MS, We identified α- and γ- tocopherols, α- and β- carotenes, six analogues of chlorogenic acid including 4 caffeoylquinic acids and 2 coumaroylquinic acids (structural and/or spatial isomers), and chlorogenic acid was used as the standard to estimate the amount of these six phenolic acids. Chlorogenic acid was used as the standard to estimate the amount of these six cumulative isomers by LC/MS. Additionally, in Moringa leaves we detected one sample containing as many as 12 flavonoids including quercetin and kaempferol glycosides with malonyl, acetyl and succinoyl acylations, among which; quercetin and kaempferol glucosides and glucoside

malonates are the major constituents based on analysis of their UV and MS data. Using LC/UV/MS, the quercetin and kaempferol aglycones of quercetin and kaempferol in acidic hydrolyzed extracts were successfully separated within 10 min and individually quantitated. A total of 25 samples were assayed for the chlorogenic acid isomers analogs (0.181 to 0.414 mg/100 g DW), tocopherols (7.1 to 116 mg/100 g DW), carotenoids (4.49 to 45.94 mg/100 g DW) and flavonoids (0.179 to 1.643 % g DW). The concentrations of these phytochemicals varied according to the environment, country of collection, genetics, and variety of *Moringa oleifera*. The nutritional and natural products characterization of Moringa conducted in this study show that the leaves of this plant can contribute significantly to the daily recommended allowance needed for many vitamins and mineral needs as well as serve as a rich source of polyphenols, confirming the importance and role that Moringa can play to improve the health and nutrition particularly in malnourished populations.

Dedication

To my parents, Emelda and Cyril Coppin and my granny, Iona Barrow, who have always been there with and for me through the uplifting times as well as the struggles. If not for you all, I would not be where I am today. I am forever indebted to you. Thank you Lord.

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CHAPTER 1

INTRODUCTION

Moringa oleifera Lam. (Moringaceae) is one of the 14 species of family Moringaceae, native to India, Africa, Arabia, Southeast Asia, South America, and the Pacific and Caribbean Islands (Iqbal et al, 2006). Because *M. oleifera* has been naturalized in many tropic and sub-tropic regions worldwide, the plant is referred to by a number of names such as horseradish tree, drumstick tree, ben oil tree, miracle tree, and "Mother's Best Friend" (Shindano & Chitundu, 2008-unpublished). According to Muluvi et al (1999), the Moringa tree was introduced to Africa from India at the turn of the twentieth century where it was to be used as a health supplement.

The Moringa plant has been consumed by humans throughout the century in diverse culinary ways (Iqbal et al, 2006). Almost all parts of the plant are used culturally for its nutritional value, purported medicinal properties and for taste and flavor as a vegetable and seed. The leaves of *M. oleifera* can be eaten fresh, cooked, or stored as a dried powder for many months reportedly without any major loss of its nutritional value (Arabshahi-D et al, 2007; Fahey, 2005). Epidemiological studies have indicated that *M. oleifera* leaves are a good source of nutrition and exhibit anti-tumor, anti-inflammatory, anti-ulcer, anti-atherosclerotic and anti-convulsant activities (Chumark et al, 2008; DanMalam et al, 2001; Dahiru et al, 2006). The investigation of the different parts of the plant is multidisciplinary, including but not limited to nutrition, ethnobotany, medicine, analytical chemistry, phytochemistry and anthropology (McBurney et al, 2004).

Botanical Background of *Moringa oleifera*

Moringa is a tree ranging in height from 5-12 m with an open umbrella-shaped crown, straight trunk (10-30 cm thick) and a corky, whitish bark. The plant (depending on climate) has leaflets 1-2 cm in diameter and 1.5-2.5 cm in length. The tree produces a tuberous tap root which explains its tolerance to drought conditions. Originally considered a tree of hot semi-arid regions (annual rainfall 250-1500 mm), Moringa is adaptable to a wide range of environmental conditions from hot and dry to hot, humid, wet conditions. The tree is tolerant to light frosts, but does not survive as a perennial under freezing conditions. Moringa grows more rapidly, reaching higher heights, when found in well-drained soils with ample water, but tolerates both sandy soils, heavier clay soils and water limited conditions. The tree can be established in slightly alkaline soils up to pH 9 as well as acidic soils as low as pH 4.5 (Shindano & Chitundu, 2008-unpublished) and is well suited for a wide range of adverse environments that would not be suitable for other fruit, nut and tree crops.

Moringa can be found in the wild or cultivated and sold as a supplement on the health market or added to drinks such as Moringa Zinga (www.zija-Moringa.org/zija-Moringa-faq.htm). In India and different parts of Africa, it is cultivated on a large scale in nurseries or orchards. Cultivation entails collection of seeds from the tree, development of plantlets in the greenhouse for 2 to 3 months and transplantation of mature stems (1-1.5 m long) to the main fields (Shindano & Chitundu, 2008-unpublished). The leaves, seeds, flowers, pods (fruit), bark and roots are all seen as a vegetable and each part is uniquely harvested and utilized. For example, fresh leaves are picked, shade dried, ground to a powder, and then stored for later as a food flavoring or

additive. Dried or fresh leaves are also used in foods such as soups and porridges (Lockett et al, 2000), curry gravy and in noodles, rice or wheat (Abilgos et al, 1999). Farmers have added the leaves to animal feed to maintain a healthy livestock (Sarwatt et al, 2002; Fahey, 2005; Sáncheza et al, 2006) while utilizing the manure and vegetable compost for crop growth (Fahey, 2005; SaveGaia International Foundation, 2005). Newer applications include the use of Moringa powder as a fish food in aquacultural systems (Dongmeza et al, 2006) and the Moringa leaves as a protein supplement for animals, such as cows. The feeding value of Moringa (Table 1.1) has been reported to be similar to that of soybeans and rapeseed meal (Soliva et al, 2005). With the leaves being rich in nutrients, pregnant women and lactating mothers use the powdered leaves to enhance their child's or children's nourishment, especially in under developed countries suffering from malnutrition (McBurney et al, 2004; Lockett et al, 2000; WHO Readers Forum, 1999).

The seeds contain much of the plant's edible oil which is used as a cooking oil for frying and as a salad oil for dressing. The fatty acid compositions of solvent and enzyme-extracted oil from *M. oleifera* seeds showed 67.9% oleic acid in the solvent extract and 70.0% in the enzyme extracts. Other prominent fatty acids in Moringa oil include palmitic (7.8% and 6.8%), stearic (7.6% and 6.5%), and behenic (6.2% and 5.8%) acids for the solvent and enzyme-extracted oils, respectively (Abdulkarima et al, 2005). Due to the high monounsaturated to saturated fatty acid ratio, Moringa seed oil could be considered an acceptable substitute for highly monounsaturated oils such as olive oil (Tsaknis et al, 2002). Moringa oil utilized as a frying oil can be a healthy alternative to other commonly used oils such as palm, canola and soybean oil when comparing fatty

acid composition (Table 1.2). Oils with high amounts of monounsaturated (oleic type) fatty acids are desirable due to an association with decreased risk of coronary heart disease (Mensink et al, 1990; Aldulkarim et al, 2007).

The whole seeds can also be eaten green, roasted or powdered, and steamed in tea and curries (Fahey, 2005). The pods and seeds, often referred to as Moringa kernels, have a taste that ranges from sweet to bitter and are most popularly consumed after frying to get a peanut-like taste (Makkar et al, 1996).

Moringa leaf has been purported to be a good source of nutrition and a naturally organic health supplement that can be used in many therapeutic ways (McBurney et al, 2004; Fahey, 2005; DanMalam et al, 2001). The leaves are a very rich source of nutrients and contain the essential vitamins A, C and E. Though not proven, it is has been considered by many to contain as much vitamin A as a carrot, vitamin C as an orange and vitamin E as a pomegranate. Leaves rich in biologically active carotenoids, tocopherols and vitamin C have health-promoting potential in maintaining a balanced diet and preventing free-radical damage that can initiate many illnesses (Smolin at al, 2007). While the provitamins cannot be identified in the leaves, they can be monitored after conversion to their respective vitamins within the body. The edible Moringa leaves contain essential provitamins, including ascorbic acid, carotenoids (Lako et al, 2007) and tocopherols (Gomez-Conrado et al, 2004; Sánchez-Machado et al, 2006).

Epidemiological studies have demonstrated that vegetables and fruits rich in carotenoids are related to a lower risk of cancer, cardiovascular disease, age-related macular degeneration and the formation of cataracts (Lakshminarayan et al, 2005; Bowman et al, 1995; Krichevsky et al, 1999). Identification of these vitamins would be a

great advantage to the nutritional attributes of the Moringa leaves. In addition to the provitamins, Moringa leaves are also considered a rich source of minerals (Gupta et al, 1989), polyphenols (Bennett et al, 2003), flavonoids (Lako et al, 2007; Siddhuraju et al, 2003), alkaloids, and proteins (Solvia et al, 2005; Sarwatt et al, 2002). These essential nutrients can help decrease the nutritional deficit and combat many chronic inflammatory diseases.

According to Hartwell (1967-1971), the leaves were used in traditional remedies for tumors (Faizi et al, 1998; Guevara et al, 1999) and extensively used as a natural sleep aid, applied as a poultice to sores, rubbed on temples for headaches, and as a purgative cleanser (Fuglie, 1999; Fahey, 2005). These applications address the use of *M. oleifera* leaves in the food industry, as a synergistic natural product applied to ethnic foods, and the medical industry, as a preventative for numerous diseases (Abilgos et al, 2003, Fahey, 2005, Miean et al, 2001; Middleton et al, 2000, Lockett et al, 2000).

A number of natural compounds have been isolated from *M. oleifera* leaves including fully acetylated glycosides bearing thiocarbamates, carbamates or nitriles (Faizi et al, 1995; Murakami et al, 1998). Glycosides containing isothiocyanates, malonates and flavonoids have also been identified and isolated in the leaves of the Moringa plant (Faizi et al, 1994; Bennett et al, 2003; Miean et al, 2001). In particular, quercetin and kaempferol glycosides are broken down to yield the natural antioxidant flavonoids, quercetin and kaempferol, indicating these glycosides can be efficiently hydrolyzed to their respective aglycones (Miean et al, 2001; Bennett et al, 2003; Wu et al, 2003). Many plant glycosides can be used as treatments for cancer or chronic conditions such as high cholesterol and atherosclerosis (Chumark et al, 2008; Ghasi et al, 2000; Murakami et al,

1998). Flavonoids exist widely in the plant kingdom and are especially common in leaves, flowering tissues and pollens. Plant flavonoids are important to the diet because of their effects on human nutrition. These phytochemicals can modulate lipid peroxidation involved in atherogenesis, carcinogenesis and thrombosis and other known properties of free radical scavenging or inhibition of hydrolytic and oxidative enzymes (phospholipidase A2, cyclooxygenase, lipooxygenase) shows strong antioxidant and anti-inflammatory activity (Siddhuraju et al, 2003). Numerous studies have indicated that flavonoids also have anti-carcinogenic, anti-viral and anti-estrogenic activities (Havsteen, 2002; Miean et al, 2001; Middleton et al., 2000). A high intake of flavonoids has been linked with a reduced risk of cardiovascular disease, osteoporosis and other age-related degenerative diseases (Havsteen, 2002; Middleton et al., 2000; Morris et al., 2006). For example, much of the interest has recently been focused on using flavonoids anti-cancerous properties (Faizi et al, 1998; Guevara et al, 1999; Miean et al, 2001) as well as using quercetin and kaempferol to fight osteoporosis (Prouilleta et al, 2004). These identified bioactive compounds in the leaves of *M. oleifera* make this an excellent candidate for nutritional and pharmaceutical supplementation.

The World Health Organization (WHO) has been studying the use of *M. oleifera* for many decades as a low cost supplement enhancer in the poorest countries around the world (WHO Readers Forum, 1999). This organization has been promoting the use of this the plant to help those countries suffering from malnutrition, which is one of the major causes of death worldwide. United Nations Food and Agriculture reported that one in twelve people worldwide is malnourished, including 160 million children under the age of 5 (United Nations Food and Agriculture Statistics, 2008).

Even though various studies have been done on the Moringa species in India, there are few experimental studies regarding the potential nutritional value of the plant grown in Africa. These includes the total vitamins, mineral, protein and phenolic content (McBurney, 2005). The research conducted in this study seeks to assess the nutritional and medicinal values of *M. oleifera* leaves from sub-Saharan Africa, specifically Ghana, Rwanda, Senegal and Zambia. Our objectives are to quantitate the antioxidant capacity, ascorbic acid, minerals, total phenols, total proteins and total alkaloids, and to individually quantify the polyphenols: chlorogenic acid analogs, ellagic acid and gallic acid reported in the leaves. A secondary objective of this study also seeks to quantitatively analyze the provitamins: β-carotene, α-carotene, α-tocopherol, γ-tocopherol, and the biologically active flavonoids: quercetin and kaempferol in the same leaf samples to get an overarching idea of the nutritional value of this species.

To accomplish these objectives, this thesis is organized into ten chapters. Here in the first chapter, we described the attributes and background of this unique plant, which can be used to improve health and nutrition and describe our objectives. In chapter 2, we present the underlying description of the Moringa material we analyzed by first characterizing the material. Here, we analyze the percent moisture in the leaves as well as the total ashes and acid insoluble ashes which indicates the amount of minerals in the leaves. Chapter 3 focuses on the some of the moringa's medicinal and bioactive aspects including the total antioxidant capacity, phenols, alkaloids and proteins present in the leaves. In chapter 4, we next turn our focus onto the nutrient quality of Moringa by presenting the total individual range of minerals is identified in the leaves. In chapters 5 through 9, we focus on vitamin C, phenolic acids, vitamin E, vitamin A and flavonoid

content, respectively. In these chapters, we identify and quantify all the vitamin E and A compounds and isomeric compounds by LC/MS. The identification and quantitation of these phytochemicals in the Moringa leaves serves to provide both the conformational and new scientific information on the nutritional and potential medicinal applications in the use of Moringa, which permits the comparison to other traditional plants used for similar purposes. We then conclude with an overall summary to tie the findings reported in the different chapters together.

Table 1.1. Composition of crude protein of dietary ingredients used for cows (Soliva et al, 2005)

Organic Matter	Crude Protein
Soybean meal	584
Rapeseed meal	391
Unextracted *Moringa oleifera* leaves	321
Extracted *Moringa oleifera* leaves	486

Values are expressed as g/kg DW

Table 1.2. Fatty acid composition of oils in a frying test (Abdulkarim et al, 2007)

Oils	Fatty Acids			
	Palmitic Acid	Oleic Acid	Linoleic Acid	Monosaturated Fatty Acids
Palm Olein	37.7	45.6	10.8	46.3
Soybean	8.9	57.4	22.8	58.6
Canola	11.3	24.8	53.5	25.1
Moringa oleifera	6.1	74.5	0.7	78.1

The results are expressed as a percentage/DW

Table 1.3. The traditional food and medicinal applications of *Moringa oleifera*

Parts of the tree	Uses	Traditional methods of preparation	Medicinal Purposes	Reference(s)
Leaves*	Salads, vegetables curries, powder for scrubbing utensils (1,5,12)	Fresh or dried leaves are ground to a powder and used to prepare salves (1, 2)	Treating tumors; as poultice for sores, reduce glandular swelling and headaches, to purge or a body cleanser, to promote digestion; traditional medicine as a hypocholesterolemic agent in obese individuals (1-12)	1. Fahey, 2005 2. Chumark, 2008; 3. Lockett et al, 2000 4. Dahiru et al, 2006 5. Hartwell, 1967-1971 6. Guevara et al,1999 7. Makkar et al, 1996 8. Murakami et al, 1998 9. Fuglie et al, 1999 10. Ghasi et al, 2000 11. Shindano & Chitundu, 2008-unpublished 12. Mcburney,
Seeds	Eaten as a snack, oil for salads, cooking, cosmetics, lubricant; water purifying(1,11,12)	Seeds are prepared green, roasted or powdered, steamed and extracted as an oil(1,12)	Treats abdominal tumors; removes harmful bacteria(1,12)	
Flowers	For honey(1,11)	Flower extracts are used for preparations(1,11)	Folk remedies for tumors(1,11)	
Bark	For tanning(1)	Decoctions for creams or emollients(1)	Promote digestion(1)	

Pods	Eaten as a fruit in salads [12]	Prepared fresh or roasted [1,12]	treat malnutrition[12]	2004
Root	As a substitute for horseradish [1,12]	Prepared as decoctions [1]	Treatment of tumors; promotes digestion [1]	

Table 1.4. Dietary Reference Intakes (DRIs): Tolerable Upper Intake Levels, Vitamins

Life Stage Group	Ca (mg/d)	K (g/d)	Mg (mg/d)	P (mg/d)	Mn (mg/d)	Fe (mg/d)	Cu (µg/d)	B (mg/d)	Al (mg/d)	Zn (mg/d)	Na (g/d)
Infants											
0-6 mo	210	0.4	30	100	0.003	0.27	200	ND*	ND	2	0.12
7-12 mo	270	0.7	75	275	0.6	11	220	ND*	ND	3	0.37
Children											
1-3 y	500	3.0	80	460	1.2	7	340	2.5*	ND	3	1.0
4-8 y	800	3.8	130	500	1.5	10	440	2.5*	ND	5	1.2
Males											
9-13 y	1300	4.5	240	1250	1.9	120	700	2.5*	ND	8	1.5
14-18 y	1300	4.7	410	1250	2.2	150	890	2.5*	ND	11	1.5
19-30 y	1000	4.7	400	700	2.3	150	900	2.5*	ND	11	1.5
31-50 y	1000	4.7	420	700	2.3	150	900	2.5*	ND	11	1.5
51-70 y	1200	4.7	420	700	2.3	150	900	2.5*	ND	11	1.3
>70 y	1200	4.7	420	700	2.3	150	900	2.5*	ND	11	1.2
Females											
9-13 y	1300	4.5	240	1250	1.6	120	700	2.5*	ND	8	1.5
14-18 y	1300	4.7	360	1250	1.6	150	890	2.5*	ND	8	1.5
19-30 y	1000	4.7	310	700	1.8	150	900	2.5*	ND	8	1.5
31-50 y	1000	4.7	320	700	1.8	150	900	2.5*	ND	8	1.5
51-70 y	1200	4.7	320	700	1.8	150	900	2.5*	ND	8	1.3
>70 y	1200	4.7	320	700	1.8	150	900	2.5*	ND	8	1.2
Pregnancy											
14-18 y	1300	4.7	400	1250	2.0	220	1000	2.5*	ND	12	1.5

19-30 y	1000	4.7	350	700	2.0	220	1000	2.5*	ND	11	1.5
31-50 y	1000	4.7	360	700	2.0	220	1000	2.5*	ND	11	1.5
Lactation											
14-18 y	1300	1.5	360	1250	2.6	290	1300	2.5*	ND	13	1.5
19-30 y	1000	1.5	310	700	2.6	290	1300	2.5*	ND	12	1.5
31-50 y	1000	1.5	320	700	2.6	290	1300	2.5*	ND	12	1.5

Table 1.5. Dietary Reference Intakes (DRIs): Recommended Dietary Allowances and Adequate Intakes, Vitamins

Life Stage Group	**Vitamins A (µg/d)**	**Vitamin C (mg/d)**	**Vitamin E (mg/d)**
Infants			
0-6 months	400*	40*	4*
7-12 months	500*	50*	5*
Children			
1-3 years	**300**	**15**	**6**
4-8 years	**400**	**25**	**7**
Males			
9-13 years	**600**	**45**	**11**
14-18 years	**900**	**75**	**15**
19-30 years	**900**	**90**	**15**
31-50 years	**900**	**90**	**15**
51-70 years	**900**	**90**	**15**
>70 years	**900**	**90**	**15**
Females			
9-13 years	**600**	**45**	**11**
14-18 years	**700**	**65**	**15**
19-30 years	**700**	**75**	**15**
31-50 years	**700**	**75**	**15**
51-70 years	**700**	**75**	**15**
>70 years	**700**	**75**	**15**
Pregnancy			
14-18 years	**750**	**80**	**15**
19-30 years	**770**	**85**	**15**
31-50 years	**770**	**85**	**15**
Lactation			
14-18 years	**1200**	**115**	**19**
19-30 years	**1300**	**120**	**19**
31-50 years	**1300**	**120**	**19**

This table (taken from Dietary Reference Intakes (DRIs): The Essential Guide to Nutrient Requirements, Institute of Medicine of the National Academies) presents Recommended Dietary Allowances (RDA) in **bold type** or Adequate Intakes (AI) in ordinary type followed by an asterisk. For healthy breastfed infants, the AI is the mean intake

CHAPTER 2 – SAMPLE PREPARATION OF *MORINGA OLEIFERA* LEAVES AND PROXIMATE ANALYSIS

2.1 INTRODUCTION

Dried leaves of *M. oleifera* were collected in 2006 from Senegal (SE61 to SE65), Ghana (Z16 and Z17) and Zambia (Z1 to Z15), and upon receiving the samples at Rutgers, were ground and stored (Table 2.1.). The initial quality control analyses for all the samples were carried out prior to this research (Juliani et al, 2007). Leaves from *M. oleifera* trees in Rwanda (07-MO-01 to 07-MO-05) and Zambia (07-MO-07 to 07-MO-09) were also collected in April, June and August of 2007. The dried Moringa leaves obtained from Rwanda and Zambia, came from the regions Gasabo, Kibungo and Kicukiro in Rwanda; and Lusaka in Zambia. The leaves were collected from nurseries and orchards, shade dried, and packaged for experimental analysis. In Lusaka, the Mitengo Womens Cooperative process Moringa leaves by drying under a shed, milling to fine grains and selling locally. Three samples of these finished products were also included in this research (07-MO-07 to 07-MO-09). For product characterization, the first initial series of quality tests were included the determination of percent moisture, total ashes and acid insoluble ashes. These tests were used to determine the moisture, mineral content and overall purity of *M. oleifera* leaves. The moisture content is a major factor since it determines the actual weight of the powdered leaves, impacts shelf life and sets the stage for the product stability or product degradation either via hydrolytic and oxidative avenues and/or the potentiation of mold and microbiological growth. Product samples

with low moisture content (less than 10%) (Food Chemical Codex, 1996) would be classified as being efficiently dried for experimental analysis. The total ashes which measure the level of impurities in a product, were obtained by burning off the organic matter and measuring the residue of ash. The remaining ashes indicated the total mineral content for each sample and gave an approximate analysis of the viable elements contained in the leaves. To examine the cleanliness of the leaves, we determined the acid insoluble ashes by boiling the ash in 2N HCl and incinerating the remaining material (Handbook of Herbs and Spices, 2000).

The dry Moringa leaves were analyzed for moisture content, mineral content and foreign matter using the methods described by the Food Chemical Codex (1996).

2.1.2 Apparatus

Crucibles, Spatulas, tweezers, aluminum foil, hardened ashless filter paper (125mm), and analytical grade hydrochloric acid (HCl) were purchased from Fisher Scientific Co. (Fair lawn, NJ). USA Standard testing sieves size A (No. 35, 500 µm) and B (No. 8, 2.36 mm) were obtained from Seedburo Equipment Company (Chicago, Il). Perten Laboratory Mill 3100 (Germany) was used to grind the leaves.

2.2 MATERIALS AND METHODS

2.2.1 Separation of leaves

The plant material was transferred to pre-weighed plastic containers and their weights were recorded. For identification, each sample was labeled with a specific sample number (Table 2.1.). USA Standard testing sieves, mesh size A and B, were used to

remove foreign matter from the leaves and stems which were collected on a white sheet of paper in separate trays. The leaves, stems and foreign matter were separately packaged and weighed in pre-weighed Ziploc bags with their corresponding sample name.

2.2.2 Sample preparation of leaves

After carefully recorded the weight, the dry *M. oleifera* leaves were ground to a fine powder with the Perten Laboratory Mill 3100 and transferred to a labeled Ziploc bag to be stored at room temperature in the dark.

2.2.3 Different variety or source of the moringa leaves

The leaves were collected from local or indigenous cultivated moringa varieties and from an introduced variety of *M. oleifera*. Seeds or mature stems were from India of the cultivars PKM-1 and PKM-2, were introduced into these countries to determine if these varieties would be better than the indigenous ones and allowed us to compare the same genetic materials grown in several sub-Saharan African countries. Another variety of Moringa, introduced into Zambia, naturalized and left to grow in the wild known as Binga was also used in this research. A complete listing of the Moringa used in this research is shown in Table 2.1.

Methods

2.2.4 Moisture

Each powdered sample was carefully weighed to 1.000 ± 0.001g and placed in a pre-weighed foil envelope, pre-marked with the sample code. Each envelope was tightly sealed to avoid spillage and sample weight was recorded. The envelopes were placed in an oven at 80-90 ^{o}C for 5 days after which they were removed from the oven and immediately placed in a dessicator for 10 mins until the weight was constant, and cooled to room temperature. Subsequently, the samples were removed, the final masses recorded and the percent moisture of each sample calculated. This was done in triplicate.

2.2.5 Total Ashes & Acid Insoluble Ashes

The crucibles were dried in the furnace at 400-600 ^{o}C for 5 hrs, cooled to room temperature and weighed. Powdered Moringa samples (2.000 ± 0.001 g) were transferred to each crucible, placed in a furnace and ignited at 650 ^{o}C for 5 hrs. After the crucibles were cooled to room temperature, the crucible (containing the sample) was removed from the furnace and placed in the dessicator for 10-20 mins. The weight of ash was recorded and the ash percent calculated from the initial sample weight.

To the weighed ashes, 25 mL of 2.7N HCl was added and boiled for 10-15 mins, the crucibles were allowed to cool and the solution was filtered through ashless filter paper. The insoluble matter collected in the filter paper was washed with distilled water and after complete drainage, folded neatly inside its respective crucible and reheated in the furnace at 650 ^{o}C for 5 hrs. When the furnace cooled to room temperature, the crucibles were removed and placed in a dessicator for 10 mins. Each crucible was weighed, and the insoluble matter and percentage of acid insoluble ash of each sample was calculated. This was done in triplicate.

