Antioxidant content and activity of selected African leafy green vegetables in the gastrointestinal tract (GIT): Determination using simulated GIT digestion

By

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DECLARATION

I, Sanele Maxine Khumalo, declare that this dissertation herewith submitted for the degree of MSc Nutrition at the University of Pretoria, has not been previously submitted by me for a degree at any university.

Signed……

Date……

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ABSTRACT

ANTIOXIDANT CONTENT AND ACTIVITY OF SELECTED AFRICAN GREEN LEAFY VEGETABLES IN THE GASTROINTESTINAL TRACT (GIT): DETERMINATION USING A SIMULATED GIT DIGESTION

Introduction

The incidence of non-communicable disease such as cancer, diabetes, and cardio vascular disease (CVD) is increasing. Major contributing factors are diets that are poor in minerals, vitamins and antioxidants. Leafy green vegetables (LGV) are an important source of these nutrients. In addition, indigenous LGV are drought resistant, robust and easy to cultivate than more exotic LGV such as spinach and cabbage. Little is known about the effects of cooking and digestion on the polyphenol, flavonoid and ß-carotene content of African LGV as well as the associated antioxidant activity. The aims of this study are firstly to evaluate the effect of pH, the digestive enzymes and the phase of digestion on the polyphenols, flavonoids and β-carotene content and associated antioxidant activity of LGVs, spinach, pumpkin, cowpea, amaranth and jute mallow. Then to determine whether following digestion the antioxidant properties of the African LGVs is better than spinach which is commercially and widely cultivated.

Spinach, pumpkin, cowpea, amaranth and jute mallow leaves were cooked for 30 minutes, the water was discarded and the remaining solid cooked LGV which is usually consumed was subjected to two methods of digestion. The first referred to as gastrointestinal model of digestion (GI) of digestion consisted only of the stomach and intestinal phase of digestion, while the second model which was more complex included the oral, stomach and intestinal (OGI) phases of digestion. Raw, cooked, pH adjusted and digested samples at each phase of digestion were collected. For each sample the total polyphenol content (TPC), the total flavonoid content (TFC) was determined with the Folin-Ciocalteu method and aluminum chloride methods respectively. The ß-carotene content was determined with HPLC. Antioxidant activity was determined with the Trolox equivalent antioxidant capacity (TEAC), the 1, 1-diphenyl–2-picrylhydrazyl (DPPH) and the oxygen radical absorbency capacity (ORAC) assays.

Cooking decreased significantly the TPC, TFC, TEAC and DPPH of spinach, pumpkin and cowpea, while the ß-carotene content of these LGV remained unchanged. For amaranth and jute mallow, cooking increased the TFC, ß-carotene and DPPH values, while their TPC and TEAC remained unchanged. All the LGVs decreased their antioxidant activity when measured by ORAC assay,

except for jute mallow which was unchanged. During digestion, the pH controls had varying effects with different assays, as it did not change the TPC of spinach, pumpkin and cowpeas, but decreased that of amaranth while the TPC of jute mallow was higher. Overall, the gastric and intestinal digestion increased the antioxidant activity of all the LGVs as measured by the ORAC assay. TPC and TEAC increased significantly for spinach, pumpkin and cowpea, while their TFC remained unchanged and antioxidant activity measured with the DPPH assay was lower. For the amaranth and jute mallow the TFC was decreased, DPPH increased, while TEAC was unchanged. Except for pumpkin the ß-carotene content was reduced following digestion. In general, the effect of pH was minimal while digestive breakdown of the LGV matrix resulted in a significant release of molecules with antioxidant activity.

In the OGI model of digestion, the oral phase of digestion contributed significantly to the levels of polyphenols and flavonoids released from the matrix. The effect on ß-carotene content was variable. Antioxidant activity for all LGV was increased (TEAC assay), variable (DPPH assay) and reduced (ORAC) assay. When considering the effect determined by the ORAC assay which is considered to be the physiologically the most relevant assay, using models that only simulate gastric and intestinal digestion may over estimate antioxidant activity.

Compared to spinach the antioxidant properties of the LGVs, although in some instances statistically different, were similar to spinach. In conclusion, LGVs evaluated in this study retained to various degrees the antioxidant activity following digestion. The biggest contributing factor to these antioxidant properties was not pH but the ability of the digestive enzymes to break down the food matrix resulting the release of antioxidant molecules. Based on the findings of this study are that African LGV are an ideal source of bioactive molecules that can prevent diseases of the GIT and if bio- available can effectively reduce the risk of diabetes, cancer and CVD.

