

**PHENOLIC COMPOUNDS FROM SELECTED NAMIBIAN LEAFY
VEGETABLES (NLV); EXTRACTION AND CHARACTERIZATION**

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AUGUSTU ASTRIDT MAVANDJE DOLLY

(200725246)

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Main Supervisor: Dr. Ahmad Cheikhyoussef

Co- Supervisor: Dr Martha Kandawa-Schulz

ABSTRACT

The purpose of this project was to study the antioxidant potential of selected Namibian Leafy Vegetables (NLV), using Total Phenolic Content (TPC), Total Flavonoid contents (TFC) as well as Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and Reducing Power (RP) assays. The study also aimed at detecting and characterizing eight phenolic acids (4-hydroxybenzoic, *trans*-ferulic, vanillic, gallic, syringic, sinapic and *para*-coumaric acids) using Gas Chromatography Mass Spectrometry (GC-MS). Thirteen leafy vegetables collected from Kavango East Region were identified as: *Hibiscus sabdarifa*, *Aeschynomene nambalensis*, *Hibiscus cannbinus*, *Oxygonum delagoense*, *Amaranthus thunbergii*, *Amaranthus hypochondriacus*, *Vigna unguiculata*, *Cleome gynandra*, *Cucurbita maxima*, *Lagenaria siceraria*, *Aeschynomene fluitans*, *Citrullus lanatus*, and *Corchorus tridens*. Organic extracts were prepared using aqueous methanol, ethanol and acetone solvents. Acid and Base hydrolyses were employed for extraction of bound phenolic acids which were analysed by GC-MS.

The best yields for each assay were as follows: TPC: methanol: $333.8 \pm 2.7 \mu\text{gGAE/g}$ in *H. sabdarifa*. TFC: methanol: $166.0 \pm 0.6 \mu\text{gQE/g}$ in *A. nambalensis*. DPPH assay: methanol: $79.3 \pm 0.6\%$ in *H. cannbinus*. ABTS assay: ethanol: $98.8 \pm 0.2\%$ in *H. sabdarifa*. RP assay: ethanol: $1227.5 \pm 106.2\%$ of *L. siceraria*. GC-MS detected phenolic acids in twenty extracts of Base Hydrolysed Samples (BHS), seven extracts of Acid Hydrolysed Samples (AHS) and thirteen extracts of Non-Hydrolysed Samples (NHS). BHS yielded the most phenolic acids among the three extraction methods, with highest yields of *p*-coumaric acid in *A. nambalensis* ($53.1 \mu\text{g/g}$).

Results obtained from the TPC, TFC and antioxidant assays showed that the selected NLVs are potential sources of phenolic compounds and antioxidants. GC-MS analysis indicate that this tool can be used for characterizing phenolic acids. This projects' work has contributed to our understanding of the phytochemical constituents of the selected NLVs and further studies are recommended on the possible industrial application of the identified phenolic acids.

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I dedicate this work to my parents: Hilde Ndumba, Astridt Ndumba and Matias Ndumba; for believing in me, loving me unconditionally and for molding me into who I am today.; to my child Elizabeth Elfriede Hazel Muyamba for giving me the courage to work hard every day for her future's sake; and to the marginalized local community people, who motivated me to choose the direction of natural products for their future health benefit.

DECLARATIONS

I, Astridt Mavandje Dolly Augustu, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution or higher education.

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ACRONYMS

ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AHS	Acid Hydrolysed Sample
ALV	African leafy vegetables
ASE	Accelerated Solvent Extraction
BHA	Butylated Hydroxyl Anisole
BHS	Base hydrolysed samples
BHT	Butylated Hydroxyl Toluenes
BSTFA	N,O-Bis (<i>trimethylsilyl</i>) trifluoroacetamide
C	Carbon
CGA	Chlorogenic Acid
CQA	Caffeoylquinic Acid
CYP3A4	Cytochrome P450 3A4
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DS	Dilution Solvent
EIC	Extracted Ion Chromatograms
FC	Folin-Ciocalteau's

FQA	Feruloylquinic Acids
GAE	Gallic Acid Equivalents
GC-MS	Gas Chromatography-Mass Spectrometry
IS	Internal Standard
ISS	Internal Standard Solution
LDL	Low Density Lipoproteins
LLP	Liquid-Liquid Partitioning
MDD	Methyldodecanoate
MAE	Microwave Assisted Extraction
MW	Molecular weight
NBRI	National Botanical Research Institute
NHS	Non-Hydrolysed Samples
NLV	Namibian Leafy Vegetables
N ₂ O ₃	Nitrogen Sesquioxide
PG	Permeability-glycoprotein
PLE	Pressurized Liquid Extraction
QE	Quercetin Equivalent
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

RP	Reducing Power
SD	Standard Deviation
SFE	Supercritical Fluid Extraction
TFC	Total Flavonoid Contents
TMS	Trimethylsilyl
TPC	Total Phenolic Contents
UNAM	University Of Namibia

CHAPTER 1: INTRODUCTION

Phenolic compounds are one of the largest and diversified groups of phytochemical compounds ubiquitous in the plant kingdom. Plant foods such as cereals, vegetables, fruits, legumes and nuts; as well as beverages such as wine, beer, tea, cocoa and ciders, are all rich sources of phenolic compounds; with phenolic levels varying greatly between cultivars even of the same species (Proestos and Komaitis, 2013). With more than 8000 phenolic compounds currently known (Trabelsi et al., 2013), these secondary plant metabolites constitute one of the most numerous and widely distributed groups of antioxidant substances in the plant kingdom. Scientists have been highly interested in extensively studying these bioactive compounds from different plant specimens because of their fascinating and diversified properties and benefits, especially their antioxidant potential (Trabelsi et al., 2013).

The balance between oxidative stress and anti-oxidation is believed to be a critical concept for maintaining a healthy biological system (Dudonne, Vitrac, Coutiere, Woillez, & Merillon, 2009). On-going clinical trials and epidemiology studies has shown that phytochemicals are important in preventing chronic and degenerative illnesses such as cancer, cardiovascular diseases, diabetes, chronic inflammation, neural degeneration among others. This is because phytochemicals have the ability to counteract excess oxidative stresses that generate from reactive oxygen species (ROS) and reactive nitrogen species (RNS); which results from an imbalance between the oxidative stress (oxidation) and the body's antioxidant status (anti-oxidation) (Campos-Vega & Oomah, 2013; Liu, 2004). The antioxidant properties of phenolic compounds; abundance in plant foods such as fruits and vegetables; and

probable roles in counteracting the aforementioned chronic diseases have attracted considerable interest in food and medical technology research in recent decades (Farhan et al., 2012).

In addition, one of the biggest challenges faced by the world is food insecurity. Therefore, food coping strategies such as planting and harvesting one's own food has strongly come into focus as a solution to poverty (Schonfeldt & Pretorius 2011). Surveys have indicated that there are more than 7000 plant species worldwide that are cultivated or harvested from the forests for food, many of these neglected and underutilised plant species are nutritionally rich and adapted to low input agriculture, therefore, studies on the health benefits of plant foods such as these can be used to reduce food insecurity by motivating rural communities to increase their cultivation and consumption of indigenous foods such as green leafy vegetables (Schonfeldt & Pretorius, 2011). This will not only contribute to their income generation strategies, but most importantly it will benefit their health due to the rich nutritional content of green vegetables. However, to recommend these traditional vegetables as improved diet and health benefiting, scientific knowledge about the nutrient and phytochemical composition of the Vegetables is required (Schonfeldt & Pretorius, 2011). This study focused on the analysis of phenolic compounds in selected Namibian leafy vegetables (NLV) from Kavango East.

1.1 Statement of the Problem

Minimal research has been done on investigating NLV phenolic compounds characterisation. Most studies done on NLV focused on the taxonomy, ecology and phytochemical content. In addition, antioxidant and antimicrobial activity studies are

being conducted, but very few and publications could be found on the profile of phenolic compounds of NLV. This gap of knowledge is very important to be filled. Therefore, the main aim of this study was to determine the phenolic contents; analyse the antioxidant potential and characterize phenolic compounds (phenolic acids) in selected NLV.

1.2 Objectives of the Research

The main objectives of this research project were:

1. To collect, and identify most frequently used Namibian leafy vegetables (NLV) from Kavango East region.
2. To screen the collected NLV extracts for the presence of phenolic compounds using the Ferric chloride qualitative test.
3. To extract the most abundant phenolic compounds using organic solvents; evaluate the total phenolic contents (TPC) and total flavonoid contents (TFC), and to evaluate their antioxidant capacities with Reducing Power (RP), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assays, with values reported as equivalents to phenolic acid standards.
4. To characterize the extracted phenolic compounds (phenolic acids) from NLV by GC-MS.

1.3 Significance of the study

Plants contain biologically active compounds, such as phenolic compounds which significantly improve one's health and reduce the chances of one getting ill. Therefore, knowledge on the nutritional value (such as the antioxidant potential) of

the selected NLV will be beneficial not only for research purposes and documentation or publishing; but more importantly it will promote the consumption of these NLV by local people who will be informed on what health benefits is gained from consuming natural indigenous plant foods. This is especially important for the marginalised people in the villages who have lost hope in cultivating foods for themselves. Communities can be informed on the outcomes of this study through social media and through the Ministry of Agriculture and Forestry's awareness strategies. The identification of foods that are enriched with phenolic compounds may lead to the development of new value-added foods or food products on the Namibian food market. The findings of this study will add to the knowledge of food nutritionists and consumers on the antioxidants present in these selected NLV.

1.4 Limitation of the study

Given the time and budget allocated to this project, the experimental part of the project would have been exhaustive if it included collecting and identifying all NLV in the country. Therefore, focus was set on the most popular/frequently consumed indigenous leafy vegetables from Kavango East Region, Ndiyona village. Also, some of these vegetables are consumed in a dry state after the rainy seasons. This project used only fresh samples for analysis, therefore the results obtained may not be the same as for dried samples, if the traditional drying methods used have an effect on the phenolic content and antioxidant potential of the vegetables.

CHAPTER 2: LITERATURE REVIEW

Phenolic compounds constitute one of the most numerous and widely distributed groups of antioxidant substances in the plant kingdom. With more than 8000 phenolic structures known (Trabelsi et al., 2013), these secondary plant metabolites have become of great interest owing to their versatile applications (Tiwari, Kumar, Kaur, Kaur & Kaur, 2011). Secondary plant metabolites are synthesized by plants as a defensive mechanism to counteract physiological and ecological challenges faced by plants, including: pathogenic and insect attack, ultraviolet radiation as well as physical wounding (Stalikas, 2007; Khoddami, Wilkes, & Roberts, 2013). Phytochemicals have health promoting properties such as antioxidant, free radical scavenging and metal chelating (Bhanja & Kuhad 2014; Ignat, Volf, & Popa, 2011), that allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and as metal chelating agents (Chirinos, Campos, Costa, Arbizu, Pedreschi, & Larondelle, 2008), that are able to protect the human body from oxidative damage. Phenolic compounds also exhibit antimicrobial, antiviral and anti-inflammatory activities (Ignat et al., 2011; Schmutzer, 2012). In the past decades, extensive studies have focused on extracting, identifying, quantifying and characterizing phenolics from plants for medical and dietary applications (Khoddami et al., 2013). Most of the past nutritional interest in polyphenolic compounds was in the deleterious effects caused by the ability of certain polyphenols to bind and precipitate macromolecules, such as dietary protein, carbohydrate and digestive enzymes, in order to reduce food digestibility (Proestos & Komaitis, 2013). However in recent decades, polyphenols have attracted considerable interest in food technology research greatly because of

their antioxidant properties, availability in plant diets and probable roles in countering deadly disease such as cancer, neuro-degenerative and cardiovascular diseases; which have been highlighted in various studies (Farhan et al., 2012). Furthermore, studies have reported substances exhibiting antioxidant properties that can be supplied as food additives or as specific preservative pharmaceuticals (Trabelsi et al., 2013).

The inadequate intake of healthy foods has led to a major dietary imbalance and is one of the major causes of chronic conditions (Luthria, Mukhopadhyay & Krizek 2006), such as obesity, diabetes mellitus, cardiovascular disease, hypertension, stroke, cancers (Khanam, Oba, Yanase & Murakami, 2012). Epidemiological studies have therefore recommended the consumption of whole foods such as fruits, vegetables, and legumes for their high nutrient content, consisting of vitamins, phenolic substances and carotenoids (Khanam et al., 2012) should be maximized as these products lower rates of chronic diseases (Bernard, 2014) such as cancer. For instance the consumption of quercetin from onions and apples was found to be inversely associated with lung cancer risk (Liu, 2004).

Experimental studies on animals and human cell lines have demonstrated that the adequate intake of natural food products have been associated with reduced rates of chronic diseases such as those mentioned above (Garcia-Salas, Morales-Soto, Segura-Carretero & Fernandez-Gutierrez, 2010). Diets rich in fruits and vegetables promote health, reducing the risk of these diseases, with five portions of fruits or vegetables being a rule of thumb for healthy lifestyle dietary habits (Rein, Renouf, Cruz-Hernandez, Actis-Goretta, Thakkar, & Pinto, 2013). The health benefits of

attributed to diets rich in vegetables and fruits primarily due to the occurrence of vitamins, minerals, and secondary phytochemicals carotenoids, anthocyanins, flavonoids, and other phenolic compounds ubiquitous throughout the plant kingdom (Luthria et al., 2006). The health effects of polyphenols depend on the amount consumed and on their bioavailability (Manach, Scalbert, Morand, Remesy & Jimenez, 2003). The major part of total antioxidant activity is from the combination of phytochemicals such as those previously mentioned. The additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities. This explains why no single antioxidant can replace the combination of natural phytochemicals in fruits and vegetables and achieve their health benefits, thus, it is evident that antioxidants are best acquired through whole food consumption and not from dietary supplements (Liu, 2004).

2.1 Namibian Leafy Vegetables

In this thesis, the word “indigenous” refers to leafy vegetables that have their natural habitat on Namibian ground and “traditional” refers to the leafy vegetables introduced over a century ago and due to long term use, have become part of the food culture in Namibia.

Vegetables are very important for human health because they occur naturally as whole foods and are rich in a large variety of nutrients. Leafy vegetables are not only good sources of minerals, but also contain vitamins, antioxidants and pigments (Farhan et al., 2012). Kavango East is one of the regions in Namibia, well-known for its diverse varieties of vegetables, among others are the thirteen species presented in this study. In comparison to Rubaihayo (1995) definition and general characteristics

of African leafy vegetables (ALV), NLV are considered as indigenous vegetables for at least three reasons:

1. NLV are easily found and harvested in their local natural habitat.
2. NLV has been consumed for many generations, reflecting their importance for local cultures in Namibia.
3. NLV contributes significantly the food security of rural populations in Namibia. They have a very high nutritional value (vitamins, proteins, minerals, etc.).

Public health awareness on health benefits obtained from non-nutrient bio-active compounds found in fruits and vegetables has prompt attention towards the importance of making traditional vegetables part of one's daily consumed diet, which, for sub-Saharan African people is significant, given that leafy vegetables have always been part of their daily consumed diet, especially as the ingredients of soups or sauces that accompany carbohydrate staples (Smith, 2007).

2.2 Phenolic Compounds

2.2.1 Phenolic Compounds in Plants

It is not by chance that the Hippocrates famous quote states "Let thy food be thy medicine and medicine be thy food" has been the marking point of food research for the past few decades (Obied, 2013). It is well known over decades that plants contain components of therapeutic value that may be used as alternative remedies for many diseases, with about $\frac{3}{4}$ of the world relying on plants and plant extracts for their health care (Farhan et al., 2012). The word "phyto-" originates from the Greek word *phyto*, which means plant. Therefore, phytochemicals are plant chemicals,

biologically defined as non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that are linked to reducing the risk of chronic diseases (Harbourne, Marete, Jacquier & O'Riordan, 2013). Vascular plants synthesize a multitude of organic molecules/phytochemicals during normal development, referred to as secondary plant metabolites. These compounds are involved in a variety of roles in the lifespan of plants; ranging from structural to protective roles such as in response to stressful conditions like infection, physical wounding and UV radiation (Khatiwora, Adsul, Kulkarni, Deshpande & Kashalkar, 2010; Khoddami et al., 2013 Stalikas, 2007).

Phenolic compounds are important determinants of sensory properties of plants, as well as in determining fruit organoleptic and nutritional quality in fruits and vegetables (Garcia-Salas et al., 2010). These diverse biological properties make them essential elements in a plant's growth and development life cycle, providing plants with the necessary protection against pathogens and predators in addition to the contribution they make to the colour and sensory characteristics (Ignat 2011). Furthermore, phenolic compounds act as phytoalexins, anti-feedants, attractants for pollinators, contributors to plant pigmentation, antioxidants and as protective agents against UV light (Ignat, 2011). The properties described above assist plants in attracting insects for dispersion and pollination; fungi; viruses and bacteria and in controlling plant hormones (Farhan et al., 2012). Plants accumulate Ultraviolet (UV) absorbing flavonoids and other phenolic compounds mainly in vacuoles of epidermal cells in order to prevent the penetration of UV-B into the deeper tissues of the plant. Also, plants secrete flavonoids from roots of legumina that activate genes of root nodule bacteria (Michalak, 2006).

Phenolic compounds such as flavonoids, phenolic acids and tannins, are stored in specific parts of the plant such as leaves, bark, flowers, seeds, fruits and roots (Khatiwora et al., 2010). They can be found in a free form, although their corresponding methyl esters and glycosides occur very commonly in free and/or bound forms. For instance, phenylpropanoids and other simple phenols (benzoic acid and benzaldehyde derivatives), are usually covalently bound to cell wall polysaccharides (predominantly ester-linked to arabinose units of hemicellulose) or to core lignin (Proestos & Komaitis, 2013). Insoluble phenolics are distributed in the plant cell walls while soluble phenolics are compartmentalized within the plant cell vacuoles. Various phenolics have been found during different maturation stages of maturation and growing conditions are known to have an impact on the phenolic content in the plant (Stalikas 2007).

2.2.2 Phenolic Acids in Plants

There are two classes of phenolic acids; both of them are derivatives of benzoic acid and those of cinnamic acids. Phenolic acids, having antioxidants properties, may act as free radicals scavengers as well as reducing and complexing agents of metal ions (Bauer, 2012).

Hydroxybenzoic acids (both esterified and free) in edible plants are found in very low quantities, except in certain red fruits, black radish and onions, in which the concentrations can reach several tens of milligrams per kilogram fresh weight (Manach et al., 2003). For this reason, this class of phenolic acids is not widely studied and not considered of great nutritional interest (Manach et al., 2003). Phenolic acids have been associated with diverse functions in plants including:

nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy (Stalikas, 2007). Both phenolic acids derivatives exist in all plant foods and are physically dispersed throughout the plant in seeds, leaves, roots, and stems. While a few exist as “free acids”; the majority of phenolic acids exist in bound form. They are bound to structural components of the plant, larger polyphenols or to smaller molecules via ester, ether, or acetyl bonds. Phenolic acids are not homogenously distributed in plant tissues (Robbins, 2003).

2.2.3 Health Benefiting Properties of Phenolic Compounds

It is believed that nature has given a cure of every disease in one way or another. Plants possess homeostatic mechanisms that allow them to keep correct concentrations of essential metal ions in cellular compartments and to minimize the damaging effects of an excess of nonessential ones (Michalak, 2006). Therefore, today researchers are emphasizing on evaluation and characterization of various plants and plant constituents against a number of diseases based on the traditional claims of the plants (Tiwari et al., 2011). The tremendous change in lifestyle has led to an increased consumption of “junk food”, has turn obesity into a worldwide epidemic, and since overweight and obesity are major predisposing factors for chronic diseases, science has focused its attention toward exploiting plants for their natural health benefiting properties; therefore during the last two decades nearly 50% of the newly introduced drugs are of plant origin or analogues thereof (Obied, 2013).

Phenolic compounds are considered nutraceuticals because the fit the definition of a nutraceuticals as coined by Stephen DeFelice; that is: a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease (Tapas, Sakarkar, & Kakde, 2008).

Natural antioxidants, particularly in fruits and vegetables have recently gained the attention of both researchers and consumers (Farhan et al., 2012) due to their fascinating health benefiting properties such as anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities (Khatiwora et al., 2010). Natural products are a better option compared to their counterpart therapeutic agents because they are abundantly present in the daily consumed diets of natural foods such as fruits and vegetables; they rarely have any side effects; they have relatively long half-life and they are easily absorbed by the intestines after ingestion (Tapas et al., 2008).

The health related effects of phenolic compounds such as those listed above; and others like antibacterial, antimutagenic, anticarcinogenic, antithrombotic and vasodilatory activities have been reported to be related to their antioxidant properties (Chirinos et al., 2008). The antioxidant capacity of phenolic compounds increases with the number of OH-groups in their structures and where they are positioned, such that when positioned on the 3'-, 4'-, 5'- position of B-ring of flavonoids, their antioxidant potential is increased compared to those with a single OH-group (Kopjar, Tadic, & Pilizota, 2015).

Several studies have shown that phenolic compounds have potent anti-amyloidogenic activities in *vitro* and in *vivo*. In fact, one of the major properties of polyphenols is the important interaction with peptides and proteins, particularly in the Alzheimer's disease, a neurodegenerative disease (Trabelsi et al., 2013).

2.2.4 Beneficial Properties of Flavonoids

The major active nutraceutical ingredients in plants are flavonoids. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators during

heavy metal stress (Michalak, 2006). They also have long been recognized to possess anti-inflammatory, anti-allergic, hepatoprotective, anti-thrombotic, antiviral, and anti-carcinogenic activities (Tapas et al., 2008). Flavonoids are potent antioxidants and have aroused considerable interest recently because of their potential beneficial effects on human health in fighting diseases (Schmutzer, 2012). Their importance as antioxidants is due to their high redox potential and ability to chelate metals (Tiwari et al., 2011).

Quercetin for instance is the most abundant dietary flavanol found in fruits, vegetables, leaves and grains. It is a potent antioxidant because it has all the right structural features for free radical scavenging activity and exhibits activities including antioxidant, radical scavenging, anti-inflammatory, anti-atherosclerotic, anticancer, and antiviral effects. Quercetin prevents the oxidation of LDL by scavenging free radicals (Farhan et al., 2012). This ability has shown a significant protection against hypertension and atherosclerosis. The antibacterial studies on quercetin has been reported to completely inhibit the growth of *Staphylococcus aureus* (Tapas et al., 2008) and is known as an inhibitor of Cytochrome P450 3A4 (CYP3A4) and a modulator of Permeability-glycoprotein (P-glycoproteins). Moreover, flavonoids may prevent endothelial dysfunction by enhancing the vasorelaxant process leading to a reduction of arterial pressure, thereby greatly preventing the development of cardiovascular diseases and the major complication of atherosclerosis and arterial thrombus formation. (Tapas, 2008). In plants, flavonoids are responsible for most of the red, yellow and blue colours in plant fruits and flowers (Tiwari et al., 2011). These colours are meant to attract pollinator animals (Khatiwora et al., 2010).