Table 2.1. Source of the Moringa grown in the selected Sub-Saharan African countries samples to be analyzed from different regions and varieties of *M. oleifera* in Africa

Country	Location	Variety	Sample Codes	Date of Collection
Rwanda	Gasabo	Indigenous	07-MO-01	June 2007
Rwanda	Kicukiro	Indigenous	07-MO-03	June 2007
Rwanda	Kibungo	Indigenous	07-MO-05	June 2007
Zambia	Lusaka (1)	Mitengo Women	07-MO-07	April 2007
Zambia	Lusaka (2)	Mitengo Women	07-MO-08	August 2007
Zambia	Lusaka (3)	Mitengo Women	07-MO-09	August 2007
Zambia[1]	PKM	PKM-1	Z1	May 2006
Zambia[1]	Mumbwa	Indigenous	Z3	May 2006
Zambia[1]	Kafue	Indigenous	Z4	May 2006
Zambia[1]	PKM	PKM-2	Z5	May 2006
Zambia[1]	Mumbwa	Indigneous	Z6	May 2006
Zambia[1]	Binga	Binga & Indigenous	Z7	May 2006
Zambia[1]	PKM	PKM-1	Z8	August 2006
Zambia[1]	Mumbwa	Indigenous	Z10	August 2006
Zambia[1]	Kafue	Indigenous	Z11	August 2006
Zambia[1]	PKM	PKM-2	Z12	August 2006
Zambia[1]	Kafue	Indigenous	Z13	August 2006
Zambia[1]	Binga	Binga & Indigenous	Z14	August 2006
Zambia[1]	PKM	PKM-1	Z15	August 2006
Ghana[1]	Dodowa	Indigenous	Z16	August 2006
Ghana[1]	Voltan Dan	Indigenous	Z17	August 2006
Senegal[1]	Dakar (1)	Indigenous	SE61	January 2006
Senegal[1]	Dakar (2)	PKM-1	SE62	January 2006
Senegal[1]	Dakar (3)	PKM-1	SE64	January 2006
Senegal[1]	Dakar (4)	Indigenous	SE65	January 2006

1 - The phenolic acids, flavonols, carotenoids and tocopherols were analyzed only. Each sample was collected from each region of different countries

PKM- : Cultivars imported into Africa from India; May, 2006 - Winter; August, 2006 – Spring

2.3 RESULTS AND DISCUSSION

Moringa samples contained consistent levels of leaves (84% to 86%), stems (14% to 15.5%) and other matter (0.25% to 0.40%) (Table 2.2.). Removal of stems and foreign matter was important to minimize contamination and error in determining the nutritional values of *M. oleifera* leaves from Sub-Saharan Africa countries. The percentage of other matter includes the parts of Moringa separated, to ensure that neither leaves nor stems as well as any sand or unwanted materials would be found in the materials that were to be analyzed. The method of analysis used was based upon prior work conducted in moringa (Juliani et al, 2006).

For the above samples, overall the moisture content ranged from 6.1 to 8.4%. With the lowest and the highest in values being collected from the Mitengo women Lusaka, Zambia (07-MO-07 and 07-MO-09). These results suggest that the leaves may have been dried at varying lengths of times and collected from different trees. Since the Mitengo women sell the leaves they may not have a consistent drying method and monitoring test to determine when the drying is complete. In addition, as their product enters into only the local market, dried products with a slightly higher moisture content result in higher profits as the products are sold by weight. Other samples showed a consistent correlation as they were within the required moisture percentage of less than 10 %. The ash content indicated that the leaves do contain minerals since there was a high percentage determined for the total ashes. Further tests were carried out to identify each element in the Moringa leaves. The insoluble ashes content was less than 1% which determined the amount of sand, dirt or environmental unknowns that would affect the

leaves in question. These values were very small in amounts which indicated the leaves were not contaminated and were relatively clean.

Table 2.2. Percentage of the leaves, stems and foreign matter of *M. oleifera* samples from Rwanda

Country	Location	% of leaves	% of stems	% of other matter
Rwanda	Gasabo	84.00 ± 0.58	15.42 ± 0.24	0.40 ± 0.01
	Kicukiro	86.05 ± 1.64	14.37 ± 2.09	0.25 ± 0.06
	Kibungo	84.62 ± 2.72	15.46 ± 2.57	0.31 ± 0.01

Values are expressed as average ± STD

Table 2.3. The percentage of the Moisture and Total and Insoluble Ashes

Country	**Location**	**% Moisture**	**Ash %**	**Acid insoluble ashes (%)**
Rwanda	Gasabo	7.29 ± 0.38	7.14 ± 0.22	0.35 ± 0.07
	Kicukiro	6.70 ± 0.44	9.12 ± 0.14	0.51 ± 0.10
	Kibungo	6.38 ± 0.52	7.43 ± 0.08	0.35 ± 0.01
Zambia	Lusaka (1)	8.40 ± 0.08	8.32 ± 0.02	0.65 ± 0.10
	Lusaka (2)	6.10 ± 0.17	8.49 ± 0.05	0.64 ± 0.08
	Lusaka (3)	6.81 ± 0.89	7.21 ± 0.08	0.58 ± 0.10

Values are expressed as average ± STD

2.4 CONCLUSIONS

To ensure the nutritional and chemical analysis reflects only the leaves and stems, proper sample preparation, including cleanliness is critical. As such, in this chapter, we described the protocols used for the initial sample preparation to remove all foreign matter. However, samples were harvested for the leaves and it was expected that the leaves would represent the majority of the biomass once the stems were separated.

The percent moisture in the samples were found to be less than 10% which indicated the samples were sufficiently dried for analysis. In the samples characterized, we can conclude that the leaves contained only 6.1 to 8.4 % water. The ash percent indicated the leaves were rich in minerals and hence it was important to carry out an elemental analysis to determine its elemental/minerals composition. Foreign matter, sand, dirt, soil and other unknown materials are common problems facing botanicals and foodstuffs; however, the results from the insoluble ash content showed that these samples were low indicating the samples were clean and free of soil and dirt. the results from these tests indicated the leaves were of high-quality and could be used for subsequent analyses.

CHAPTER 3

ANTIOXIDANT CAPACITY, TOTAL PHENOLS, ALKALOIDS AND PROTEIN ANALYSIS

3.1 INTRODUCTION

Epidemiological studies have shown that foods rich in vitamins provide protection against degenerative diseases including cancer, coronary heart disease and even Alzheimers's disease (Ames et al, 1993). Plants containing antioxidants like vitamin C, vitamin E, carotenes, polyphenols, and many other compounds reduce these disease risks. Most of the antioxidant compounds in a typical balanced diet are derived from plant sources with a wide variety of biological and chemical properties (Scalbert et al, 2005). Synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used as food additives; but recent reports have expressed safety concerns allowing natural antioxidant to be the focus of intense interest (Sun et al, 2005; Wilson, 1999). Plants are rich sources for natural antioxidants, the best known are tocopherols, flavonoids, vitamin C and other phenolic compounds (Laandrault et al, 2001). Other contributors to the antioxidant activity include alkaloids, proteins, minerals and other vitamins such as the carotenoids and vitamin B_6, B_{12} and K (Smolin et al, 2007).

Polyphenols scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and inhibit the oxidative mechanisms that can lead to degenerative diseases. There are a number of clinical studies confirming the powerful anti-cancerous and anti-

heart disease properties of polyphenols (Prakash et al, 2007; Bajpai et al, 2005, Siddhuraju et al, 2003).

Alkaloids, responsible for bio-enhancing properties and the therapeutic effect of many plants, can help the body's central nervous system and absorption in the gastrointestinal tract. Many alkaloids are toxic and subsequently banned for use in medications (Sreevidya et al, 2003). While there have been no studies or reports suggesting that Moringa leaves may contain alkaloids, part of this work was to examine and determine whether Moringa was free of alkaloids. Amino acids also play a central role both as building blocks of protein and intermediates in the metabolic process. Humans can produce 10 of 20 essential amino acids *in vivo* while the others must be supplied by a diet of plants or animals. Failure to obtain enough of any one of the 10 essential amino acids that are not formed, results in degradation of the body's protein and muscle (Institute of Medicine, 2006; Smolin et al, 2007). Plants rich in proteins can be ingested and be broken down metabolically into amino acids, which can supplement the body's need for the 10 essential amino acids which it cannot produce on its own.

The objective of this chapter will be to determine the total antioxidant capacity, phenols, alkaloids and proteins as part of the larger examination as to the plant properties relative to health and nutrition.

3.2 ANTIOXIDANT CAPACITY

The antioxidant capacity was determined by the spectrophotometric ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) method at an absorbance of 734 nm. Results

were expressed as g of Trolox equivalent (a water soluble analog of vitamin E) per 100 g dry weight of the plant (% g DW) (Gao et al, 2000; Anonymous, (2002).

3.2.1 MATERIALS AND METHODS

3.2.1.1 Materials

Automatic pipettes and tips (20 µl & 1000 µl) were purchased from Daigger & Co. (Vernon Hills, IL). Plastic cuvettes for alcoholic mixtures, Erlenmeyer 250 mL flask, volumetric flasks (10 & 25 mL), 1.5 mL centrifuge tubes and analytical grade ethanol (EtOH) plastic were purchased from Fisher Scientific Co. (Fair Lawn, NJ). ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and potassium perusulfate was purchased from Sigma Aldrich (St. Louis, MO). HPLC-grade water (18M Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

3.2.1.2 Equipment

Hewlett Packard 8453 UV/Visible spectrophotometer (Hewlett Packard Laboratories, Palo Alto, CA) with UV Visible Chem Station Software was used for spectrophotometric analysis.

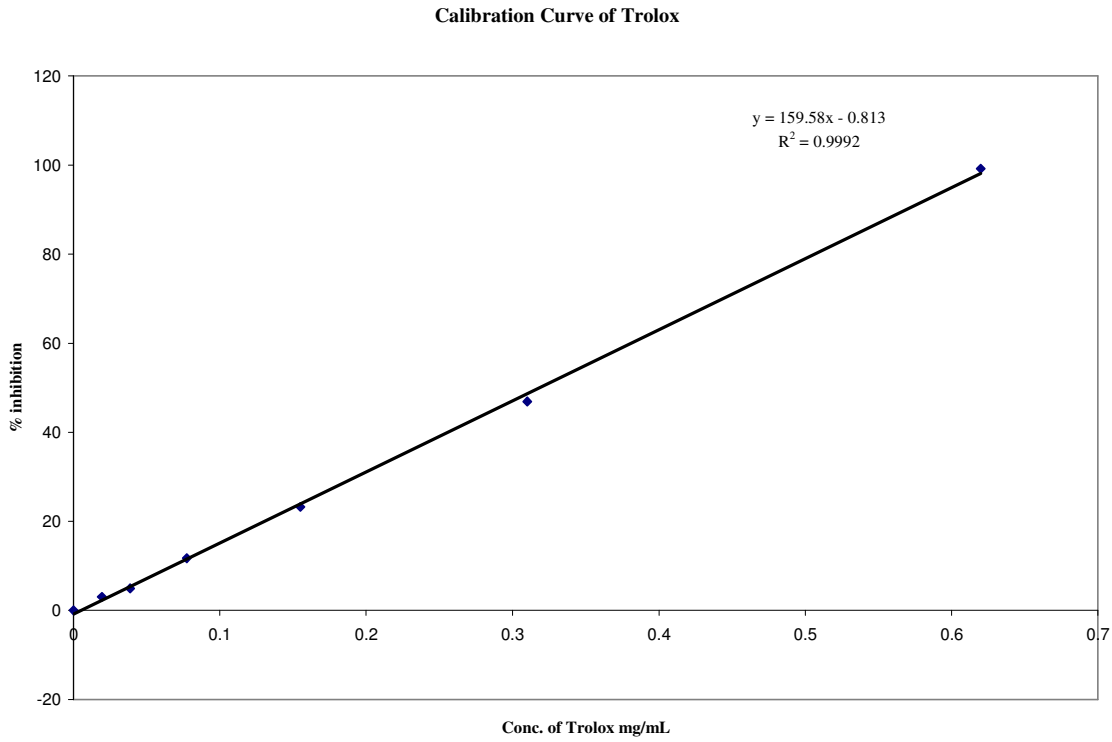
3.2.1.3 Method

The ABTS radical reagent was prepared by adding 38.4 mg of ABTS and 6.6 mg of potassium persulfate in 10 mL of water. The ABTS radical reagent was mixed well and placed in the dark for 16-20 hrs to allow the radical to fully develop; the radical is stable

in this form for more than a day when stored in the dark at room temperature. The ABTS reagent was diluted with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 30 ^{o}C. 1.3 mL of ABTS reagent was added to 100 mL ethanol with more ethanol (5 mL at a time) or ABTS reagent (30 µL at a time) was added to adjust the absorbance to a range of 0.68 - 0.72.

About 100 mg of Moringa powder was extracted by sonicating in 10 mL of water for 1 hour. 10 µL of this extract and 990 µL of ABTS solution (0.71 Abs) were combined in a centrifuge tube and allowed to react at room temperature for 20-30 min. Each sample was transferred to a cuvette and read spectrophotometrically at 734 nm. Samples were then measured against a blank (1 mL of ethanol) and a reference sample, made by combining 10 µL ethanol with 990 µL of ABTS solution.

A calibration curve was prepared by dissolving 15.5 mg of trolox in 25 mL of pure ethanol to make the standard stock solution. A series of six dilutions of ranging from concentrations of 0.62 mg/mL to 0.01938 mg/mL and a blank were prepared as the plant samples above and read spectrophotometrically to give the equation $y = 159.58x - 0.813$ ($r^2 = 0.9992$). This procedure was done in triplicate.



Graph 3.1. Calibation Curve of of Trolox

3.3 TOTAL PHENOLS

Using the Folin Ciocalteu's Reagent, total phenols in each Moringa sample were measured at an absorbance of 752 nm. The results are expressed as g gallic acid equivalents per 100 g dry weight (% g DW).

3.3.1 MATERIALS AND METHODS

3.3.1.1 Materials

Plastic cuvettes for alcoholic mixtures, 250 mL Erlenmeyer flask, 25 mL volumetric flasks, plastic 1.5 mL centrifuge tubes, spatulas and methanol (HPLC grade) were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Folin Ciocalteu's reagent (2 N), sodium bicarbonate (Na_2CO_3) was purchased from Sigma Aldrich. Automatic pipettes

and tips (200 µl & 1000 µl) were purchased from Daigger & Co. (Vernon Hills, IL). HPLC-grade water (18 MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

3.3.1.2 Equipment

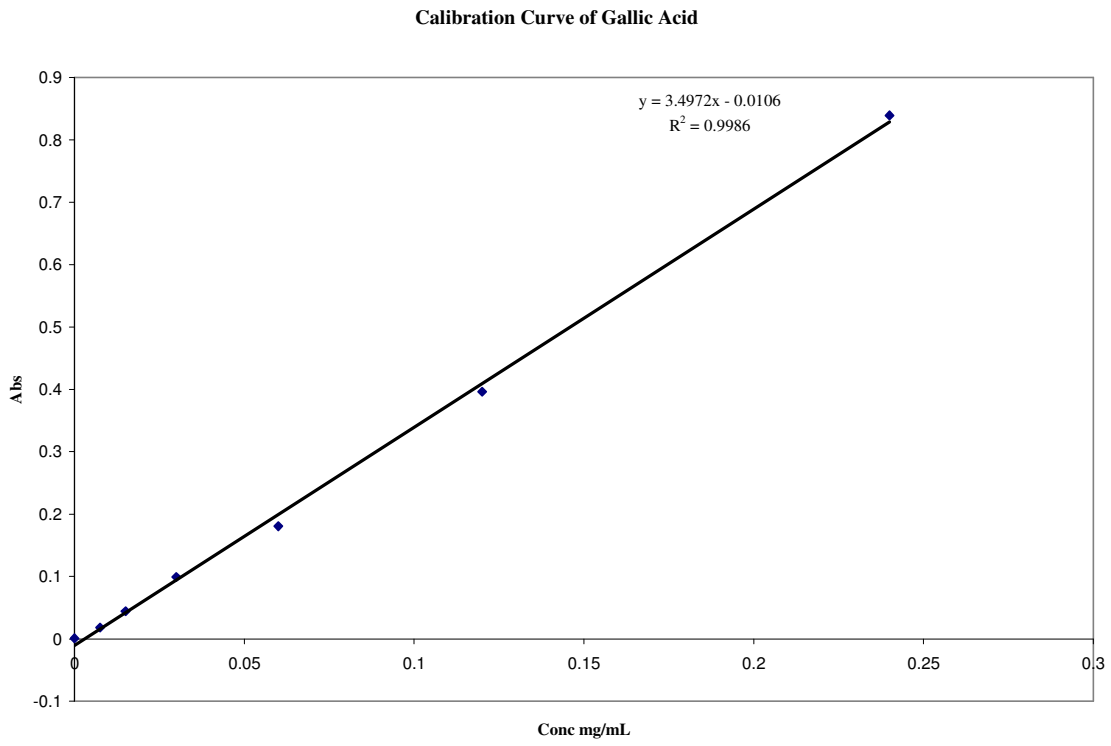
Hewlett Packard 8453 UV/Visible spectrophotometer (Hewlett Packard Laboratories, Palo Alto, CA) with UV Visible Chem Station Software was used for analysis.

3.3.1.3 Method

First, 2 mL Folin Ciocalteu's Reagent in 20 mL of water was prepared to form a stock solution of Folin Ciocalteu's reagent solution. Saturated solution of Na_2CO_3 (15%) was prepared by dissolving 7.5 g of Na_2CO_3 in 50 mL of water.

About 100 mg of the powdered *M. oleifera* was placed in a 25 mL volumetric flask and 20 mL of 60% methanol in water (v/v) was added and sonicated for 25 minutes. After sonication, the flasks were filled to volume with water and 40 µL of the extract was transferred to a centrifuge tube with 900 µL of Folin Ciocalteu's Reagent solution and set aside for five minutes. 400 µL of 15% Na_2CO_3 was added to the mixture, allowed to react for 45 minutes and was measured at 752 nm.

To develop the calibration curve, 6.0 mg of gallic acid was added into 25 mL of 60% methanol solution to provide the standard solution. Six dilutions of concentrations ranging from 0.24 mg/mL to 0.0075 mg/mL and a blank were prepared as the plant samples above and gave the equation $y = 3.4972x - 0.0106$ ($r^2 = 0.999$). 40 µL of each dilution was also used for the calibration curve. This was done in triplicate.



Graph 3.2. Calibation Curve of Gallic Acid

3.4 TOTAL ALKALOIDS

The quantitation of alkaloids precipitable with Dragendorff's Reagent was used for the detection of total alkaloids (Sreevidya et al, 2003). This rapid, simple and efficient spectrophotometric method was used to determine the total alkaloid content for each of the Moringa sample. The amount of bismuth present was estimated after precipitation of the alkaloids with Dragendorff's Reagent and the results expressed as µg of Bismuth per 100 mg of dried plant material (DW) (Sreevidya et al, 2003).

3.4.1 MATERIALS AND METHODS

3.4.1.1 Materials

Bismuth nitrate pentahydrate, thiourea, disodium sulfide were purchased from Sigma Aldrich (St. Louis, MO). Analytical grade glacial acetic acid and cuvettes was obtained by Fisher Scientific Co. (Fair Lawn, NJ). HPLC-grade water (18M Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

3.4.1.2 Equipment

Hewlett Packard 8453 UV/Visible spectrophotometer (Hewlett Packard Laboratories, Palo Alto, CA) with UV Visible Chem Station Software and an Accumet Basic AB 15 pH meter from Fisher Scientific Co. was used.

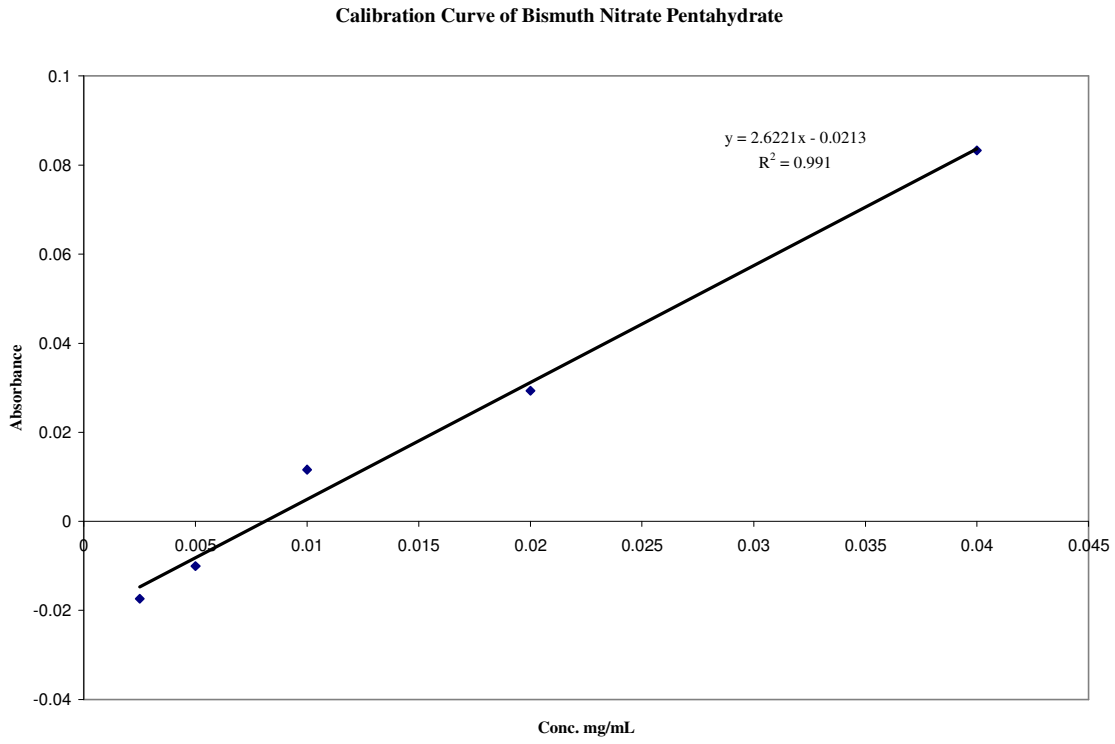
3.4.1.3 Reagent Solutions

Dragendorff's Reagent was prepared by dissolving bismuth nitrate pentahydrate (0.8044 g) in 40 mL of water and 10 mL of glacial acetic acid and combined with a solution of 8.0 g of potassium iodide in 20 mL water. 10.0 mg bismuth nitrate pentahydrate in 5 mL concentrated nitric acid was diluted in 100 mL with distilled water to make a standard bismuth nitrate solution. 3.00 g of thiourea was dissolved in 100 mL of water to form a thiourea solution and a stock solution of disodium sulfide was prepared by dissolving 1.00 g of disodium sulfide in 100 mL of water.

3.4.1.4 Method

About 200 mg of dried Moringa leaves was mixed with 10 mL 60% ethanol: 40 % acetic acid (10 mL in 100mL of water) and extracted at room temperature for 20 minutes by sonication. The extract solution was brought to a pH of 2.5 with dilute HCl, vortexed to

dislodge the precipitation; 1.5 mL was transferred to a microcentrifuge tube and centrifuged for 20 minutes at 13000 rpm. 0.5 mL of the supernatant was transferred to a new tube, 0.2 mL of Dragendorff's reagent was added and the tube was vortexed to dislodge any precipitate from the wall of the tube. This solution was then centrifuged for 40 minutes at 13000 rpm. The residue was dissolved in 0.2 mL concentrated nitric acid and 0.8 mL water. 0.2 mL was pipetted out and added to 1 mL of the thiourea solution. The absorbance was measured at 464 nm against a blank containing nitric acid and thiourea. The absorbance values were compared against the calibration curve obtained with five dilutions of 40% Bismuth nitrate pentahydrate in water to determine the concentration of total alkaloids. The calibration curve concentration ranged from 0.0025 mg/mL to 0.04 mg/mL and a blank giving the equation $y = 2.6221x - 0.0213$ ($r^2 = 0.9910$). The procedure was done in duplicate.



Graph 3.3. Calibation Curve of Bismuth Nitrate Pentahydrate

3.5 TOTAL PROTEINS

Total proteins were quantitated by using Bradford Reagent experiment by spectrophotometer method and measured at wavelength 595 nm. The results were expressed as g protein (Albumin) on a dry weight basis (g Albumin/100 g DW).

3.5.1 MATERIALS AND METHODS

3.5.1.1 Materials

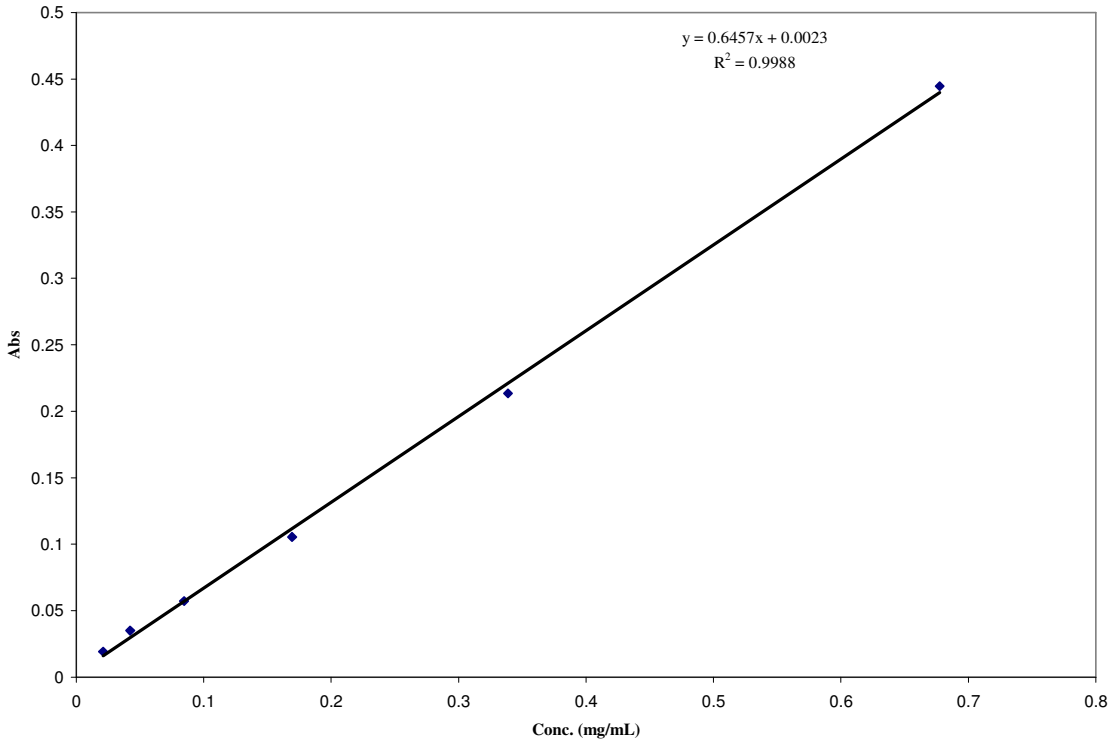
Bis-Tris-Hydrochlorine-Glycerol, Bradford reagent, Albumin was purchased from Sigma Aldrich (St. Louis, MO). HPLC-grade water (18M Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

3.5.1.2 Equipment

Hewlett Packard 8453 UV/Visible spectrophotometer (Hewlett Packard Laboratories, Palo Alto, CA) with UV Visible Chem Station Software and an Accumet Basic AB 15 pH meter from Fisher Scientific Co. was used.