Key words: Antioxidants, leafy green vegetables, gastrointestinal tract digestion, polyphenols, flavonoids, TEAC, DPPH, ORAC

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LIST OF ABBREVIATIONS AND FORMULAS

µM	Micromolar
A	
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
A- GI	Amaranth gastric intestinal digestion
A - OGI	Amaranth oral gastric intestinal digestion
A0	Uncooked amaranth
A30	Cooked amaranth
ABTS	2,2-Azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diaminium salt
$AlCl_3$	Aluminum chloride
C	
C0	Uncooked cowpea leaves
C30	Cooked cowpea leaves
C-GI	Cowpea gastric intestinal digestion
C - OGI	Cowpea oral gastric intestinal digestion
CVD	Cardiovascular disease
D	
$dddH_2O$	Double distilled deionized water
DM	Dry mass
DPPH	2,2-Diphenyl-1-picrylhydrazyl
E	
ET	Electron transfer
F	
FAO	Food and Agricultural Organization
G	
GLOBOCAN	Global Burden of Cancer Study
GAE	Gallic acid equivalents
H	
HAT	Hydrogen atom transfer
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
I	Food and Agricultural Organization

IDD	Iodine deficiency disorders

J

JM0	Uncooked jute mallow
JM30	Cooked jute mallow
JM-GI	Jute mallow gastric intestinal digestion
JM- OGI	Jute mallow oral gastric intestinal digestion

K

kJ	kiloJoules

L

LSD	Least significant difference
LGV	Leafy green vegetables

M

mg	Milligrams
min	Minutes
mM	Millimolar
MTBE	Tert-butyl-methyl ether

N

Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
$NaNO_3$	Sodium nitrate

O

OH^-	Hydroxyl radical
ORAC	Oxygen radical absorbance capacity

P

P0	Uncooked pumpkin
P30	Cooked pumpkin
P-GI	Pumpkin gastric intestinal digestion
P- OGI	Pumpkin oral gastric intestinal digestion
PBS	Phosphate buffered saline
PEM	Protein energy malnutrition
pH	Logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution

R

r^2	Square correlation
RDA	Recommended daily allowances
$ROO^{\cdot}$	Peroxyl radical
ROOH	Hydroperoxide
ROS	Reactive oxygen species

S

S0	Uncooked spinach leaves
S30	Cooked spinach leaves
S-GI	Spinach gastric intestinal digestion
S- OGI	Spinach oral gastric intestinal digestion
SEM	Standard error of mean

T

TAC	Total antioxidant capacity
TE	Trolox equivalence
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TPC	Total polyphenol content

V

VAD	Vitamin A deficiency

W

WHO	World Health Organization

CHAPTER 1: INTRODUCTION

A report by WHO (2011), stated that in 2008 an estimate of 36.1 million people died from diseases such as cancer, diabetes and heart disease and 80% of this population are from low or middle-income countries. Major contributing factors include poor diets, physical inactivity and smoking. Cancer alone constitutes an enormous burden to society and its occurrence and prevalence is increasing (Torre *et al.,* 2015). About 14.1 million cancer cases were reported worldwide and 8.2 million ended up dying in 2012, as per GLOBOCAN estimates (Torre *et al*., 2015). In 2012 in southern Africa alone the overall cancer incidence and mortality rate was as high as 117.5 and 112.5 per 100 000 respectively (Torre *et al*., 2015).

Central to the development of these chronic diseases is the formation of reactive oxygen species (ROS), which are a natural byproduct of the normal metabolism of oxygen. During times of stress ROS increases dramatically causing damage to lipids, proteins and DNA. These effects result in the dysfunction of biochemical pathways, inhibition of enzyme activity and structural changes to protein and DNA. Cellular dysfunction leading either to cell and/or tissue death or DNA mutational changes will lead to altered cell proliferation and the development of cancer (Uttara *et al*., 2009). A practical and cost-effective way to reduce ROS formation is to increase dietary sources of endogenous antioxidants such as polyphenols and β-carotene. Several studies have shown that a diet rich in vitamins with antioxidant activity and polyphenols reduces the risk of disease (Pandey and Rizvi, 2009; Bouayed and Bohn, 2010; Habauzit and Morand, 2012). African LGVs being one of the diets have been found to contain more nutrients when compared to the exotic vegetables, like spinach and cabbages, especially related to antioxidants, vitamins and mineral content (Chipurura, 2010).