2.2.5 Beneficial Properties of phenolic acids

Phenolic acids are phenolic compounds with one phenol ring, bound with one or more hydroxyl groups. Just as polyphenols, cinnamic acids exhibit strong antioxidant properties. For instance 1,5-diCQA has been revealed to be an hepato-protector when challenged by carbon tetrachloride, where radical scavenging is involved in the mechanism of action (Pereira, Valentao, Pereira, & Andrade, 2009). Chen, Inbaraj, & Chen (2012) reported on how 9-10 phenolic acids in Kale seeds and leaves were demonstrated to have properties of scavenging DPPH free radicals and inhibiting the growth of different bacteria species such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis* and *Moraxella catarrhalis*. In addition to that, chlorogenic acids are the substrate for enzymatic oxidation leading to browning, particularly in apples and potatoes.

The antioxidant efficiency of mono-phenols is strongly enhanced by the introduction of a second hydroxyl group at the *ortho*- or *para*- positions, and is increased by one or two methoxy substitution in the *ortho*- position with respect to the hydroxyl group (Pereira et al., 2009). Biological activities such as promotion of bile secretion and reduction in blood loss have been reported for some phenolic acids (Ghasemzadeh & Ghasemzadeh, 2011). Consumption of foods rich in chlorogenic acid (CGA) has been associated with several benefits; among others is the reduction of the risk of cardiovascular disease, diabetes type 2, Alzheimer's disease, antibacterial and anti-inflammatory activities. Their lactones have been shown to exert positive effects in rats such as enhancement of insulin action. The production of nutraceuticals from green coffee is due to the abundance of CGA in green coffee (Farah, Monteiro, Donangelo & Lafay, 2008).

Ferulic acid is another abundant phenolic acids in plants and might be found in high concentrations in foods such as navy bean, corn bran, wheat bran, eggplant, artichokes and beets. Ferulic acid (FA) is an important biological and structural component of the plant cell wall. Due to its ability to stop radical chain reactions by resonance followed by polymerization, this phenolic acid offers protection against UV-radiation and is responsible for cross-linking polysaccharides and other cell wall polymers. Ferulic acid has recently been shown to competitively inhibit HMG-CoA reductase and activate glucokinase, contributing to reduce hypercholesterolemia and hyperglycemia, respectively (Paiva, Goldbeck, Santos & Squina, 2013).

2.3 Applications of Phenolic Compounds

Epidemiological studies have consistently shown that regular consumption of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases (Liu, 2004). Phenolic compounds are of great use in the food and cosmetic industries as they influence multiple sensorial food properties such as flavour, astringency and colour; and they contribute to the aroma and taste of numerous food products of plant origin (Kilci & Gocmen, 2014). Phenolics have been reported to have excellent properties of food preservation (Trabelsi et al., 2013; Ignat 2011). Therefore, these compounds are industrially used as food preservatives, food colorants and for the production of substances such as paint, paper and cosmetics (Ignat, 2011).

However, while some phenolics have great potential as nutraceuticals and others are powerful antioxidants (Kilci & Gocmen, 2014); there are phenolics such as chlorinated phenols which are potentially carcinogenic and are considered as environmental contaminants (Santana, Ferrera, Padron, & Rodriguez, 2009). The

application of phenols in the production of pesticides, dyes, drugs, textiles; and in agriculture as herbicide, insecticides and fungicides leads to the distribution of potentially hazardous phenols in soils and sediment; which may end up contaminating waste waters and ground waters, in some instances affecting the taste of drinking ground waters (Santana, et al., 2009). In addition, studies have indicated that synthetic antioxidants such as butylated hydroxyl toluenes (BHT) and butylated hydroxyl anisole (BHA) that are widely used in the food industry may be responsible for liver damage and carcinogenesis. Therefore, recent interest is in finding natural antioxidants from plants to replace synthetic ones (Trabelsi et al., 2013).

The quantity of phenolic compound in food is influenced by factors such as genotype (cultivar or variety), agronomic practices (irrigation, fertilization, and pest management), maturity at harvest, post-harvest storage and climatic conditions (Luthria, 2006).

2.3.1 Application of Phenolic acids

Phenolic acids have greatly contributed to the food industry. In food quality, phenolic acids have been associated with colour, sensory qualities, and nutritional and antioxidant properties of foods; particularly in organoleptic properties (flavour, astringency, and hardness) of foods (Robbins, 2003; Tapas et al., 2008). Other studies have focused on the effect of phenolic acids on fruit maturation, prevention of enzymatic browning and their roles as food preservatives (Robbins, 2003).

2.4 Classification of Phenolic Compounds

There are approximately 8000 naturally occurring compounds belonging to the group of phenolics (Stalikas, 2007), of which flavonoids and phenolic acids make up the

most pervasive groups of plant phenolics (Ghasemzadeh & Ghasemzadeh, 2011), the former comprising of 60%, and the latter 30% of phenolics known to date (Garcia-Salas et al., 2010). These are produced from phenylalanine and tyrosine via the shikimic acid pathway (Farhan et al., 2012). Phenolic compounds have an aromatic ring bearing one or more hydroxyl groups, structurally varying from a simple phenolic molecule (one phenol), to a complex high-molecular mass polymer (two or more phenol rings) (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012). However, each compound differs from another in the number of phenol rings and hydroxyl groups in their structures (Tiwari et al., 2011).

Phenolic compounds vary in structure; hydroxybenzoic acids and hydroxycinnamic acids have a single-ring structure while flavonoids comprise of 3 ring structures and can be further classified into anthocyanins, flavan-3-ols, flavones and flavonols (Chirinos et al., 2008). Although a large variety of plant phenols exist, most of them arise from a common origin: the amino acids phenylalanine or tyrosine, which are delaminated to cinnamic acids which enter the phenylpropanoid pathway (Pereira, 2009). The key step in this biosynthetic route is the introduction of one or more hydroxyl groups into the phenyl ring; as a result, these compounds are derived from a common carbon skeleton building block: the C₆-C₃ phenylpropanoid unit (Pereira et al., 2009).

The biosynthesis of phenolic compounds through the phenylpropanoid pathway produces the large variety of plant phenols including cinnamic acids (C₆-C₃), benzoic (C₆-C₁), coumarins (C₆-C₃), stilbenes (C₆-C₂-C₆), lignins [(C₆-C₃)_n], lignans (C₆-C₃-C₃-C₆), flavonoids (C₆C₃C₆), proanthocyanidins (C₆-C₃-C₆)_n (Pereira et al., 2009);

all have one or more hydroxyl groups directly linked to the aromatic ring (Tiwari et al., 2011; Schmutzer, 2012). Refer to figure below.

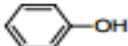

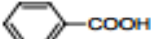
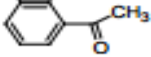



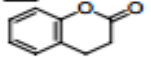


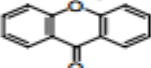
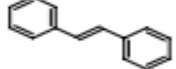
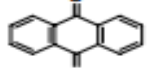
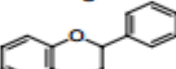

<i>Carbon numbers</i>	<i>Class</i>	<i>Basic structure</i>
C_6	Simple phenols	
	Benzoquinones	
C_6-C_1	Benzoic acid	
C_6-C_2	Acetophenones	
	Phenylacetic acid	
C_6-C_3	Cinnamic acid	
	Phenylpropene	
	Coumarins	
	Chromones	
C_6-C_4	Naphthoquinones	
$C_6-C_1-C_6$	Xanthenes	
$C_6-C_2-C_6$	Stilbenes	
	Anthraquinones	
$C_6-C_3-C_6$	Flavonoids	
$(C_6-C_3)_2$	Lignans, neolignans	
$(C_6-C_1)_n$	Hydrolysable tannins	Heterogeneous polymer composed of phenolic acids and simple sugars
$(C_6-C_3)_n$	Lignins	Highly crosslinked aromatic polymer

Figure 1 Classification of families of Phenolic Compounds (Garcia-Salas 2010)

Tannins, both hydrolysable and non-hydrolysable refer to polyphenols that possess three or more phenol subunits (Stalikas, 2007). This diverse chemistry is what makes it challenging to design one extraction technique applicable to all phenolic compounds (Garcia-Salas et al., 2010).

Phenolic Compounds can further be classified into water -soluble and water insoluble compounds. Water soluble phenolics include phenolic acids, phenylpropanoids, flavonoids, and quinones while water insoluble ones include condensed tannins, lignins and cell-wall bound hydroxycinnamic acids (Haiminiuk et al., 2012).

2.4.1 Flavonoids

Flavonoids are the most common group of polyphenolic compounds (Khatiwora et al., 2010). They account for approximately two-thirds of dietary phenols, with more than 6000 flavonoid molecules identified in plants (Tiwari et al., 2011). Flavonoids are planar molecules in structure, formed from aromatic amino acids phenylalanine, tyrosine and malonate.

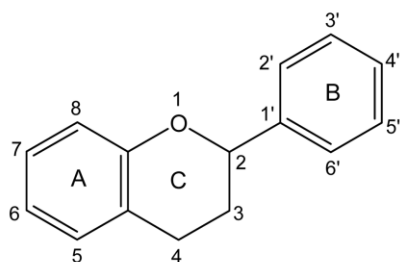


Figure 2 Basic structure of *flavonoids* (Stalikas, 2007)

The basic flavonoid structure is that of diphenylpropanes called the flavan nucleus which consists of 15 carbon atoms arranged in three rings ($C_6-C_3-C_6$) carbon framework; a phenylbenzopyran functionality (Tiwari et al. , 2011), that usually form

an oxygenated heterocycle (Manach et al., 2003). Biogenetically, the A ring usually comes from a molecule of resorcinol or phloroglucinol synthesized in the acetate pathway, whereas the B ring is derived from the Shikimate pathway (Proestos & Komaitis, 2013). Differences in the generic structure of the heterocycle C ring classify them (figure 3) as flavonols, flavanones, flavan-3-ols, isoflavone, flavanols (catechins), flavones and anthocyanidins; with the flavones (apigenin, luteolin, diosmetin, etc.); flavonols (quercetin, myricetin, kaempferol, etc.) (Proestos & Komaitis, 2013); flavanols (naringenin); anthocyanidins and isoflavonoids (genistein) being the most common in diets (Tiwari et al., 2011; Proestos & Komaitis, 2013). They occasionally occur in plants and food products as aglycones, especially as a result of food processing, but are most commonly found as glycoside derivatives in nature (Proestos & Komaitis, 2013). Flavonoids linked to one or more sugar molecules are referred to as flavonoid glycosides but when they are not connected to any sugar molecule, they are called aglycones. The degree of glycosylation directly affects the antioxidant capacity of flavonoids. Usually, the aglycone forms of myricetin and quercetin are more active than the glycoside form (Tiwari et al., 2011). They are observed in the phenotype of plants when expressed in blue, orange, scarlet colours (Garcia-Salas et al., 2010), purple, yellow and red (Khoddami et al., 2013) in different parts of the plants. Many different glycosides have been found in nature; more than 80 different sugars have been discovered bound to flavonoids. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper (Michalak, 2006). The more hydroxyl groups a compound has in its structure, the higher its antioxidant activity (Haminiuk et al., 2012).

Their structural variation emanates, in part, from the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation (Stalikas, 2007). Flavonols occur as *O*-glycosides while flavone *O*-glycosides and *C*-glycosides are very common, with the latter characterized for having a carbon-carbon linkage between the anomeric carbon of a sugar molecule and the C-6 and C-8 carbon of the flavone nucleus. Unlike *O*-glycosides, sugars in and *C*-glycosides are not cleaved by acid hydrolysis (Proestos & Komaitis, 2013).

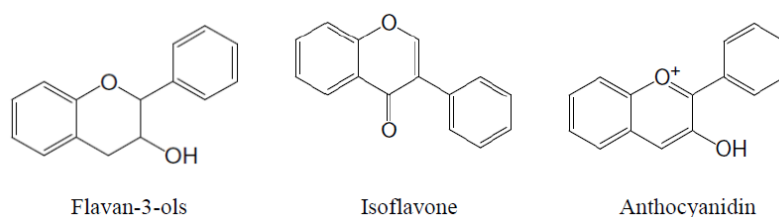


Figure 3 Structures of some flavonoids (Ghasemzadeh & Ghasemzadeh, 2011)

2.4.2 Phenolic Acids

The second most important group of phytochemicals is the group of phenolic acids, which account for almost the remaining third of the dietary phenolics. This group is divided into two major subgroups: hydroxybenzoic (protocatechuic, vanillic, syringic and gallic acids) and hydroxycinnamic (caffeic, *p*-coumaric and ferulic acids) acids (Figure 4). These two groups unlike other phenolic compounds present an acidic character owing to the presence of one carboxylic group in the molecule (Stalikas, 2007).

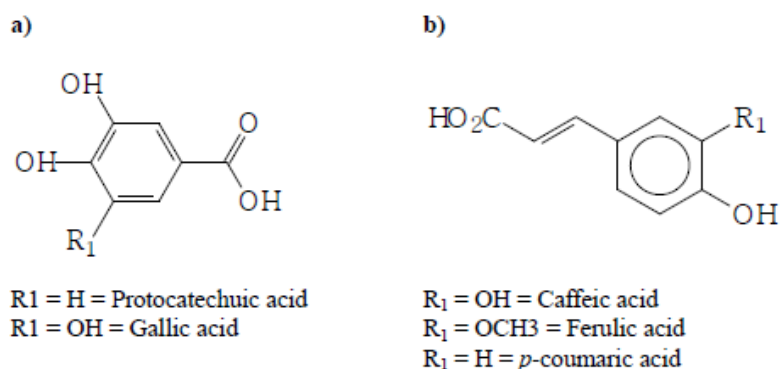


Figure 4 Chemical structures of hydroxybenzoic and hydroxycinnamic acid

Hydroxycinnamic acid compounds are usually present as derivatives having a C6-C3 skeleton. Pre-eminent amongst cinnamic acids, as far as natural occurrence is concerned is chlorogenic acid (5-*O*-caffeoylquinic acid) (CGA) which is caffeic acid esterified with quinic acid (Manach et al., 2003; Stalikas, 2007). Caffeic acid is formed through the esterification of these cinnamic acids (Farah et al., 2008).

Phenolic acids, rarely occur in a free state (aglycones) in nature (Haminiuk et al., 2012), they are mainly found in the bound form, linked to cell-wall structural components, such as cellulose, lignin and proteins via ester bonds (Liu, 2004), glycosides, (Haminiuk et al., 2012) or as amides (Khoddami et al., 2013). Hydroxycinnamic acids are predominantly bound to arabinoxylans in corn fiber and wheat bran (Bauer, 2012). Ferulic acid primarily occurs in the seeds and leaves of plants; in many cases, they are bound to plant-cell-wall polysaccharides, glycoproteins polyamines, lignin, and insoluble carbohydrate bipolymers.

Other acids such as gentisic and syringic are only present in selected natural sources. Caffeic acid, both free and esterified, is generally the most abundant phenolic acid and represents about 75% of the total hydroxycinnamic acid content of most fruits,

with highest levels on the outer parts of ripe fruits, and is found chiefly in the ‘*trans*’ form (Manach, 2003). Curcumin, which is responsible for yellow pigment in mustard, is made of two ferulic acids linked by a methylene in a di-ketone structure.

2.4.3 Lignins

Lignin is a strengthening material found in cell walls which acts as a matrix for cellulose micro-fibrils. This plant polymer is formed by phenolic oxidative coupling of hydroxycinnamoyl alcohol monomers (figure 5), brought about by peroxidase enzymes; the most important of these monomers being 4-hydroxycinnamoyl alcohol (*p*-coumaroyl alcohol), coniferyl alcohol and sinapoyl alcohol; though the monomers used vary among different plants (Pereira et al., 2009).

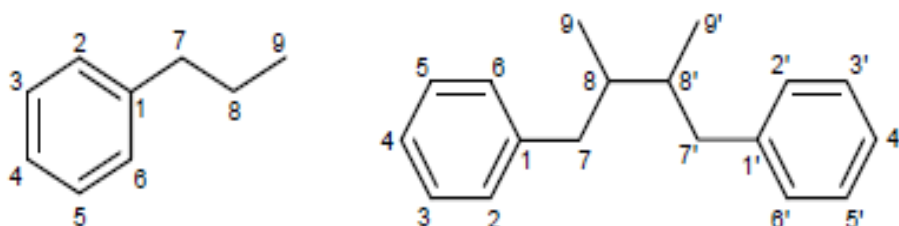


Figure 5 Structures of phenylpropanoid unit and lignin

2.4.4 Lignans

Lignans are organic compounds that result from the establishment of a link between β carbons of the side chain of two 1-phenylpropane derivatives (8-8' link), usually occurring as glycosides (Tiwari et al., 2011).

Lignans are one of the major classes of phytoestrogens, which are oestrogen-like chemicals. In the gastrointestinal tract, these molecules are converted into compounds (enterodiols and enterolactone) that have both oestrogenic and anti-

oestrogenic properties. The average intake of lignans from fruits and vegetables is estimated to be 233.6 μg per day⁻¹. The highest amounts of these compounds are found in flaxseed (linseed) (Tiwari et al., 2011), with over 1000 times as that found in other plant foods such as vegetables (garlic, asparagus, carrots), leguminous plants (lentils), cereals (triticale and wheat) and fruits (pears, prunes) (Manach, 2003). Most lignin compounds (figure 6) possess cytostatic and antimitotic properties; although only hemisynthetic derivatives of podophylotoxin have been explored in therapeutics. Among others; properties such as inhibition of AMPc phosphodiesterase and of enzymes from the respiratory chain, as well as antihypertensive activities, have been reported (Pereira et al., 2009).

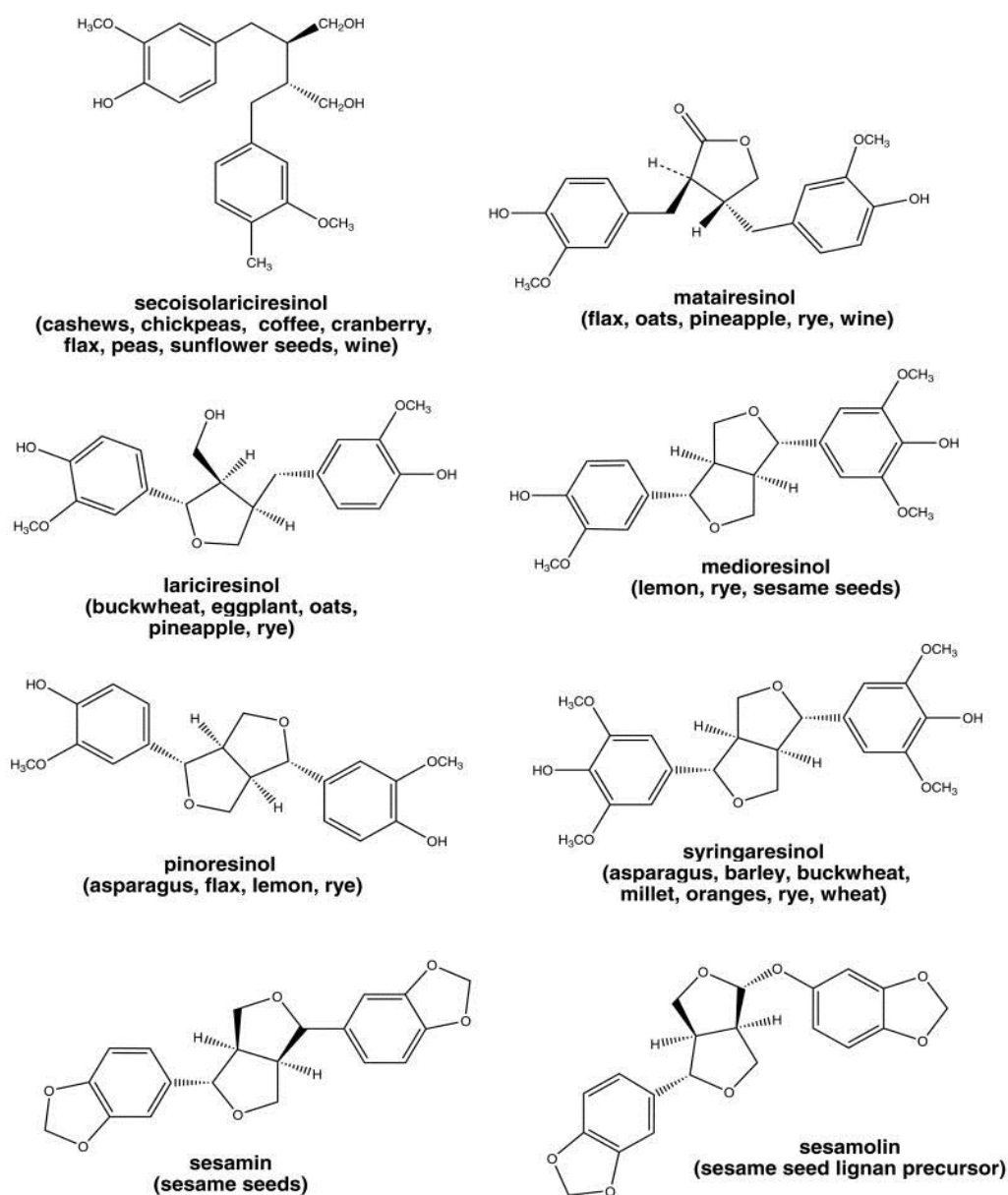


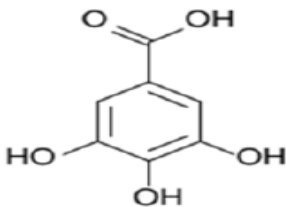
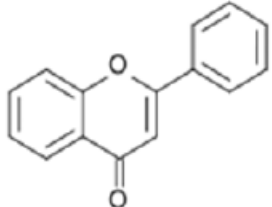
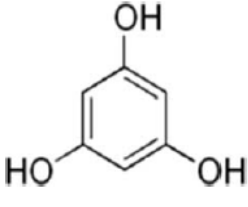
Figure 6 Structures and sources of individual lignans in common foods

2.4.5 Tannins

Tannins are the third class of polyphenols. This group includes two distinct chemical groups: hydrolysable tannins (polymers of ellagic acid, or of gallic acid, with glucose); and condensed tannins, which result from the condensation of monomers of flavan-3-ol units (Pereira et al., 2009, Tiwari et al., 2011). Tannins are astringent and

bitter substances of different molecular weight, and some of them, especially the hydrolysable tannins, are soluble in water (Tiwari et al., 2011). These compounds can combine with proteins of animal hide, preventing their putrefaction and converting them into leather. They exert their antioxidant activity by scavenging free radicals, chelating trace metals and by binding proteins with suppression of enzymatic activity (Pereira et al., 2009). The scavenging activity of tannins increases with an increase in the number of galloyl groups and molecular weight; as well as in the presence of an *ortho*-dihydroxy structure. The hydroxyl groups are responsible for the chelating and radical scavenging properties of these compounds (Pereira et al., 2009).