3.5.1.3 Method

A total of 50 mg of the ground leaves were placed in a 15 mL centrifuge tube along with 10 mL of 50mM Bis-Tris-Hydrochlorine-Glycerol (in water). This was carefully mixed until all the material was extracted for 30 mins, after which 1.5 mL of Bradford reagent was added to each tube. After 20 minutes, the absorption was measured at 595 nm and the results were expressed as g of protein on a dry weight basis (g Albumin/100 g DW). A calibration curve of six dilutions with concentrations ranging from 0.678 to 0.0212 mg/mL and a blank were plotted and gave the equation $y = 0.6457x + 0.0023$ ($r^2 = 0.9988$).



Graph 3.4. Calibration Curve of Albumin

3.6 RESULTS AND DISCUSSION

M. oleifera leaves have a relatively low antioxidant capacity when compared to the known high antioxidant capacity of *Camellia sinensis*. The calculated values are not as high as drinks which contain green tea, giving values as high as 33-45% antioxidant capacity, but there are many compounds within the leaves which contribute to this moderate value. Leaves obtained from Rwanda in 2007 had a consistent antioxidant value in the range of 5.00 to 5.98 % while those bought from the Mitengo women in Lusaka, Zambia were slightly lower with one variety, 07-MO-08, having the highest value at 6.24%. Even though the antioxidant capacity may not be as high as when extracted in 60-80% methanol solution (Bajpai et al, 2005; Prakash et al, 2007), it was

important to calculate the antioxidant capacity of the leaves in a water extraction to address the main use of the plant being consumed as a food or supplement.

While some reports indicate extraction with methanolic solutions gives higher concentrations of phenols (Bajpai et al, 2005), other reports (Juliani et al, 2007) showed higher values of total phenols were obtained when Moringa was extracted with pure water. Because the total phenol concentration of these samples is addressing the antioxidant capacity of Moringa following cooking methods, we decided to use the water extraction for quantitation. The leaves collected from Rwanda (Gasabo, Kibungo and Kicukiro) were shown to be consistent in values ranging from 2.69 to 2.73 %. Values obtained from the Mitengo women in Lusaka, Zambia increased to 4.81 %.

The total alkaloids observed in the leaves were quite low across the range of samples (0.0956 – 0.148 mg/100 g DW) in comparison to other tests shown; therefore, we concluded the alkaloids are not a major contributor to the antioxidant capacity.

Previous studies (Juliani et al, 2007) showed that higher values were obtained when Moringa was extracted with pure water for the quantitation of total proteins gave higher concentrations than by methanolic extractions. The leaves collected in Rwanda: Gasabo, Kicukiro and Kibungo, all had relatively high total protein values when extracted with water compared to the total alkaloids and phenols in which the sample from Kicukiro, Rwanda was the highest. The samples collected from Lusaka fluctuated in values with a decrease in total proteins from Lusaka, Zambia.

Table 3.1. Moringa grown in the selected Sub-Saharan African countries and analyzed from its antioxidant capacity, total phenols, total alkaloids and total proteins

		Antioxidant Capacity (%)	Total Phenols (%)	Total Alkaloids (mg/ 100 g DW)	Total Proteins (%)
Country	Location	Trolox equivalent	Gallic acid equivalent	Bismuth Nitrate pentahydrate equivalent	Albumin equivalent
Rwanda	Gasabo	5.00 ± 0.15	2.69 ± 0.04	0.101 ± 0.04	5.83 ± 0.06
	Kicukiro	5.36 ± 0.07	2.61 ± 0.42	0.126 ± 0.08	5.95 ± 0.05
	Kibungo	5.98 ± 0.61	2.73 ± 0.48	0.0956 ± 0.02	5.48 ± 0.01
Zambia	Lusaka (1)	4.66 ± 0.38	2.51 ± 0.03	0.148 ± 0.12	5.43 ± 0.03
	Lusaka (2)	6.24 ± 0.48	3.80 ± 0.44	0.0981 ± 0.09	4.04 ± 0.01
	Lusaka (3)	4.98 ± 0.08	4.18 ± 0.19	0.0959 ± 0.08	3.88 ± 0.03

Values are expressed as average ± STD. % expressed as g/100 g DW

3.7 CONCLUSIONS

The total phenols, alkaloids and proteins were measured in the leaves of moringa and among the samples analyzed the range was narrow both within and between countries. Moringa leaves contained low levels of alkaloids while moringa could be considered a rich or high source of both total phenols and proteins. Additional phytochemicals are likely to contribute to the overall antioxidant capacity of *M. oleifera*. These methods were done in water in part to more easily and accurately estimate the ROS or antioxidant value of moringa as may be found in household cooking. Therefore, if 100 g of *M. oleifera* leaves were used for cooking, 0.0956 to 0.148 mg/100 g DW of alkaloids, 2.51 to 4.18 % of phenols and 3.88 to 5.95 % of proteins would be consumed by an individual. Later

chapters will provide evidence showing that Moringa contains additional phytochemicals such as vitamins, flavonoids, and minerals, which contribute to the plants antioxidant capacity.

CHAPTER 4

ELEMENTAL ANALYSIS

4.1 INTRODUCTION

Minerals are inorganic elements needed by the body as structural components and regulators of body processes. They may combine with other elements in the body, while retaining their chemical identity. Unlike vitamins, they are not destroyed by heat, oxygen, or acid. The ash that remains after a food or plant is combusted at extreme temperatures, is a simple test used to determine the content of the minerals present in the food. In some foods, the amounts of minerals naturally present are predictable because they are regulated by components of the plant or animal. For instance, magnesium is component of chlorophyll, so it is found in consistent amounts in leafy greens; calcium is a component of milk, therefore, drinking a glass of milk reliably provides a known amount (Smolin et al, 2007).

To maintain health, enough of each mineral must be consumed and the total diet must contain all the minerals in the correct proportions (Table 1.4.). An inadequate amount of any one mineral can cause a deficiency or a toxic reaction. For some minerals, too much or too little causes obvious symptoms that impact short-term health. Deficiencies of other minerals might only cause symptoms for long term health. For example, a low calcium intake has no short-term consequences, but over the long term reduces bone density, increasing the risk of fractures later in life (Institute of Medicine, 2006). Nutritional status and nutrient intake can affect mineral transport in the body. When protein intake is deficient, transport proteins (and proteins in general) cannot be

synthesized; therefore, even if a mineral is adequately found in the diet, it cannot be transported to the cells where it is needed (Institute of Medicine, 2006; Smolin et al, 2007).

Together, the macronutrients and micronutrients provide energy, structure, and regulation which are needed for growth, maintenance, repair, and reproduction (Smolin et al, 2007). Each nutrient provides one or more of these functions, but all nutrients together are needed to maintain human health (Institute of Medicine, 2006).

The samples were sent to the Agricultural Analytical Services Lab, Penn State University to determine the amounts of 11 elements in the *M. oleifera* leaves, specifically the macronutrient phosphorus, potassium, calcium, magnesium, and the micronutrients manganese, iron, copper, boron, aluminum, zinc and sodium. The results were expressed for the macronutrients as g of element per 100 g on a dry weight bases (% g DW) and the micronutrients, mg of element per 100g on a dry weight basis (mg element/100 g DW).

4.2 MATERIALS AND METHODS

4.2.1 Materials

Muffle Furnace and Inductively Coupled Plasma Optical Emission Spectrometer (ICP) was used at Penn State University for analysis in the Agricultural Analytical Lab.

4.2.2 Method

0.2 g of the moringa sample was placed in a small dish in a muffle furnace and heated slowly to 500 ^{o}C for 2 hr to burn-off all the carbon leaving the minerals in ash. The temperature was kept constant at this level to avoid losing elements or leaving excess

carbon behind. The remaining ash was dissolved in 10ml of 1N HCl and analyzed in the Inductively Coupled Plasma Optical Emission Spectrometer (ICP) which analyzes the major elements in percentage (%/DW) and traces of the minor elements in µg/g DW (Miller, 1998).

4.3 RESULTS AND DISCUSSION

The Moringa samples from Zambia contained relatively high amounts of total minerals. As the data shows for these samples, the highest value of minerals was collected from the Mitengo women in Lusaka (07-MO-O8), in which it was found to be a good source of calcium (1.61 %), potassium (0.52 %), magnesium, (0.60 %), iron (40.65 mg/100 g) manganese (14.60 mg/100 g) and copper (0.95 mg/100 g) all of which are minerals essential to the diet. Nutritionally, 31.1 g of fresh ground leaves of *M. oleifera* from Lusaka, Zambia would provide 100% of the recommended daily allowances (RDA's) for calcium, iron, magnesium and manganese for a child between the ages of 1 to 3 (Table 1.4.). From only around 50 g of the same variety, Lusaka (2) sample, Moringa will offer the same nutritional value to a child from 4 to 8 years old. For copper, 47 g of fresh from this variety would be enough to provide the RDA for children ages 1 to 8 (Table 4.1).

To satisfy all the daily requirements of iron for a child between the ages of 1 to 3 and 4 to 8 years, only 18 g of the leaves and 69 g of the leaves from Zambia would be needed, respectively. For calcium, 25 g of ground leaves of the Zambian Moringa would be sufficient for the RDA of children aged 1 to 3 years old.

For copper, 36 g of the leaves will be needed for children 1 to 3 years to meet their daily requirement while only 47 g are necessary for those children aged 4 to 8 years to provide the requirement.

This research suggests that samples bought from the Mitengo women of Lusaka, Zambia were excellent sources for the recommended daily requirements of calcium, phosphorus, iron, magnesium, manganese, copper, aluminum and sodium for children as well as pregnant and lactating women. With 192 and 250 g of the Lusaka (07-MO-08) samples, the RDA's of calcium, iron and magnesium for pregnant and lactating women, respectively, between the ages of 14 to 50 years old can be also satisfied.

While we recognize that the observations relative to RDA's are based upon assumptions first of what ones considers to be the appropriate RDA, and thus, are calculated by age only, not taking into account body weight and other factors, the data should still be viewed in a comparative manner to other sources of the same nutrients and in a relative rather than absolute manner using the same assumptions.

Table 4.1. Elemental composition of the leaves of different *M. oleifera* varieties from Africa

	Percent*				mg/100 g of DW						
Location	P	K	Ca	Mg	Mn	Fe	Cu	B	Al	Zn	Na
Gasabo	0.44 ±0.1	1.78 ±0.04	1.12 ±0.04	0.38 ±0.02	8.65 ±0.66	12.88 ±1.19	0.73 ±0.05	2.73 ±0.28	2.63 ±0.25	3.28 ±0.21	4.00 ±0.18
Kicukiro	0.44 ±0.03	1.93 ±0.13	2.06 ±0.09	0.33 ±0.01	9.70 ±1.96	26.85 ±7.57	0.70 ±0.00	4.40 ±0.64	10.65 ±1.28	3.10 ±0.35	2.80 ±0.18
Kibungo	0.47 ±0.01	1.93 ±0.05	1.14 ±0.02	0.35 ±0.015	6.43 ±0.33	10.23 ±0.34	0.80 ±0.00	2.70 ±0.12	3.85 ±0.35	2.73 ±0.05	1.83 ±0.39
Lusaka	0.40 ±0.00	1.97 ±0.00	1.86 ±0.01	0.26 ±0.00	12.40 ±0.14	19.40 ±0.42	0.80 ±0.00	2.50 ±0.00	9.85 ±0.05	1.80 ±0.00	4.05 ±0.05
Lusaka	0.52 ±0.01	1.76 ±0.02	1.61 ±0.02	0.60 ±0.00	14.60 ±0.00	40.65 ±0.92	0.95 ±0.07	1.85 ±0.70	34.95 ±0.92	3.20 ±0.00	11.40 ±0.14
Lusaka	0.43 ±0.01	1.53 ±0.21	1.33 ±0.01	5.35 ±0.01	11.80 ± 0.14	31.95 ±0.92	0.90 ±0.00	1.25 ±0.07	29.30 ±0.00	2.60 ±0.00	10.50 ±0.00

Values are expressed as average ±STD

*Percentage is expressed as the amount in 1g of the dried *M. oleifera* leaves

4.4 CONCLUSIONS

The data in this chapter shows that Moringa leaves can provide the needed minerals for undernourished and malnourished people, particularly children and pregnant and lactating women in Sub-Saharan Africa. Our findings also confirm and further support the use of Moringa as a source of minerals, particularly calcium, potassium, iron, magnesium and phosphorus for our diet. With these essential minerals in the leaves, and in the absence of any adverse reporting in the use of moringa leafs, seeds, oil and commercial product and in the light that moringa has and is used as a traditional foodstuff, we suggest there is a high probability the plant is safe for human consumption, even in a concentrated formula.

CHAPTER 5

QUANTITATION OF VITAMIN C BY UV/VIS SPECTROPHOTOMETRY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.1 INTRODUCTION

Vitamin C or ascorbic acid (AA) acts as an antioxidant that, along with other vitamins, protects the body from oxidative stress, maintains the immune system and aids in the absorption of iron. Additionally, ascorbic acid neutralizes any radicals, damaged lipids and DNA in the blood that may cause illnesses to emerge while regenerating the active antioxidant capacity from vitamin E (Smolin et al, 2007). Ascorbic acid is highly sensitive to various modes of processing. There are factors that can influence the degradation of vitamin C; these include temperature, salt and sugar concentration, pH, oxygen, enzymes and metal catalysts (Bineesh et al, 2005). This water soluble compound is extracted from the *M. oleifera* with water over a short period of time to analyze the ascorbic acid content.

This spectrophotometric method does not measure the actual ascorbic acid but the total dehydroascorbic acid after oxidation, that reacts with *o*-phenylenediamine to give the quinone which is measure at 358 nm as illustrated in Figure 5.1. (Raghu et al, 2007, Esteban et al, 1997).

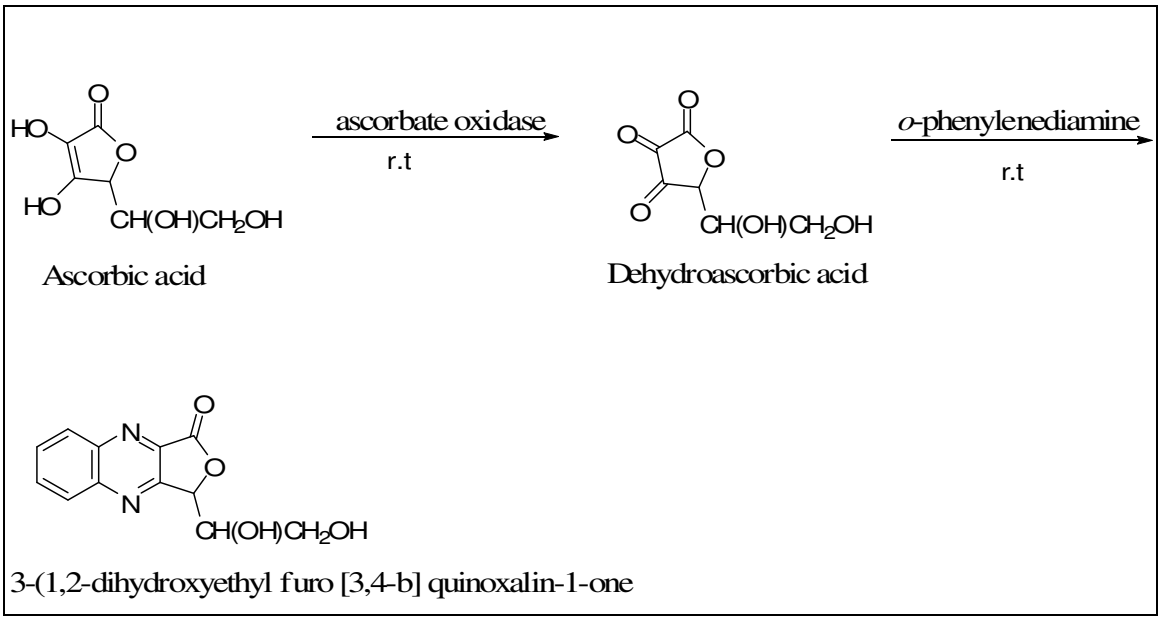


Figure 5.1. Proposed oxidation of ascorbic acid catalyzed by the ascorbate oxidase enzyme and the subsequent reaction to produce the quinoxaline chromophore

5.2 MATERIALS AND METHODS

5.2.1 Materials

Automatic pipettes and tips (20, 200 µl & 1000 µl) were purchased from Daigger & Co. (Vernon Hills, IL). Plastic cuvettes, volumetric flasks (10 mL), *o*-phosphoric acid were purchased from Fisher Scientific Co. (Fair Lawn, NJ). *O*-phenylenediamine (OPDA), ascorbate oxidase (250 units) potassium dihydrogen, hydrochloric acid (HCl) and potassium hydroxide (KOH) was purchased from Sigma Aldrich (St. Louis, MO). HPLC-grade water (18M Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

5.2.2 Equipment

Hewlett Packard Agilent 1100 Series LC/MS (Agilent Technologies, Waldbronn, Germany) equipped with autosampler, quaternary pump system, DAD detector, multiple wavelength detector, degrasser, MSD trap with an electrospray ion source (ESI), and HP ChemStation Software was used.

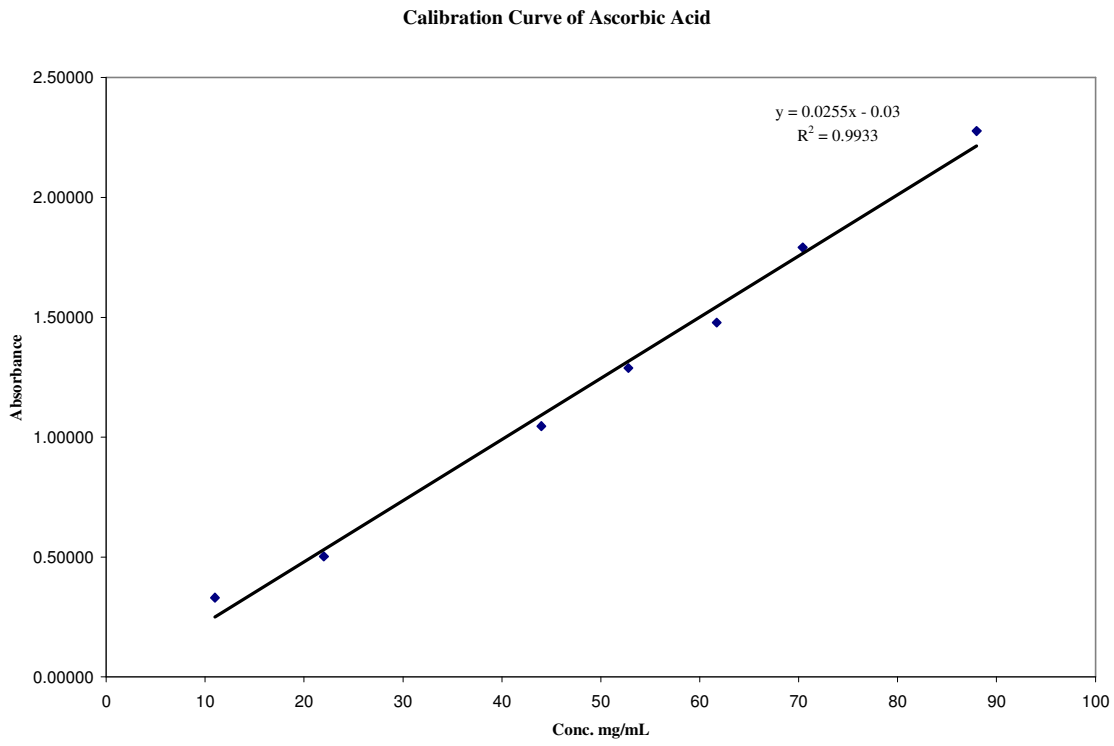
5.2.3 Standards

Ascorbic Acid was purchased from Sigma Aldrich (St. Louis, MO).

5.2.4 Method by UV Spectrophotometer

Ascorbic acid (0.5284 g) was dissolved in 300 mL of water to make a stock solution of 0.010 M. Potassium dihydrogen (5.44 g) was added to 200 mL of water and stirred to dissolve. HCl and KOH were added while stirring to get a phosphate buffer of pH 4.0. The ascorbate oxidase (250 units) was dissolved in 50 mL of water to obtain a solution of 5 units/mL. All the solutions were sealed and refrigerated before use. The ascorbate oxidase enzyme was separated into 1.5 mL microtubes and stored in a freezer. Into 10 mL volumetric flasks, 1000 μL OPDA, 1000 μL phosphate buffer, 100 μL ascorbate oxidase, and varying volumes of ascorbic acid stock solution were combined. The flasks were then filled to volume with water and allowed to react at room temperature for 24 hours. A blank, containing only buffer and the enzyme, was similarly prepared. The absorbance of each sample was read at 358 nm (Esteban et al, 1997). Using the standard stock solution of ascorbic acid, a calibration curve was established of seven dilutions

across a range of concentrations 11µg/mL to 88µg/mL giving the equation y = 0.0255x - 0.03 (r^2 = 0.9933).



Graph 5.1. Calibration Curve of Ascorbic Acid

5.2.5 Method by LC/MS

Approximately 500 mg of each Moringa sample was placed in conical tubes; into which 20 ml of 4% *o*-phosphoric acid was added and sonicated for 5 mins. The samples were protected from light using aluminum foil and were stored at -15 ^{o}C overnight. After 24 hrs the samples were thawed at room temperature, the extraction mixture was filtered and approximately 1 mL of the extract was transferred into light sensitive HPLC vials (Raghu et al, 2007; Burini, 2007; Tausz et al 1996).

5.3 RESULTS AND DISCUSSION

The two methods utilized for quantitation of vitamin C included UV spectrophotometry and HPLC/DAD/ESI-MS. The UV- Vis spectrophotometric method gave high vitamin C values for PKM-1 leaves (SE62) from Senegal at 0.749 mg/100 g while PKM-1 leaves from Zambia (Z1) were much lower (0.424 mg/100 g). Samples collected from Zambia in April 2007 showed a range from 0.351 to 0.441 mg/100 g and no significant difference in vitamin C content was observed among these samples. Zambian samples collected from the Mitengo women showed slightly higher vitamin C amounts than those collected in April 2007.

The HPLC/MS method was included to validate the results from the spectrophotometric method and was especially important because of the low concentrations of vitamin C observed across the Moringa samples. However, using this method, we could not detect the presence of vitamin C by LC/MS, most likely due to the small amount originally present followed by the rapid degradation of vitamin C. This is a possible indication that the UV spectrophotometric method overestimated the vitamin C content due to cross reactions. The LC/DAD/MS method is very sensitive and should provide a more accurate/sensitive determination than the spectrophotometric method.

Table 5.1. Total ascorbic acid content achieved by UV spectrophometric method

Country	Location	Ascorbic Acid mg/100g
Rwanda	Gasabo	0.359 ± 0.04
	Kibungo	0.351 ± 0.03
	Kicukiro	0.441 ± 0.01
Zambia	Lusaka (1)	0.372 ± 0.01
	Lusaka (2)	0.531 ± 0.02
	Lusaka (3)	0.425 ± 0.014
PKM	PKM-1	0.424 ± 0.00
Zambia	Mumbwa	0.567 ± 0.00
Ghana	Dodowa	0.378 ± 0.00
Senegal	Dakar (PKM-1)	0.749 ± 0.01
Senegal	Dakar (PKM-1)	0.573 ± 0.00

Values are expressed as average ± STD

5.4 CONCLUSIONS

With the conflicting results of the two methods for quantitating vitamin C, we cannot conclude the levels of vitamin C in the dried leaves of *M. oleifera*. However, we can suggest that both methods resulted in real differences and that vitamin C content in fresh leaves is expected to be far different than the content that would be found in dried leaves. The level of vitamin C degradation during the drying and storage stages may be quite significant. Other reports show fresh Moringa leaves as having high concentrations of ascorbic acid (Siddhuraju et al, 2003). Researchers used fresh leaves which were either freeze-dried and stored at -18 ^{o}C or used immediately after collection. They quantitatively determined the amount of ascorbic acid by using a titration method with the extracted samples and 2,6-dichlorophenolindophenol dye to measure the oxidation of

ascorbic acid. In this research, the dried leaves gave an extremely low concentration of ascorbic acid compared to values as high as 5500 mg/100 g of dry weight determined by titration from fresh materials (Siddhuraju et al, 2003). Additionally, other reports indicate the vitamin C content ranges around 204 mg/100 g of fresh weight (Iqbal et al, 2006; Sreeramula et al, 2003). Previous research on *M. oleifera* over a period of 48 years (1951-1999) the data consistently showed, with one exception that fresh Moringa leaves contain approximately 220 mg/100 g of ascorbic in the leaves (McBurney et al, 2004). While the methodology did not specify whether leaves were fresh or dry; the results showing moringa leaves to be a source of vitamin C appear to only be coming from research using fresh leaf tissue, not dry leaves, and as such, we are presuming that this range cited above also reflects fresh tissue only. In one study on degradation kinetics of ascorbic acid in Moringa when fresh leaves are cooked, the vitamin C content decreased considerably, as high as 98.5%, for more dilute samples (Sreeramula et al, 1983; Binessh et al, 2005). However, when the leaves were cooked in smaller volumes of water for shorter periods of times, the loss of vitamin C was minimized (Sreeramula et al, 1983). Studies have shown that ascorbic acid is highly sensitive to a number of factors, including temperature, light, salt and sugar concentration, pH, oxygen, enzymes and metal catalysts. The lack of ascorbic acid in processed food has been explained mostly by heat, oxygen and light (Bineesh et al, 2005). Therefore, as ascorbic acid has a very short half-life and the leaves collected in this analysis were dried, the ascorbic acid should be far lower than that which would be found in fresh tissue.

CHAPTER 6

POLYPHENOLS OF MORINGA

6.1 INTRODUCTION

Phenolic acids are universally found in plants and have been the subject of a great number of chemical, biological, agricultural, and medicinal studies. Recent interest in phenolic acids stems from their potential protective role through ingestion of plants. Like vitamins, phenolic acids also help with many ailments such as coronary heart disease, blood clots which can lead to strokes and cancers (Prakash et al, 2007). People take many vitamins and health supplements to build the body's resistance in fighting various biological threats (Scalbert et al, 2005). For phenolic acids, the total dietary intake could be as high as 1 g/day which is much higher than other classes of phytochemicals and known dietary antioxidants.

Until the mid-1990's, the most widely studied antioxidants were vitamins, carotenoids, and minerals (Smolin et al, 2007). The phenols were not truly researched until after 1995, when researchers looked at the antioxidant properties and effects in disease prevention (Prakash et al, 2007; Bajpai et al, 2005). Clinical trials and epidemiologic studies have suggested that polyphenols clearly improve the status of different oxidative stress biomarkers. Much uncertainty persists, however, regarding both the relevance of these biomarkers as predictors of disease risk and the suitability of the different methods used (Bowman et al, 1995; Scalbert et al, 2005). Today, it is well established that some polyphenols, administered as supplements or in food, improve

health status as indicated by several biomarkers closely associated with cardiovascular diseases (Bowman et al, 1995; Smolin et al, 2007).