Nutritious diets have become one of the most important aspects of health and well-being and in recent years developed countries have experienced problems of over nutrition and an associated increase in non-communicable diseases, whereas in developing countries the problems were mostly to do with under-nutrition. This however is changing as recent reports have identified the co-existence of under- and over-nutrition in developing countries (Tzioumis and Adair, 2014; dos Santos *et al.,* 2014). This increase in nutritional health related problems has led to increased research into the identification and evaluating the health

benefits of functional foods. An important field of research is the identification of non-nutrient bioactive compounds found in fruits and vegetables (Smith and Eyzaguirre, 2007). Indigenous plants are valuable sources of these nutrients and are often widely available, easily cultivated, pest resistant and are adapted to the climatic conditions of the region. Due to these advantages, it was reported that in Kenya, the cultivation and consumption of indigenous vegetables, were favoured (Cernansky, 2015). These vegetables can also be beneficial in school feeding and community nutrition intervention programs, used to address nutritional deficiencies (Lawson, 2012). Besides being nutritionally relevant, they can also be financially beneficial for both settings. Of special interest in this study are vegetables especially indigenous African LGV.

Around the world, LGVs are an important component diets and have been identified as a significant source of vitamins and other nutritional components that can increase the nutritional value of African diets (Mroso, 2003). LGVs are often used as a relish in most rural households or served as a side dish (Mulokozi, Hendren and Svanberg, 2004). In South Africa, the cultivation of African LGVs is encouraged (van Vuuren, 2013), however the most commonly cultivated LGV is spinach. According to van Vuuren (2013), the tradition of growing spinach alone is gradually changing, as in many communities, other species are now being introduced and are cultivated as part of a mixed cropping system. This was done after realizing that growing traditional African LGVs is easier, compared to exotic LGV as cultivation of these LGV require fewer resources such as water, are pest resistant and more robust than their exotic counterparts. Therefore, the African LGVs discussed in the study include amaranth, pumpkin leaves, cowpea leaves and jute mallow.

LGVs also contribute significantly to the micronutrient intake in most African diets. According to the WHO (2003), micronutrient deficiencies are now recognized as an important contributor to the global burden of disease and iron deficiency alone has caused millions of deaths among children. Vitamin A is a common deficiency (Veda *et al.*, 2006) and in South Africa about 33% of children between the age of 3 and 5 are vitamin A deficient (Faber, van Jaarsveld and Laubscher, 2007). The carotenoids specifically β-carotene found in photosynthetic tissues are the important precursors of vitamin A (Tee and Lim 1990). Animal sources such as liver and eggs are good sources of preformed vitamin A, but most African diets are dependent on plant food as source of carotenoids especially β–carotenes to fulfil their vitamin A requirement.

Many studies focus on the identification of vegetables and fruit that have high levels of polyphenols and that can scavenge ROS. However, little is known whether, when these vegetables are consumed the antioxidant activity is retained, enhanced or lost. Identified factors that will affect bio-availability are the method of processing LGV and the digestive process which includes the effect of pH and enzymatic digestion of the LGV matrix. Therefore, the aims of this study are to determine the effect of cooking on the polyphenols, flavonoids, β-carotene content and associated antioxidant activity of the selected LGVs and then to evaluate the effect of digestion on the polyphenols, flavonoids and β-carotene content and associated antioxidant activity of the LGVs. Lastly to determine whether following digestion, the antioxidant properties of African LGVs are better than spinach, a commercially widely cultivated LGV.

CHAPTER 2: LITERATURE REVIEW

2.1 Green leafy vegetables

According to van Rensburg, *et al.*, (2007), green leafy vegetables (LGV) are plant species that are used as vegetables and includes the leafy part, and often also the young succulent stems, flowers and very young fruits. For sub-Saharan African populations, the reliance on LGVs as important dietary components is significant, as these vegetables have been part of their diets and are usually part of traditional sauces that accompany carbohydrate dishes (Smith and Eyzaguirre, 2007). Little is known about patterns of African LGV production, processing and consumption, and whether the processing and digestion affects the nutritional properties of these LGV.

Raw African LGV have been found to contain antioxidants, and therefore can be used as an affordable source for combating cellular oxidative damage (Uusiku *et al.*, 2010). Some of the African LGVs include: *Amaranthus creuntus L.*, *Corchorus oritorius L.*, *Curcumbita maxima*, *Moringa oleifera*, *Ipomea batatas* and *Vigna unguiculata*. A majority of these LGV have been domesticated or grown in Africa for the past centuries and have been recently replaced by new commercially cultivated species such as spinach, cabbage and kale. For this study four LGV that commonly consumed were identified and these are *Amaranthus creuntus L.*, *Corchorus oritorius L.*, *Curcumbita maxima* and *Vigna unguiculata* (L) Walp. The contribution of these LGV to addressing the antioxidant requirements of the southern African population will be evaluated and compared to commercially cultivated *Spinacia olerancia L.*

2.1.1 *Amaranthus cruentus L.* (Amaranth)

Amaranth is one of the most important African LGV species which belongs to the Amaranthaceae family. A mature amaranth plant has a height of between 0.3 m and 2 m (van Rensburg, *et al.*, 2007). It grows naturally under warm conditions and it is rarely cultivated. The leaves have a high content of essential micronutrients, especially vitamin A and C, iron, calcium and folate (Leon-Camacho, Garcia-Gonzalez and Apirocio, 2001). Although it is loaded with micronutrients, their absorption in the human gut is dependent to the quality, preparation method, the combination with other foods and the health of the consumer. Cooking time is between 5–10 minutes in lightly salted water. After cooking amaranth,

usually the cooking water is discarded thus some nutrients are lost, especially vitamin C, niacin, riboflavin and thiamin (Adefegha and Oboh, 2011).