Table 1: Three Classes of Tannins

Base unit			
Name	Gallic acid	Flavan-3-ol	Phloroglucinol
Class/ Polymer	Hydrolyzable Tannins	Condensed Tannins	Phlorotannins

2.4.6 Stilbenes

Stilbenes are a group of phenylpropanoid-derived compounds characterised by a 1,2-diphenylethylene backbone (C6-C2-C6) (Tiwari et al., 2011). Low quantities of stilbenes are present in the human diet (Manach et al., 2003), and their main representative is resveratrol, mostly found in the glycosylated form. Resveratrol is a

phytoalexin which is produced in grapevines in response to injury and fungal infection. Studies on medicinal plants have shown that resveratrol has anticarcinogenic effects (Manach et al., 2003); thus is able to prevent cancer and coronary, neurological and degenerative disease (Tiwari et al., 2011).

2.5 Phenolic compounds and Oxidative stress

Oxidation is an essential process in life which results in the production of free radicals in food, chemicals and even in living systems. Free radicals are important in food spoilage, chemical material degradation and various human disorders (Barku, Opoku-Boahen, Owusu-Ansah, & Mensah, 2013). ROS are natural by-products of the normal metabolism of oxygen in living organisms (Oliveira et al., 2012). They consist of free radicals and non-free radicals. Free radicals include: superoxide anion, hydroxyl, hydroperoxyl, peroxy and alkoxy; while non-free radicals are such as hydrogen peroxide, hypochlorous acid, ozone and singlet oxygen. RNS are mainly nitric oxide, peroxynitrite and nitrogen dioxide (Oliveira et al., 2012). Oxidative stress may occur due to an imbalance between oxidants and ant-oxidative defence system of the human body (Bhanja & Kuhad, 2014).

The oxidative stress induced by free radicals is involved in the aetiology of a wide range of chronic diseases such as cancer, multiple sclerosis, autoimmune disease and Parkinson's disease (Haiminiuk et al., 2012) because they induce oxidative damage in biological molecules such as DNA (deoxyribosenucleic acid), proteins, lipids and carbohydrates (Bhanja & Kuhad, 2014), which play important roles in conditions such as aging, cancers, cardiovascular disease, and inflammation (Barku et al., 2013). Oxidative damage also causes significant molecular and physical damage to cells,

thereby leading to numerous skin conditions (Bhanja & Kuhad, 2014; Lin, 2005). Ultraviolet (UV) radiation generates oxidative stress in skin creating photodamage. Mechanistically, a photon of radiation interacts with *trans*-urocanic acid in skin generating singlet oxygen (Lin, 2005).

Free radicals are well known for depleting antioxidants of the immune system, change in gene expression, and inducing the production of abnormal proteins (Barku et al., 2013). Because oxidation is a normal process that continuously occurs in the body, the body is designed to deal with oxidative stress. This is achieved by employing a series of low molecular weight antioxidants that neutralize the reactive oxygen species before they can produce oxidative changes in the body cells (Lin, 2005).

Antioxidants refer to compounds that can delay or inhibit oxidation of lipids or other molecules by inhibition of initiation or propagation of oxidation chain reactions caused by free radicals and thereby can prevent, reduce or repair damage in the human body cells (Farhan et al., 2012; Trabelsi et al., 2013). These antioxidants scavenge and neutralize the free radicals described above, by donating an electron to the free radical and converting it into an innocuous molecule before it can attack body cells; thereby preventing damage to lipids, proteins, enzymes, carbohydrates and DNA (Bhanja & Kuhad, 2014, Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012). Phenolics are reported to have the ability to neutralise free radicals by quenching the unpaired oxygen radicals (Sani, 2012). For instance some flavonoids and phenylpropanoids are oxidized by peroxidase enzyme and act in peroxide (H₂O₂)-scavenging (Michalak, 2006). When plant roots are exposed to heavy metal,

they exude high levels of phenolics which inactivate iron ions through metal chelating additionally suppressing the superoxide-driven Fenton reaction, which is believed to be the most important source of ROS (Michalak, 2006).

Studies have suggested that a diet rich in phenolic compounds (fruits and vegetable) can avoid oxidative damage, keeping equilibrium between oxidants and antioxidants in the body (Bhanja & Kuhad, 2014). Although the use of synthetic antioxidants such as BHT and BHA, which are now restricted due to their negative effects, existed and have been documented, Barku et al. (2013) reported that the major part of total antioxidant activity is from the combination of phytochemicals. Therefore, no single antioxidant can replace the combination of natural phytochemicals obtained from a diet rich in fruits and vegetables and achieve their health benefits.

2.6 Extraction of Phenolic Compounds

2.6.1 Overview

Plant foods (fruits, cereal grains, legumes and vegetables), as well as beverages, are major sources of phenolic compounds in the human diet. The preparation and extraction of these compounds from this wide range of samples depends on many factors such as the nature of the sample matrix, chemical properties of the phenolic (molecular structure, polarity, concentration, number of aromatic rings and hydroxyl groups) (Khoddami et al., 2013). Phenolic compounds present acylation with other phenolics or aliphatic acids, which complicates the identification task. Some flavonoids, such as flavan-3-ols can be found in the form of dimers, trimers and polymers (Chirinos et al., 2008). The number, type and concentration of phenolics in plants exhibit extreme diversity. This diversity is one of the contributing factors to

the complexity of the analysis of phenolic acids (Stalikas, 2007), resulting in varying suitability to different extraction conditions and different susceptibilities to degradation during extraction (Ross & Beta, 2009).

The amount of phenolic compounds in a plant is strongly dependant on several factors such as climate, soil composition, geographic location and storage conditions (Tiwari et al., 2011). The complexity of the phenolic mixtures present in plant materials requires a preliminary clean-up and fractionation of the crude extract (Chirinos et al., 2008).

2.6.2 Hydrolysis

Since most phenolic compounds exist in bound forms, these linkages can be hydrolysed by addition of enzymes (such as cellulase and amylase), promoting the release of the bound phenolics during sample preparation for detection (Khoddami et al., 2013; Ross & Beta, 2009). Acid and Alkaline hydrolysis are commonly employed during phenolics extraction; and are important for the stability of the phenolics in the extract (Khoddami et al., 2013); In most cases, basic hydrolysis (NaOH ranging from 2-10 N) is preferred over acid hydrolysis as the latter can be too harsh and lead to decomposition of phenolics, thus, acid hydrolysis is sometimes only applied to liberate bound phenolics that have not been previously hydrolysed (Chirinos et al., 2008; (Ross & Beta, 2009). For instance a study by Ross & Beta (2009) on phenolic in beans showed that the majority of phenolic acids were detected in the base the hydrolyzed fraction, however acid hydrolysis released compounds present in legumes seeds that were not released with base hydrolysis. Also, in a study to compare samples of basic hydrolysis, acidic hydrolysis and those that were not

hydrolysed; Sani, Iqbal, Chan & Ismail (2012) reported that acid hydrolysis gave the highest concentration of phenolics.

While alkaline hydrolysis has been commonly applied to estimate total contents of bound ferulic acid and other hydroxycinnamates; it is also capable of extracting ferulic acid. For instance, the industrial application of alkaline hydrolysis of corn fibre and wheat bran releases high amounts of bound ferulic acid which is used to produce extracts enriched with hydroxycinnamates; which in turn are used either as antioxidants in foods or for the isolation of ferulic acid which may be used as precursor of flavour production like vanillin (Bauer, 2012).

2.6.3 Sample preparation

Sample preparation is very crucial for a successful analytical method. Complexes with proteins, carbohydrates or other elements hinder complete extraction of some phenolics (Khoddami et al., 2013). As mentioned before, crude plant matrices are very complex and thus it is unrealistic to develop standard protocol for all type of samples (Tsao & Li, 2013). The aim of the sample preparation is to either concentrate or dilute the sample so that the compounds of interest can be detected and quantified within the detection limit and linear range and get rid of unwanted substances that may cause interference during the detection of the analyte (Tsao & Li, 2013). Extraction of phenolic compounds is influenced by several parameters, and the initial step of a preliminary experiment is to select the most appropriate extraction conditions (Tiwari et al., 2011) depending on the target compounds. Sample preparation plays an important role in the quantification of phytochemicals from plant materials. Ideally, the extraction of polyphenols should be performed

using fresh samples; however, because of seasonality, perishability, shelf-life and quality, many researchers have used freezing and drying processes to preserve the plant material against degradation by microbes and for concentrating the antioxidant compounds (Tiwari et al., 2011). Drying is a technique used to reduce the availability water content in a material by heating or using chemical substances. The heat sources can be electric or global radiation, the traditional method of drying, known as sun drying or open-air drying, involves simply laying the product in the sun on mats, roofs or drying floors (Bernard, 2014).

In addition, dried plant material is sometimes preferable in order to inhibit the metabolic processes which can cause degradation of active compounds (Harbourne et al., 2013). However, some researchers have reported that the freezing process and long-term frozen storage cause important losses in the amount of phenolic compounds and vitamins from the plant material (Tiwari et al., 2011). Some preparation protocols involve the samples to be freeze-dried, air-dried or oven dried, with different reasoning. For instance, some researchers have found that shade air-drying yield more phenolics than oven drying (Khoddami et al., 2013). Milling or grinding of dried samples is also commonly practiced in order to reduce the particle size, thereby improving enzymatic action and extraction. Defatting is also a common sample preparation procedure that removes oil from lipid-containing samples. Generally, most experiments include these procedures (drying, milling and defatting), in the sample preparation step prior to extraction (Khoddami et al., 2013). It is also important to note that the content of phytochemicals may vary depending on the species or organ (e.g. roots, leaves, flowers, fruits); therefore, extraction conditions used may also vary (Harbourne et al., 2013). While liquid samples such as

wines require minimal manipulation, solid sample such as vegetables may need to be homogenised (using a blender) in a solvent, freeze-dried or ground into fine powder before extraction (Robbins, 2003). An ideal extraction method should be designed in such a way that it maximizes the yield of the compound of interest, while minimizing the extraction of undesirable compounds (Harbourne et al., 2013).

Depending on the objectives of the study, clean-up or pre-treatment may be needed for the analysis to get rid of unwanted substances. Therefore, extraction methods usually involve liquid-liquid partitioning (LLP) or solid phase extraction (SPE) to fractionate a specific phenolic compound from a crude mixture (Chen et al., 2012). LLP separates compounds depending on the lipophilicity or hydrophilicity of the compounds of interest. For instance, methanol or ethanol can be used to extract polyphenols, which are then partitioned against hexane in order to remove highly fat-soluble components such as plant sterols and fatty acids (Tsao & Li, 2013). In addition to obtaining better chromatographic separation, LLP is crucial for removing interference for spectrophotometric analysis of phytochemicals such as Total Phenolic Content (TPC), thereby reducing the risk of obtaining over estimated results (Tsao & Li, 2013). Moreover, while freely existing phenolic compounds can be readily extracted into solvents, other compounds such as phenolic acids existing in a bound form need to be released from their bound nature before extraction. This can be achieved through, among others acid/basic hydrolysis. In acid hydrolysis for instance the acidification neutralises polyphenols partitioning them into an organic solvent, from which they are extracted with another solvent such as ethyl acetate (Tsao & Li, 2013). Solid Phase Extraction in the same way partitions compounds from a crude mixture. This method involves the use of adsorbents such as porous

resins (e.g. C18, LH-20). Batch soaking using these adsorbents is rare, more commonly used are columns or cartridges pre-packed with these adsorbent resins. After passing the sample through the cartridge/ column, the compounds of interest are eluted with the appropriate solvent (Tsao & Li, 2013).

2.6.4 Conventional Extraction Techniques (solid-liquid extraction)

There are several methods employed for extracting phytochemicals from plants. Conventional methods include maceration, infusion and Soxhlet extraction (Harbourne et al., 2013); these are discussed below. The initial stage of conventional extraction typically utilises aqueous organic solvents, targeting soluble/extractable phenolic acids (free, soluble esters, and soluble glycosides) (Ross & Beta, 2009).

In these methods, the solvent diffuses into the plant material, dissolving the compounds of interest inside the plant matrix (phytochemicals); which finally diffuses out of the plant matrix into the solvent solution (Harbourne et al., 2013). Although conventional methods have some advantages, their biggest disadvantages include:

1. The use of large volumes of hazardous organic solvents which are environment pollutants and health hazards;
2. They are time consuming; and
3. Their processes consistently undergo interference with, and degradation of target compounds as a result of external factors such as light, air, high temperatures and enzymatic reactions (Khoddami et al., 2013).

Other modern extraction systems currently employed in the extraction of polyphenols include: pressurized liquid extraction (PLE), microwave assisted

extraction (MAE) and supercritical fluid extraction (SFE). PLE, also known as accelerated solvent extraction (ASE), is similar to the conventional solvent systems, except that ASE employs the combination of high pressure and temperature with highly reduced solvent volume, thus, resulting in high recovery compared to the conventional techniques (Tsao & Li, 2013).

2.6.4.1 Maceration

In this method, the plant material is soaked in an organic solvent, in a closed container (Tiwari et al., 2011), at ambient temperature (Khoddami et al., 2013). Occasional stirring can be employed in order to increase the rate of extraction. After extraction, the mixture is filtered and the procedure is usually repeated several times to maximize extraction. This method is safe and useful for heat labile compounds as it is carried out at room temperature (Harbourne et al., 2013); however, it is a very time and solvent consuming process (Khoddami et al., 2013), ranging from hours to several weeks for a single complete extraction. Phenolic extracts require in many cases a hydrolysis pre-treatment, although significant phenolic losses may occur due to decomposition of some phenolic compounds (Harbourne et al., 2013).

2.6.4.2 Infusions

Infusion is very similar to maceration; however, it is carried out at a specific temperature (higher than the room temperature but lower than 100°C) and a specific set time, ranging from minutes to hours. Infusions usually use water (traditionally boiling water) as the extraction solvent. After extraction is complete, the solution is filtered and the procedure repeated as in maceration. An example of maceration is making a cup of tea (Harbourne et al., 2013).

2.6.4.3 Soxhlet extraction

The Soxhlet method has been used to extract phytochemicals from plant materials for many years. It is often used as a reference for evaluation other solid-liquid extraction methods or new non-conventional methods (Harbourne et al., 2013); or when the desired compound has a limited solubility in a solvent (Tiwari, 2011). The procedure is normally performed at 90°C for several hours (Khoddami et al., 2013). It is not suitable for thermolabile compounds as prolonged heating may lead to degradation of compounds (Tiwari et al., 2011). The system consists of a thimble-holder with perforated sides in which the plant material is put; and a bottom so that liquid can flow through. It has a collection flask below the thimble and a reflux condenser above it. When heat is applied to the flask containing the solvent, the solvent evaporates, travelling to the condenser where it condenses and falls into the thimble. The solvent in the thimble eventually reaches a certain level when it is unloaded back into the solvent flask. The solution is distilled to separate the solute from the solvent while fresh solvent passes through the plant material. The procedure is repeated until complete extraction is attained (Harbourne et al., 2013). The biggest advantage of the Soxhlet system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled (Tiwari et al., 2011).

2.6.5 Factors affecting extraction methods

The efficiency of the solid-liquid extraction methods are affected by several factors including: solvent type, solvent: plant material ratio, temperature, extraction time and the structure of the matrix (e.g. particle size, plant organ) (Harbourne et al., 2013).

2.6.5.1 Solvent type

The type of solvent used for extraction is very important because different phytochemicals dissolve in different solvents differently. The solubility of phenolic compounds in a certain solvent depends on the polarity of the solvent, polymerisation of the phenolics and their interactions with other compounds present in the extract (Kopjar et al., 2015). Recently, the use of organic solvents such as ethanol, methanol, acetone, or ethyl acetate either alone or their respective aqueous extracts for the extraction of phytochemicals has become very popular (Chen, 2012; Ross & Beta, 2009); focusing on less toxic solvents (Harbourne et al., 2013 et al., 2013). Common combinations of organic solvents with water that have been reported include: methanol/acetone:water, acetone:water, methanol:water, ethanol:water, and acidified methanol (Ross & Beta, 2009). Water is also used very commonly in extracting phenolics. In this case, depending on the polarity of the compounds being extracted, the water will extract the more polar compounds while ethanol will extract the more hydrophobic compounds (Harbourne et al., 2013). In addition, the pH of the solvent can be changed to selectively extract or improve the extraction of certain plant bioactive compounds. For instance, anthocyanins are usually extracted at an acidic pH as they are unstable at neutral or alkaline pH (Harbourne et al., 2013).

2.6.5.2 Temperature

In general, the extraction of phytochemicals at a higher temperature causes an increase in the rate of diffusion of the analyte into the solvent, thereby reducing extraction time. The effect of temperature can be in the increase in the solubility of the compounds from the plant matrix into the solvent solution or in breaking down cellular constituents, thereby resulting in the release of the phytochemicals. In

addition, the increase in temperature may also enzymatic activities, therefore resulting in an increase in the yield of bioactive compounds. The temperature is set depending on the boiling point of the solvent being used. For instance while acetone boils between 56-57°C, water boils at 100°C (Harbourne et al., 2013). While an increase in temperature works well on heat stable compounds, it has a major limitation on heat liable compounds such as anthocyanins (Harbourne et al., 2013), which tend to degrade under high temperatures (Tsao & Li, 2013).

2.6.5.3 Time

When considering extraction time, the compromise lies between complete extraction and having a process which is both cost and time effective. Extraction of various phytochemicals vary depending plant species, plant organ and particle size. In general, extraction of phytochemicals from leaves is faster than the extraction from harder materials such as the roots and bark of the plant (Harbourne et al., 2013).

2.6.5.4 Particle size

Grinding or milling are the most common procedures applied to plant material in order to reduce particle size and increase surface area. The smaller the particles, the faster the solvent travels to the analyte, thereby reducing extraction time. These procedures also cause damage to the plant cells, which makes it easier to extract the compounds of interest. However, the disadvantage of grinding and milling plant material is that the finely ground powder can block filters, resulting in lengthy experimental procedures and possibly wastage of extract (Harbourne et al., 2013).

2.6.6 Detection of phenolic compounds

The development of chromatographic methods for separation and analysis of unknown sample is one of the challenges currently faced by analytical chemists (Cabooter, Broeckhoven, Kalili, Villiers & Desmet 2011). Colorimetric methods including thin layer chromatography, gas chromatography (GC), GC-MS and high performance liquid chromatography (HPLC) have been employed for the analysis of phenolic compounds. Chromatographic techniques, coupled with absorption spectrum analysis and mass spectrometry provides definitive information for identification and quantification of phenolic compounds (Chang, Yang, Wen, & Chern, 2002). However, because these methods require advanced instruments, authentic standards and the analysis is time consuming, colorimetric methods are still widely employed for routine analyses (Chang et al., 2002) .

CHAPTER 3: METHODOLOGY

3.1 Research Design

The selected Namibian Leafy Vegetable (NLV) samples were collected from the Kavango region. Fresh samples were air-dried and different extracts of each plant prepared using organic solvents (acetone, methanol and ethanol). The ferric chloride test was performed on all plant extracts to examine the presence of phenolic compounds in each extract. Acid and base hydrolysis was applied during extraction of some of the extracts.

The sample extracts were quantitatively screened for phenolic content with the following assays: Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and analysed for antioxidant potential with the following assays: Reducing Power (RP), DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). The absorbance values of the extract were obtained using a microplate reader (TPC, TFC) and a UV spectrophotometer (RP, DPPH and ABTS). Finally, qualitative and semi-quantitative analyses of the hydrolysed and non-hydrolysed methanol extracts were performed on the GC-MS to characterize specific phenolic acids present in each sample using phenolic acids standards.

3.2 Research Instruments, Solvents and Reagents

- Grinder
- GC- MS (Thermo Scientific Focus)
- Freeze dryer (Christ, UK)

- Rotary evaporator (Heidolph Instruments GmbH & Co.KG, Germany)
- Universal Stirred Water bath (Biosan Ltd, EU)
- Digital Oven (Scientific series 2000, South Africa)
- SpectraMax ® M2 Multi-mode Microplate Reader (Molecular Devices, Deigned in California, USA; made in China)
- Millipore Millex- HN, Nylon 0.45 µm filters (Millipore, Merck, Germany)
- Filtration pump (Heidolph, Germany)
- Digital Ultrasonic Cleaner (EUmax, LABOTEC)
- Universal Stirred Water bath (Biosan Ltd, EU)
- Labcon Shaking Incubator (Laboratory Marketing Services, Maraisburg)
- Shaker (DHZ, China)
- Centrifuge (Eppendorf AG, Hamburg, Germany)
- Analytical weighing balance (Mettler Toledo, BioScientific (Pty) Ltd; Switzerland)
- Analytical weighing balance (OHAUS, China)
- UV spectroscopy (Helios gamma Spectronic Unicam, England)
- Vortex (BOECO, Germany)

3.3 Sample Collection and Storage

Two field trips were undertaken to collect 13 NLV samples from the Kavango East region in the Ndiyona constituency. These were namely: *Derere*, *Mutete (waugeha)*, *Mutete (waukenu)*, *Mutete kashashu*, *Mboga*, *Mpungu*, *shinuti*, *Rupotera*, *Ntangavidi*, *Makunde*, *Livuvu*, *Namayara*, and *Mukugho* (local language: Rumanyo). The collection sites as well as the indigenous name(s) of the collected traditional

vegetables were documented; specimens were pressed, voucher specimen numbers were assigned and digital photos taken and sent to the National Botanical Research Institute (NBRI) for validation and scientific identification. The plant samples were collected during the rainy season (January and April 2014) whilst still fresh. Collection was done by obtaining several leaves from a whole plant. The freshly collected vegetable samples were brought to the Department of Chemistry and Biochemistry of the Faculty of Science, University of Namibia (UNAM); Windhoek campus for further processing. The finely ground powdered samples were stored in the fridge (-15 and -20 °C) until further analysis.