Moringa oleifera has been reported to contain phenolic acids such as gallic acid, chlorogenic acid, ferulic acid, caffeic acid and ellagic acid (Prakash et al, 2007; Bajpai et al, 2005; Siddhuraju et al, 2003). In this study, the phenolic acids of Moringa leaves from Africa were identified and quantitated using LC/UV/MS.

6.2 MATERIALS AND METHODS

6.2.1 Materials

HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC grade formic acid was obtained from Acros Organics (NJ). HPLC-grade water (18M Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

6.2.2 Equipment

HPLC separation was performed on a Prodigy ODS3 5µm, 150 x 3.2mm 5 micro (Phenomenex Inc. Torrace CA). Hewlett Packard Agilent 1100 Series LC/MS (Agilent Technologies, Waldbronn, Germany) equipped with autosampler, quaternary pump system, DAD detector, multiple wavelength detector, degrasser, MSD trap with an electrospray ion source (ESI), and HP ChemStation Software.

6.2.3 LC/MS condition for analysis of phenolic acids

The mobile phase for chromatographic separation consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile, v/v) for the following gradient: 0 to 10 % B in 0-10 mins, 30% B in 10-30 mins, and 50% in 30-40 mins at a flow rate of 1.00 mL/min. The injection volume was 10 µL and the UV detector was set to 330 nm. The injection volume was 10 µL and the UV detector was set to 330nm. The electron ion mass spectrophotometer (ESI-MS) was operated under positive ion (Threshold, 30000) and optimized collision energy level of 35%, scanned from *m/z* 100 to 600. ESI was conducted using a needle voltageof 3.5 kV. High-purity nitrogen (99.999%) was used as dry gas and at a flow rate of 12 L/min, capillary temperature at 350 ^{o}C. Nitrogen was used as Nebulizer at 40 psi. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity.

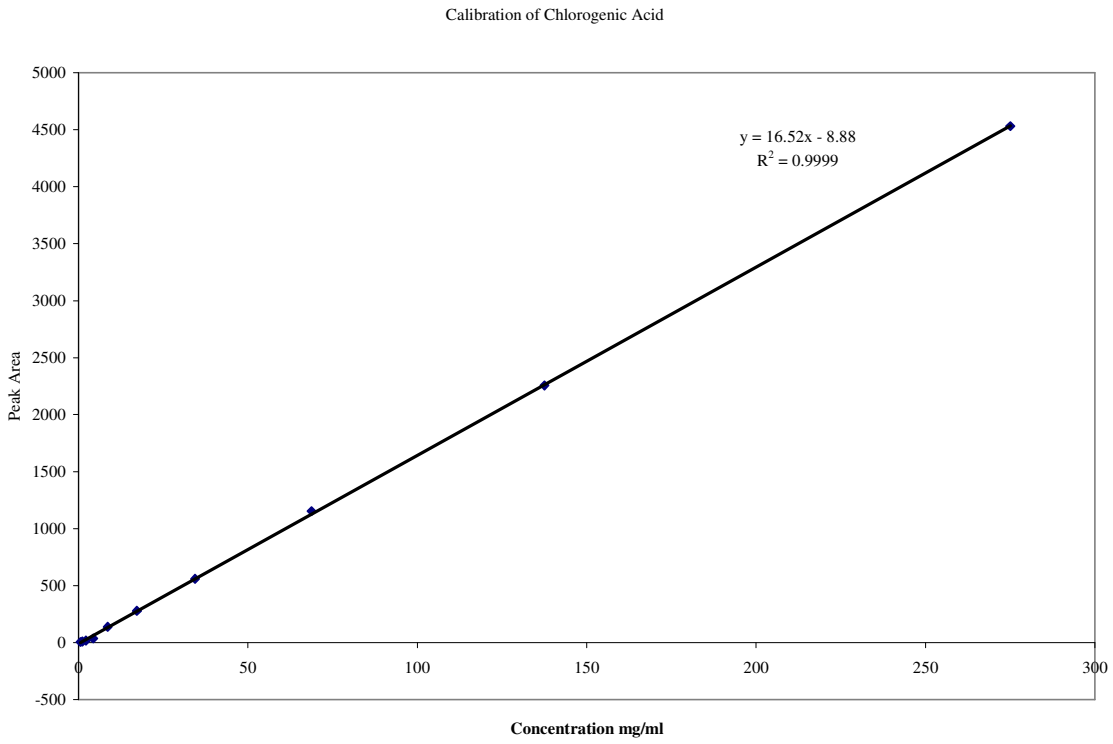
6.2.4 Standards

Standard compounds, gallic acid, chlorogenic acid, caffeic acid, ellagic acid and ferulic acid were purchased from Sigma Aldrich Co. (St. Louis, MO).

6.2.5 Method

About 100 mg samples of the powdered Moringa sample was placed in 25 mL glass vials, and extracted with 25 mL of 70% methanol for 30 minutes. Approximately, 1 mL of the extract was filtered into light sensitive HPLC through a 0.45 µm filter and stored at -20 ^{o}C in the dark (Siddhuraju et al, 2003, Standard Operating Protocol (SOP), 2001). Next, 5.5 mg chlorogenic acid was dissolved in 10 mL of 70% MeOH to make a standard stock

solution which was then diluted to make work solutions of 11 concentration ranging from 275 µg /mL to 0.537 µg/mL for a calibration curve giving the equation y = 0.045x – 0.102 (r^2 = 0.9999).



Graph 6.1. Calibration Curve of Chlorogenic Acid

6.3 RESULTS AND DISCUSSION

Moringa extract was chemically profiled using LC/DAD/MS in comparison with phenolic acid standards. This led to the identification of four isomers (structural and/ or spatial, peaks 1, 2, 5, and 6 of Figure 6.1-A.) and 2 analogs (dehydroxy, peaks 3 and 4 of Figure 7.1-A.) of chlorogenic acid based on analysis of the UV (λ_{max} 330 nm) and MS data. The six identified peaks (Figure 6.1.) did not have retention times matching that of the chlorogenic acid standard; however, peaks 1, 2, 5 and 6 analyzed by mass spectrometry, had the same molecular weight as that of chlorogenic acid while peaks 3 and 4 differed by a molecular weight of 16 (Figure 6.2.). In absence of authenticated pure materials, it was necessary to use chlorogenic acid to estimate the peak areas of each peak for quantitative purposes. The MS spectra of the standard compound chlorogenic acid is illustrated in Figure 6.2-A, while the phenolic acids detected in Moringa leaves can be seen in Figure 6.2-B, which shows the adduct molecular ions ([M+Na]+) and characteristic fragment ions. The proposed structures of the six phenolic acids identified in Moringa leaves are presented in Figure 6.3 and proposed fragments (Figure 6.4.). Under optimized HPLC conditions, all six phenolic acids were individually quantitated by UV detection at 330 nm. The contents of phenolic acids in the Moringa samples were calculated according to the calibration formula calculated with chlorogenic acid as external standard and a correction factor for peaks 1, 2, 5 and 6 (1.000) and peaks 3 and 4 (0.955, ratio of MW), respectively.

The UV chromatograms (Figure 6.1.) show that isomers of chlorogenic acid, or 5-caffeoylquinic acid (CQA), were the only phenolic acids found in the *M. oleifera* leaf. Chlorogenic acids are a large family of polyphenols characterized by an ester bond

between quinic acid and one to four residues of specific trans-cinnamic acids (Figure 6.5.), most commonly caffeic, p-coumaric and ferulic acid. They function as dietary neurosignalling compounds at adenosine receptors and as antioxidants (Nakatani et al, 2000). Looking at some of the isomers of chlorogenic acid, we can see that peaks 1, 2, 5 and 6 could be compounds 2, 3 and 5 or isomers thereof, while peaks 3 and 4 are assumed to be isomers of compound 4 (Table 6.1.).

Overall, sample 07-MO-09 from Zambia (2007) had the highest concentration of chlorogenic acid isomers while Z4 from Zambia (2006) had the lowest. The total analogs content varied between Moringa varieties. However, a t-test showed that there was no significant difference between the PKM and indigenous varieties. We then compared the samples for the PKM-1 variety from Lusaka, Zambia and Dakar, Senegal, and the indigenous variety, specifically Z3, Z4, Z6, Z10 and Z11 from Mumbwa, Kafue and Lusaka, Zambia and SE61 and SE 65 from Dakar, Senegal. The PKM-1 varieties had values between 0.223 to 0.358 % with the samples from Dakar, Senegal, exhibited the highest concentrations at 0.388 and 0.358 %. The PKM-2 varieties (Z5 and Z12) were in the same range as the PKM-1 varieties. There were slight variations seen in the indigenous varieties with a range of 0.181 to 0.388 % with the highest collected from Dakar, Senegal (SE61) and the lowest from Zambia (Z4).

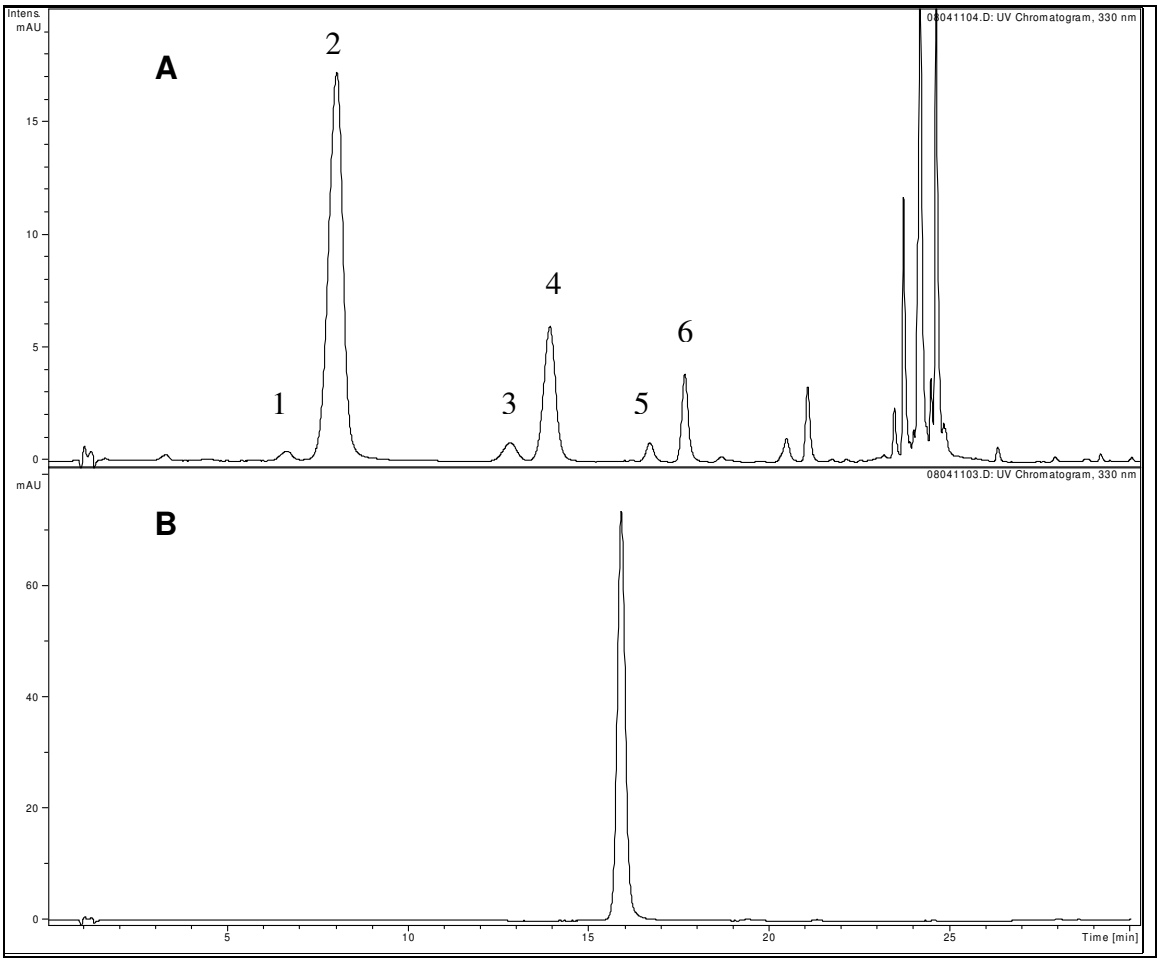


Figure 6.1. UV chromatograms (330 nm) of (A) Moringa extract and (B) standard compound chlorogenic acid

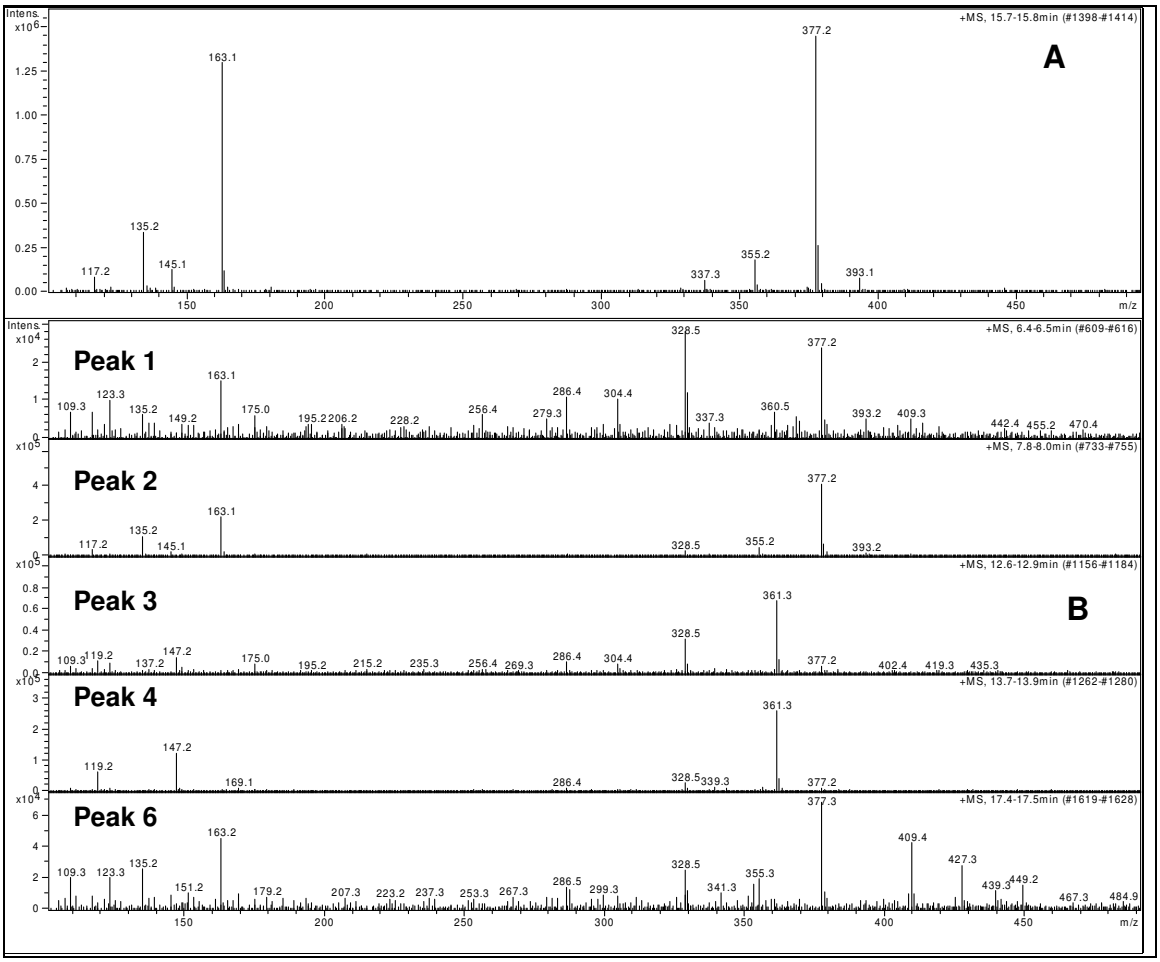


Figure 6.2. MS spectra of (A) standard compound chlorogenic acid and (B) the major analogues.

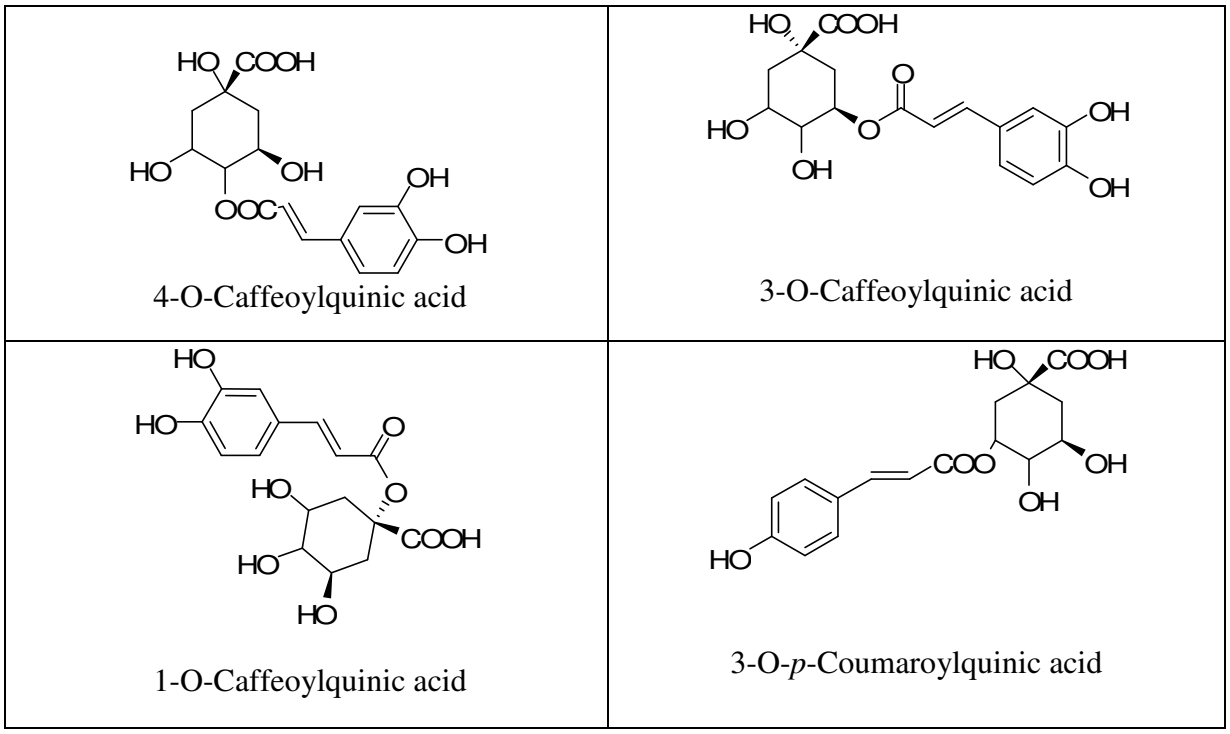
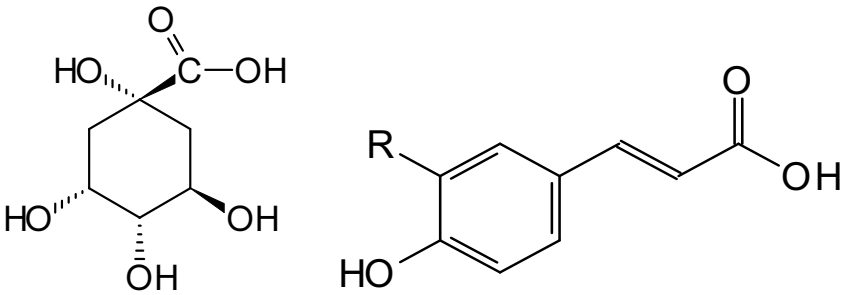


Figure 6.3. Isomers and Analogs of Chlorogenic Acid
Peaks 1, 2, 5 and 6 (Figure 6.1.) can potentially be compounds 1, 3, or 4- caffeoylquinic acid and while peaks 3 and 4 (Figure 6.1.) can potentially be 3-O-*p*-coumaroylquinic acid



quinic acid R=OH (3, 4-dihydroxy cinnamic acid, peak 1, 2, 5 and 6)

R=H (4-hydroxy cinnamic acid, peak 3 and 4)

Figure 6.4. Proposed structure of the phenolic acids detected in Moringa leaves, which are the esters formed between cinnamic acids and quinic acid. Chlorogenic acid is 5-O-caffeoylquinic acid

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Table 6.1. Analogs of chlorogenic acids in *Moringa oleifera* leaves

	Content (% g dry wt)						
Location	1	2	3	4	5	6	Total chlorogenic acid*
Gasabo	T	0.016	0.027	0.074	0.041	0.078	0.236
Kibungo	T	0.014	0.021	0.053	0.028	0.092	0.207
Kicukiro	T	0.015	0.030	0.120	0.042	0.152	0.358
Lusaka (1)	T	0.017	0.021	0.048	0.033	0.095	0.214
Lusaka (2)	0.024	0.013	0.038	0.162	0.036	0.066	0.339
Lusaka (3)	0.026	0.015	0.043	0.209	0.032	0.089	0.414
PKM	0.021	0.019	0.020	0.051	0.036	0.093	0.240
Mumbwa	0.020	0.014	0.019	0.047	0.034	0.089	0.223
Kafue	0.021	0.013	T	0.025	0.028	0.095	0.181
PKM	0.021	0.014	0.022	0.046	0.032	0.090	0.223
Kafue	0.022	0.015	0.023	0.070	0.030	0.091	0.252
Binga	0.024	0.014	T	0.038	0.029	0.113	0.218
PKM	0.024	0.017	0.020	0.046	0.035	0.105	0.248
Mumbwa	T	0.018	0.020	0.058	0.041	0.122	0.260
Kafue	0.021	0.021	0.026	0.090	0.046	0.138	0.343
PKM	0.025	0.015	0.021	0.051	0.036	0.099	0.246
Kafue	0.021	0.014	0.027	0.093	0.047	0.139	0.340
Binga	0.024	0.013	0.020	0.05	0.040	0.126	0.273
PKM	0.021	0.015	0.018	0.042	0.035	0.097	0.228
Dodowa	0.021	0.014	0.029	0.098	0.028	0.149	0.339
Volta Dan	T	0.015	0.025	0.097	0.039	0.094	0.271
Dakar	0.027	0.019	0.032	0.086	0.042	0.181	0.388
Dakar	0.023	0.018	0.028	0.072	0.028	0.189	0.358
Dakar	0.022	0.017	0.023	0.048	0.027	0.160	0.296
Dakar	0.023	0.016	0.026	0.074	0.041	0.078	0.326

The values are expressed as average in % g dry wt. *Total analogs of chlorogenic acid in the study refer only to the additions of the analogs of chlorogenic acid. Locations from Rwanda: Gasabo, Kicukiro, Kibungo; Zambia: Binga,Lusaka, Mumbwa, Kafue; Ghana: Dodowa, Volta Dan; Senegal: Dakar
T=trace amounts detected only

6.4 CONCLUSIONS

Six phenolic acids were identified based on the UV and MS data. Analysis of the chromatograms (Figure 6.1.) did not lead to the identification of gallic acid, caffeic acid, ellagic acid, or ferullic acid as was shown in previous studies. However, four of the compounds had the same molecular weight as that of chlorogenic acid while the other two had a molecular weight similar to that of 3-O-*p*-coumaroylquinic acid while all had slightly varying retention times when compared to the standard chlorogenic acid. The detected compounds were determined to be analogs of chlorogenic acid and therefore effectively quantitated with chlorogenic acid as an external standard, utilizing a correction factor of the ratio of the respective molecular weights. This discovery of chlorogenic acid analogs in the leaves give a promising indication that the leaves have medicinal use in maintaining a healthy liver and gall bladder function as well as for other medicinal applications. When compared to fruits such as prunes and coffee beans, both of which give high concentrations of chlorogenic acid and analogs, Moringa has a low concentration; however, these analogs can be of good use to the body and Moringa can be seen as a new source. Recent reports have associated consumption of the analog forms of chlorogenic acid to have protective benefits to the central nervous system. This gives further evidence that Moringa leaves are promising for both nutrition and health purposes.

CHAPTER 7

QUANTITATION OF THE TOCOPHEROLS BY HPLC ANALYSIS

7.1 INTRODUCTION

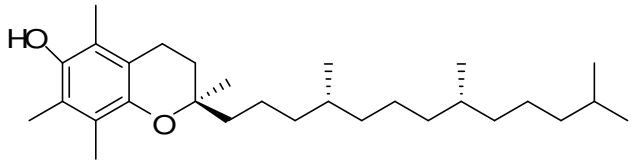
Vitamin E is a fat-soluble vitamin with strong antioxidant capacity and is a combination of tocopherol isomers α, β, γ, δ–tocopherol (Ching et al, 2001; Sánchez-Machado et al, 2005). Although vitamin E has been promoted as a cure for infertility, an anti-scar treatment (Thiele et al,2007), a defense against air pollution, a compound that exhibits anti-ageing characteristics as promoted in cosmetics and skin care products (Guaratini et al, 2006), research has not shown it to be useful for all these purposes. There are several naturally occurring forms of vitamin E found in foods, but only α-tocopherol meets the body's vitamin E requirement (Institute of Medicine, 2006).

Vitamin E functions as a chain-breaking antioxidant in the body by preventing free radicals and protecting cell membranes (Smolin et al, 2007). It is important in maintaining the integrity of red blood cells and cells in the nervous tissue and immune system (Smolin et al, 2007). Vitamin E defends cells damaged by heavy metals, such as lead and mercury, and toxins - carbon tetrachloride, benzene, and a variety of drugs (Ames et al, 1997). A number of vitamin E's roles are hypothesized to reduce the risk of heart disease and as an antioxidant; it helps protect low density lipoprotein (LDL) cholesterol oxidation (Sánchez-Machado et al, 2002, Ching et al, 2001; Lui et al, 2008).

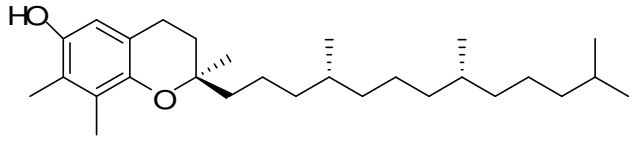
In vitro studies indicate that vitamin E inhibits a number of events critical to the development of atherosclerosis. (Smolin et al, 2007; Institute of Medicine, 2006; National Academy Press, 2000.).

Because of the recognized importance of vitamin E in health and nutrition, the Institute of Medicine of the National Academies has established recommended daily guidelines for vitamin E intake. The recommended dietary allowance (RDA) for adult men and women is set at 15mg/day of α-tocopherol (Table 1.5.). Vitamin E deficiency is usually characterized by neurological problems associated with nerve degeneration; symptoms observed in humans include poor muscle coordination, weakness, and impaired vision (Institute of Medicine, 2006). However, because vitamin E is plentiful in various food supplies and is stored in many of the body's tissues, vitamin E deficiency is rare in developed countries occurring largely in those unable to absorb the vitamin E due to fat malabsorption. In the USA, dietary supplements containing vitamins, promotes hair growth; alleviate fatigue; maintain immune function; enhance athletic performance; prevent heart disease and cancer; and treat a host of other medical problems (Smolin et al, 2007).