3.4 Chemicals and Reagents

Chemicals used in this research were purchased from ACROS Organics, New Jersey, USA. The organic solvents (methanol, ethanol, acetone, ethyl acetate); reagents and water for chromatography were purchased from Merck, Germany.

3.4 Sample Preparation

The dried sample leaves were finely ground in an industrial, electric grinder at the Ministry of Agriculture, Water and Forestry (MAWF) in Windhoek. All dried samples tested were defatted with *n*-hexane in the ratio 1:5 w/v (5g of a sample was mixed with 25mL *n*-hexane). The mixture was properly mixed on a vortex (BOECO, Germany) for 1 min, allowed to stand for 10 minutes and filtered on Whatman No.1 filter paper. This process was repeated three times, the samples were air-dried for 48 hours in open air (room temperature) and stored in the freezer (-15 and -20 °C) for further extraction.

3.5 Extraction of Free Phenolic Compounds

Free phenolic compounds were extracted according to the method described by Su et al. (2014) with a few modifications. Each NLV sample had 3 beakers, 1 beaker for each organic solvent, namely 80% acetone, 80% methanol and 80% ethanol. 0.5g of defatted dried powdered was weighed out for each vegetable sample and extracted with each organic solvent in the ratio 1:10 w/v respectively. Extraction was carried out at 50°C for 60min in a universal stirred water bath (Biosan, EU), and was repeated three times. The mixture was centrifuged and filtered on Whatman No.1 filter paper at each step, and the final filtrate pooled together and further filtered using 0.45 Millipore filters (Merck, Germany) for purification.

The extracts were concentrated by evaporating the organic solvent with a rotary evaporator (Heidolph, Germany) at 40°C, and further dried in the freeze dryer to remove all the water.

The dried residues were stored at -80 °C before analysis. During analysis, the residues were re-constituted in 10mL of the respective solvents (pure) and refrigerated at 4°C for analysis of qualitative (ferric chloride test), quantitative (TPC, TFC); and antioxidant (RP, DPPH and ABTS) tests. The concentration of the extracts was 0.05g/mL.

3.6 Hydrolysis of Bound Phenolics

For extraction of bound phenolics, 0.5g of defatted powdered samples were weighed out in two flasks for each vegetable sample. One flask was labelled Acid Hydrolysed Sample (AHS) and the other was labelled Base Hydrolysed Sample (BHS). These samples were first extracted with methanol remove free phenolics, filtered on

Whatman No.1 filter paper and the residues discarded, this process was repeated three times. The filtrates were dried and subject to acid and base hydrolysis conditions respectively.

Hydrolysis conditions subjected to each sample were as previously described by Cheng et al. (2014), Proestos & Komaitis (2013) and Su et al. (2014) with some modifications. Comparison was done between the two hydrolysis methods and further compared to methanol extracts in GC-MS analysis to see the effect of hydrolysis on the presence and quantity of phenolic acids in each extract.

3.6.1 Acid Hydrolysis of Bound Phenolic Compounds

Acid hydrolysis protocol was modified from Su et al. (2014) and Cheng et al. (2014). The following conditions were set for the Acid Hydrolysed Samples (AHS): liquid/solid ratio 21:1 v/w; Methanol/ 12N H₂SO₄ 90:10 v/v. The mixture was sonicated in a Digital Ultrasonic Cleaner Sonicator (EUmax, LABOTEC) for 15 min and refluxed in a water bath at 90 °C for 2 h. The mixture was then filtered and made up to 100 mL with methanol. This process was performed three times. After hydrolysis, the residues were pooled together and extracted six times with 10mL ethyl acetate; each time centrifuging and the residue re-extracted. The organic fractions were combined, filtered with 0.45 µm Millipore filters and evaporated to dryness at 35°C with a rotary evaporator (Heidolph, Germany). Dry extracts were reconstituted in 10mL methanol and stored in the freezer (-4°C) for GC-MS analysis. The concentration of the extracts was 0.05g/mL. Anhydrous sodium sulphate was added to the samples to remove moisture. For each sample, 100 µL of the extract was

evaporated under nitrogen gas stream and derivatised before analysis on the gas chromatography mass spectrometer.

3.6.2 Base Hydrolysis of Bound Phenolic Compounds

Basic hydrolysis was performed according to a modified version of Proestos & Komaitis (2013). Basic hydrolysis conditions were as follow: liquid (extraction solvent)/solid (dried sample) ratio 20:1 v/w; extraction solvent was methanol: 6N NaOH 90:10 v/v; extraction time of 4.5 hours at room temperature. Extraction was repeated three times. After hydrolysis, the residues were pooled together and extracted six times with 10mL ethyl acetate; each time centrifuging and the residue re-extracted with ethyl acetate. Sodium sulphate was added to samples to remove moisture. The organic fractions (ethyl acetate) were then combined, filtered with 0.45 µm Millipore filters to remove tiny solid particles and evaporated to dryness at 40°C at a pressure of 206 mmHg with a rotary evaporator. The dry extracts were reconstituted in 10mL methanol and stored in the freezer for GC-MS analysis. The concentration of the extracts was 0.05g/mL.

3.7 Qualitative Colorimetric Assay (Ferric Chloride Test)

The Ferric Chloride Test was performed to indicate the presence of phenolic compounds in all the prepared samples. The procedure followed was according Harbourne et al. (2013). 1mL of each extract was transferred into a test tube; then few drops of Ferric Chloride Solution were added to each test tube. A colour change to dark green indicated the presence of phenolic compounds in each plant extract (Tiwari et al., 2011). Gallic acid was used as the positive control (standard) of phenolic compounds and deionized water as a negative control.

3.8 Quantitative Colorimetric Assays: Introduction

Phenolic compounds are polar in nature, therefore, they need a polar medium to dissolve in such as organic solvents. However these solvents also vary in polarity. Theoretically, the more polar the solvent, the better it is at extracting phenolic compounds. Methanol is one of the commonly used organic solvent for extracting phenolic acids (Chanda & Dave, 2009). It is well known to enhance the extraction of complex mixtures of antioxidant compounds (Sani, 2012).

3.8.1 Total Phenolic Contents (TPC)

In TPC, the phenol in the alkaline medium reacts with phosphomolybdic acid of the Folin-Ciocalteu's (FC) reagent producing a blue coloured complex (Corpuz, Osi & Santiago 2013).

The total phenolic contents of each sample were determined using the (FC) reagent according to the method of Sultana (2012), with a few modifications. An aliquot of 0.5mL of each sample extract at a concentration of 0.05g/mL was mixed with 2.5mL of a 10 fold dilute FC reagent and 2mL of 7.5% Na_2CO_3 solution. The mixture was covered with foil and incubated for 30 minutes at room temperature (ca. 25°C) in the dark, after which 200 μL was loaded on the microplate reader wells. Distilled water was loaded on the plate as the blank. The control consisted of all the above reagents except the sample or standard. The blank and standard solutions were treated as if they were samples for control purpose.

The absorbance was measured at 760 nm using a SpectraMax ® M2 Multi-mode Microplate Reader (Molecular Devices, USA). The absorbance value of the blank was subtracted from all the absorbance values of the samples obtained from the

microplate reader since all the samples were loaded on the 96 well plate at ones and the microplate reader was not set to zero (blanked) before taking sample readings.

A standard curve was prepared using gallic acid as the standard phenolic compound with concentrations of 0, 10, 20, 30, 40, 50 and 60 $\mu\text{g}/\text{mL}$. The amount of total phenolic content was expressed as μg Gallic Acid Equivalents (GAE)/g of dry weight of samples.

The total phenolic content was calculated with the formula:

$$C = [(C_i * V * D.F) / W_s];$$

Where C is the TPC in μg GAE/g; C_i is concentration from the calibration curve of gallic acid in $\mu\text{g}/\text{mL}$; V is the volume of extract in mL and W_s is the weight of plant sample in grams. All experiments were performed in triplicate analysis.

3.8.2 Total Flavonoid Contents (TFC)

The aluminium chloride colorimetric method is widely adopted in almost all published work to measure the total flavonoid content of plants. The principal is based on the acid-stable complex between aluminium chloride, and the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols (Corpuz et al., 2013).

The total flavonoid contents of the samples were estimated using the method described by Corpuz et al. (2013). 100 μL of 2% AlCl_3 ethanol solution was added to 100 μL of 0.05g/mL sample extract. Absorbance was read at 420nm using a microplate reader. Quercetin was used as the standard flavonoid compound. The standard solutions and blank were treated the same way as the plant extracts. The

control consisted of AlCl₃ ethanol solution and ethanol was used as the blank. The absorbance value of the blank was subtracted from all the absorbance values of the samples obtained from the microplate reader since all the samples were loaded on the 96 well plate at ones and the microplate reader was not set to zero (blanked) before taking sample readings.

A standard curve was prepared using quercetin as the standard phenolic compound with concentrations of 0, 10, 20, 30, 40, 50 and 60 µg/mL. The amount of TFC was expressed as µg quercetin equivalent (QE)/g of dry weight of samples.

The TFC was calculated using the same formula as for TPC;

$$C = [(C_i * V * D.F) / W_s];$$

Where C is the TFC in µgQE/g; C_i is concentration of from the quercetin calibration curve, calculated in µg/mL; V is the volume of extract in mL and W_s is the weight of plant sample in grams.

3.9 Antioxidant Assays

3.9.1 DPPH Radical Scavenging Activity

DPPH radical scavenging is used as an index to evaluate the antioxidant potential of many plants. DPPH radical scavenging activity assay assesses the capacity of the extract to donate hydrogen or to scavenge free radicals (Barku, 2013). DPPH is a purple coloured stable free radical which upon reduction, at room temperature becomes the clear yellow diphenyl picryl hydrazine (Garcia, Oldoni, Alencar, Reis, Loguercio & Grande, 2012).

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity of each extract was measured according to a procedure described by Farhan et al (2012). 100µL of 0.15mM DPPH in ethanol was added to 100µL sample extract (0.05g/mL) and standard (ascorbic acid solutions at various concentrations) respectively. The mixture incubated in the dark for 30 minutes. Absorbance was read at 517nm using a microplate reader.

The DPPH solution was freshly prepared before the experiment. Ascorbic acid was used as a standard, ethanol was used as a blank and the control was a mixture of 100µL DPPH with 100µL ethanol instead of the sample. The absorbance value of the blank was subtracted from all the absorbance values of the samples obtained from the microplate reader since all the samples were loaded on the 96 well plate at ones and the microplate reader was not set to zero (blanked) before taking sample readings.

Radical scavenging activity for each extract was calculated using the following formula:

$$\% \text{ of inhibition} = [(A_c - A_s) / A_c] \times 100,$$

Where A_c is the absorbance of the control and A_s is the absorbance of the test sample.

3.9.2 ABTS Radical Scavenging Activity

The ABTS free radical-scavenging capacity was studied using the ABTS radical cation decolorization assay; which is based on the reduction of $ABTS^{\cdot+}$ radicals by plant extracts tested. ABTS was performed on methanol and ethanol extracts according to the method described by Sani et al (2012) with a few modifications. A stock solution of $ABTS^{\cdot+}$ radical cation was prepared by adding 2.45mM of

potassium persulfate with 7.0mM ABTS (dissolved in deionized water) in the ratio 1:1; and incubated for 16 hours in the dark. A working solution was prepared by diluting the resulting cation with methanol to give an absorbance of 0.7 ± 0.02 at 734nm.

To 100 μ L sample extract (0.05g/mL), 900 μ L working solution was added and the first reading taken at 734nm using a UV spectrophotometer (Helios gamma Spectronic Unicam, England). The samples were then placed in the dark for 10minutes, after which the decrease in absorbance was measured at the same wavelength (734nm). The procedure was applied to the standard phenolic acid, which in this case was gallic acid solutions at various concentration.

The blank was phosphate buffer with no ABTS and the control was ABTS radical solution with methanol. Radical scavenging activity was calculated as follows:

$$\text{Radical scavenging activity (\%)} = [(A_c - A_s) / A_c] * 100$$

Where A_c is the absorbance of the control (initial radical solution with no test sample) and A_s the absorbance of the sample at 734 nm.

3.9.3 Reducing Power

Compounds with a high reducing power act as secondary antioxidants because they donate electrons in order to reduce oxidized intermediates (Chanda & Dave, 2009).

The reducing power was determined following the method by Chanda & Dave (2009) with a few modifications. Briefly, 2.5mL phosphate buffer (pH 6.6, 200mM) was added to 1mL sample extract at a concentration of 0.05g/mL. The mixture was incubated at 50°C for 20 minutes, after which 2.5mL trichloroacetic acid (TCA)

(600mM) was added and centrifuged for 20 minutes at 3000rpm. Then, 2.5mL of the upper layer was transferred into a clean test tube, 2.5mL dH₂O and 0.5mL FeCl₃ (6mM) added and well mixed. Then, 200μL was loaded on a microplate reader and absorbance read at 700nm. Ascorbic acid was used as the standard. The control consisted of all reagents except the sample extract or standard. Phosphate buffer was used as a blank. The absorbance value of the blank was subtracted from all the absorbance values of the samples obtained from the microplate reader since all the samples were loaded on the 96 well plate at ones and the microplate reader was not set to zero (blanked) before taking sample readings.

The reducing capacity was expressed in percentage (%) and calculated as follows:

$$\text{Reducing capacity (\%)} = 100 - [(A_c - A_s / A_c) * 100];$$

Where A_c is the absorbance of the control and A_s is the absorbance of the test sample.

3.10 Gas Chromatography - Mass Spectrometry (GC-MS) Analyses:

Introduction

Mass spectrometry is a powerful analytical technique based on ions separation; therefore ionization is of key importance for high sensitivity and selectivity. The ionization efficiency depends on the physico-chemical properties of a molecule, and also on the conditions established in the ionization interface (Silvestro, Tarcomnicu & Savu, 2013). In this case, GC-MS was employed for the detection (qualitative analysis) of eight phenolic acids (4-hydroxybenzoic acid, Vanillic acid, Syringic acid, *p*-Coumaric acid, Gallic acid, *trans*-Ferulic acid, Caffeic acid and Sinapic acid)

in NLV samples collected from Kavango East region and semi-quantification (quantitative analysis) of the present phenolic acids.

3.10.1 Phenolic Acids Standard Solution (SS)

Standard solutions of each phenolic acid standard (4-hydroxybenzoic acid, Vanillic acid, Syringic acid, *p*-Coumaric acid, Gallic acid, *trans*-Ferulic acid, Caffeic acid and Sinapic acid; purchased from Sigma-Aldrich, Germany; were prepared at 50ppm. Furthermore, one SS containing all eight phenolic acids, each at 50ppm each was prepared.

3.10.2 Internal Standard Solution (ISS)

In this experiment, methyl dodecanoate (MDD), was used as the Internal Standard (IS). The purpose of the Internal Standard (IS) is to counteract for any shifts in the mass spectra of the target compounds. Therefore, it is added to all samples including the standards and is expected to elute at the same retention time in all samples. Therefore if there is a shift in the retention time of any compound, the retention time of the IS would also shift with the same magnitude.

The ISS was prepared in ethyl acetate at 5ppm in a 25 mL volumetric flask, by using the formula $\text{density} = \text{mass}/\text{volume}$ to obtain the volume of MDD required.

3.10.3 Dilution Solvent (DS)

The dilution solvent was a diluted solution of the ISS at a concentration of 1.25ppm in a 25mL volumetric flask by transferring 6.25mL of the 5ppm ISS into a 25 mL volumetric flask and filling in up to the mark with ethyl acetate.

The DS was prepared at a concentration such that when added to each standard solution prepared at various concentrations for the purpose of plotting a calibration curve, the concentration of the IS would remain the same in all the standard solution samples. Derivatives

3.10.4 Derivatization Procedure (Silylation)

Derivatization is a process that is applied to high molecular weight non-volatile compounds, with the aim of reducing the polarity and increasing volatility of the compounds, leading to symmetric peaks and an increase in sensitivity (Schmutzer, 2012).

Silylation is a derivatization procedure ideal for GC-MS analysis of non-volatile and thermolabile compounds. During silylation of phenolic acids, a trimethylsilyl (TMS) group replaces an active hydrogen of an –OH group on the phenolic acid compound, through a nucleophilic substitution reaction, producing TMS derivatives that are more volatile, less polar and more thermotolerant than the parent compound (Proestos & Komaitis, 2013). N,O-Bis (*Trimethylsilyl*) Trifluoroacetamide (BSTFA) was the derivatization reagent used in this experiment.

Derivatization procedure was performed according to a modified version of the method described by Proestos & Komaitis (2013). An aliquot of 50 μ L of the 50ppm Standard solution was transferred into a new dry vial and dried under nitrogen gas. Then 150 μ L of the derivatization reagent, BSTFA was added to the vial and placed in the oven for 45 minutes at 80°C to allow for the derivatization reaction to take place. After the 45 minutes, the vial was left to cool at room temperature before

opening it, and then 50 μ L of the ISS was added to the vial and labelled as vial 1, with an actual SS concentration of 25ppm.

A series of dilutions was prepared from vial 1(25ppm), to make five more vials at 20, 15, 10, 5 and 1.25ppm concentrations of SS, by diluting the content of the previous vial with the DS respectively, for the purpose of plotting a calibration curve that would be used to calculate the concentration of each phenolic acid in each vegetable extract as a pilot study of GC-MS quantification. After that, an amount of 1 μ L was injected on the GC-MS (Focus Thermo-Fisher, USA) from each vial for analysis.

3.10.5 GC-MS Analysis Conditions

Gas Chromatography-Mass Spectrometry (GC-MS) analyses were performed on a Thermo Scientific Focus GC coupled to an ITQ 700 MS using Xcalibur Software, version 2.1, for data acquisition. A SGE BP5MS capillary GC column (30 m x 0.25 mm i.d., film thickness of 0.25 μ m) was used with helium as carrier gas at a flow rate of 1.0 mL/min (constant flow). The GC injector was maintained at a temperature of 220 °C. Samples were injected in the split mode using a split ratio of 1:10. The oven temperature was programmed at 5 °C/min from 40 °C to 300 °C. Electron ionization-Mass Spectrometry (EI-MS) data were acquired at 70 eV and a mass range of m/z 25 to 625 was scanned. Ion source and interface temperatures of 200 and 250 °C, respectively, were used for the analysis. Each analysis ran for 30 minutes with an extra 15 minutes of cooling, making it a total of 45 minutes to 1 hour before the next sample can be loaded.

3.10.6 Identification of Phenolic Acids by GC-MS

The focus of the GC-MS analysis was on the qualitative analysis, to detect and characterise individual phenolic acids present in NLV samples extracts based on retention time of authentic reference standard phenolic acids. The presence or absence of each phenolic acid was manually searched for using the Quantitative Browser at the elution time of each phenolic acid. An extracted ion chromatogram (EIC) of the sample mass spectra at the expected retention time was compared to the EIC of the phenolic acid and the compound structure in the mass spectra library to verify the phenolic acid.

3.10.7 Quantification of Phenolic Acids by GC-MS

The phenolic acid standards prepared at various concentrations were used to quantify phenolic acids present in the NLV samples. Standard curves were prepared for each standard compound and the phenolic acid concentration was calculated using the formula:

$$\text{Phenolic acid Sample } (\mu\text{g/g}) = \left[\left(\frac{A_s}{A_i} \right)^{a+b} \right] * (C_i * V * DF) / W_s$$

Wherein A_s is the peak area of the standard phenolic acid; A_i : peak are of the IS; a : slope of the calibration curve of the specific phenolic acid; b : intercept of calibration curve; C_i : concentration of IS; V : volume of extract (mL); DF : Dilution factor; R : recovery and W_s : weight of the sample (g).

3.11 Data analysis

All measurements were done in triplicates; means and standard deviation (SD) were also calculated. One way analysis of variance (ANOVA) was conducted using

Microsoft office excel 2013 to compare the yield values and concentration of different phenolic compounds in different plant extracts and solvents.

3.12 Research Ethics

A research permit was obtained from the postgraduate school of UNAM to carry on the research activities reported in this thesis. The collection of leafy vegetables was done during the rainy season of 2014. The NLV collected were sent to NBRI for identification. Positive and negative controls were included to all experiments were applicable.

CHAPTER 4 RESULTS

4.1 Collection and Identification of NLV

Thirteen NLVs were collected from Ndiyona village in Ndiyona constituency, Kavango East region and identified at the National Botanical Research Institute (NBRI) in Windhoek. Their scientific names and specimen vouchers numbers are shown in Table 1. Pictures of the respective NLVs taken during the field trip are provided below (figures 8-19). More than 10g each vegetable species was collected.

Table 2 Namibian Leafy Vegetables (NLVs); indigenous (*Rumanyo*), scientific and family names.