In this chapter, the presence and content of both α-tocopherol and γ-tocopherol in the Moringa leaves was quantitated by HPLC analysis.



α-tocopherol



γ-tocopherol

Figure 7.1. Chemical Structures of α-tocopherol and γ-tocopherol

7.2 MATERIALS AND METHODS

7.2.1 Materials

HPLC grade acetonitrile (ACN), methanol (MeOH), butanol (BuOH), ethyl acetate (EtOAc) and hexane were obtained from Fisher Scientific (Fair Lawn, NJ). Pyrocatechol was purchased from Sigma Aldrich (St.Louis, MO). HPLC grade formic acid was obtained from Acros Organics (NJ). HPLC-grade water (18M Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

7.2.2 Equipment

HPLC was performed on a Luna 5μm Phenyl-Hexyl 150 × 4.6 mm, Phenomenex Co., Alliance HPLC equipped with Waters 2795 Separation module and Waters 2487 UV Dual λ Absorbance Detector, and Waters Millennium Software.

7.2.3 HPLC condition

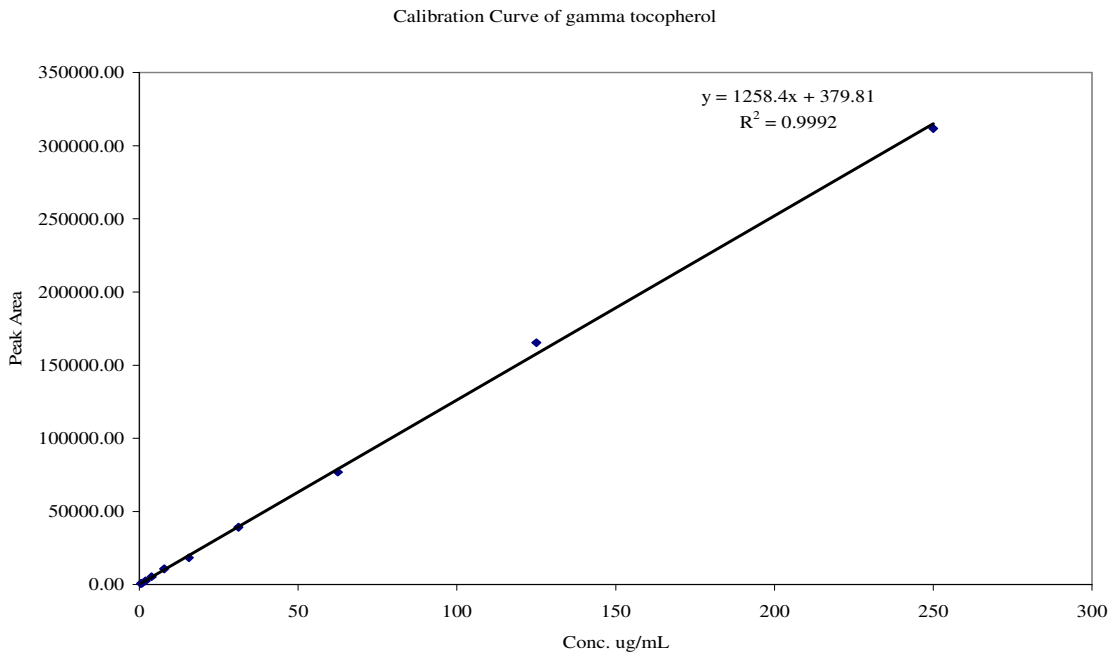
The mobile phase for chromographic separation consited of solvent A (water), solvent B (ACN) and solvent C (MeOH in 0.1% formic acid) for the following gradient: 10:40:50 (A:B:C) at 0 min, 7.5:42.5:50 (A:B:C) at 10 mins and 10:40:50 (A:B:C) 40 mins at a flow rate of 1.0 mL/min. The injection volume was 10 μL and the UV detector was set at 300nm.

7.2.4 Standards

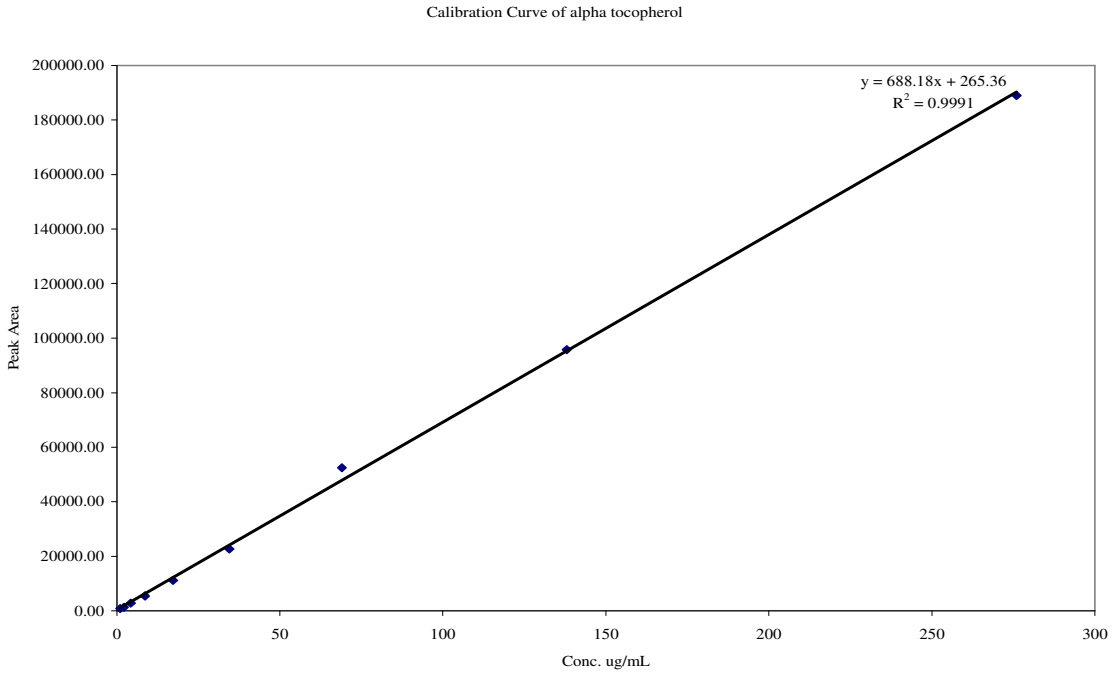
Standard compounds, α-tocopherol and γ-tocopherol were purchased from Sigma Aldrich Co. (St. Louis, MO).

7.2.5 Method

The procedure for sample preparation was slightly modified from that used by the method of Sánchez-Machado et al, 2006. KOH solution was prepared in methanol (0.5M) and the pyrocatechol solution (1 g in 5 mL of methanol) was prepared fresh daily and stored at -20 ^{o}C in the dark. About 0.4g (± 0.001g) was weighed out in a screw-top assay tube. Two hundred microlitres of pyrocatechol solution and 5 ml of KOH solution (0.5M in methanol) were added, and immediately vortexed for 20 s. The tubes were placed in a water bath at 80 ^{o}C for 15 min, removing them every 5 min, removing them every 5 min and vortexing again for 15s. After cooling in a ice bath, 1 ml of water and 5 ml of hexane was added, and the mixture was rapidly vortexed for 1 min, then centrifuged for 5 mins, afterwards 3 ml of the diluent (20:80, v/v MeOH:ACN) was then membrane filtered. Before injection, the extracts were maintained at -10 ^{o}C in the dark. Next, 6.9 mg and 12.5mg of α- and γ-tocopherol were dissolved in 25 mL of a mixture of BuOH:EtOAc:Hexane to achieve the standard stock solution which was then diluted to make work solutions of 10 concentrations 276 µg/mL to 0.539 µg/mL and 250 µg/mL to 0.488 µg/mL for the calibration curve, giving the equations $y = 688.18x + 265.36$ ($r^2 = 0.9991$) and $y = 1258.4x + 379.81$ ($r^2 = 0.9992$) for α- and γ-tocopherol respectively.



Graph 7.1. Calibration Curve of γ- Tocopherol



Graph 7.2. Calibration Curve of α- Tocopherol

7.3 RESULTS AND DISCUSSION

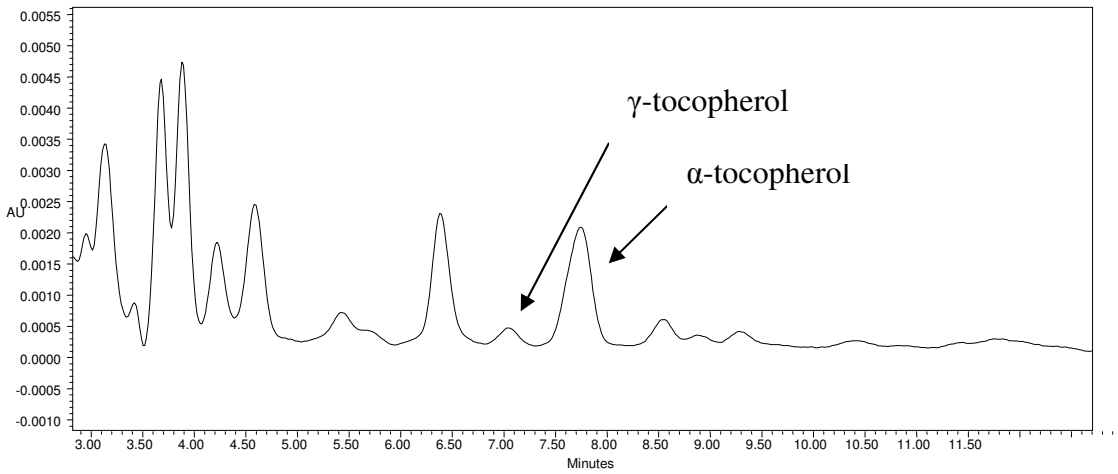


Figure 7.2. A representative HPLC chromatogram of γ- and α- tocopherols extracted from the leaves of *Moringa oleifera* grown and collected in sub-Saharan Africa.

There are many studies which optimize the extraction of α- and γ-tocopherols from natural substances by saponification or lipid extraction. In this research, saponification gave higher concentrations of tocopherols from the moringa leaves. We were able to identify both α- and γ-tocopherols by comparison of retention times with the UV absorption spectra of corresponding standards. Peaks were observed at 7.10 min for γ-tocopherol and 7.83 min for α-tocopherol (Figure 7.2.)

Comparing the samples collected in 2006 with those collected in 2007, we found that the sample with the highest concentration of total tocopherols was a finished commercial product from Lusaka, Zambia (07-MO-07), at a concentration of 116.79 mg/100 g DW. The vitamin E RDA for children aged 1 to 3 years and 4 to 8 years is 6 and 7 mg per day, respectively (Table 1.5.). Therefore, children aged 1 to 3 years would need 5.5 g of Moringa from Lusaka, Zambia while children of ages 4 to 8 years would need 6.0 g to acquire the daily amount. In contrast, the lowest concentration of

tocopherols found in Moringa samples, 7.11 mg/100g DW from Mumbwa, Zambia (Z3), the children aged 1 to 3 years would need to consume 84.4 g DW and children aged 4 to 8 years would need 98.5g DW to meet the RDA values.

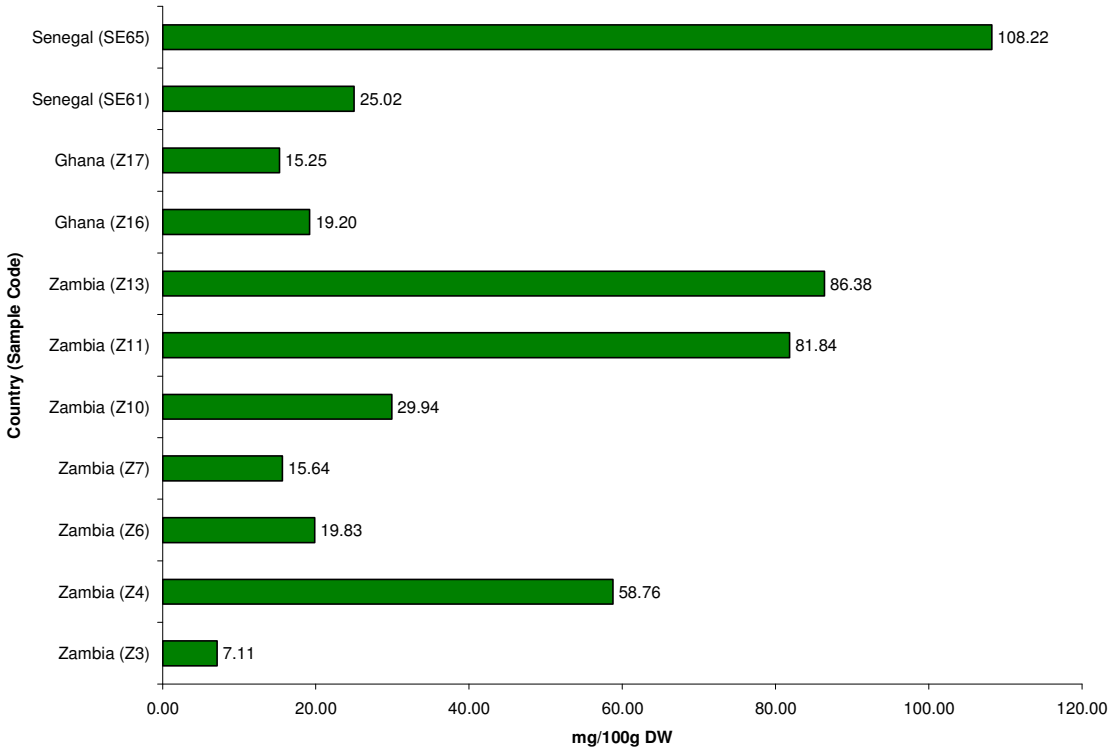


Figure 7.3. Content of γ- and α- tocopherols in the indigenous Moringa from the different countries in sub-Sahara Africa, 2006.

For the moringa collected in 2006, we found that the amount of total tocopherols varied from 7.11 to 112.04 mg/100 g DW with the highest collected from Zambia (Z14), the variety Binga; and the lowest from Mumbwa, Zambia (Z3), an indigenous moringa. The Moringa samples collected from Zambia and Senegal gave an inconsistent relationship in tocopherol concentrations. Indigenous samples collected in 2006 from Kafue, Zambia, showed consistent amounts of tocopherols in the dried leaves (Figure 7.3.).

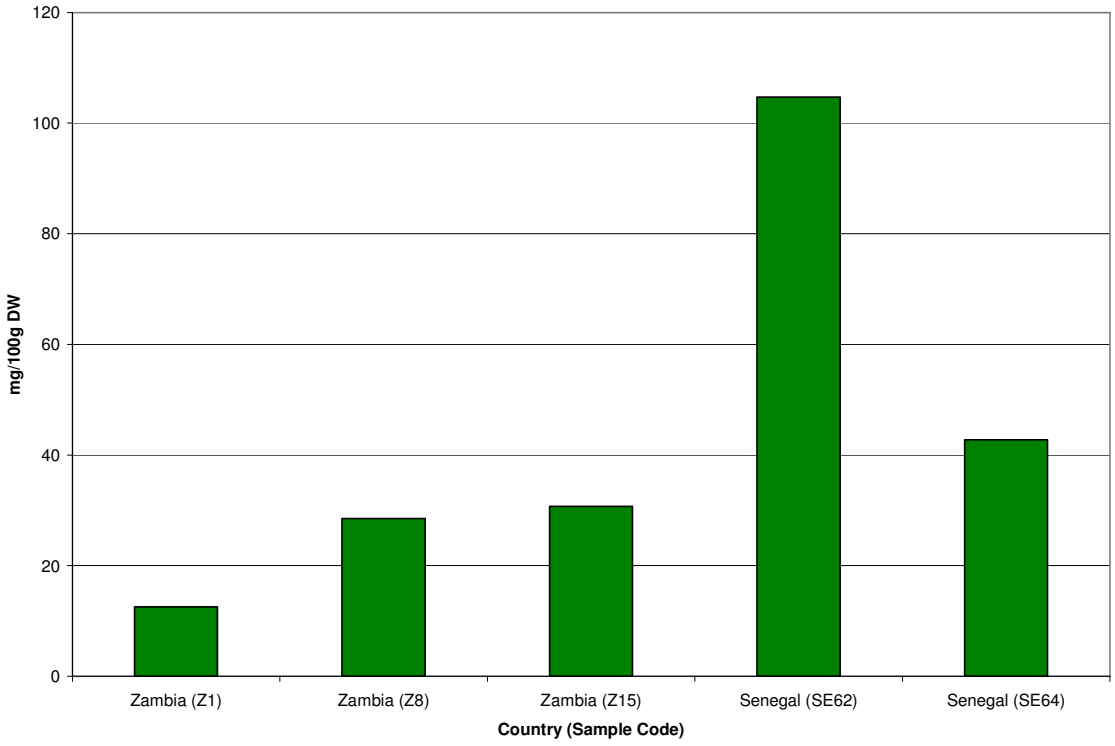


Figure 7.4. Content of γ- and α- tocopherols in the Moringa variety, PKM-1 from different locations in sub-Sahara Africa.

For the PKM-1 variety, the γ- and α- tocopherols concentrations varied by country, with the highest from Dakar, Senegal (SE62) at 104.11 mg/100 g and the lowest PKM-1 variety was from Zambia (Z1) at 42.73 mg/100 g (Figure 7.4.). The concentrations of γ- and α- tocopherols were consistently high in samples from Dakar, Senegal, specifically SE62 and SE64. For the PKM-2 variety growing in Zambia, samples showed to be relatively low in γ- and α- tocopherols with concentrations of 12.77 and 14.03 mg/100 g DW. There was no significant difference for the mean amounts of total tocopherols between PKM-1 and PKM-2 dried leaves.

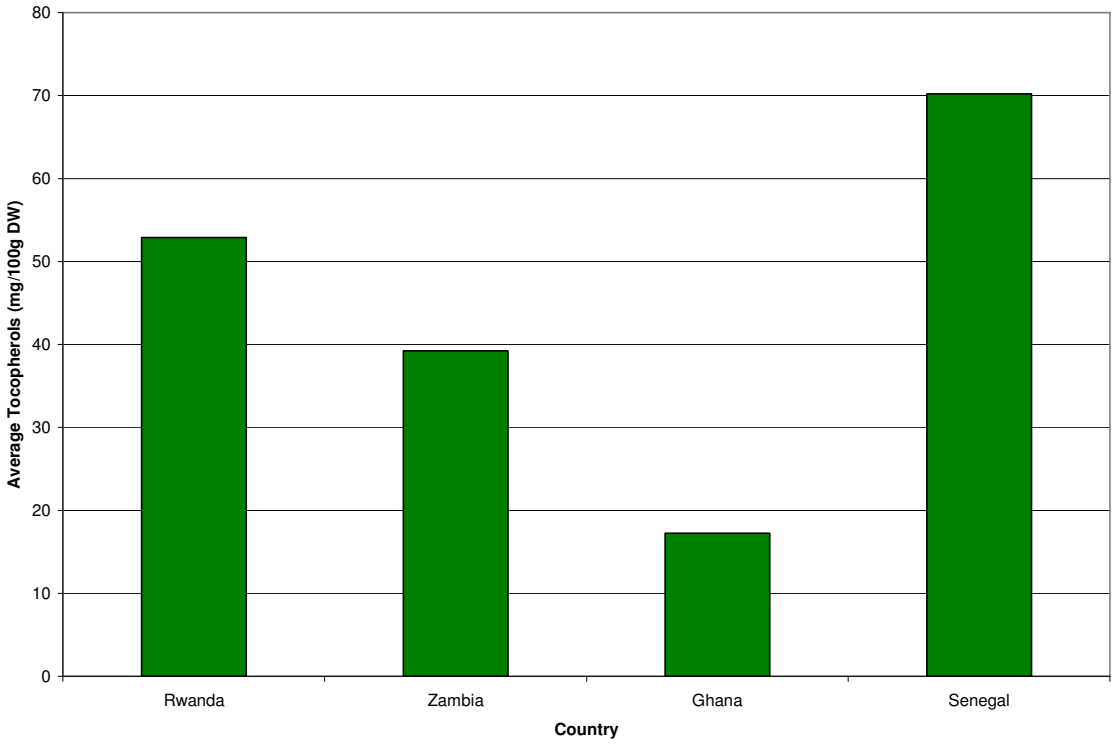


Figure 7.5. Average tocopherol content from different countries in sub-Sahara Africa.

Samples collected from Rwanda, Zambia and Senegal showed a significant difference in total tocopherols (defined in this thesis as the addition of γ- and α- tocopherol) when compared to the moringa samples from Ghana which were considerably lower (Figure 7.5.). No difference in γ- and α- tocopherols were found between the moringa samples from Rwanda, Senegal, and Zambia. Overall, leaves collected from Ghana had the lowest amounts of tocopherols per gram of dry weight (mg/100g DW).

Table 7.1. Contents of tocopherol isomers in *Moringa oleifera* leaves

Country	Location	γ -tocopherol	α-tocopherol	Total tocopherols*
Rwanda	Gasabo	2.82	34.11	36.93
	Kibungo	5.75	43.77	49.52
	Kicukiro	3.24	39.84	43.08
Zambia	Lusaka	5.46	111.34	116.79
	Lusaka	1.79	14.94	16.73
	Lusaka	1.56	33.43	35.00
	PKM	0.66	11.91	12.58
	Mumbwa	0.67	6.44	7.11
	Kafue	7.40	51.35	58.76
	PKM	0.91	11.86	12.77
	Kafue	1.60	18.23	19.83
	Binga	1.38	14.26	15.64
	PKM	2.97	25.60	28.57
	Mumbwa	1.49	28.45	29.94
	Kafue	7.76	74.08	81.84
	PKM	1.05	12.98	14.03
	Kafue	7.73	78.65	86.38
	Binga	10.54	101.51	112.04
	PKM	4.06	26.70	30.76
Ghana	Dodowa	1.73	17.47	19.20
	Volta Dan	2.10	13.14	15.25
Senegal	Dakar	1.45	23.57	25.02
	Dakar	6.60	98.11	104.71
	Dakar	2.98	39.75	42.73
	Dakar	3.55	104.67	108.22

Values are expressed as average in mg/100g DW

*Total tocopherols in the study refer only to the additions of both α-tocopherol and γ-tocopherol

PKM – improved sample from India grown in Lusaka

7.4 CONCLUSIONS

The method described allows the rapid isolation and quantitative determination of α- and γ-tocopherol in samples of the *M. oleifera* leaves. Since this method (Sánchez-Machado et al, 2006) is a simple method of preparing samples, it can be used for the analysis of vegetable foods which are very lipophilic. Even though the samples gave varying amounts of γ- and α- tocopherols (and we report the addition of both forms as the total tocopherol content, though we recognize there are other forms not included) in the leaves, the moringa from Rwanda, Senegal and Zambia were on par while the collected samples from Ghana was the lowest in tocopherols. However, in all cases, the leaves of moringa were shown to provide a considerable source of this vitamin. For a child to obtain the recommended dietary allowance would need to intake at least 5.5 g of *M. oleifera* leaves.

CHAPTER 8

QUANTITATIVE ANALYSIS OF THE CAROTENOIDS BY HPLC

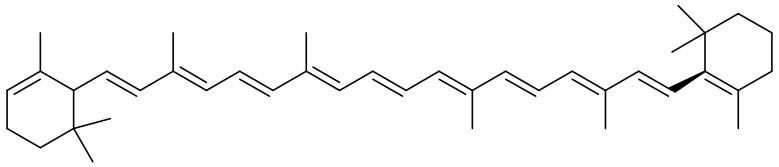
8.1 INTRODUCTION

Carotenoids are the natural yellow, orange or red pigments found in plants and are abundant in deeply colored fruits and vegetables (Breithaupt et al, 2000). The most prevalent carotenoids are α-carotene, β-carotene, lycopene, lutein, zeaxanthin and crypotoxanthin which can be converted into retinol (Vitamin A) in the body. These are collectively referred to as provitamin A carotenoids. However, lycopene, lutein, and zeaxanthin have no vitamin A activity and are called non-provitamin A carotenoids (Institute of Medicine, 2006; Smolin et al, 2007).

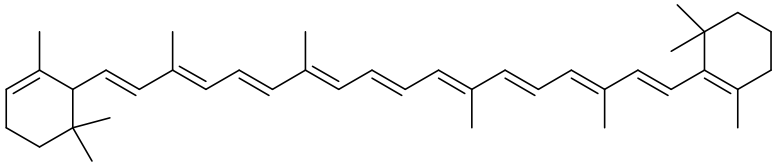
β-carotene is the most potent precursor found in the Vitamin A. Approximately 50-55 % of β-carotene has provitamin A activity while α-carotene has approximately 40-50 % of the pro-A activity of β-carotene (Murray, 1996). Both preformed vitamin A and carotenoids are bound to proteins in foods. To be absorbed, each must be released from the protein pepsin and other protein digesting enzymes. In the small intestine, the released carotenoids and retinol combine with bile acids (binds to the bile for absorption) and other fat-soluble food components to form micelles, which facilitate their diffusion into mucosal cells. Absorption of preformed vitamin A is efficient-70 to 90% of what is consumed. A diet that is very low in fat (less than 10g/day) can reduce vitamin A absorption. This is rarely a problem in industrialized countries, where typical fat intake ranges from 50 to 100 grams per day. However, in populations with low dietary fat intakes, vitamin A deficiency may occur due to poor absorption. Diseases that cause fat

mal-absorption, as well as some medication, can also interfere with vitamin A absorption and cause deficiency. The recommended dietary allowance (RDA) for the average male or female (14-70 yrs) is 600 μg/d, which can be taken without toxic side effects or poor absorption.

Carotenoids are found in ripe yellow fruits and vegetables, and appear to be more efficiently converted into vitamin A than those found in dark green, leafy plants (Rajyalakshmi et al, 2001). The objectives of this study is to quantitate β-carotene and α-carotene in the Moringa leaves by HPLC and to ascertain the relative contribution that Moringa leaves may provide to overall health and nutrition.



α-carotene



β-carotene

Figure 8.1. Chemical structures of α and β carotene

8.2 MATERIALS AND METHODS

8.2.1 Materials

HPLC grade Acetonitrile (ACN), Methanol (MeOH), 1-Butanol (BuOH), Ethyl Acetate (EtOAc), Acetic Acid (AcOH) and Hexane were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC grade formic acid was obtained from Acros Organics (NJ). HPLC-

grade water (18M Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

8.2.2 Equipment

HPLC was performed on a Luna 5μm Phenyl-Hexyl 150 × 4.6 mm, Phenomenex Co., Alliance HPLC equipped with Waters 2795 Separation module and Waters 2487 UV Dual λ Absorbance Detector, and Waters Millennium Software. The powdered plant samples were weighed on a calibrated analytical balance accurate to ± 0.1 mg.

8.2.3 HPLC condition

The mobile phase for chromographic separation consited of solvent A (water), solvent B (ACN) and solvent C (MeOH) for the following gradient: 10:40:50 (A:B:C) at 0 min and 0:50:50 (A:B:C) at 35 mins at a flow rate of 1.0 mL/min. The injection volume was 10 μL and the UV detector was set at 450 nm.

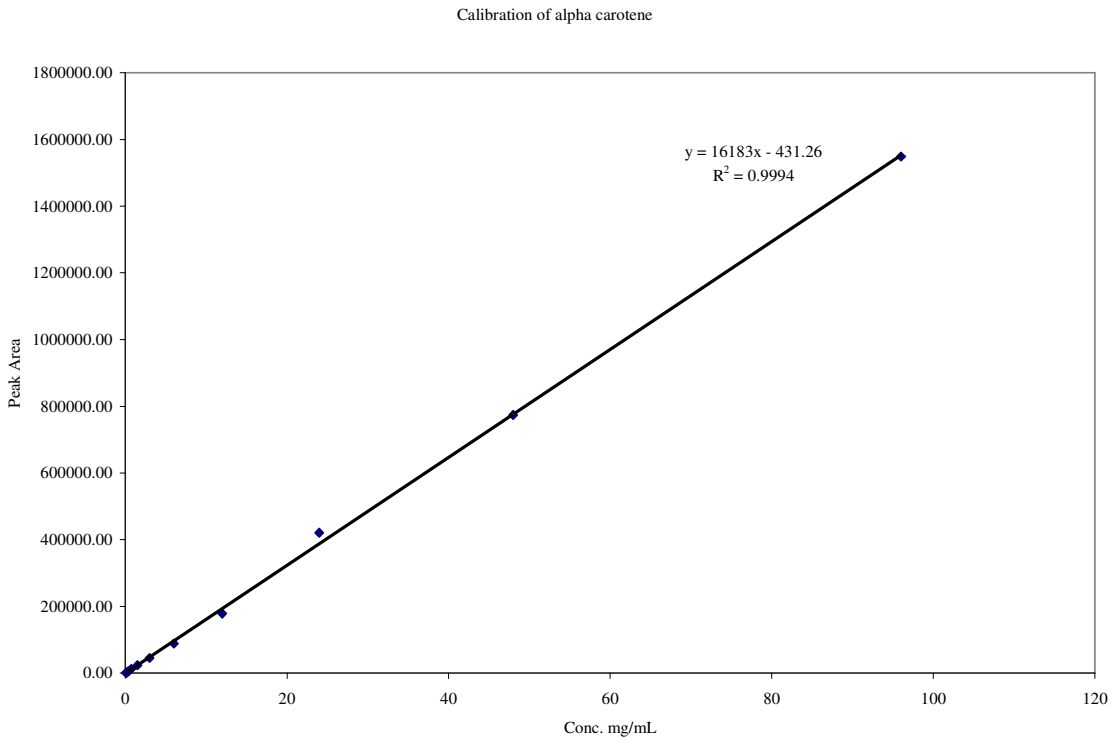
8.2.4 Standards

Standard compounds, β-carotene and a mixture of β & α carotene (2:1) were purchased from Sigma Aldrich Co. (St. Louis, MO).

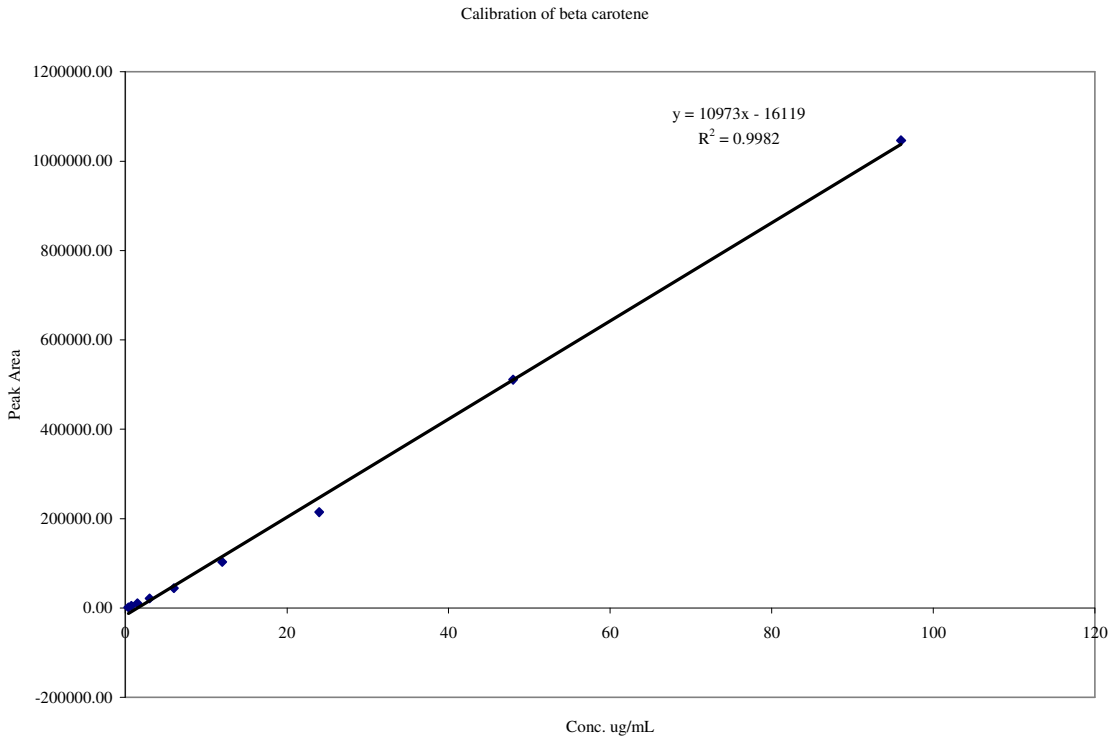
8.2.5 Method

The procedure for sample preparation of the tocopherol was used as described for the determination of carotenoids (Sánchez-Machado et al, 2006). 7.2mg of α- and β-carotenoids in a 1:2 ratio were dissolved in 25 mL of a mixture of BuOH:EtOAc:Hexane

to obtain the standard stock solution which was then diluted to make work solutions of 11 concentration ranging from 96 µg/mL to 0.09375 µg/mL for α-carotene and 192 µg/mL to 0.375 µg/mL for β-carotene for the calibration curve, giving the equation y = 16183x – 431.26 (r^2 = 0 .9994) and y = 10973-16119 (r^2 = 0 .9982) respectively.



Graph 8.1. Calibration Curve of α- Carotene



Graph 8.2. Calibration Curve of β- Carotene

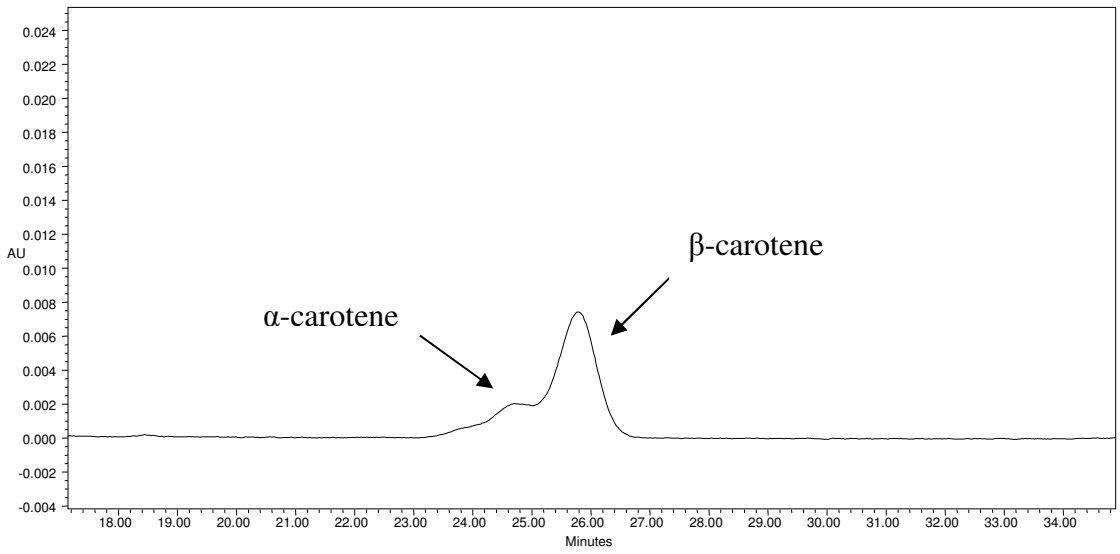


Figure 8.2. HPLC chromatogram of carotenoids in extract of Moringa leaves

8.3 RESULTS AND DISCUSSION

Results from the analysis showed that *M. oleifera* leaves contain both α- and β-carotene with consistently higher amounts of β-carotene. No direct relationship between the values obtained for α- and β-carotene was noted. That is, samples that exhibited high concentrations of β-carotene did not necessarily have neither high nor low concentrations of α-carotene. Moringa samples collected in 2007 showed higher concentrations of carotenoids than samples collected in 2006. The 2007 samples collected from the indigenous varieties and the finished commercial samples varied in carotenoid amounts, but again no consistent relationship between the samples could be observed. Specifically, indigenous samples collected from Kicukiro, Rwanda, and samples from Lusaka, Zambia (07-MO-07), a finished commercial sample, showed the highest of concentrations of carotenoids for all the samples tested. Samples collected in 2006 showed the same variation as those collected in 2007, and while the variation was consistent, it is important to note that the moringa can serve as a valuable source of α- and β-carotene. The leaves collected from Dakar, Senegal were higher in carotenoid concentration than those collected in both Zambia and Ghana. When comparing the different PKM-1 samples (Figure 8.3), the total carotenoids were highest in concentration for samples collected in Dakar, Senegal, at 16.32 and 12.26 mg/100 g DW for SE64 and SE62, respectively.

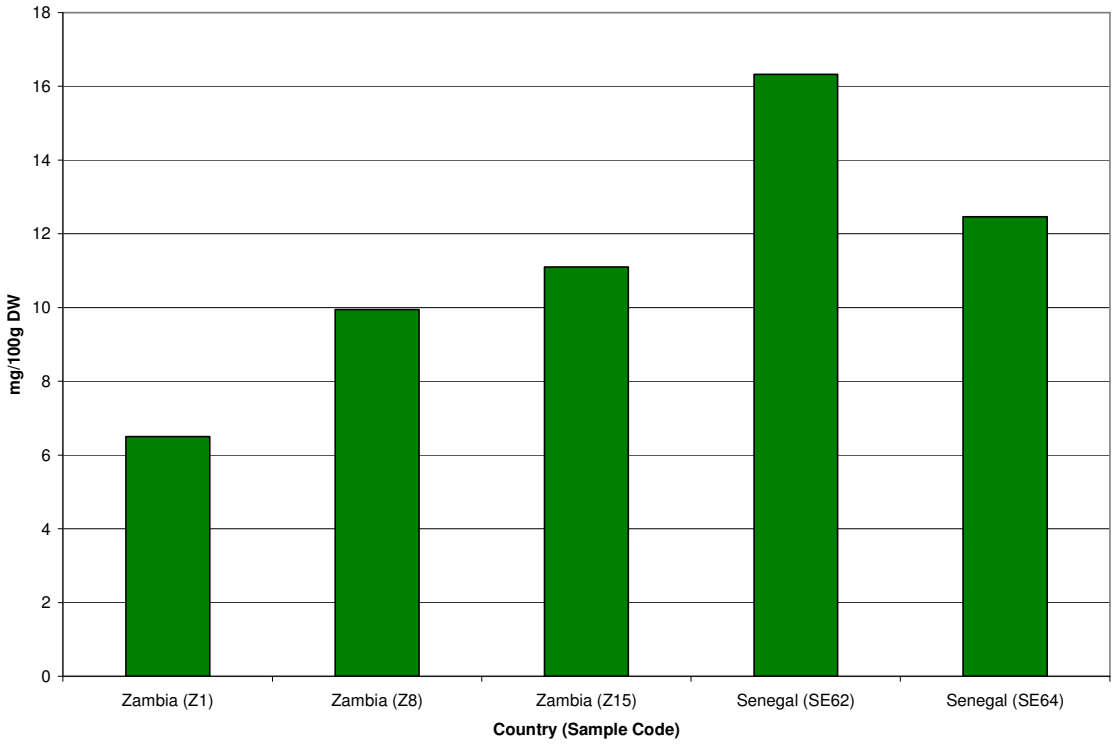


Figure 8.3. Total carotenoids in *Moringa oleifera* for PKM-1 variety grown and collected in Senegal and Zambia.

The leaves collected from Dakar, Senegal had the highest amount of total carotenoids for the PKM-1 variety (Figure 8.3.). From Zambia, the varieties Binga and PKM-2 showed only slight differences between each variety. *Moringa oleifera* plants from Zambia, Ghana and Senegal also varied but there was no observed statistical difference. Samples grown in Ghana gave the lowest concentrations for all the indigenous varieties with the exception of one sample from Mumbwa Zambia (Figure 8.4.).

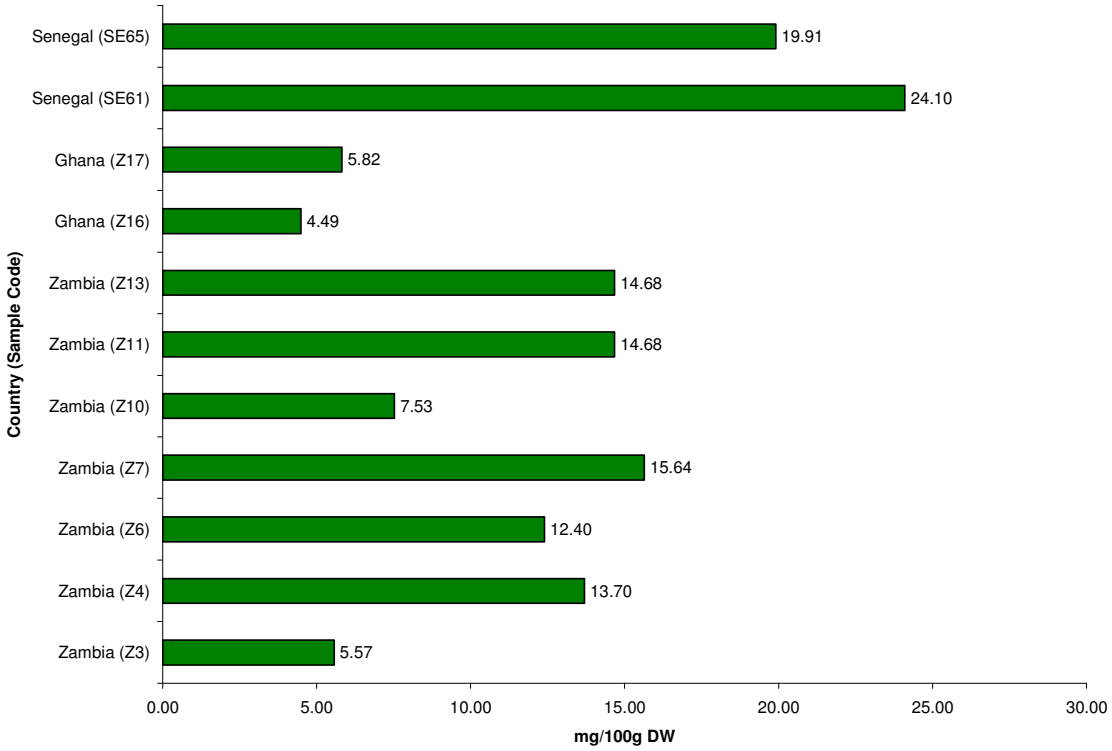


Figure 8.4. Total carotenoids in indigenous varieties of *Moringa oleifera* from the different countries in sub-Sahara Africa collected in 2006.

Table 8.1. Contents of carotenoids in Moringa leaves from sub-Sahara Africa.

Country	Location	α - Carotene	β –Carotene	Total Carotenoids*
Rwanda	Gasabo	2.54	20.48	23.02
	Kibungo	5.44	40.50	45.94
	Kicukiro	2.58	26.86	29.44
Zambia	Lusaka	4.85	38.69	43.53
	Lusaka	0.34	18.55	18.89
	Lusaka	0.64	19.60	20.24
	Lusaka	0.16	6.34	6.50
	Mumbwa	0.08	5.49	5.57
	Kafue	0.22	13.48	13.70
	PKM	0.14	13.54	13.69
	Kafue	0.20	12.29	12.49
	Binga	0.10	7.46	7.56
	Lusaka	0.15	9.80	9.95
	Mumbwa	0.29	7.24	7.53
	Kafue	0.51	13.88	14.39
	PKM	0.10	7.60	7.70
	Kafue	0.46	14.23	14.68
	Binga	0.35	9.75	10.10
	Lusaka	0.15	10.95	11.10
Ghana	Dodowa	0.31	4.17	4.49
	Volta Dan	0.16	5.66	5.82
Senegal	Dakar	0.57	23.53	24.10
	Dakar	0.59	15.73	16.32
	Dakar	0.30	11.96	12.26
	Dakar	0.68	19.23	19.91

Values expressed as average in mg/100g DW

*Total carotenoids in the study refer only to the additions of both α-carotene and β-carotene.

8.4 CONCLUSIONS

In comparing the samples collected in 2007 and 2006, the Moringa leaves collected in 2007 exhibited higher concentrations of carotenoids than those collected in 2006. Of the *M. oleifera* leaves collected in 2006, the sample with the highest concentration of total carotenoids were collected from Dakar, Senegal while the leaves collected in 2007 gave varied results but were the highest of most samples (Table 8.1.). Taking an average sample, such as SE62 from Dakar, Senegal with a value of 16.32 mg/100 g DW, a person between the ages of 14 and 70 who consumed 3.68g of dried *M. oleifera* leaves a day would meet the RDA of 600 μg/d. For children aged 0 to 6 months and 7 to 12 months taking 2.45 g and 3.06 g DW respectively and children aged 1 to 3 years and 4 to 8 years taking 1.84 g and 2.45 g DW a day would meet the RDA values of 300, 400 and 500 μg/d. Providing children between the ages of 6 months and 60 months with moringa as a vitamin A supplement can substantially reduce child mortality in vitamin A-deficient countries.

CHAPTER 9

DETERMINATION OF THE FLAVONOIDS BY LC/MS

9.1 INTRODUCTION

Flavanols are a sub-group of the flavonoids which are a class of water soluble compounds found in plants. Flavonols like the vitamins act as antioxidants boosting the effects of vitamins, regulating nitric oxide and scavenging free radicals while regulating the blood flow and keeping the heart healthy (Hertog et al, 1996). As antioxidant, some flavonoids such as quercetin, protect LDL "bad" cholesterol from oxidative damage and are found in a wide range of food, for instance flavones are found in citrus, isoflavanones in soy products (Ho et al, 2002) and flavans in apples (Guyot et al, 2002).

Flavonoids demonstrate a wide range of biochemical and pharmacological effects including anti-oxidation, anti-inflammation, anti-platelet, anti-thrombotic action and anti-inflammation (Miean et al, 2001). In vegetables, quercetin glycosides predominate, but glycosides of kaempferol are found in trace quantities. Prior chemical research has reported several flavonoid glycosides detected in Moringa leaves (Bennett et al, 2003; Miean et al, 2001).

In this study, using HPLC combines with UV and MS the flavonoid profile from 25 samples of Moringa leaves were examined. Under optimized conditions, 12 flavonoid glycosides were malonylglycosides, acetylglycosides and succinnoylglycosides, among which the quercetin and kaempferol glucosides and glucoside malonates are the major constituents. This quantitative study allowed the two flavonods aglycones quercetin and

kaempferolin acidic hydrolyzed Moringa leave extracts to be successfully quantified individually using RP-HPLC with UV detector.

9.2 MATERIALS AND METHODS

10.2.1 Materials

HPLC grade Acetonitrile (ACN), Methanol (MeOH), and Acetic Acid (AcOH) were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC grade formic acid was obtained from Acros Organics (NJ). HPLC-grade water (18M Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA).

9.2.2 Equipment

HPLC separation was performed on a Prodigy ODS3 5µm, 150 x 3.2mm 5 micro (Phenomenex Inc. Torrace CA). Hewlett Packard Agilent 1100 Series LC/MS (Agilent Technologies, Waldbronn, Germany) equipped with autosampler, quaternary pump system, DAD detector, multiple wavelength detector, degrasser, MSD trap with an electrospray ion source (ESI), and software of HP ChemStation, Bruker Daltonics 4.2 and Data Analysis 4.2.

9.2.3 LC/MS conditions for qualitative identification

HPLC separation was performed with the mobile phase containing solvent A and B in gradient, where A 0.1% in formic acid (v/v) in water and B (0.1% in formic acid (v/v) in acetonitrile. for the following gradient: 10 % to 30 % B in 10 - 20 mins and 30 % B in 20 – 30 mins at a flow rate of 1.0 mL/min. The injection volume was 10 µL and the detector

was set at 370 nm. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) under positive ion mode, and the samples were scanned from *m/z* 100 to 800. ESI was conducted by using a needle voltage of 3.5KV under optimum collision energy level of 60%. High-purity nitrogen (99.999%) was used as dry gas and at a flow rate of 12 L/min capillary temperature at 350 ^{o}C. Nitrogen was used as Nebulizer at 40 psi.

HPLC conditions for quantitative study

The mobile phase for chromatographic separation consisted of solvent A (0.1 % in formic acid (v/v) in water) and solvent B (0.1 % in formic acid (v/v) in acetonitrile) for the following gradient: 5 % B in 10 mins, 15 % B in 20 mins, and 75 % B in 30 mins at a flow rate of 1.0 mL/min. The injection volume was 10 µL and the detector was set at 370 nm.

9.2.5 Standards

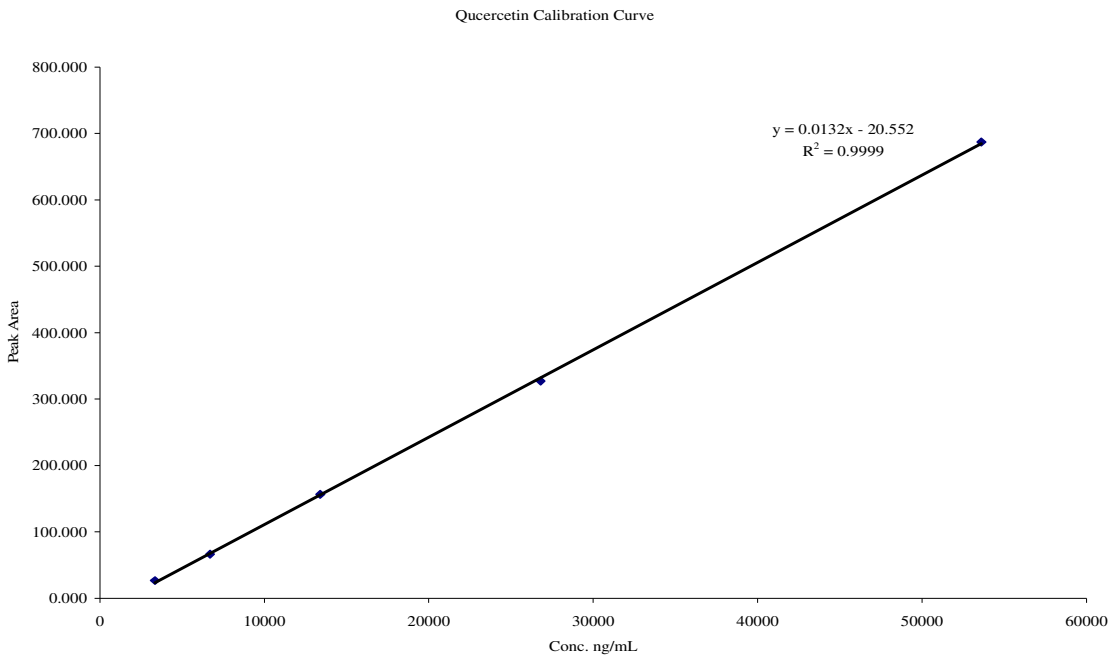
Standard compounds, quercetin and kaempferol were purchased from Sigma Aldrich Co. (St. Louis, MO).

9.2.6 Method

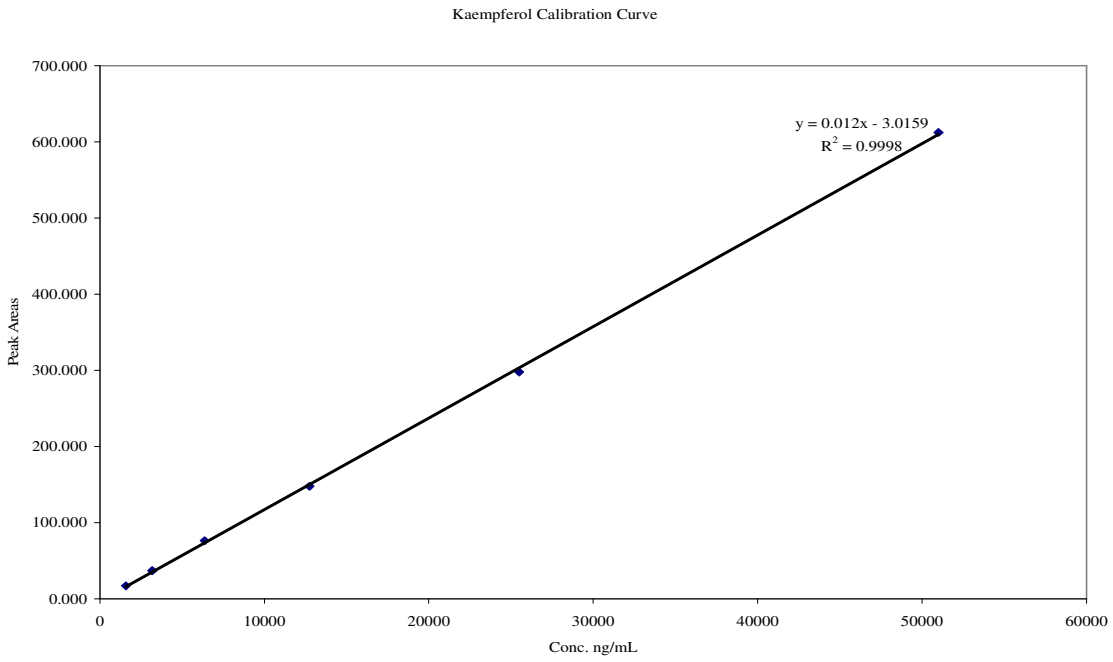
For the first qualitative study, 100 mg of the powdered Moringa leaf samples were placed in 30 mL glass vials and were extracted with 25mL of 70% methanol in water with 0.1% glacial acetic acid and water (methanolic solution) for 25 minutes and was left for 2 hours at room temperature. The samples were stored in the freezer at -20 ^{o}C overnight. The extracted solution was allowed to warm to room temperature and approximately 1 mL

was filtered through 0.45 μm filter and transferred into light sensitive HPLC vials (Wu et al, 2003; Bennett et al, 2003; Ho et al, 2002).