	Collection no.	Indigenous name	Scientific name	Family name
1	IKSTF 0500	<i>Mutete waugaha</i>	<i>Hibiscus sabdarifa</i>	Malvaceae
2	IKSTF 0501	<i>Livuvu</i>	<i>Aeschynomene nambalensis</i>	Fabaceae
3	IKSTF 0502	<i>Mutete waukenu</i>	<i>Hibiscus cannbinus</i>	Malvaceae
4	IKSTF 0503	<i>Mutete Kashashu</i>	<i>Oxygonum delagoense</i>	Polygonaceae
5	IKSTF 0504	<i>Mboga</i>	<i>Amaranthus thunbergii</i>	Amaranthaceae
6	IKSTF 0505	<i>Mukuwo</i>	<i>Amaranthus hypochondriacus</i>	Amaranthaceae
7	IKSTF 0506	<i>Makunde</i>	<i>Vigna unguiculata</i>	Fabaceae
8	IKSTF 0507	<i>Mpungu</i>	<i>Cleome gynandra</i>	Cleomaceae
9	IKSTF 0508	<i>Shinuti</i>	<i>Cucurbita maxima</i>	Cucurbitaceae
10	IKSTF 0509	<i>Rupotera</i>	<i>Lagenaria siceraria</i>	Cucurbitaceae
11	IKSTF 0510	<i>Namayara</i>	<i>Aeschynomene fluitans</i>	Fabaceae
12	IKSTF 0511	<i>Ntangavidi</i>	<i>Citrullus lanatus</i>	Cucurbitaceae
13	IKSTF 0512	<i>Derere</i>	<i>Corchorus tridens</i>	Malvaceae

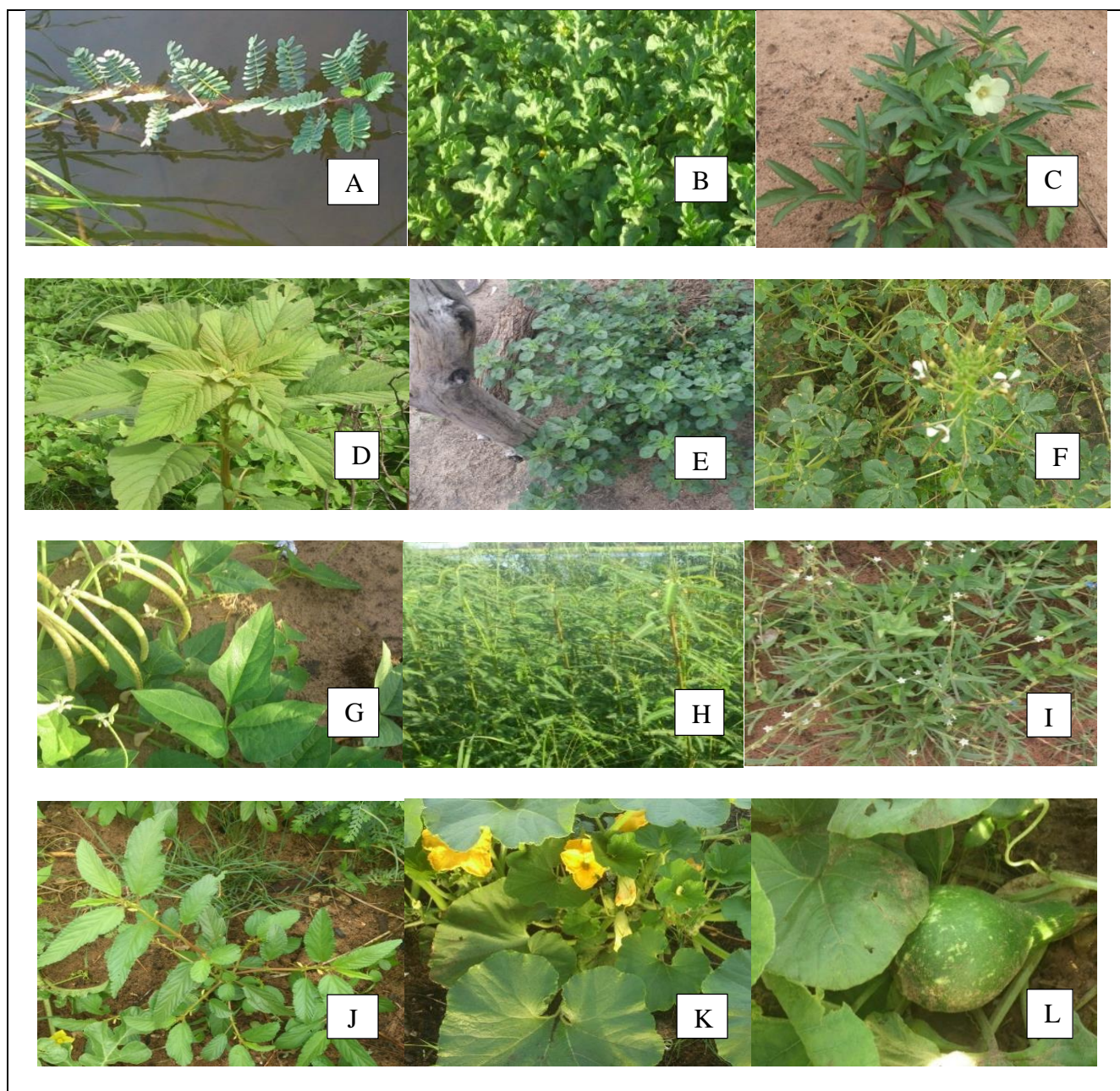


Figure 7 Selected Namibian Leafy Vegetables collected for this project

A: *Aeschynomene fluitans*; B: *Citrullus lanatus*; C: *Hibiscus sabdarifa* and *H. cannabinus*; D: *Amaranthus hypochondriacus*; E: *Amaranthus thunbergii*; F: *Cleome gynandra*; G: *Vigna unguiculata*; H: *Aeschynomene nambalensis*; I: *Oxygonum delagoense*; J: *Corchorus tridens*; K: *Cucurbita maxima*; L: *Lagenaria siceraria*.

4.2 Qualitative Colorimetric Analysis

4.2.1 Ferric Chloride Test

All NLV extracts gave positive results with the Ferric chloride test for the presence of phenolic compounds. This is as expected because all plants contain phenolic compounds.

4.3 Quantitative Colorimetric Analyses

4.3.1 Total Phenolic Content (TPC)

TPC was performed on 80% aqueous acetone, methanol and ethanol extracts. A calibration curve of gallic acid was produced, R^2 value = 0.9822 (Fig 8).

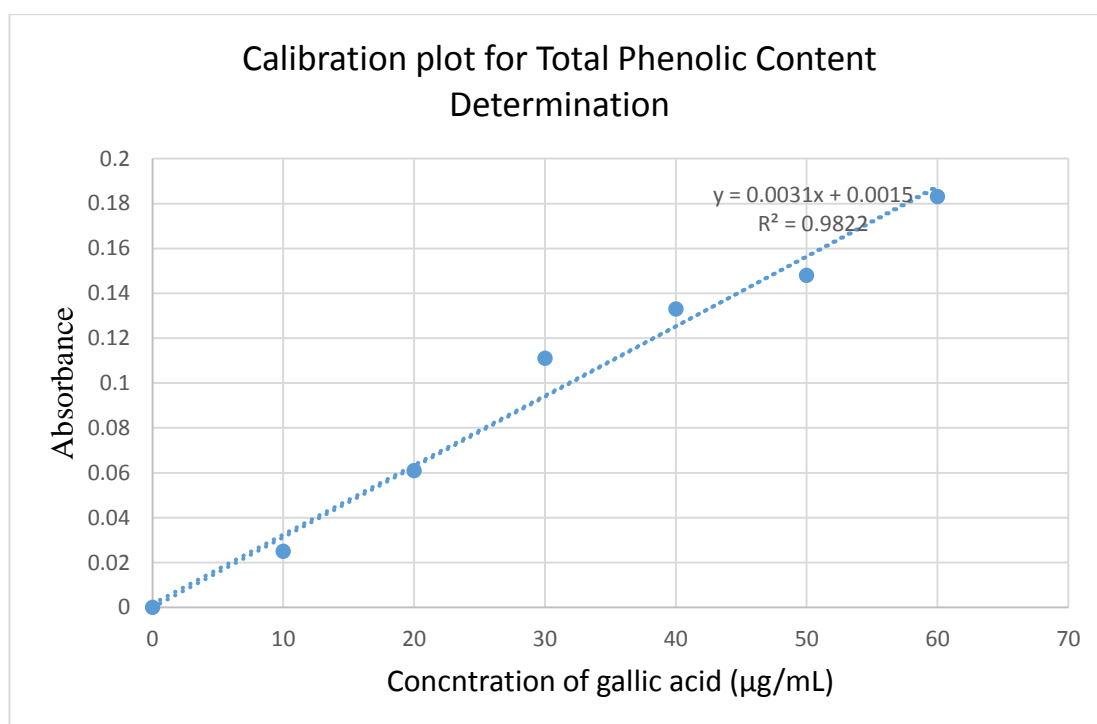


Figure 8 Gallic acid Standard Curve for TPC

The TPC values ranged from 159.8 ± 15.3 to 333.8 ± 9.4 µgGAE/g for acetone extracts; 194.9 ± 4.5 to 324.4 ± 9.9 µgGAE/g for methanol extracts and 210.0 ± 7.5 to

320.4± 3.8 µgGAE/g for ethanol extracts, showing a variation of 2-fold among acetone extracts and 1.5-fold variation among methanol and ethanol extracts. The highest yields of TPC in each solvent was as follows: acetone extracts: 333.8± 9.4 µgGAE/g in *A. nambalensis*; methanol extracts: 333.8± 2.7 µgGAE/g in *H. sabdarifa*; and ethanol extracts: 334.9± 4.3 µgGAE/g in *H. cannbinus*. The results obtained from the TPC analysis are displayed in table 3 below.

Table 3 Total Phenolic Contents (TPC) of NLV; aqueous acetone (80%), methanol (80%) and ethanol (80%) extracts.

Name of Plant	80% Acetone	80% Methanol	80% Ethanol
<i>Corchorus tridens</i>	290.9± 37.9	239.3± 9.8	271.8± 9.6
<i>Oxygonum delagoense</i>	218.4± 10.4	230.7± 15.6	224.0± 5.5
<i>Aeschynomene nambalensis</i>	333.8± 9.4	324.4± 9.9	320.4± 3.8
<i>Vigna unguiculata</i>	184.8± 18.0	225.2± 6.1	243.3± 12.4
<i>Amaranthus thunbergii</i>	226.4± 17.5	194.9± 4.5	210.0± 7.5
<i>Cleome gynandra</i>	229.5± 15.6	228.8± 4.1	230.0± 14.6
<i>Amaranthus hypochondriacus</i>	159.8± 15.3	179.9± 2.5	151.9± 3.7
<i>Hibiscus sabdarifa</i>	299.1± 28.1	333.8± 2.7	332.6± 7.0
<i>Hibiscus cannbinus</i>	343.8± 13.1	302.2± 20.7	334.9± 4.3
<i>Citrullus lanatus</i>	204.9± 21.4	323.4± 10.0	280.9± 19.2
<i>Lagenaria siceraria</i>	183.2± 16.4	251.4± 16.9	258.9± 3.3
<i>Cucurbita maxima</i>	103.9± 2.1	183.2± 8.6	185.3± 3.5

4.3.2 Total Flavonoid Contents (TFC)

TFC like TPC was performed on 80% aqueous acetone, methanol and ethanol extracts. A calibration curve of quercetin was produced, R^2 - value = 0.9902 (Fig 9).

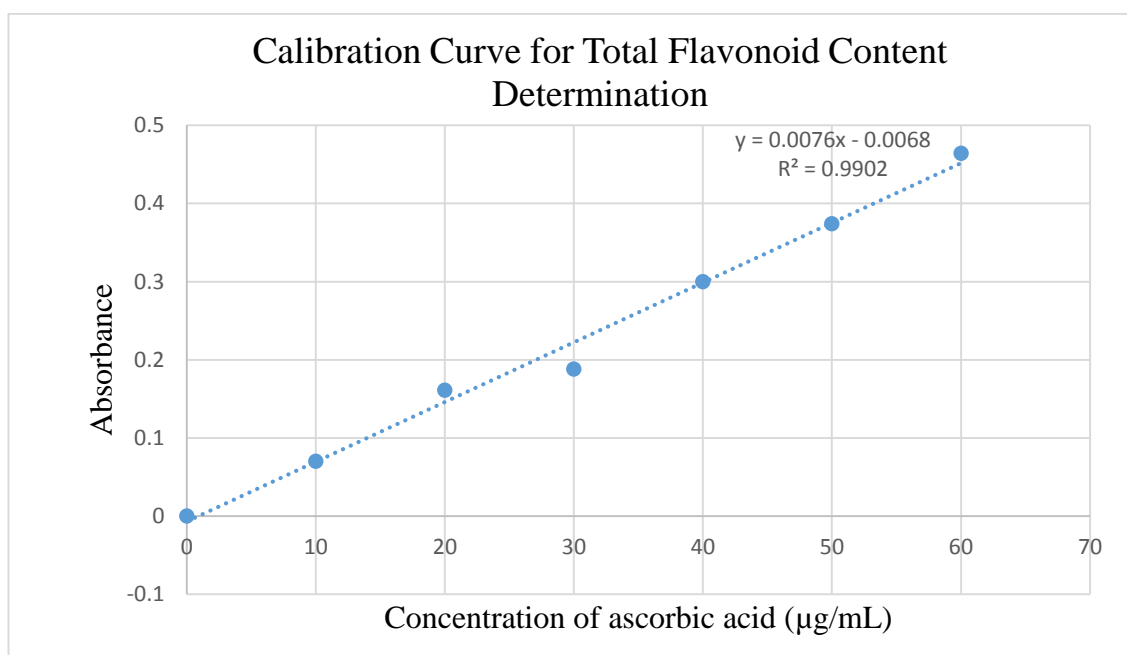


Figure 9 Ascorbic acid standard curve for TFC

TFC concentrations values are in μg Quercetin equivalence/g. Values ranged between 143.8 ± 4.2 to 164.7 ± 2.3 $\mu\text{gQE/g}$ for acetone extracts; 98.4 ± 3.1 to 166.0 ± 0.6 $\mu\text{gQE/g}$ for methanol extracts and 140.9 ± 0.1 to 156.2 ± 0.5 $\mu\text{gQE/g}$ for ethanol extracts. The highest yields in each solvent was as follow: Acetone: 164.7 ± 2.3 $\mu\text{gQE/g}$ and Methanol: 166.0 ± 0.6 $\mu\text{gQE/g}$ both in *A. nambalensis* extracts; Ethanol: 156.2 ± 0.5 $\mu\text{gQE/g}$ in *H. sabdarifa*. TFC results are displayed in table 4.

Table 4: Total Flavonoid Contents (TFC) of acetone, methanol and ethanol NLV extracts.

Name of Plant	80% Acetone	80% Methanol	80% Ethanol
<i>Corchorus tridens</i>	157.3± 1.4	152.7± 1.0	144.4± 0.5
<i>Oxygonum delagoense</i>	150.6± 0.1	149.9± 0.8	142.0± 1.3
<i>Aeschynomene nambalensis</i>	164.7± 2.3	166.0± 0.6	150.6± 0.4
<i>Vigna unguiculata</i>	157.7± 1.4	149.7± 0.2	145.6± 0.2
<i>Amaranthus thunbergii</i>	148.1± 2.3	114.6± 2.7	142.0± 1.3
<i>Cleome gynandra</i>	148.2± 0.8	146.4± 0.3	146.9± 0.7
<i>Amaranthus hypochondriacus</i>	143.8± 4.2	98.4± 3.1	140.9± 0.1
<i>Hibiscus sabdarifa</i>	152.5± 0.6	164.9± 0.1	156.2± 0.5
<i>Hibiscus cannbinus</i>	151.6± 0.5	163.4± 0.2	155.4± 0.7
<i>Citrullus lanatus</i>	151.6± 2.3	155.2± 0.8	144.4± 0.5
<i>Lagenaria siceraria</i>	153.8± 0.9	143.9± 0.5	141.6± 0.3
<i>Cucurbita maxima</i>	148.5± 1.1	138.9± 2.7	143.2± 0.1

4.4 Antioxidant Analyses

Three antioxidant assays were employed in this study, the reduction of DPPH, ABTS assay and Reducing Power which were at a concentration of 0.05g/mL.

4.4.1 DPPH Radical Scavenging Antioxidant Analysis

Results of the DPPH radical scavenging capacity tested on methanoic and ethanoic extracts are shown in table 5.

DPPH radical scavenging activities of plant extracts varied from $37.8 \pm 3.7\%$ to $79.3 \pm 0.6\%$ for methanoic extracts and $11.0 \pm 6.8\%$ to $55.4 \pm 31.2\%$ for the ethanoic extracts; which represents a variation of 2-folds for methanol extracts and about 5-folds for ethanol extracts. For methanol extracts, *H. cannbinus* showed the highest antioxidant capacity ($79.3 \pm 0.6\%$ of DPPH inhibition), followed by *A. thunbergii* ($78.0 \pm 0.4\%$) and *H. sabdarifa* (75.8 ± 0.2). *O. delagoense* showed the lowest ABTS inhibition of $37.8 \pm 3.7\%$. Ethanol extracts showed highest antioxidant capacity in *H. cannbinus* extracts ($71.6 \pm 1.0\%$ of DPPH inhibition) followed by *H. sabdarifa* ($67.6 \pm 1.8\%$) and *O. delagoense* ($55.4 \pm 31.2\%$). *A. thunbergii* extract showed the lowest antioxidant capacity of $11.0 \pm 6.8\%$.

In this experiment, aqueous methanol showed to be a better solvent for extracting DPPH scavenging antioxidant compounds compared to aqueous ethanol. Results are displayed in table 5.

Table 5 DPPH radical scavenging in Methanol and Ethanol NLV extracts.

Name of plant	80% Methanol	80% Ethanol
<i>Corchorus tridens</i>	45.7± 3.0	49.6± 2.2
<i>Oxygonum delagoense</i>	37.8± 3.7	55.4± 31.2
<i>Aeschynomene nambalensis</i>	40.4± 2.0	30.8± 3.1
<i>Vigna unguiculata</i>	61.0± 1.4	17.5± 3.6
<i>Amaranthus thunbergii</i>	78.0± 0.4	11.0± 6.8
<i>Cleome gynandra</i>	57.7± 2.0	41.5± 26.5
<i>Amaranthus hypochondriacus</i>	58.2± 0.7	12.9± 0.7
<i>Hibiscus sabdarifa</i>	75.8± 0.2	67.6± 1.8
<i>Hibiscus cannbinus</i>	79.3± 0.6	71.6± 1.0
<i>Citrullus lanatus</i>	53.1± 0.7	29.5± 2.9
<i>Lagenaria siceraria</i>	62.1± 1.1	45.0± 2.0
<i>Cucurbita maxima</i>	55.6± 7.3	49.6± 2.2

4.4.2 ABTS Radical Scavenging Antioxidant Analysis

ABTS radical scavenging capacity results of selected Namibian leafy vegetable extracts are shown in table 6. In this assay, methanol extracts values ranged from $21.3 \pm 17.9\%$ to $98.6 \pm 0.5\%$, showing a variation of almost 5-fold; while values of ethanol extracts ranged from $40.7 \pm 6.2\%$ to $98.8 \pm 0.2\%$, representing a variation of approximately 2-fold.

The highest scavenging activities for methanol extracts was observed in *C. maxima* ($98.6 \pm 0.5\%$ of ABTS inhibition), followed by *A. hypochondriacus* ($96.6 \pm 3.4\%$), *H. sabdarifa* ($88.5 \pm 18.0\%$) and *H. cannbinus* ($87.5 \pm 18.9\%$). Ethanol extracts showed the highest ABTS inhibition in *A. hypochondriacus* ($98.8 \pm 0.2\%$), followed by *C. maxima* (98.7 ± 0.8), *H. sabdarifa* (98.6 ± 0.7), *O. delagoense* ($97.6 \pm 0.9\%$), *A. nambalensis* ($91.3 \pm 4.0\%$) and *C. tridens* ($90.5 \pm 6.3\%$). The results obtained for the ABTS assay are opposite of what was obtained in the DPPH assay, in this case showing that aqueous ethanol extracted ABTS-radical scavenging antioxidants better than aqueous methanol. ABTS assay results are shown in table 6.

Table 6 ABTS scavenging activity of methanol and ethanol extracts of NLVs.

Name of Plant	80%Methanol	80%Ethanol
<i>Corchorus tridens</i>	72.8± 23.8	90.5± 6.3
<i>Oxygonum delagoense</i>	55.3± 21.1	97.6± 0.9
<i>Aeschynomene nambalensis</i>	63.7± 22.5	91.3± 4.0
<i>Vigna unguiculata</i>	63.2± 22.8	77.4± 3.3
<i>Amaranthus thunbergii</i>	36.6± 26.2	56.1± 0.7
<i>Cleome gynandra</i>	21.3± 17.9	40.7± 6.2
<i>Amaranthus hypochondriacus</i>	96.6± 3.4	98.8± 0.2
<i>Hibiscus sabdarifa</i>	88.5± 18.0	98.6± 0.7
<i>Hibiscus cannbinus</i>	87.5± 18.9	81.6± 7.3
<i>Citrullus lanatus</i>	74.9± 17.0	74.2± 2.3
<i>Lagenaria siceraria</i>	45.1± 38.2	86.3± 3.0
<i>Cucurbita maxima</i>	98.6± 0.5	98.7± 0.8

4.4.3 Reducing Power (RP)

The RP values of Ascorbic acid standard shown in table 7 indicate that the antioxidant potential of the vegetable extracts are much higher than that of the standard ascorbic acid at the same concentration (0.05g/mL). This might be due to the presence of many other phenolic compounds in the plant sample extract unlike in the case of the standard, which is represented by a single compound.

Table 7 Reducing Capacity of Ascorbic acid (Positive control)

Concentration (g/mL)	% inhibition
0.1	153.3
0.075	150.6
0.05	145.9
0	-

Reducing power (PR) was analysed on all the three organic extracts (acetone, methanol and ethanol). Table 8 shows the results obtained thereafter. RP values ranged between from $220.2 \pm 17.5\%$ to $837.0 \pm 88.6\%$ for acetone extracts; $259.8 \pm 3.1\%$ to $723.7 \pm 26.3\%$ for methanol extracts; and $181.5 \pm 1.8\%$ to $1485 \pm 32.2\%$ for ethanol extracts.

The highest RP values were observed in the extracts of *L. siceraria* for acetone ($837.0 \pm 88.6\%$) & ethanol ($1227.5 \pm 106.2\%$) solvents; and in *H. sabdarifa* ($723.7 \pm 26.3\%$) for methanol solvent.

Table 8 Reducing Power of selected of NLVs extracts at 0.05g/mL.

Reducing power (%)	80%Acetone	80%Methanol	80%Ethanol
<i>Corchorus tridens</i>	295.8± 2.5	362.0± 20.6	1127.3± 36.3
<i>Oxygonum delagoense</i>	271.6± 3.4	550.1± 37.7	181.5± 1.8
<i>Aeschynomene nambalensis</i>	729.4± 22.6	259.8± 3.1	1485± 32.2
<i>Vigna unguiculata</i>	310.1± 49.8	551.3± 49.5	859.3± 45.2
<i>Amaranthus thunbergii</i>	283.7± 1.8	388.0± 11.0	617.7± 64.2
<i>Cleome gynandra</i>	321.2± 12.3	301.0± 11.6	1197.8± 18.4
<i>Amaranthus hypochondriacus</i>	483.5± 19.2	273.2± 8.2	883.7± 17.9
<i>Hibiscus sabdarifa</i>	252.3± 55.1	723.7± 26.3	569.2± 1.7
<i>Hibiscus cannbinus</i>	220.2± 17.5	298.0± 19.9	613.7± 12.3
<i>Citrullus lanatus</i>	411.5± 38.5	552.4± 0.6	848.6± 29.2
<i>Lagenaria siceraria</i>	837.0± 88.6	692.4± 0.4	1227.5± 106.2
<i>Cucurbita maxima</i>	677.7± 72.4	487.2± 2.0	1224.0± 13.3

4.5 GC-MS Identification and Quantification

In the TPC, TFC and antioxidant assays described above, the quantification obtained is not compound specific. The percentage inhibition reflecting the amount of antioxidant is based on the presence of any other antioxidant compounds present in the sample extract, thus only a portion of it is phenolic compounds, even a smaller portion of that is phenolic acids. The GC-MS analysis on the other hand, identifies and quantifies single compounds from an extract of plant metabolites depending on how easily detectable they are; and quantifies them individually based on their retention times (qualitative) and peak areas (quantification) relative to those of the internal and external phenolic acid standards.