For the second study on quantitation, approximately 100 mg of each Moringa leaf sample was placed in a 125 mL round bottom flasks; then 25 mL of the methanolic solution was added and sonicated for 15 minutes. Concentrated HCl (1.5 mL) was added to the each mixture and refluxed for 2 hrs. The samples were allowed to cool to room temperature and placed in a 25 mL volumetric flask to keep volume constant. Approximately 1 mL of the extraction was filtered through a 0.45 μm filter and transferred into light sensitive HPLC vials. The samples were analyzed by LC/MS (Agilent 1100 Series) (Shen et al, 2006). A calibration curve of six dilutions of the standards quercetin and kaempferol in 70% methanol was achieved in duplicate analysis. The calibration concentrations ranged from 120 μg/mL to 1.875 μg/mL and the equation $y = 0.0132x - 20.552$ ($r^2 = 0.9999$) for quercetin and concentrations from 102 μg/mL to 6.375 μg/mL with the equation $y = 0.012x - 3.0159$ ($r^2 = 0.9998$) for kaempferol. The recoveries were validated by spiking known quantities of isoflavone standards corresponding approximately to 100%, 75% and 50% of the expected values in the Moringa sample 07-MO-09 and then together extracted according to the quantitative extraction method.



Graph 9.1. Calibration Curve of Quercetin



Graph 9.2. Calibration Curve of Kaempferol

9.3 RESULTS AND DISCUSSION

Representative UV chromatograms, MS total ion chromatogram and processed chromatograms of 70% Moringa leaves extract are presented in Figure 9.2. The identities, retention time, [M+ H^+] ions and other characteristic fragment ions for individual peaks are shown in Table 9.1. Based upon these UV and MS analyses, 12 flavonoids were simultaneously identified as quercetin and kaempferol glycosides, including malonyl glycosides, acetyl glycosides and succinoyl glycosides. Of these identified compounds, quercetin and kaempferol glucosides and glucosides malonates are the major constituents. The structures of the flavonoid aglycones quercetin and kaemperferol are illustrated in Figure 9.3, while Figures 9.4 and 9.5 illustrate the MS spectra of the 12 flavonoids detected in Moringa leaves.

The qualitative analysis (Figures 9.4 and 9.5) revealed that *M. oleifera* leaves contained large amounts of flavonoids, including quercetin and kaempferol glycosides, malonylglycosides, acetylglycosides and succinoylglycosides. The objective of this study was to accurately evaluate and quantitate the total flavonoids in Moringa leaves. Plant samples were hydrolyzed during extraction to facilitate the chromatographic separation. Under optimized HPLC conditions, the two main flavonoids, quercetin and kaempferol were successfully quantified in hydrolyzed extracts by UV detection. Using the HPLC conditions optimized under multiple preliminary assays, this approach enabled total separation of the two target flavonoid aglycones within 10 min. The successful separation can be seen in the representative chromatograms of a standard mixture and a typical hydrolyzed Moringa leaf extract under UV protection at wavelength of 370nm

(Figure 9.6.). Peak assignments were made with single compounds injection and MS spectral data. Baseline separation was successfully achieved for analytes.

The precision of this method was evaluated by carrying out triplicate determinations on the same day with different concentrations of the standard compound. The samples were spiked with known concentrations of the flavonoid standards, quercetin hydrate and kaempferol, corresponding approximately to 100%, 75% and 50% of the expected values in a representative sample, 07-MO-09. No considerable differences were found in recovery when spiked at different levels (<5.95 %). The mean recoveries at the different concentrations were 96.2, 101.3 and 107.5 % for quercetin (Table 9.3) and 92.6, 96.1 and 99.3 % for kaempferol (Table 9.4.) which gave an overall mean of 101.7 % for quercetin and 96.0 % for kaempferol. The results indicated the analytical method used to determine the flavonoids was precise and accurate.

The leaves of Moringa contained high amounts of flavonoids including that of quercetin and kaempferol with quercetin being the higher of the two. The amount of quercetin in the leaves ranged from 1.62 to 0.066 % while for kaemperol the percentage was lower ranging from 0.673 to 0.054 %. Overall, samples collected in 2006 and 2007 both showed high amounts of flavonoids with no major significance between the data. The sample with the highest content of flavonoids was found to be 07-MO-08 (1.643 %) from Lusaka Zambia, a finished commercial sample, while the lowest sample was SE65 (0.179 %) a sample from Dakar, Senegal.

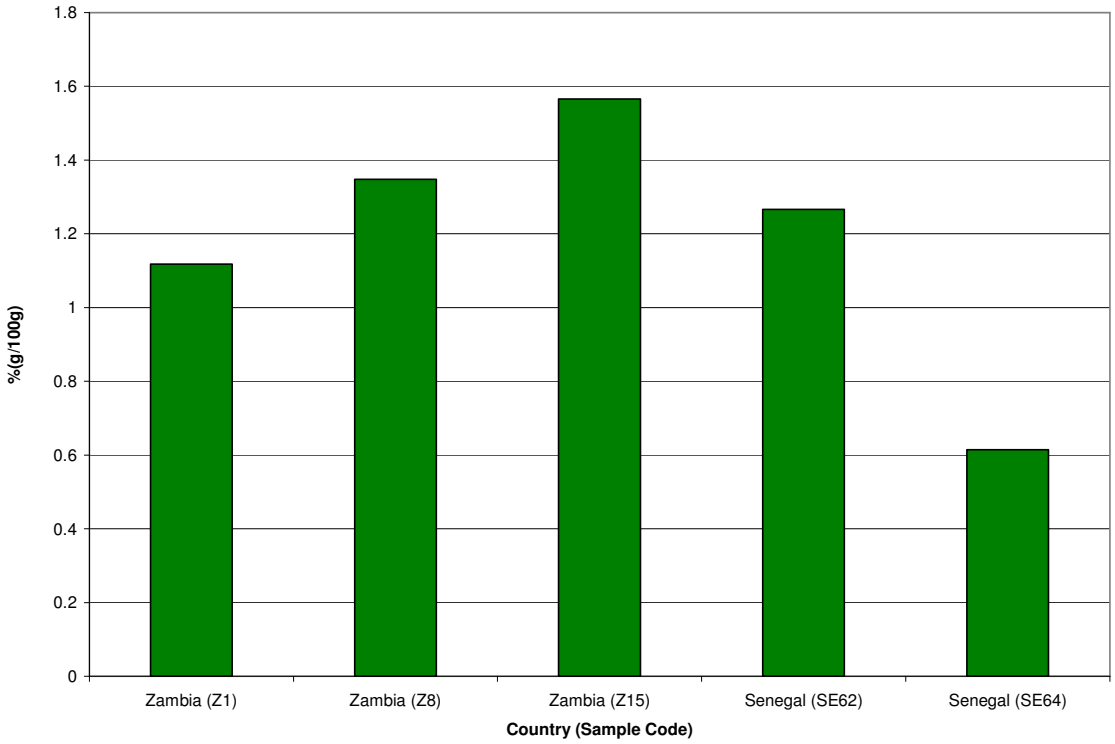


Figure 9.1. Total flavonoids in *Moringa oleifera* for the PKM-1 variety grown and collected in Senegal and Zambia.

The PKM-1 variety contained a high percentage of flavonoids (1.565-0.614% DW) as shown in Figure 9.6. However, compared to the Binga, PKM-2 and indigenous varieties there was no major difference between the varieties and in most situations would be considered as rich sources of these flavonoids independent of the Moringa variety. The samples collected from Dodowa and Volta Dan, Ghana had the lowest amount of total flavonoids while samples obtained from Zambia both in 2006 and 2007 exhibited very high concentrations (Table 9.4.).

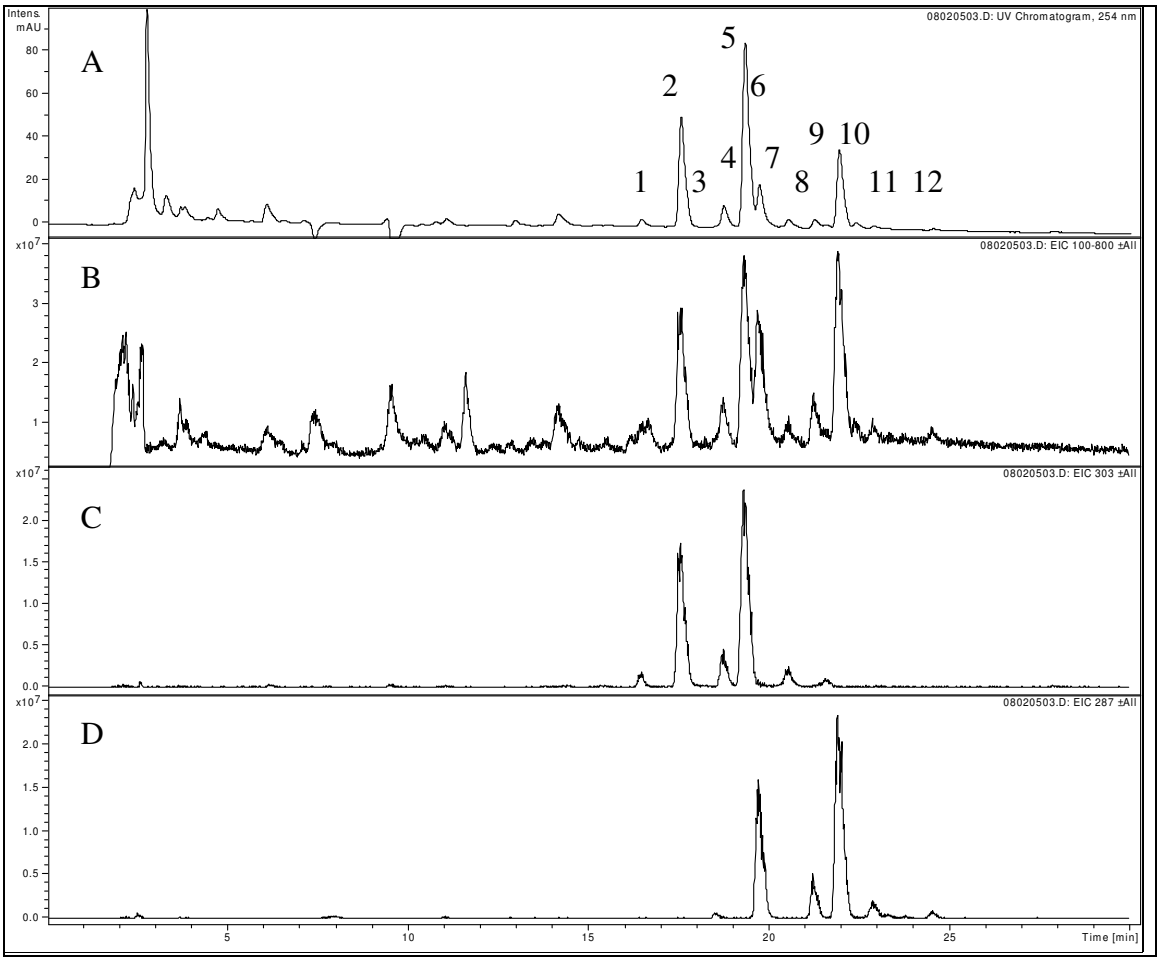
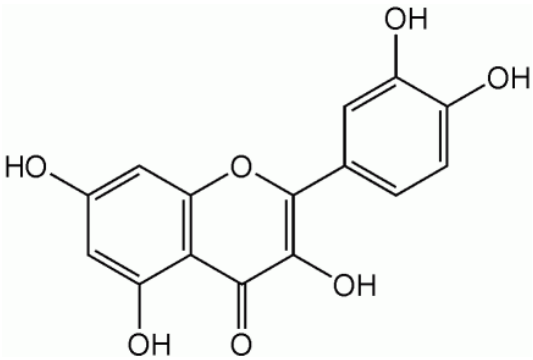


Figure 9.2. Representative UV and MS chromatograms of Moringa extract. (A) UV chromatogram (254 nm). (B) Total ion chromatogram. (C) Extracted ion chromatogram for Quercetin derivatives (EIC 303). (D) Extracted ion chromatogram for Kaempferol derivatives (EIC 287). The peaks of flavonoids are labeled in A and the identities are listed in Table 9.1.

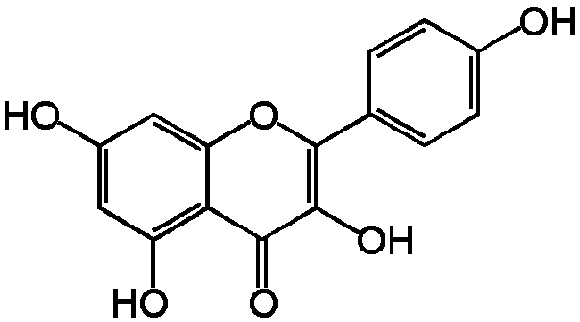
Table 9.1. Peak assignments for the analysis of *Moringa oleifera*.

Peak	t_R (min)	$[M+H/Na]^+$ (*m/z*)	MS fragment ion (*m/z*)	Identities
1	16.4	611	465, 303	Quercetin-G-Rha
2	17.5	465	303	Quaercetin-G
3	18.5	595	449, 287	Kaempferol-G-Rha
4	18.7	609	303	Quercetin-Xyl-Api-Ac
5	19.3	551	303	Quercetin-G-M
6	19.7	449	287	Kaempferol-G
7	20.4	551	303	Quercetin-G-M
8	21.2	593	287	Kaempferol-Xyl-Api-Ac
9	21.5	565	303	Quercetin-G-S
10	21.9	535	287	Kaempferol-G-M
11	22.9	535	287	Kaempferol-G-M
12	24.5	549	287	Kaempferol-G-S

G: Glucosyl/Galactosyl moiety; Rha: Rhamnosyl; Ara: Arabiosyl; Api: Apiosyl; M: Malonyl; S: Succinoyl



Quercetin



Kaempferol

Figure 9.3. Structure of the flavonoid aglycones quercetin and kaempferol.

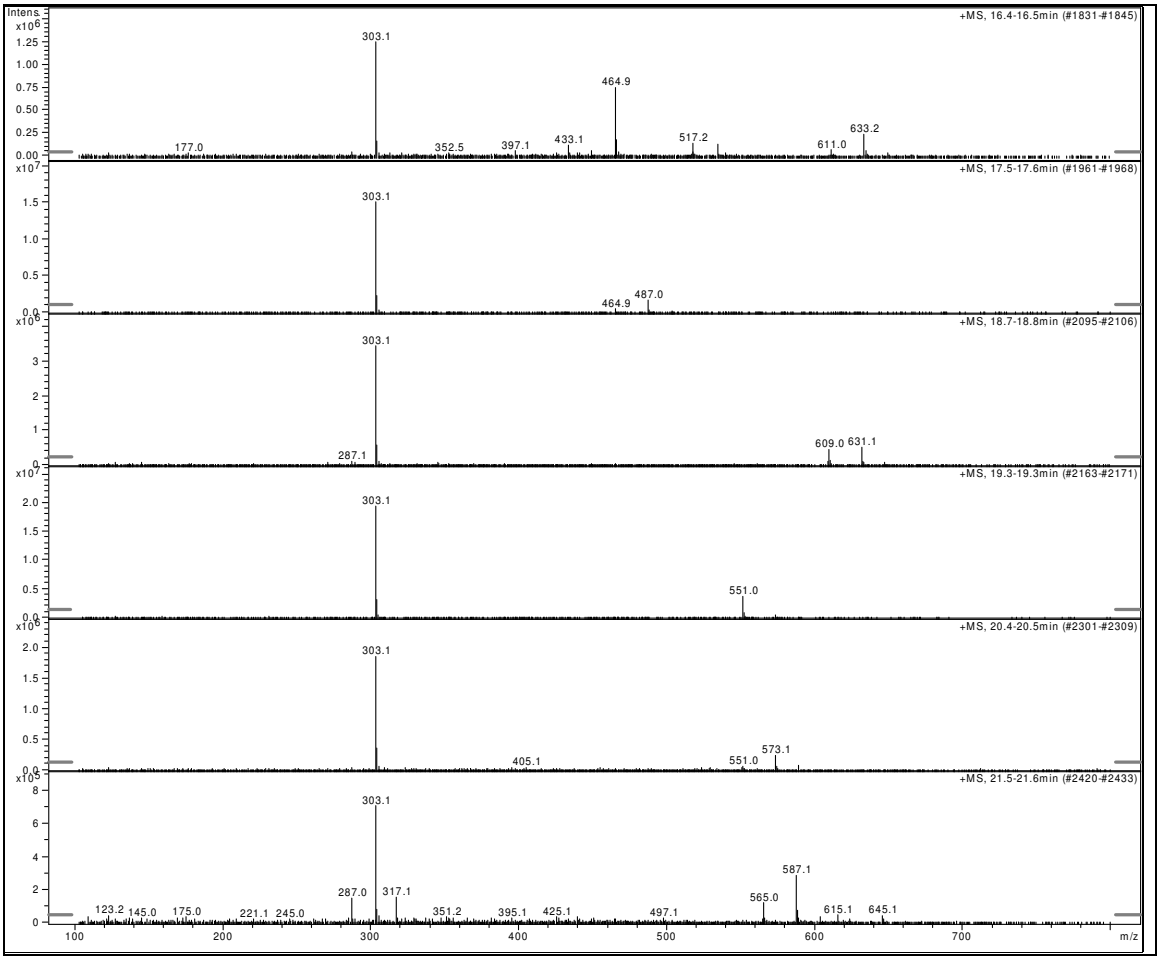


Figure 9.4. MS spectra of six Quercetin derivatives as found in Moringa grown and collected in sub-Sahara Africa.

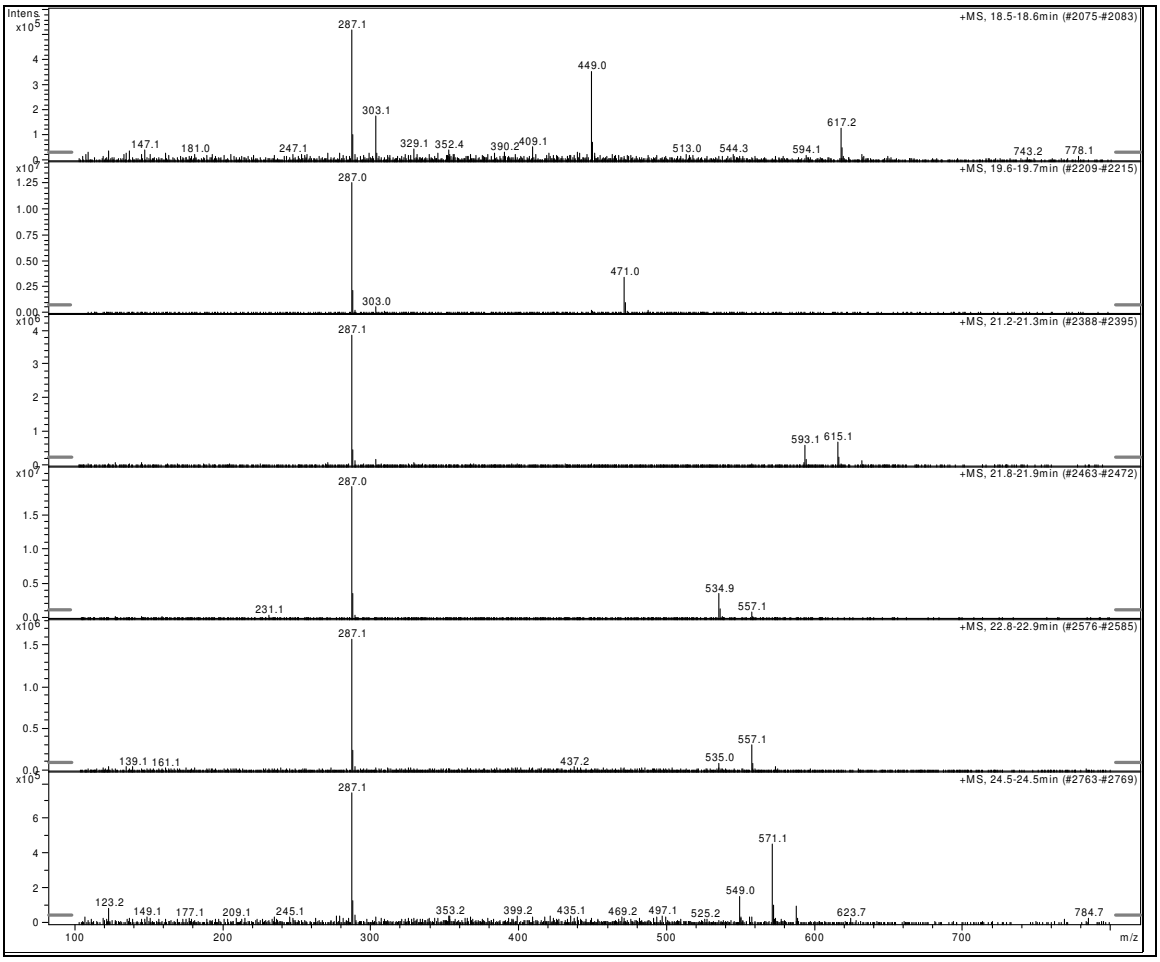


Figure 9.5. MS spectra of six kaempferol derivatives as found in Moringa grown and collected in sub-Sahara Africa.

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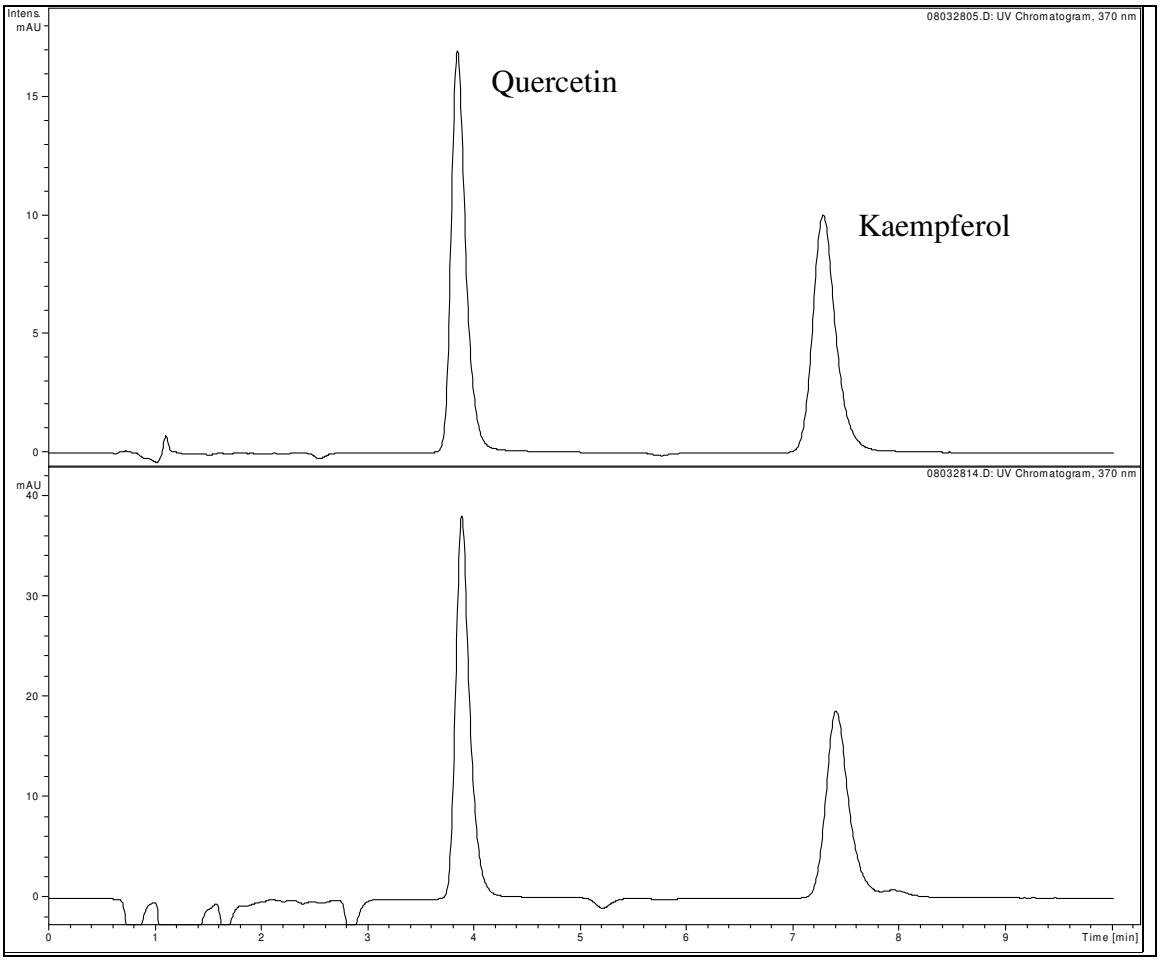

Figure 9.6. UV chromatograms (370 nm) of (A) standard mixture of quercetin and kaempferol; and (B) Moringa hydrolyzed extract.

Table 9.2. Contents % (g/g) of flavones in Moringa samples grown and collected in sub-Sahara Africa.

Country	Location	[1]Quercetin %	[1]Kaempferol %	Total Flavonols*
Rwanda	Gasabo	0.687	0.093	0.780
	Kibungo	0.244	0.173	0.417
	Kicukiro	0.774	0.427	1.202
Zambia	Lusaka	0.560	0.663	1.223
	Lusaka	1.057	0.586	1.643
	Lusaka	0.699	0.559	1.258
	Lusaka	0.916	0.202	1.118
	Mumbwa	0.959	0.234	1.193
	Kafue	0.114	0.673	0.787
	PKM	0.766	0.202	0.968
	Kafue	0.868	0.289	1.156
	Binga	0.066	0.479	0.545
	Lusaka	1.072	0.276	1.348
	Mumbwa	0.844	0.297	1.141
	Kafue	0.884	0.669	1.553
	PKM	1.024	0.328	1.352
	Kafue	0.811	0.645	1.457
	Binga	0.498	0.539	1.037
	Lusaka	1.262	0.303	1.565
Ghana	Dodowa	0.526	0.054	0.579
	Volta Dan	0.510	0.105	0.615
Senegal	Dakar	0.573	0.165	0.738
	Dakar	1.118	0.148	1.266
	Dakar	0.509	0.106	0.614
	Dakar	0.116	0.063	0.179

1-Values are expressed an average in % or g/g DW
*Total flavonoids in the study refer only to the additions of both quercetin and kaempferol.

Table 9.3. Recovery of quercetin in different concentrations

% of Quercetin Added	Found (mg/g)	Added (mg/g)	Recovery %	Average Recovery %	STD
100%	13.20	6.26	92.42		
	14.16	6.24	99.21		
	13.87	6.26	97.03	96.22	3.470
75%	12.72	4.69	99.97		
	13.01	4.69	102.26		
	12.95	4.69	101.77	101.34	1.205
50%	12.67	3.12	113.63		
	11.85	3.11	106.31		
	11.44	3.11	102.63	107.52	5.595

Table 9.4. Recovery of kaempferol in different concentrations

% of Kaempferol Added	Found (mg/g)	Added (mg/g)	Recovery %	Average Recovery %	STD
100%	11.83	6.00	92.35		
	11.92	5.99	93.19		
	11.83	6.01	92.34	92.63	0.491
75%	10.96	4.50	97.00		
	10.77	4.50	95.33		
	10.86	4.50	96.04	96.12	0.839
50%	10.31	2.99	105.19		
	9.56	2.99	97.66		
	9.32	2.98	95.18	99.34	5.215

9.4 CONCLUSIONS

This study showed that *M. oleifera* contained high levels of quercetin and kaempferol, specifically in samples from Zambia. High performance liquid chromatography combined with ultraviolet and electrospray ionization-mass spectrometric detector (HPLC-UV-ESI-MSD) has been applied to the study of flavonoids in the *M. oleifera* leaves. The flavonoids consist mainly of the quercetin and kaempferol derivatives. These derivatives are the glycosides, glycoside malonates all of which contains glucosyl/galactosyl moieties, rhamnosyl, arabiosyl, apiosyl; malonyl and succinoyl groups. The validation of this method was performed on precision control and recovery test, suggesting it is sensitive, reliable and precise. This method can be used to determine flavonoids, quercetin and kaempferol, from their corresponding flavonoid aglycones or derivatives in *M. oleifera* leaves. Considering that several botanical products are on the commercial market as sources for these flavonoids, it would be of interest to assess the potential of using moringa extracts as a new natural source of these bioactive natural products.

CHAPTER 10

CONCLUSIONS

This research sought to characterize and quantitate the nutritional benefits of Moringa, also known as the Miracle Tree, from trees grown in several sub-Saharan Africa. While many studies have examined the myriad of applications of Moringa, there are fewer studies that have systematically and rigorously examined the chemistry and health applications of moringa. This study both confirmed and extended the breadth of information and knowledge we have come to understand about this tree that is so well adapted to a wide range of environmental conditions and whose properties continue to appear so promising to aid in improving the nutritional status of malnourished populations. The *M. oleifera* leaves have many phytochemical which are responsible for its antioxidant activity. Antioxidant systems (endogenous and dietary) have evolved to help defend the body against free radicals which are commonly produced within the body under normal circumstances. The phytochemicals found within the plant can be used for bioactive purposes in the medical and industrious fields. The examination of the phytochemicals in Moringa species affords the opportunity to examine a range of fairly unique compounds. In particular, this plant is rich in polyphenols and flavonoids, which we characterized in great detail; in vitamin E; in the carotenoids which lead to vitamin A; and in minerals such as iron and others. Much can be learned from the moringa analyses reported from India, where the most scientific work appears to be have been conducted and where moringa is well recognized both as a food and an excellent nutritional addition in school lunch programs. The Moringa plants grown in sub-Sahara Africa countries appear as nutritious and as rich in the major antioxidant phytochemicals as found in India

and reported in prior studies. As such, this thesis has both confirmed the popular and some of the scientific research which has shown the nutritional benefit and attributes of moringa, while extending the work on the nutritional and bioactive polyphenols in moringa. In addition, this work sought to systematically examine the nutritional and polyphenol content of moringa grown and collected in sub-Sahara Africa to provide a scientific basis by which others can judge and assess the value and application of this most interesting plant which can be used in so many ways to improve the health and nutrition of African populations that today face malnourishment.

The benefits for the treatment, prevention of disease or infection may accrue from dietary or tropical administration of moringa preparations as shown in Table 1.3. Moringa plant has been consumed by humans in many diverse culinary ways for it thought to be therapeutic properties. In this research, we found that the leaves consist of the flavonoid derivatives especially the aglycones quercetin and kaempferol, the carotenoids, α- and β-carotene, the tocopherols, γ- and α- tocopherols, and the chlorogenic acid derivatives. The 12 flavonoid derivatives which we have identified and report from African-grown Moringa can be used to justify the medicinal potential of *Moringa oleifera* leaves and therefore would be appropriate to test the leaves for many activities. To assist the evalution of moringa for food preparation, a modified acid hydrolysis method was used to quantitate the two aglycones, quercetin and kaempferol in the leaves. We found that the combination of these two compounds gives a high total content for each M. oleifera variety. For the vitamins A and E which have high antioxidant activity, we found that β-carotene a vitamin A analogue and α-tocopherol a vitamin E were the potent pro-vitamins found within the plants. Even though the

recommended dietary intake of the leaves would be relatively high for one person to intake, if considered being used as a food product or additive, once concentrated in large amount of powdered leaves, the plant would administer the nutritional value in one serving for an individual. In the dry leaves there is little to no vitamin C being found within the leaves, however many factors contributes to this findings and many cross reactions as well as other nutrients in the leaves will cause this reduction to be seen. Environmental factors such as heat and oxygen can also degradation to occur. It is believed however that the amount of vitamin C in the dried leaves are tremendously lower than that of fresh leaves.

The data from our research permits us to conclude that the leaves of Moringa grown and collected in Ghana, Rwanda, Dakar, Senegal and Zambia are a rich source of Vitamin A and E, total mineral including iron, a high flavonoid content and a moderate source of phenols and yet the dried leaves were a low source of vitamin C. Comparatively, we can also conclude that the Moringa samples collected in 2007 from Rwanda and Zambia exhibited a higher content of all nutrients tested within the leaves. In varieties there was no significant difference between the improved samples from India (PKM) and samples grown either indigenous or naturalized (Binga), however PKM-1 Moringa grown in Dakar, Senegal consistently was higher in nutrients than those from Zambia.

These findings together demonstrate that moringa is an excellent plant candidate to be used to improve the health and nutrition of communities and also appears to be a most promising candidate from which specific nutraceutical bioactive products could be developed.

Reference:

1. Abdulkarim, S. M.; Long, K.; Lai, O. M.; Muhammad, S. K. S.; Ghazali, H. M. Some physico-chemical properties of *Moringa oleifera* seed oil extracted using solvent and aqueous enzymatic methods. *Food Chemistry*. **2005**, 93, 2, 253-263
2. Abdulkarim, S. M.; Long, K.; Lai, O. M.; Muhammad, S. K. S.; Ghazali, H. M. Frying quality and stability of high-oleic *Moringa oleifera* seed oil in comparison with other vegetable oils. *Food Chemistry.* **2007**, 105, 1382–1389
3. Abilgos, R. G.; Barba, C. V. C. Utilization of Malunggay (*M. oleifera* Lam.) leaves in rice (Orya sativa L.) flat noodle-production. *Philippine J. Science*. **1999**, 128, 79-84.
4. Ames, B. N. The prevention of DNA damage: The role of nutrition. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. **1997**, 379, 1, 1, S172
5. An End to World Hunger: Hope for the future. United Nations Food and Agriculture. (http://library.thinkquest.org/C002291/high/present/stats.htm)
6. Anonymous. Committee on Food Chemical Codex. Food Chemical Codex: National Acedemy Press: Washington, DC. **1996**
7. Anonymous. Dietary Reference Intake: Recommended Intakes for Individuals. United States Department of Agriculture-National Agricultural Library. Washington DC. **2002**

8. Anonymous. SOP No: CB0103. Standard Operating Protocol (SOP) Subject: To determine the contents of catechins and gallic acid in green tea products by HPLC Analysis. **2001**
9. Arabshahi-D, S.; Devi, D. V.; Urooj, A. Evaluation of antioxidant activity of some plant extracts and their heat, pH and storage stability. *Food Chemistry*. **2007**, 100, 1100-1105.
10. Ames, B. N.; Shigenga, M. K.; Hagen, T. M. Oxidants, antioxidants and degenerative diseases of aging. *Proceeding of the National Academy of Sciences of the United States of America.* **1993**, 90, 7915-7922
11. A Supplementation: A Decade of Progress, UNICEF. **2007**
12. Bajpai, M.; Pande, A.; Tewari, S. K.; Prakash, D. Phenolic contents and antioxidant activity of some food medicinal plants. *Int. J. of Food Sci. Nutr.*. **2005**, 56, 4, 287-291.
13. Bennett, R. N.; Mellon, F. A.; Foidl, N.; Pratt, J. H.; Dupoint, S. M.; Perkins, L. Kroon, P. A. Profiling Glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *M. oleifera* L. (Horseradish Tree) and *Moringa stenopetala* L. *J. Agri. Food Chem*. **2003**, 51, 3546-3553.
14. Bineesh, N. P.; Singhal R. S.; Pandit, A. B. A study on degradation kinetics of ascorbic acid in drumstick (*Moringa oleifera*) leaves during cooking. J. *Sci. Food and Agric.* **2005**, 85, 1953-1958
15. Bowman, P. E.; Morbarhan, S.; Evidence from cancer intervention and biomarker studies and the development of biochemical markers. *Am. J. Clin. Nut.* **1995**, 62, 1403S-1409S.

16. Breithaupt, D. E.; Schwack, W. Determination of free and bound carotenoids in paprika (Capsicum annum L.) by LC/MS. *Eur. Food Res. Technol*. **2000**, 211, 52-55

17. Bulletin of the World Health Organization, Readers Forum. 1999, 77 (9)

18. Burini, G. Development of a quantitative method for the analysis of the total L-ascorbic acid in foods by high-performance liquid chromatography. *J. Chromatography A*. **2007**, 1154, 97-102.

19. Clifford, M. N.; Wu, W.; Kuhnert, N. The chlorogenic acids of Hemerocallis. *Food Chem*. **2006**, 95, 574- 578

20. Ching, L. S.; Mohammed, S.; Alpha-Tocopherol content in 62 edible tropical plants. *J. Agric. Food Chem*. **2001**, 49, 3101-3105

21. Chumark, P.; Khunawat, P.; Sanvarinda, Y.; Phornchirasilp, S.; Morales, P.N.; Phivthong-ngam, L.; Ratanachamnong, P.; Srisawat, S.; Pongrapeeporn, K. S. The *in vitro* and *ex vivo* antioxidant properties, hypolipidaemic and antiatherosclerotic activities of the water extract of *Moringa oleifera* Lam. leaves. *J. of Ethnopharmacology*. **2008**, 116, 439-446

22. Dahiru, D.; Obnubiyi, J. A.; Umaru, H. A. Phytochemical screening and antiulcerogenic effect of Moringa. *African Journal of Traditional, Complimentary and Alternatives Medicines*. **2006**, 3, 3, 70-75

23. DanMalam, H. U.; Abubakar, Z.; Katsayal, U. A. Pharmacognostic studies on the leaves of *Moringa oleifera*. *Nigerian Journal of Natural Product and Medicine*. **2001**, 5, 45-49.

24. Dongmeza, E.; Siddhuraju, P.; Francis, G.; Becker, K. Effects of dehydrated methanol extracted Moringa (*Moringa oleifera* Lam.) leaves and three of its fractions on growth performance and feed nutrient assimilation in Nile tilapia (Oreochromis niloticus (L.)). *Aquaculture*. **2006**, 261, 1, 407-422.

25. DRI, Dietary Reference Intakes. The Essential Guide to Nutrient Requirements. Institute of Medicine of National Academies. The National Academies Press. Washington, D.C. (**2006**).

26. Esteban, M.R.; Ho, C. Enzymatic spectrophotometric determination of ascorbic acid in commercial vitamin C tablets. *Microchem. J.* **1997**, 56, 122-129

27. Fahey J. W. *M. oleifera*: A Review of the Medical Evidence for Its Nutritional, Therapeutic, and Prophylactic properties. Part 1. *Trees for Life Journal* **2005**, 1:5

28. Faizi, S.; Siddiqui, B. S.; Saleem, R.; Siddiqui S.; Aftab K.; and Giliani A.H.; Isolation and structure elucidation of new nitrile and mustard oil glycosides from *M. oleifera*. *J. Nat. Prod.* **1994,** 57, 1256-1261.

29. Faizi, S.; Siddiqui, B. S.; Saleem, R.; Siddiqui S.; Aftab K.; and Giliani A.H. Fully acetylated carbamate and hypotensive thiocarbamate glycosides from *M. oleifera*. *J. Phytochemistry.* **1995,** 38, 957-963.

30. Faizi, S.; Siddiqui, B. S.; Saleem, R.; Siddiqui S.; Shaheen, K.; Aftab K.; and Giliani A.H. Bioactive compounds from the leaves and pods of *Moringa oleifera*. New trends in natural products chemistry. **1998**, 175-183.

31. Fuglie, L. J. **1999**. The Miracle Tree: *Moringa oleifera*: Natural Nutrition for the Tropics. Church World Service, Dakar. 68pp.; revised in 2001 and published as The Miracle Tree: The Tree Multiple Attributes of Moringa, 172 pp.

http://www.echotech.org/bookstore/advanced_search_result.php?keywords=Miracle+Tree.)

32. Gao, X.; Bjork, L.; Trajkovski V.; Uggla, M. Evaluation of antioxidant activities of rosehip ethanol extracts in different test systems. *J. Sci. Food and Agric.* **2000**, 80, 2021-2027

33. Ghasi, S.; Nwobodo, E.; and Ofili, J. O.; Hypocholesterolemic effects of crude extracts of leaf of *M. oleifera* Lam in high-fat diet fed wistar rats. *J. Ethnopharmacology.* **2000**, 69, 21-25

34. Gomez-Coronado, D. J. M.; Ibanez, E.; Ruperez. F. J.; Barbas, C. Tocopherol measurement in edible products of vegetable origin. *J. Chromatography A*. **2004**, 1054, 227-233.

35. Guaratini, T.; Gianeti, M D.; Campos, P. M. B. G. M. Stability of cosmetic formulations containing esters of Vitamins E and A: Chemical and physical aspects. *Int. J. Pharm.*. **2006**, 327, 1-2, 12-16

36. Guevara, A. P.; Vargas, C.; Sakurai, H.; Fujiwara, Y.; Hashimoto, K.; Maoka, T.; Kozuka, M.; Ito, Y.; Tokuda, H.; Nishino, H. An antitumor promoter from *Moringa oleifera* Lam. *Mutation Research*. **1999**, 440, 181-188

37. Gupta, K.; Barat, G. K.; Wagle, D. S.; Chawla, H. K. L. Nutrient contents and antinutritional factors in conventional and non-conventional leafy vegetables *Food Chem.* **1989,** 31, 2, 105-116

38. Guyot, S.; Le Bourvellec, C.; Marnet, N.; Drilleau, J. F.; Procyanidins are the most abundant polyphenols in dessert apples at maturity *Lebensmittel-Wissenschaft und-Technologie*, **2002**, 35, 3, 289-291

39. Hartwell, J. L. Plants used against cancer. A survey. Lloyodia **1967**- 1971, 30-34.

40. Havsteen, B.H. The biochemistry and medical significance of the flavonoids. *Pharmacol. and Ther.* **2002**, 96, 67–202.

41. Hertog, M. G. L.; Hollman, P. C. H. Potential health effects of the dietary flavonoid quercetin. *Eur. J. Clin. Nutr.* **1996**, 50, 63-66

42. Ho, H. M.; Chen, R. Y.; Leung, L.K.; Chan, F. L.; Huang, Y. Chen, Z. Difference in flavonoid and isoflavone profile between soybean and soy leaf. *Biomed Pharmacother,* **2002**, 56, 289-295

43. Iqbal, S.; Bhanger, M. I. Effect of season and production location on antioxidant activity of *Moringa oleifera* leaves grown in Pakistan. *J. of Food Comp. and Anal.* **2006**, 19, 544-551.

44. Juliani, R.; Fonseca, Y.; Simon, J. E. Assessment of the nutritional value of *Moringa oleifera* species from Zambia. **2007**, Unpublished.

45. Krichevsky, S. B.; β-carotene, carotenoids and the prevention of coronoary heart disease. *J. Nutr*. **1999**, 129, 5-8.

46. Lako, J.; Trenerry, V. C.; Wahlqvist, M.; Wattanapenpaiboon, N.; Southeeswaran, S.; Premier, R. Phytochemical flavonols, carotenoids and the antioxidant properties of a wide selection of Fijian fruit, vegetables and other readily available foods. *Food Chemistry*. **2007**, 101, 4, 1727-1741

47. Longo, L.; Scardino, A.; Vasapollo, G. Identification and quantification of anthocyanins in the berries of Pistacia lentiscus L., Phillyrea latifolia L. and Rubia peregrina L. Innovative *Food Sci. & Emerging Technologies* **2007**, 8, 3, 360-364

48. Laandrault, N.; Pouchert, P.; Ravel, P.; Gase, F.; Cros, G.; Teissedro, P.-L. Antioxidant activities and phenolic level of French wines from different varieties and vintages. *J. Agric. and Food Chem*. **2001**, 49, 3341-3343.

49. Lachance, P. A.; Nakat, Z.; Jeong, W.; Beyond Deficiency: New Roles for Vitamins. Antioxidants: An Integrative Approach. *Nutrition*. **2001**, 17, 835-838.

50. Lakshminarayana, R.; Raju, M.; Krishnakantha, T. P.; Baskaran, V.; Determination of Major Carotenoidsin a Few Leafy Vegetables by High Performance Chromotography. *J. Agric. Food Chem*. **2005**, 53, 2838-2842.

51. Liu, D.; Shi, J.; Ibarra, A. C.; Kakuda, Y.; Xue, S. J. The scavenging capacity and synergistic effects of lycopene, vitamin E, vitamin C, and β-carotene mixtures on the DPPH free radical *LWT Food Science and Technology*. **2008**, 41, 7, 1344-1349

52. Lockett, C. T.; Calvert, C. C.; Grivetti L. E. Energy and micronutrient composition of dietary and medicinal wild plants consumed during drought. Study of rural Fulani, northeastern Nigeria. *Int. J. Food Sci. Nutr*. **2000**, 51, 195-208

53. Makkar, H. P. S.; Becker, K. Nutrional value and whole and ethanol antinutritional components ofextracted *Moringa oleifera* leaves. *Animal Feed Science Technology*. **1996**, 63, 211-228.

54. McBurney, R. P. H.; Griffin, C.; Paul, A. A.; Greenberg, D. C. The nutritional composition of African wild food plants: from compilation to utilization. *Journal of Food Composition and Analysis*. **2004**, 17, 277-289.

55. Mensink, R. P.; Katan, M. B. Effect of dietary trans-fatty acids on high-density and low-density lipoprotein cholesterol levels in healthy subjects. *New England Journal of Medicine*. **1990**, 323, 7, 439–445.

56. Middleton Jr., E.; Kandaswami, C.; Theoharides, T.C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews.* **2000**, 52, 673–751.

57. Miean, H. K.; Mohamed, S. Flavonoid (Myricetin, Quercetin, Kaempferol, Luteolin, and Apigenin) Content of Edible Topical Plants. *J. Agri. Food Chem*. **2001**, 49, 3106-3112.

58. Miller, Robert O. 1998. High-Temperature Oxidation: Dry Ashing. In Y.P. Kalra (ed.) Handbook and Reference Methods for Plant Analysis. CRC Press, New York

59. Monograph on *M. oleifera* (As a Nutritional Supplement) prepared by Church World Service by Phyto-Riker Pharmaceuticals Ltd. July **2002**

60. *M. oleifera* – Food, Medicine and Industrial Products – A Review by John Shindano and Kasase Chitundu-2008. Unpublished**.**

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61. Morris, M.E.; Zhang, S. Flavonoid–drug interactions: Effects of flavonoids on ABC transporters Marilyn. *Life Sciences.* 2006, 78, 2116–2130

62. Murakami, A.; Kitazono, Y.; Jiwajinda, S.; Koshimizu, K.; Ohigashi, H.; Naizimin, A thiocarbamate from the leaves of *M. oleifera*, holds a strict structural requirement for inhibition of tumor-promoter-induced Epstein-Barr virus activation. *Planta Med*. **1998**, 64, 319-323.

63. Murray, M. *Encyclopedia of Nutritional Supplements*. Roseville, CA: Prima Vitamin Publishing, **1996**.

64. Muluvi G.M.; Sprent J.I.; Soranzo N.; Provan J.; Odee D.; Folklard G.; McNicol J.W.; and Powell W. Amplified fragment length polymorphism (AFLP) analysis of genetic variation in *M. oleifera* Lam. *J. of Mol. Ecol.* **1999**, 8, 463-470.

65. Nakatani, N.; Kayano, S.; Kikuzaki, H. Sumino, K.; Katagiri. K; Mitani, T. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (prunus domestica L.). *J. Agric. Food Chem.* **2000**, 48, 5512-5516

66. Neto, M. M.; Neto, M. A.; Filho, R. B.; Lima, M.A.S.; Silveira, E.R. Flavonoids and alkaloids from leaves of Bauhinia Ungulata L. *Biochemical Systematics and Ecology*. **2008**, 36, 3, 227-229.

67. Prakash, D.; Suri, S.; Upadhyay, G.; Singh, B.N. Total phenol, antioxidant and free radical scavenging activities of some medicinal plants. *Int. J.f Food Sci. Nutr.* **2007**, 58, 1, 18-28.

68. Prouilleta, C.; Mazie`reb, J.; Mazie`reb, C.; Wattela, A.; Braziera, M.; Kamela, S. Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. *Biochem. Pharmacol.* **2004**, 67, 1307–1313

69. Pulido, R.; Bravo, L.; Saura-Calixto, F.; Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem*. **2000**, 48, 3396-3402

70. Raghu, V.; Platel, K.; Srinavasan, K. Comparison of ascorbic acid content of Emblica offcinalis fruits determined by different analytical methods. *J. Food Comp. Anal*. **2007**, 20, 6, 529-533.

71. Rajyalakshmi, P.; Venkatalaxmi, K.; Venkatalakshmamma, K.; Jyothsna, Y.; Devi, K.; Suneetha, V. Total carotenoids and beta-carotene contents of forest green leafy vegetables consumed by tribals of South India. *Plant Foods for Human Nutrition*. **2001**, 56, 225-238.

72. Risso, E. M.; Péres, R. G.; Amaya-Farfan, J. Determination of phenolic acids in coffee by micellar electrokinetic chromatography. *Food Chem*. **2007**, 105, 1578-1582

73. Sánchez, N.R.; Spörndly, E.; Ledin, I. Effect of feeding different levels of foliage of *Moringa oleifera* to creole dairy cows on intake, digestibility, milk production and composition. *Livestock Sci.* **2006**, 101, 1-3, 24-31

74. Sánchez-Machado, D. I.; Lopez-Cervantes, J.; Rios Vasquez, N.J. High performance liquid chromatography method to measure α- and γ-tocopherol in leaves, flowers and fresh beans from *M. oleifera. J. Chromatogr. A*. **2006**, 1105, 1-2, 111-114.

75. Sánchez-Machado, D. I.; Lopez-Hernandez, J.; Paseiro-Losada, P. High performance liquid chromatographic determination of α-tocopherol in microalgae. *J. Chromatogr. A*. **2002**, 976, 1-2, 277-284.

76. Sarwatt, S. V.; Kapange, S. S.; Kakengi A. M. V. Substituting sunflower seed-cake with *Moringa oleifera* leaves as a supplemental goat feed in Tanzania *Agroforestry Systems*. **2002**, 56, 3, 241-247

77. SaveGaia International Foundation, 2005: http://savegaia.blogspot.com/2005/06/wondertree-100-facts-Moringa-fact-04.htmlz

78. Scalbert, A.; Johnson, I. T.; Saltmarsh. M. Polyphenols: antioxidants and beyond. *Am. J. Clin.l Nutr.* **2005**, 8 (Suppl), 215S-2175S.

79. Scheiber, A.; Marx, M.; Carie, Reinhold. Simultaneous determination of carotenes and tocopherols in ATBC drinks by high-performance liquid chromatography. *Food Chem*. **2002**, 76, 3, 357-362.

80. Shen, D.; Wu, Q.; Sciarappa, W. J.; Lavoie, E.J.; Simon, J. E. An analytical survey of isoflavones in Tofu-type soybeans cultivated in North America. **2006.** New Use Agriculture and Natural plant Products Program, Department of Plant Biology and Pathology, School of Environmental and Biological Sciences, Rutgers University, New Brunswick, New Jersey 08901, USA

81. Siddhuraju, P.; Becker, K. Antioxidant properities of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*M. oleifera* Lam.) Leaves. *J. Agric. Food Chem.* **2003**, 51, 2144-2155

82. Smolin, L. A. Grosvenor, M. B.Nutrition: Science & Application. **2007**

83. Soliva, C.R.; Kreuzer, M.; Foidl, N.; Foidl G.; Machmüller, A.; Hess, H.D. Feeding value of whole and extracted *Moringa oleifera* leaves for ruminants their effects on ruminal fermentation in vitro. *Anim. Feed Sci. Technol.* **2005**, 118, 47-62

84. Sreeramula, N.; Ndossi, G. D.; Mtotomwema, K. Effect of cooking on the nutritive value of common food plants of Tanzania: Part 1-Vitamin Cin some of the wild green leafy vegetables. *Food Chem*. **1983**, 10, 3, 205-210

85. Sreevidya, N.; Mehrotra, S.; Spectrophotometric method for estimation of alkaloids precipitable with dragendorff's reagent in plant materials. *J. AOAC Int.* **2003**, 86, 6, 1124-1127.

86. Sun, T.; Ho, Chi-Tang. Antioxidant activities of buckwheat extracts. *Food Chem*. **2005**, 90, 743-749.

87. Tausz, M.; Kranner, I.; Grill, D. Simultaneous determination of ascorbic acid and dehydroascorbic acid in plant materials by high performance liquid chromatography. *J. Phytochem. Anal.*. **1996**, 7, 68-72

88. Thiele, J. J.; Ekanayake-Mudiyanselage, S. Vitamin E in human skin: Organ-specific physiology and considerations for its use in dermatology. *Molecular Aspects of Medicine*. **2007**, 28 , 5-6, 646-667

89. Tsaknis, J.; Lalas, S. Stability during Frying of *Moringa oleifera* Seed Oil Variety ‘‘Periyakulam 1’’ *J. Food Comp. Anal*. **2002** 15, 79–101

90. Wilson, T. Whole foods, antioxidants and health. In Antioxidants, Human Health and Health Diseases. **1999**. 141-150.

91. Wu, Q.; Wang, M. Simon, J.E. Determination of isoflavones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. J. Chromotography A, **2003**, 1016, 195-209.