The substitution of trimethylsilyl (TMS) groups on the parent molecules through derivatization means that during analysis, the phenolic acids are identified by their respective TMS derivative standards.

4.5.1 Identification and Quantification of Phenolic Acid Standards

The results obtained from the standard solution run are shown in Figure 10; which is a Total Ion Chromatogram (TIC) of the TMS derivatives of the 8 phenolic acid standards at 20ppm, with the internal standard (MDD) and their retention times. Table 9 is a summary of figure 10, showing the molecular mass (MW) and molecular ions of these TMS derivative structures. In addition, figure 11 shows representative Chromatograms of each individual phenolic acid standards with the respective retention time at 40ppm.

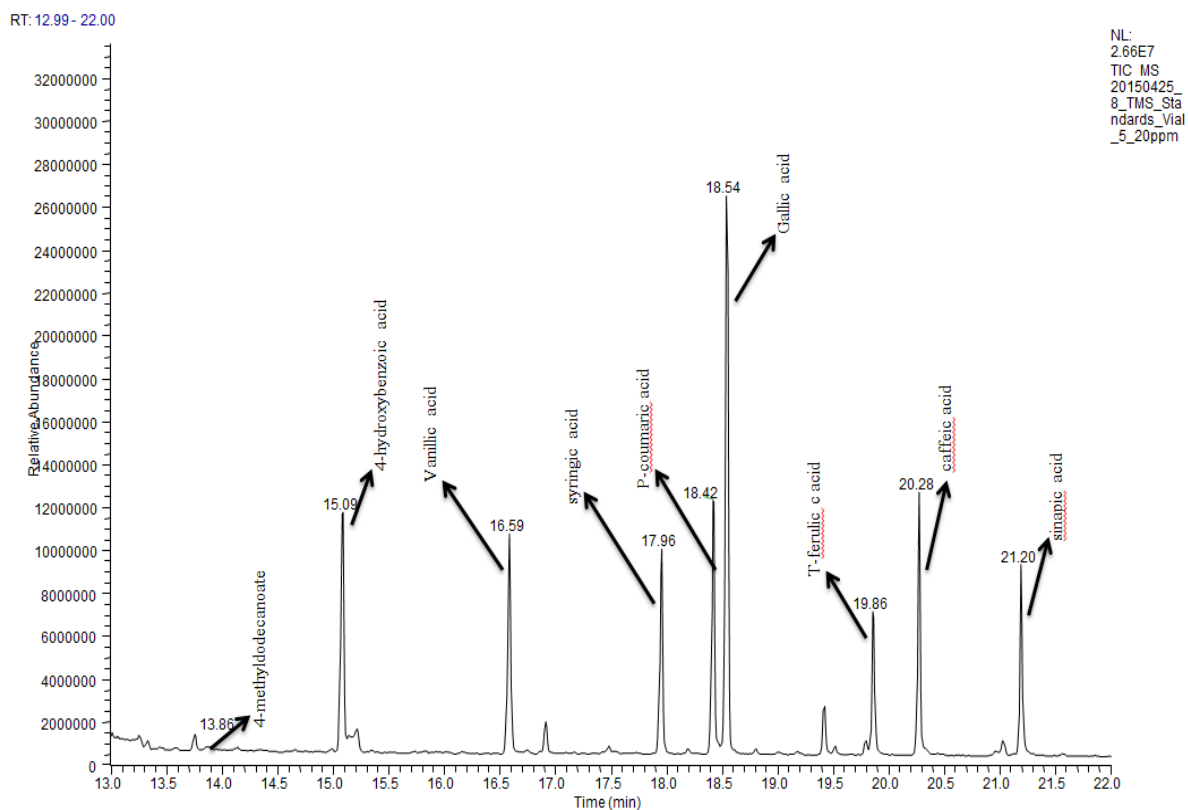


Figure 10 TIC of phenolic acid standards at 20ppm

Table 9 Molecular weight (MW) and important ions present in the mass spectra of silylated phenolic compounds (identified as TMS derivatives) in the examined plant extracts by GC-MS.

Compound (TMS derivative)	Retention time (min)	MW (Silylated compounds)	Molecular ion $[M]^+$
Internal standard (MDD)	13.86	214	214
4-Hydroxybenzoic acid	15.09	282	282
Vanillic acid	16.59	312	312
Syringic acid	17.96	342	342
<i>p</i> -coumaric acid	18.42	308	308
Gallic acid	18.54	458	458
<i>t</i> -Ferulic acid	19.86	338	338
Caffeic acid	20.28	396	396
Sinapic acid	21.2	368	368

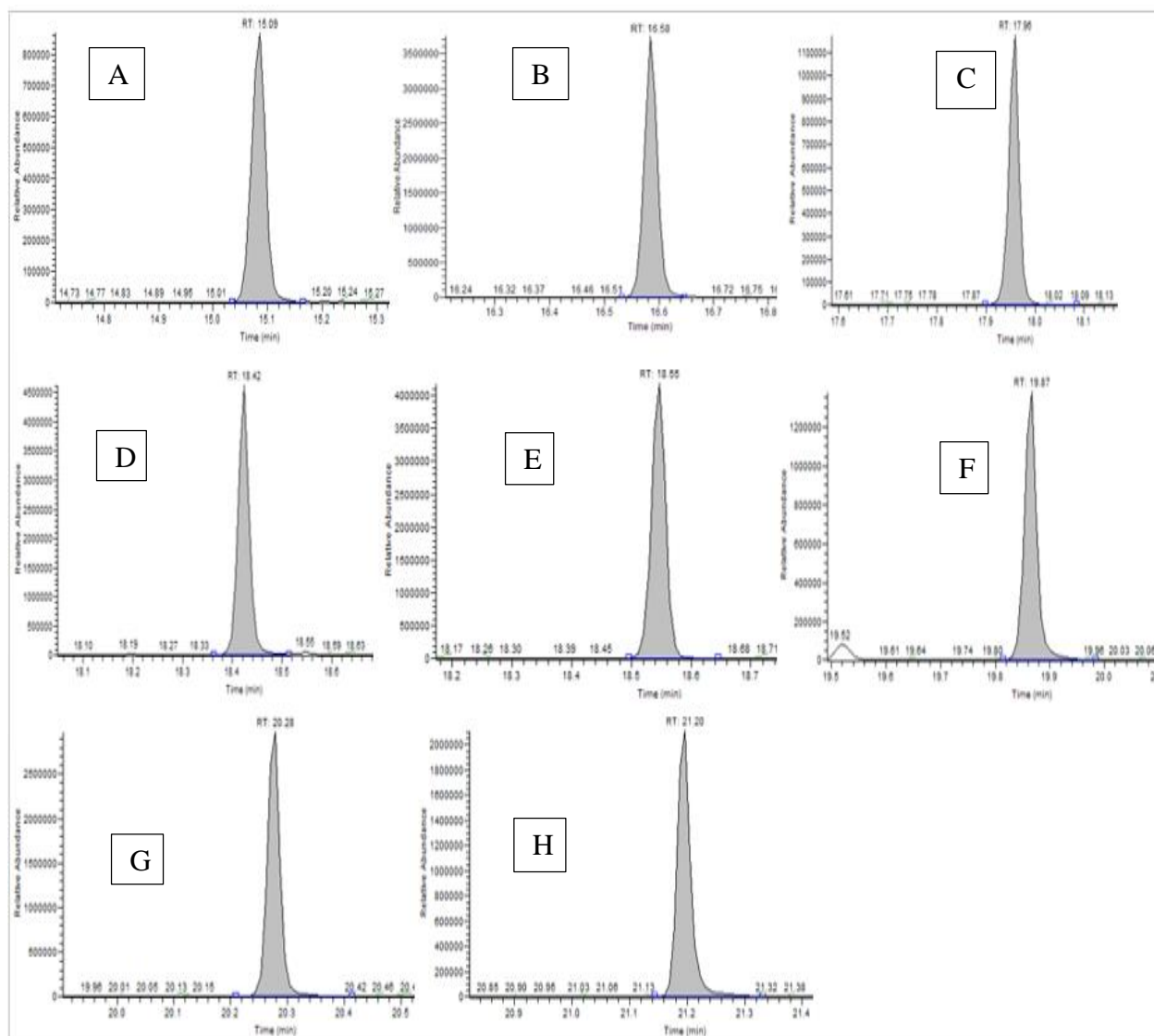


Figure 11 Chromatograms of TMS phenolic acid standards at 40ppm

A: 4-Hydroxybenzoic acid, Rt 15.09 min; B: Vanillic acid, Rt: 16.59min; C: Syringic acid, Rt: 17.96min; D: *p*-Coumaric acid, Rt: 18:42min; E: Gallic acid, RT: 18:55; F: *t*-Ferulic acid, Rt: 19.87min; G: Caffeic acid, Rt: 20:28min; H: Sinapic acid, Rt: 21:20 min. Rt is the retention time that the compound (phenolic acid) elutes in minutes (min).

4.5.2 Identification and Quantification of Phenolic Acids in Plant Extracts

Using the X-calibur software of the GC-MS, phenol acids in the plant extracts were manually searched for in the qualitative and quantitative browsers for each vegetable extract. Among all present phenolic acids, only the samples that clearly depicted a visible internal standard (IS) peak were quantified.

Three extracts were analysed for each sample. The methanoic extracts which are referred to as the non-hydrolysed samples (NHS); acid hydrolysed samples (AHS) and the base (alkaline) hydrolysed samples (BHS). Each extract had 12 extracts which had to be analysed for the presence of the 8 phenolic acids, making a total of 96 manual searches in total. Phenolic acids were detected in 40 of the analysis performed (20 in BHS, 7 in AHS and 13 in NHS). Results for each extraction yield are discussed below.

Tables 10-12 show the results for the detected phenolic acids obtained from the GC-MS analysis for each solvent. The presence of the compound of interest (phenolic acid) in the qualitative browser that could not be quantified is represented by the letter “p” and the absence thereof by the letter “a”. If quantification was done, the concentration in μg is indicated.

4.5.3 Identification and Quantification of Non-hydrolysed Samples (NHS)

The NHS were the methanoic extracts, selected among the three organic extracts since methanol is one of the most common organic solvent used for phenolic extraction (Sani et al., 2012). Phenolic acids were detected in thirteen sample extracts, and eleven of those were quantified (Table 10). Two phenolic acids (*trans*-Ferulic and Caffeic acids) that were detected in the extract of *H. sabdarifa* (Table 10)

through the qualitative browser but the quantitative browser could not detect them properly; therefore there are no chromatograms for them. Chromatograms of the 11 phenolic acids that were visible and quantified in BHS are shown in figures 12 and 13 below.

Table 10 GC-MS Analysis of Non-Hydrolysed Samples (NHS) of NLV.

Plant name	4-hydroxybenzoic acid	Vanillic acid	Syringic acid	p-Coumaric acid	Gallic acid	t-Ferulic acid	Caffeic acid	Sinapic acid
<i>Corchorus tridens</i>	a	4.6	a	a	a	a	a	a
<i>Oxygonum delagoense</i>	a	a	a	a	a	a	a	a
<i>Aeschynomene nambalensis</i>	a	1.3	a	1.7	a	a	a	a
<i>Vigna unguiculata</i>	a	3.9	a	6.5	a	a	a	a
<i>Amaranthus thunbergii</i>	a	a	a	a	a	a	a	a
<i>Cleome gynandra</i>	a	a	a	a	a	a	a	a
<i>Amaranthus hypochondriacus</i>	a	a	a	a	a	a	a	a
<i>Hibiscus sabdarifa</i>	a	a	a	a	a	p	p	a
<i>Hibiscus cannbinus</i>	a	1.1	a	3.5	0.5	1.9	1.0	a
<i>Citrullus lanatus</i>	a	a	a	a	a	a	a	a
<i>Lagenaria siceraria</i>	a	a	a	a	a	a	a	a
<i>Cucurbita maxima</i>	a	a	a	6.6	a	a	a	a

In Table 10 above, “a” means compound is absent and “p” means compound is present

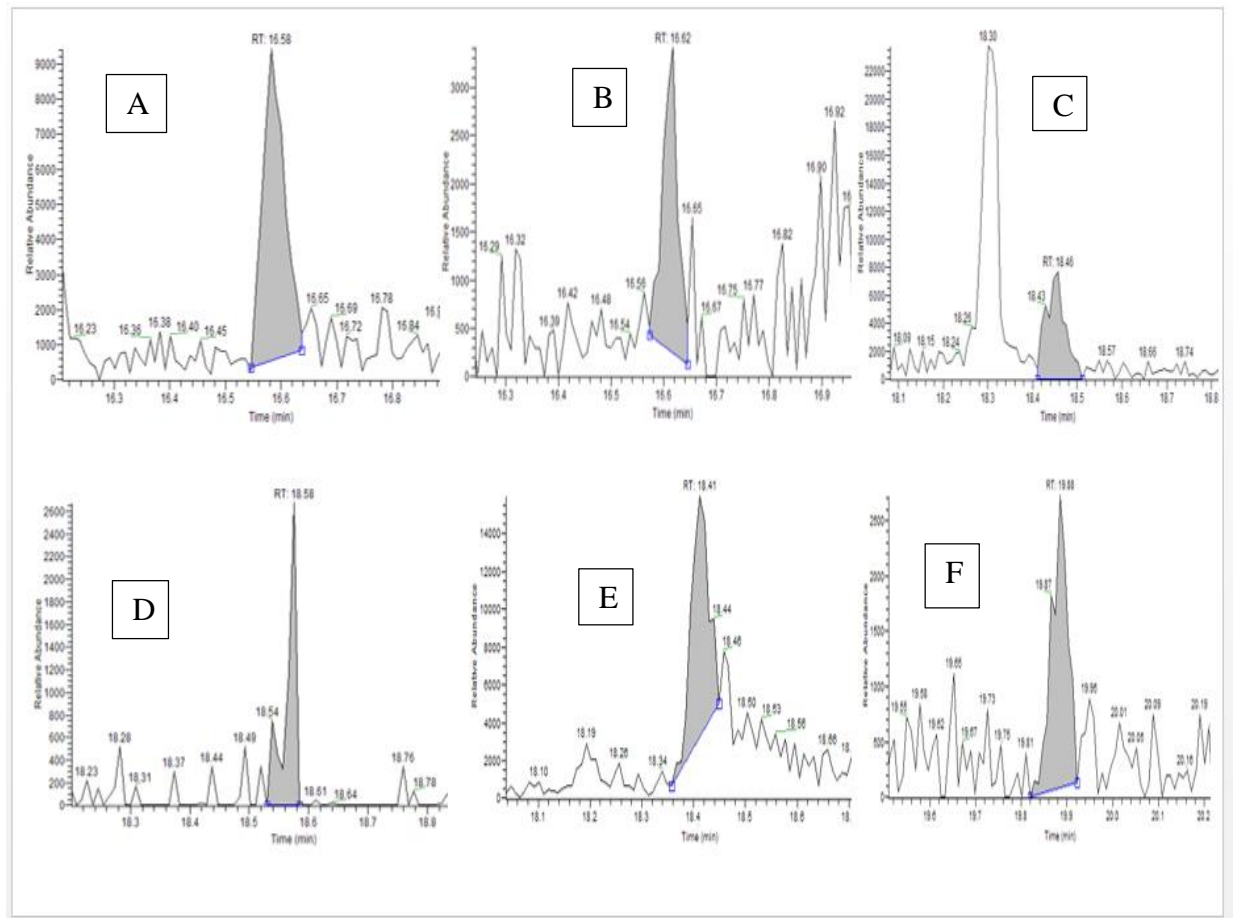


Figure 12. Chromatograms of Non-Hydrolysed Samples (NHS)

A: Vanillic acid, RT: 16.58 in *V. unguiculata*; B: Vanillic acid, RT: 16.62 in *H. cannbinus*; C: p-Coumaric acid, RT: 18.46 in *H. cannbinus*; D: Gallic acid, RT 18.58 in *H. cannbinus*; E: p-Coumaric acid, RT: 18.41 in *V. unguiculata*; F: Ferulic acid, RT: 19.88 in *H. sabdarifa*. RT is the retention time that the compound (phenolic acid) elutes in minutes (min).

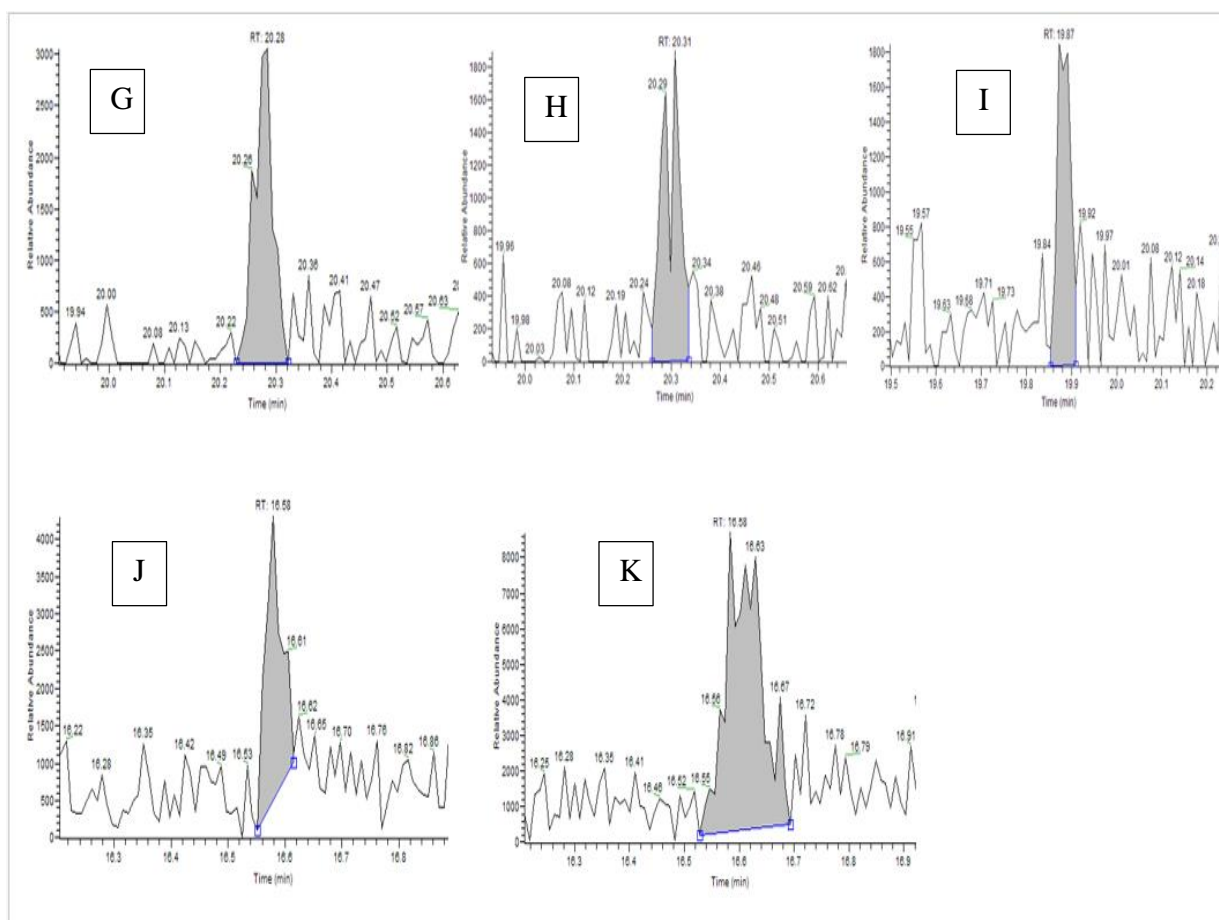


Figure 13 Chromatograms of NHS continued

G: Caffeic acid, RT: 20.28 in *H. sabdarifa*; H: Caffeic acid, RT: 20.29 in *H.cannbinus*; I: Ferulic acid, RT: 19.87 in *H.cannbinus*; J: Vanillic acid, RT: 16:5 in *A. nambalensis*; K: Vanillic acid, RT: 16:58 in *C. tridens*. RT is the retention time that the compound (phenolic acid) elutes in minutes (min).

4.5.4 Identification and Quantification of Acid Hydrolysed Sample (AHS)

In the Acid hydrolysed samples (AHS), phenolic acids were only detected in seven of the sample extracts and only vanillic acid could be quantified in *C. gynandra*, with a value of 4.4 μ g/g (Table 11). All phenolic acids that were detected in the qualitative browser were visible in the quantitative browser. However, only one could be quantified as the internal standard was not well visible in the other samples. Thus, confirmation was only based on retention time and the qualitative browser library. The chromatograms of these 7 compounds are shown in figure 14 below.

Table 11 GC-MS Analysis of Acid Hydrolysed Samples (NHS) of NLV

Plant name	4-hydroxybenzoic acid	Vanillic acid	Syringic acid	p-Coumaric acid	Gallic acid	t-Ferulic acid	Caffeic acid	Sinapic acid
<i>Corchorus tridens</i>	a	a	a	a	a	a	a	a
<i>Oxygonum delagoense</i>	a	a	a	a	p	p	a	a
<i>Aeschynomene nambalensis</i>	a	a	a	p	a	a	a	a
<i>Vigna unguiculata</i>	p	a	a	a	a	a	a	a
<i>Amaranthus thunbergii</i>	a	a	a	a	a	a	a	a
<i>Cleome gynandra</i>	a	a	a	a	a	a	a	a
<i>Amaranthus hypochondriacus</i>	a	4.4	a	a	a	a	p	a
<i>Hibiscus sabdarifa</i>	p	a	a	a	p	a	a	a
<i>Hibiscus cannabinus</i>	a	a	a	a	a	a	a	a
<i>Citrullus lanatus</i>	a	a	a	a	a	a	a	a
<i>Lagenaria siceraria</i>	a	a	a	a	a	a	a	a
<i>Cucurbita maxima</i>	a	a	a	a	a	a	a	a

In Table 11: “a” means compound is absent and “p” means compound is present

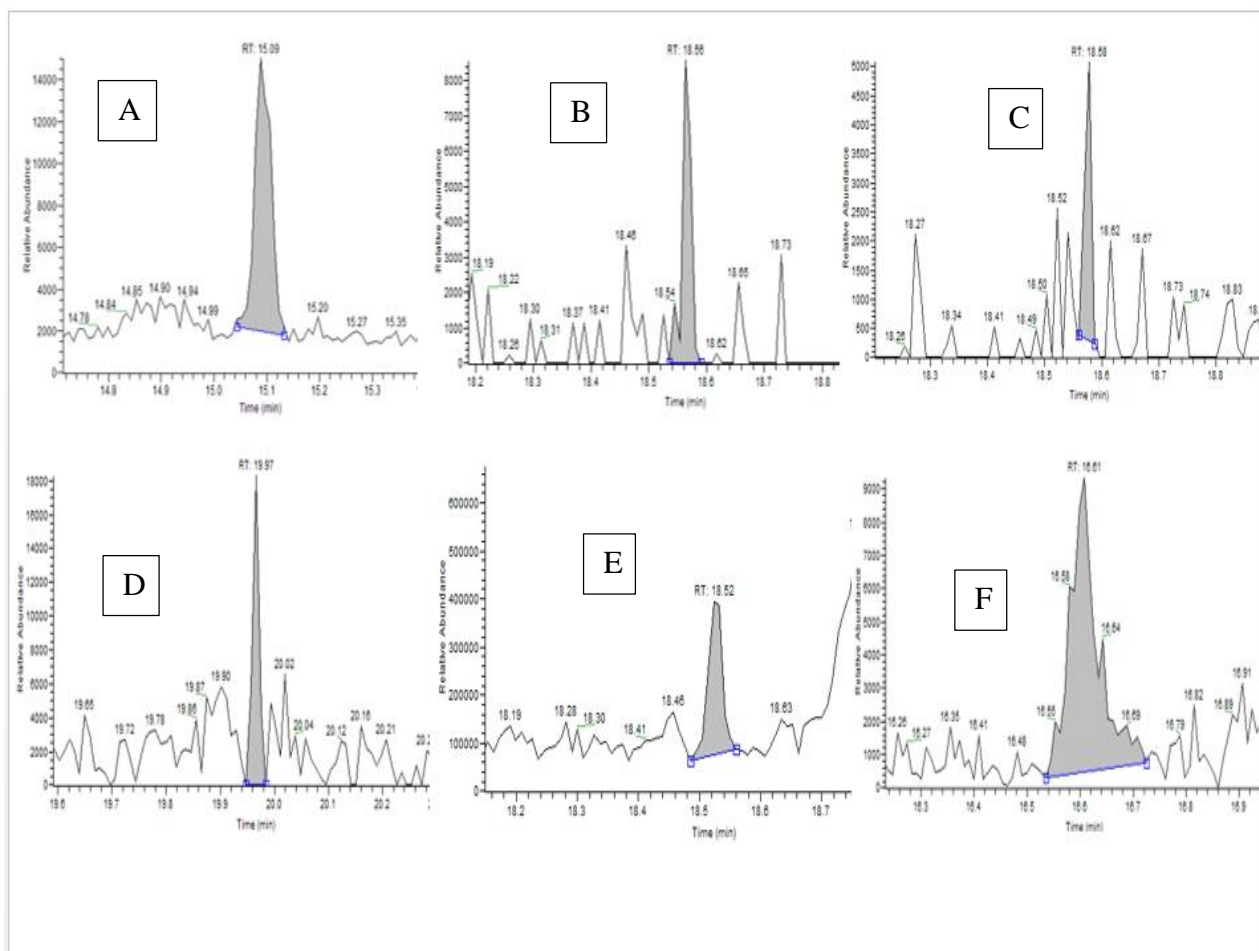


Figure 14 Chromatograms of Acid Hydrolysed Samples

A: 4-hydroxybenzoic acid, RT: 15.09 in *V. unguiculata*; B: Gallic acid, RT: 18.56 in *H. sabdarifa*; C: Gallic acid, RT: 18.58 in *O. delagoense*; D: Ferulic acid, RT: 19.97 in *O. delagoense*; E: p-Coumaric acid, RT: 18.42 in *A. nambalensis*; F: Vanillic acid, RT: 16.61 in *A. hypochondriacus*. RT is the retention time that the compound (phenolic acid) elutes in minutes (min).

4.5.5 Identification and Quantification of Base Hydrolysed Samples (BHS)

Basic hydrolysis yielded the best results among the three extraction methods. Phenolic acids were identified in 20 samples and 15 of them were quantified. Results are shown in table 12. Figures 15 and 16 below show chromatograms of 17 phenolic compounds that were identified with visible peaks. The rest were either identified by qualitative browser only or the internal standard was not visible, thus could not be quantified.

Table 12 GC-MS Analysis of Base Hydrolysed Samples (NHS) of NLV

Plant name	4-hydroxybenzoic acid	Vanillic acid	Syringic acid	p-Coumaric acid	Gallic acid	t-Ferulic acid	Caffeic acid	Sinapic acid
<i>Corchorus tridens</i>	a	a	a	a	a	a	a	a
<i>Oxygonum delagoense</i>	a	p	6.3	p	a	34.5	1.5	a
<i>Aeschynomene nambalensis</i>	a	p	a	53.1	a	14.2	a	a
<i>Vigna unguiculata</i>	a	a	a	48.7	a	4.8	a	a
<i>Amaranthus thunbergii</i>	a	a	a	a	a	a	a	a
<i>Cleome gynandra</i>	a	a	a	a	a	a	a	a
<i>Amaranthus hypochondriacus</i>	10.2	1.8	a	5.7	a	30.3	0.7	a
<i>Hibiscus sabdarifa</i>	a	a	a	p	a	a	a	a
<i>Hibiscus cannbinus</i>	a	1.8	4.4	a	a	3.9	a	a
<i>Citrullus lanatus</i>	a	a	a	a	a	a	a	a
<i>Lagenaria siceraria</i>	a	a	a	a	a	a	a	a
<i>Cucurbita maxima</i>	a	p	a	a	a	a	a	a

In Table 12 above, “a” means compound is absent and “p” means compound is present

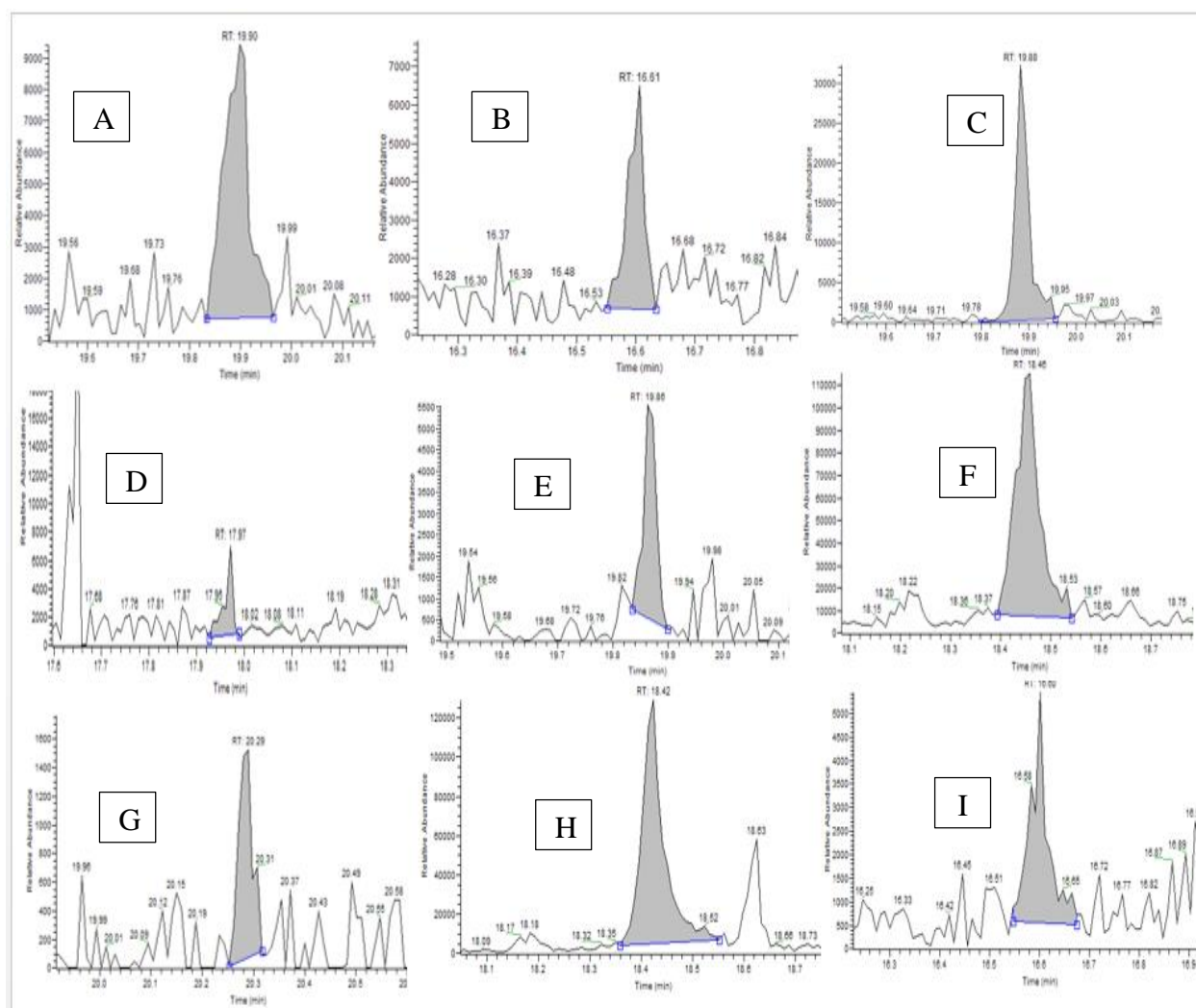


Figure 15. Chromatograms of phenolic acids from Basic Hydrolysed Samples (BHS)

Ferulic acid, RT: 19.90 in *A. nambalensis*; B: Vanillic acid, RT: 16.61 in *A. hypochondriacus*; C: Ferulic acid, RT: 19.88 in *A. hypochondriacus*; D: Syringic acid, RT 17.97 in *H. cannbinus*; E: Ferulic acid. RT: 19.86 in *H. cannbinus*; F: p-Coumaric, RT 18.46 in *A. nambalensis*; G: Caffeic acid, in RT 20.29 *A. hypochondriacus*; H: P-coumaric, in RT 18:42 *V. unguiculata*; I: Vanillic acid, RT: 16.60 in *H. cannbinus*. RT is the retention time that the compound (phenolic acid) elutes in minutes (min).

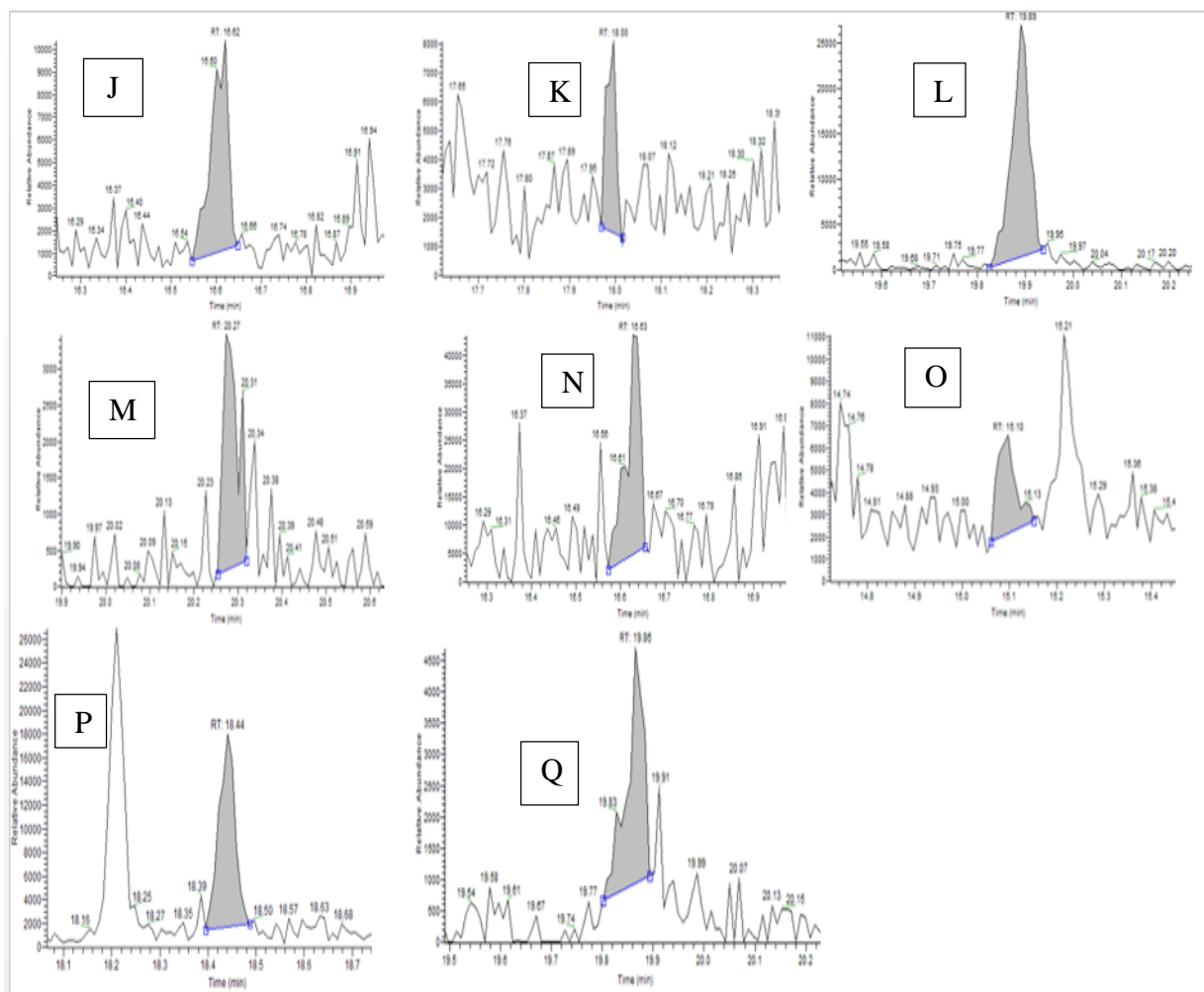


Figure 16. Chromatograms of phenolic acids from BHS continued

J: Vanillic acid, RT: 16.62 in *O. delagoense*; K: Syringic acid, RT: 18.00 in *O. delagoense*; L: Ferulic acid, RT: 19:89 in *O. delagoense*; M: Caffeic acid, RT: 20:27 in *O. delagoense*; N: Vanillic acid, RT: 16:63 in *C. maxima*; O: 4-Hydroxybenzoic acid, RT: 15:10 in *A. hypochondriacus*; P: Coumaric acid, RT: 18.44 in *A. hypochondriacus*; Q: Ferulic acid, RT: 19.86 in *V. unguiculata*. RT is the retention time that the compound (phenolic acid) elutes in minutes (min).

4.5.6 Extracted Ion Chromatograms (EIC) of Phenolic acids from the NLV vegetable samples

Provided below (figures 17 to 23) are EIC obtained from the GC-MS qualitative browser library, each one representing one phenolic acid from one of the NLV sample extracts. The EIC is used to confirm the presence of the compound of interest because it specifically indicates the molecular weight (mass to charge ration) of the TMS derivative standard phenolic in the sample, and upon the library search, the specific structure of the compound is shown with its molecular weight for confirmation.

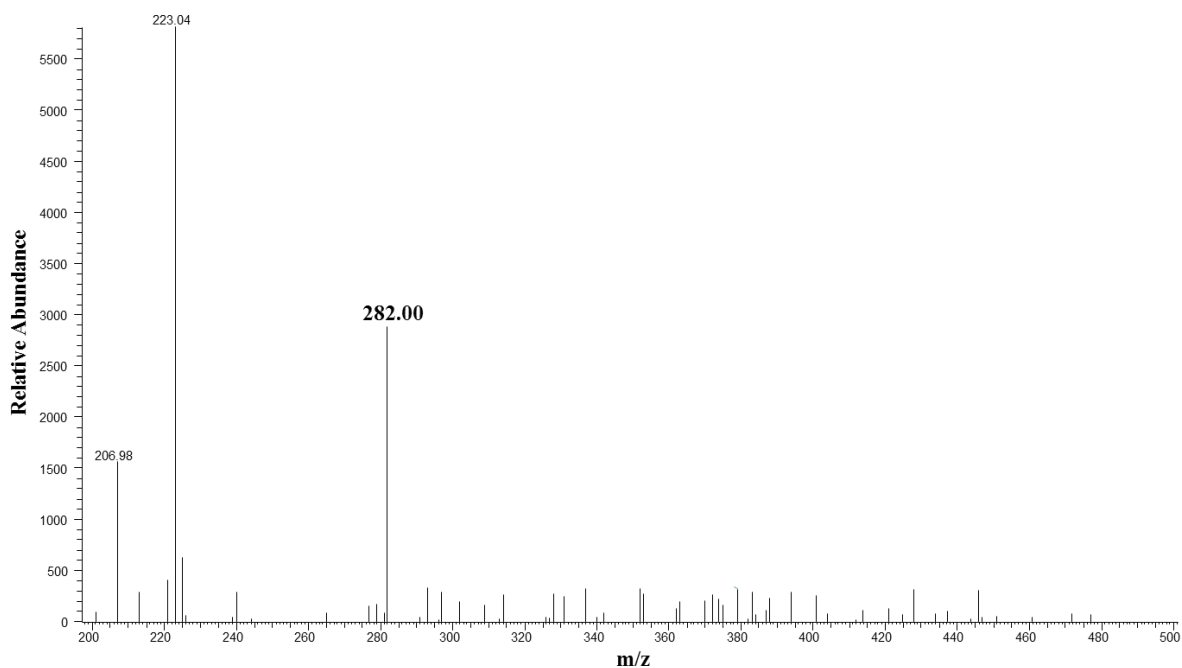


Figure 17: Extracted ion chromatogram of 4- hydroxybenzoic acid, MW 282, in the acid hydrolysed extract of *H. sabdarifa*

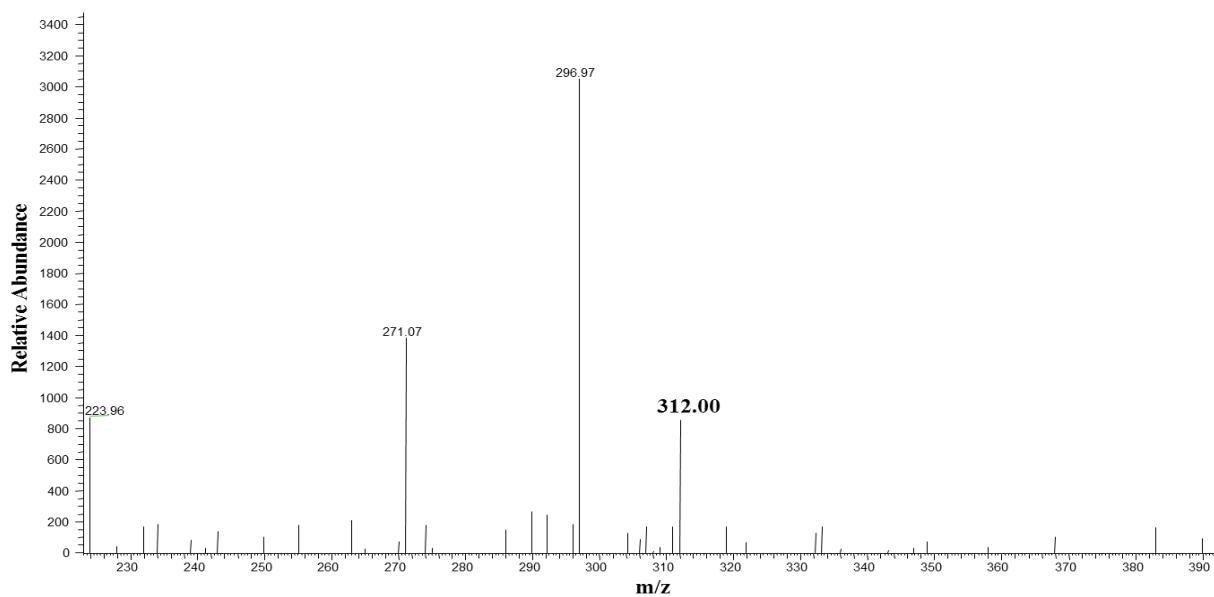


Figure 18: Extracted ion chromatogram of vanillic acid, MW 312, in the non-hydrolysed methanol extract of *V. unguiculata*,

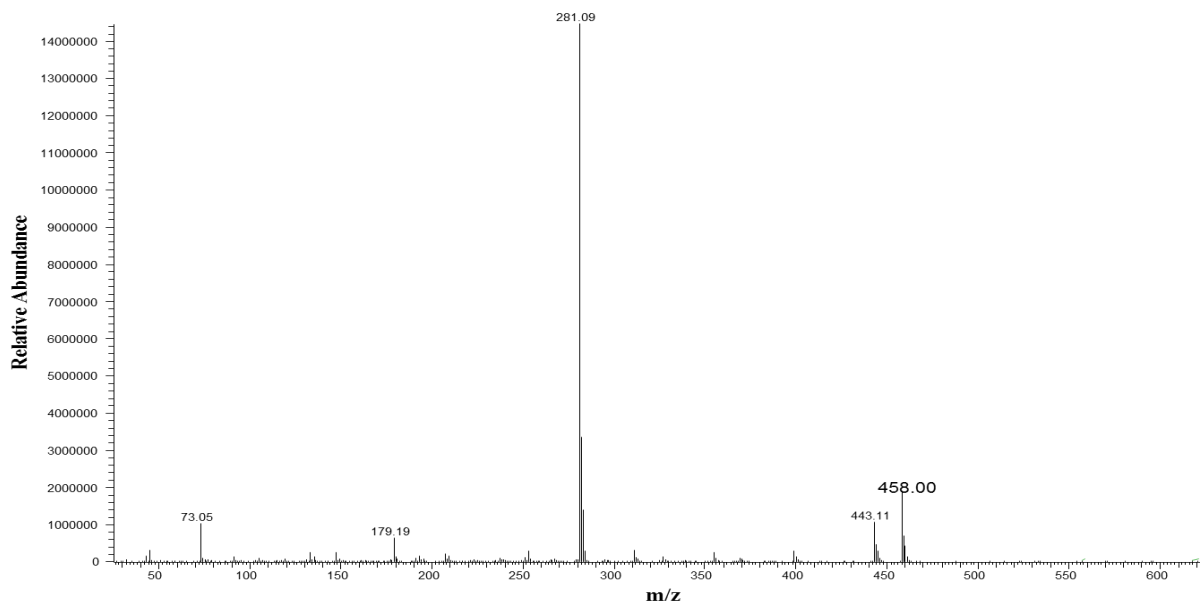


Figure 19: Extracted ion chromatogram of Gallic acid, MW 458, in the acid hydrolysed extract of *O. delagoense*.

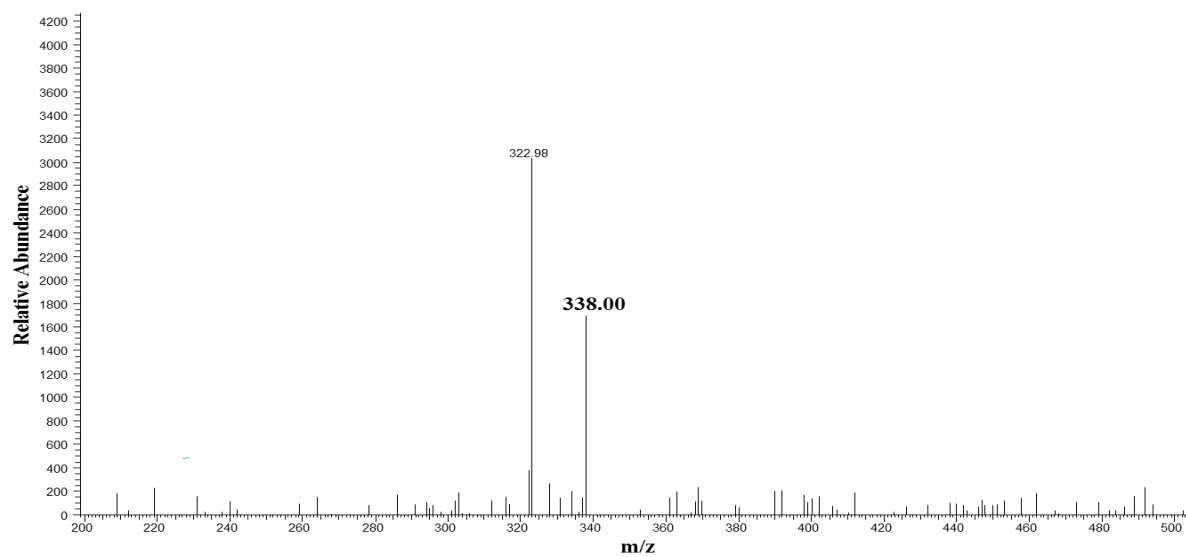


Figure 20: Extracted ion chromatogram of t-Ferulic acid, MW 338, in the basic hydrolysed extract of *H. cannabinus*.

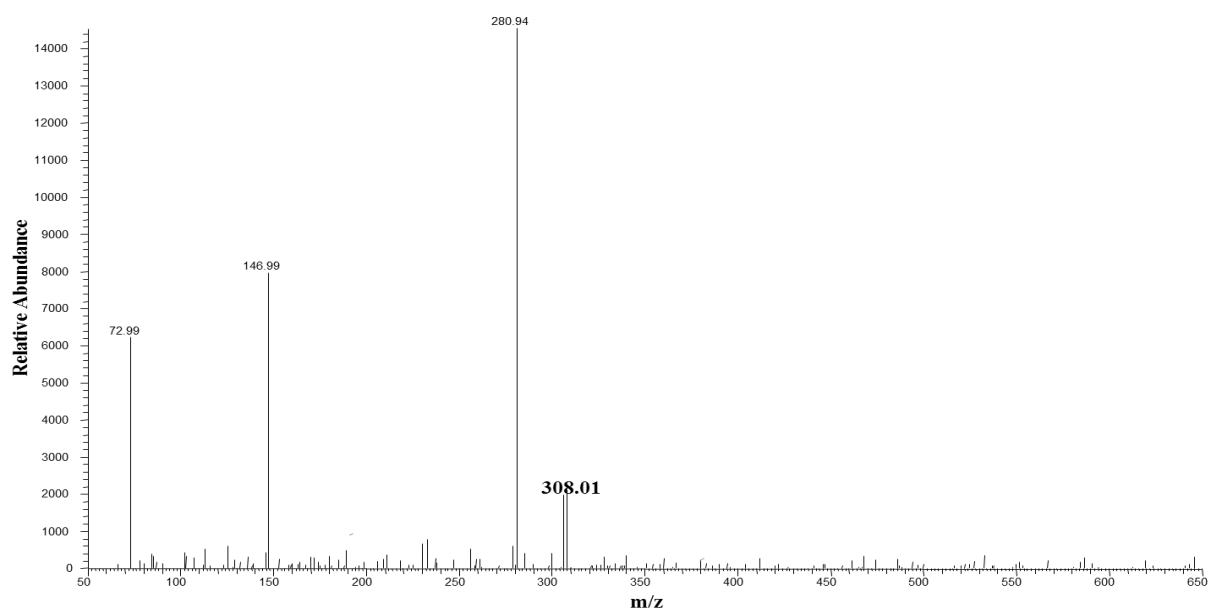


Figure 21: Extracted ion chromatogram of P-coumaric acid, MW 308 in the non-hydrolysed methanol extract of *V. unguiculata*.

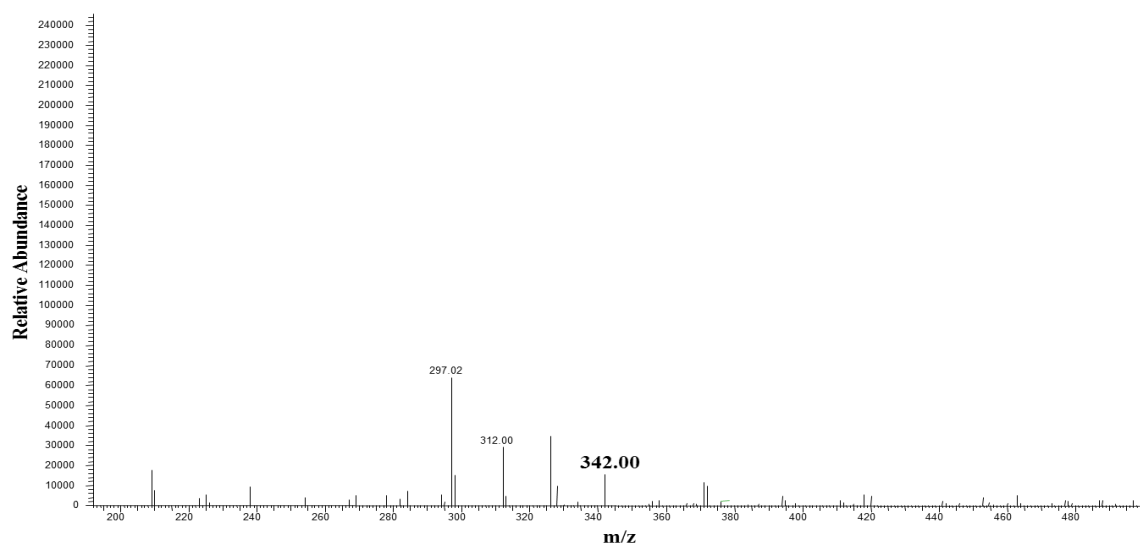


Figure 22: Extracted ion chromatogram of Syringic acid, MW 342 in the basic hydrolysed extract of *H. cannbinus*.

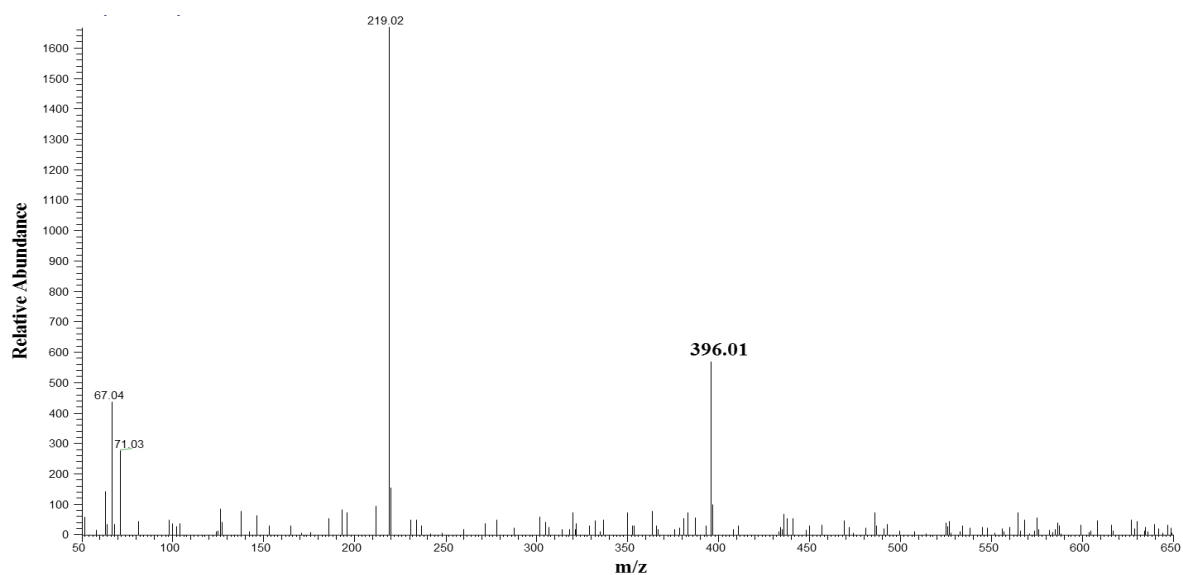


Figure 23: Extracted ion chromatogram of Caffeic acid, MW 396, in the non-hydrolysed methanol extract of *H. cannbinus*.

CHAPTER 5 DISCUSSION

5.1 Identified Namibian Leafy Vegetables

Among the selected NLV, *C. maxima* (pumpkin leaves), *V. unguiculata* (green beans), *C. maxima*, *C. lanatus* and *L. siceraria* which can be cultivated throughout the year as long as they are given enough water. The rest (*C. tridens* (Jew's Mallow), *C. gynandra* (Spider flower), *O. delagoense*, *C. tridens*, *A. hypochondriacus*, *A. thunbergii*, *A. nambalensis* and *A. fluitans*) only grow during rainy seasons. *A. nambalensis* and *A. fluitans* grow when the river waters rise and fill up valleys. Knowledge on the nutritional value of these vegetables (especially those that can be cultivated), can greatly contribute to community awareness programmes emphasizing on the intake of these leafy vegetables; consequently, contributing to poverty reduction and the well-being status of local communities.

5.2 Colorimetric Analysis: Total Phenolic and Flavonoid Contents

The TPC of the NLV extracts were highest in both species of *Hibiscus cannbinus*, followed by *A. nambalensis* in all three organic extracts. The lowest yield was the acetone extract of *C. tridens*. All three solvents seem to have extracted phenolic compounds very well. Therefore, the TPC does not show a significance difference among the three organic solvents in the extraction of phenolics from these vegetable samples. However, a significance difference is seen among the vegetables extracts. Methanol extracts had a slightly better yield of phenolic content compared to acetone and ethanol since its lowest value is $179.9 \pm 2.5 \mu\text{gGAE/g}$, while the lowest values of ethanol and acetone are 151.9 ± 3.7 and $103.9 \pm 2.1 \mu\text{gGAE/g}$ respectively. This

observation goes hand in hand with a study by Orhan, Senol, Demirci, Ozturk, Baser & Sener (2012). Moreover, the TPC of the NLV are higher than the TPC values of 8 selected leafy vegetables in a study by Khanam (2012), which ranged between 80.84 ± 4.76 to $317.48 \pm 5.05 \mu\text{g FAE/g}$. Hence, indicating that NLV have great potential as antioxidants. The highest TPC values among the NLV was observed in the following order of top five species: *H. cannabinus* > *A. nambiensis* > *H. sabdarifa* > *C. tridens* > *C. lantana*.

The TFC results were much lower than the TPC results. This is as expected since TFC is a portion of TPC. TFC results shows similarity between the three extraction solvents across a single extract, in exception of *C. gynandra* and *A. hypochondriacus*. However, close inspection shows that acetone and methanol gave better results than ethanol extracts. The TFC values of the selected NLV, ranged between 98.4 ± 3.1 to $166.0 \pm 0.6 \mu\text{gQE/g}$, showing that they are higher than the TFC values of the 8 selected vegetables (Komatsuna, Mizuna, Poi choi, Mitsuba, Salad spinach, Lettuce, Red amaranth and Green amaranth) study by Khanam (2012); which ranged between 29.56 ± 1.09 to $96.97 \pm 0.88 \mu\text{gFAE/g}$. TFC values among the NLV was observed in the following order of top five species: *A. nambiensis* > *H. sabdarifa* > *C. tridens* > *C. lantana*.

5.3 Antioxidant Assays

TAC results reflect their ability to inhibit the formation of radicals. TAC was measured using DPPH, ABTS and RP assays.

The ability to inhibit DPPH free radicals was highest in both the two *Hibiscus* species samples showed the very high inhibition capacities ranging from $67.6 \pm 1.8\%$ to $79.3 \pm 0.6\%$. The methanol extract of *A. thunbergii* also yielded a high percentage inhibition of $78.0 \pm 0.4\%$ in contrast to its ethanoic extract which was the lowest antioxidant potential of $11.0 \pm 6.8\%$ in the entire assay. The inhibition ability of methanol extracts ranged between 37.8 ± 3.7 - $79.3 \pm 0.6\%$; most of them above 50% inhibition. However, the inhibition ability of ethanoic extracts ranged between 11.0 ± 6.8 - $71.6 \pm 1.0\%$; most of them below 50%. Therefore, in this assay, methanol had better inhibition capacity compared to ethanoic extracts.

In the ABTS radical scavenging assay, both methanoic and ethanoic extracts showed very good scavenging activities with the highest antioxidant inhibition activities seen in the two *Hibiscus* species, *Lagenaria siceraria*, *Cucurbita maxima* , and *Aeschynomene fluitans*; ranging between 88.5 ± 18.0 - $98.8 \pm 0.2\%$. However, in this assay, ethanoic samples seems to have better scavenging capacity compared to the methanol extract. This is seen especially in: *Oxygonum delagoense* ($72.8 \pm 23.8\%$; $90.5 \pm 6.3\%$); *Aeschynomene nambalensis* ($55.3 \pm 21.1\%$; $97.6 \pm 0.9\%$); *Vigna unguiculata* ($63.7 \pm 22.5\%$; $91.3 \pm 4.0\%$) and *Lagenaria siceraria* (45.1 ± 38.2 ; 86.3 ± 3.0) in methanol and ethanol extracts respectively

Reducing power is an important assay that reflects the ability of an extract to donate electrons in order to reduce oxidized intermediates of lipid peroxidation processes, thereby acting as primary and secondary antioxidants (Chanda & Dave, 2009). The higher the RP value, the higher their reducing capacity. The highest inhibition was observed in *L. siceraria*. Among the three organic solvents, ethanol extracts showed

the highest RP capacity in most of the extracts such as *L. siceraria* ($1227.5 \pm 106.2\%$), *C. maxima* ($1224.0 \pm 13.3\%$) and *C. tridens* ($1127.3 \pm 36.3\%$). RP is a good indicator of the antioxidant potential of a sample. Comparing the RP values of ascorbic acid and of the extracts at the same concentration show that the selected NLV are potential electron donors, hence contain a good portion of antioxidant compounds.

In these experiments, methanol yielded the best results in the TPC analysis compared to both acetone and ethanol solvents. In TFC, both methanol and acetone showed similar extraction abilities compared to ethanol which yielded the lowest TFC values. The same is applicable for antioxidant assays. Methanol was the best extraction solvent in the DPPH assay, while ethanol yielded very high values of RP compared to both methanol and acetone. In DPPH, both ethanol and methanol show similar results of DPPH % inhibition.

5.4 GC-MS Analysis

The GC-MS analysis allowed the detection and characterization of individual phenolic acids. While p-coumaric was the most abundant phenolic acid, especially in BHS; 4-hydroxybenzoic acid and gallic acid was mostly detected in AHS qualitatively. Sinapic acid was not observed in any sample. BHS gave the highest yield for the extraction of phenolic compounds, with phenolic acids identified in 20 extracts while in AHS, only 7 were identified. This could be due to the mild conditions of basic hydrolysis as compared to acidic hydrolysis conditions which may have led to decomposition of compounds of interest. As literature stated previously, acid hydrolysis is usually applied to release bound phenolic compounds

that were not previously released through hydrolysis (Chirinos et al., 2008; Ross & Beta, 2009). In addition, phenolic acids are easily released during extraction, thus, harsh conditions may not be necessary for their extraction.

The same is observed when comparing the AHS to the non-hydrolysed samples (NHS), where the NHS gave better results than acid hydrolysed samples. Showing that acid hydrolysis conditions could have led to the loss of phenolic acids in the sample extracts.

5.4.1 Analysis of chromatographic peaks (Matrix Effect)

The shapes of the chromatographic peaks from the sample extracts clearly show that there was a lot of interference with the detection of the analyte compounds as opposed to the standards. The shapes of the standard peaks are clean, perfect and exact with retention time, traits that are not observed in many of the peaks from the sample extracts. This is due to a phenomenon known as “matrix effect”, which is the interference (direct or indirect) in the detection of the compound of interest (phenolic acids) due to the presence of unintended compounds or substances in the extract (Silvestro et al., 2013). Thus, identification was strongly emphasized on the qualitative library analysis in order to prevent considering peaks of other compounds that might elute at the same time as the compounds of interest. The extracted ion chromatograms such as those in figures 15 to 21 for each analyses are the determinants of the compound represented by the peak of interest. Not only does it show the molecular weight of the represented compound but it is supported with the library structures of the respective compound. Matrix effects have always presented a

challenge to GC-MS analyses and thus are widely described as one of the major sources of errors in GC-MS analysis (Rimayi, 2015).

5.4.2 Non-Hydrolysed Samples

In Non-hydrolysed Samples (NHS), phenolic acids were detected in 40 extracts (20 in BHS, 7 in AHS and 13 in NHS). The most abundant phenolic acids in these extracts were p-coumaric acid (1.7 - 6.6µg/g) with the highest values in *C. tridens* (6.6µg/g) and *V. unguiculata* (6.5µg/g) extracts; followed by vanillic acid (1.1-4.6µg/g) with highest values in *C. tridens* (4.6µg/g) and *V. unguiculata* (3.96µg/g). *H. cannabinus* shows the detection of most of the phenolic acids, the highest being p-coumaric acid (3.5µg/g). Two phenolic acids (t-ferulic and caffeic acids) that were detected in the extract of *H. sabdarifa*.

5.4.3 Acid Hydrolysed Samples (AHS)

Only vanillic acid could be quantified in *C. gynandra*, with a value of 4.4µg/g (Table 9). All phenolic acids that were detected in the qualitative browser were visible in the quantitative browser. However, only one could be quantified as the internal standard was not well visible in the other samples. Thus, confirmation was only based on retention time and the qualitative browser library

5.4.4 Base Hydrolysed Samples (BHS)

The highest yields were of p-coumaric acid in *A. nambalensis* (53.1 µg/g) and *V. unguiculata* (48.7 µg/g); followed by *trans*-ferulic acid in *O. delagoense* (34.5µg/g) and *A. hypochondriacus* (30.3µg/g). *Trans*-ferulic and vanillic acids was also well detected in a number of samples, and t-ferulic was quantified in all these extracts.

CHAPTER 6 CONCLUSIONS

All collected samples were identified and validated at the National Botanical Research Institute (NBRI). All the tests performed in this study indicate the Namibian Leafy Vegetables (NLV) can be seen as potential sources of phenolic compounds that can contribute to good health and wellbeing of people when consumed in adequate quantities. The Hibiscus species for instance showed the highest yields of phenolic and flavonoid content as well as the highest antioxidant activity on average compared to the other vegetables. TPF, TFC and antioxidant assays (DPPH, ABTS and RP) results showed that organic solvents are good extraction mediums for phenolic compounds, however each solvent yields different results for different assays, thus, there is not a specific solvent that can be said to be the best for extracting phenolic compounds across all assays. In these experiments, methanol seems to have be the best extraction solvent as it yielded the best results in most of the assays.

GC-MS detected almost all the standard phenolic acids (4-hydroxybenzoic, *trans*-ferulic, vanillic, gallic, syringic and *para*-coumaric acids) except sinapic acid which could not be picked up in both the qualitative and quantitative browsers. Detection was based on the retention times of external authentic phenolic acids and an internal standards (methyl dodecanoate). The GC-MS library search verified the certainty of the compounds of interest with molecular structures. Most abundant detected phenolic acid detected and quantified by GC-MS in the extracts was *p*-coumaric acid, followed by *trans*-ferulic and caffeic acids, these are all well-known antioxidants used in industries such as in the cosmetic and food industries. Base hydrolysed

samples yielded the best extraction of phenolic acids. GC-MS quantitative analysis was performed as a pilot study to support the qualitative analysis results obtained from the qualitative browser and this was a great success. While, this part of the experiment was a trial and preliminary analysis, it added a better explanation of what the qualitative results showed, hence the results obtained from the single analysis were good enough to show the need for further studies to the NLVs. GC-MS can therefore be used to effectively detect and quantify phenolic acids from vegetable samples. The research outcomes addressed the possible establishment of phenolic acids library to identify Namibian Leafy Vegetables; and contributes positively to the advancement of phytochemistry of NLV.

CHAPTER 7 RECOMMENDATIONS

There is definitely a need for further research work on NLV in term of increasing the solvent extraction system, screening new sample preparation techniques and GC-MS analysis conditions. Further studies on the identified phenolic acids for their possible role as natural food preservatives or applications in other industries are needed to confirm their potential for industrial application. Studies on the effect of traditional drying, storage and preparation (cooking) methods on the phenolic content is highly recommended in order to compare if there are any major loses in phenolic content when subject to these treatments, as it stands, these vegetables are mostly dried and stored before consumption; and they not consumed without the application of heat (cooking).

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