Figure 2.1: The LGV, *Amaranthus cruentus L.*

2.1.2 *Corchorus oritorius L.* (Jute mallow)

Corchorus oritorius L, commonly called Jew's mallow or jute mallow in English is used as a leafy mucilaginous vegetable which belongs to the Tiliaceae family. When cooked it forms a slimy sticky sauce, which is found suitable for easy consumption of starchy foods made from cassava, yam or millet, especially in Nigeria and from mealie meal in the most southern parts of Africa. Drying the leaves is one of the preservation methods used when preserving jute mallow (Ndlovu and Afolayan, 2008) and it is a good source of vitamins and minerals. According to Woomer and Imbumi, (2003) jute mallow is often cooked with cowpeas, pumpkin, cocoyam leaves, sweet potato, milk and butter and meat. This is especially done in east Africa. The soil fertility and climatic conditions influences the micronutrient content of the jute mallow. Nitrogen fertilizer greatly improves the micronutrient content, i.e iron, phosphorus, calcium, carotene and vitamin C (Ndlovu and Afolayan, 2008). Jute mallow leaves contain antioxidative phenolic compounds, of which 5-caffeoylquinic acid is the most abundant (Chipurura *et al.*, 2009).

Figure 2.2: The LGV, *Corchorus olitorius L*.

2.1.3 *Curcumbita maxima Duchesne* (Pumpkin leaves)

Curcumbita maxima Duchesne, also known as pumpkin leaves is a member of the Curcurbitaceae family. The young succulent leaves and stems, with a hair-like surface are frequently harvested and are widely consumed in Africa as a LGV relish accompanying starchy staple foods. Pumpkin leaves are a good source of vitamins and minerals, as well as antioxidant. The fruit of this LGV is also widely consumed and is a rich source of antioxidants (Gacche *et al*., 2010), such as α-carotene and β–carotene (Ikpe, 2013). In addition, preliminary phytochemical screening of pumpkin leaf extract has revealed the presence of tannin, flavonoid, alkanoids and saponin which according to Akande and Yahaya (2010) has antimicrobial activity.

Figure 2.3: The LGV, *Curcumbita maxima* Duchesne

2.1.4 *Vigna unguiculata L.* (Cowpea leaves)

Vigna unguiculata L is also known as a cowpea and belongs to the Leguminosae family. According to Zia-UI-Haq *et al.,* (2013), the tender shoots and leaves can be used or consumed at the seedling stage. In the cowpeas plant, the fresh leaves, immature pods and seeds are used as vegetables, while the dry grain is used to prepare main dishes and snacks or grounded to make cowpea cakes or consumed as is (Zia-UI-Haq *et al*, 2013). The cowpea leaves are under exploited especially related to nutritional value and the effects of various preservation methods on nutritional quality is unknown. There are different varieties of cowpea and the variety that is commonly used as a LGV is the prostate type. According to Kiminywe *et al.,* (2007), cowpea leaves were found to boost appetite and solve digestive problems. A study by Vats *et al*., (2012), evaluated the phytochemical, antioxidants and antimicrobial properties of cowpeas, concluded that the callus culture of *V.unguiculata* is a potential source of flavonoids, phenolics and antimicrobial agents.

Figure 2.4: The LGV, *Vigna unguiculata L.*

2.1.5 *Spinacia olerancia* (Spinach)

Spinacia olerancia, commonly called spinach is an exotic LGV that has gained popularity in African diets. It is consumed after cooking either fresh or frozen. Spinach is rich in micronutrients, especially vitamins A (from β-carotene), C, K and folate, and the minerals, calcium, iron and potassium. It also provides fibre and is low in calories (Hedges and Lister, 2007). As such spinach provides an excellent control vegetable for studies evaluating the nutritional value of African LGV. Several studies have showed that spinach has a strong antioxidant activity and high levels of antioxidant compounds such as phenolics and

carotenoids (Pandey and Rizvi, 2009). Lutein and zeaxanthin are major compounds in spinach which has health benefits as they protect against eye diseases such as macular degeneration (Gil, Ferreres and Tomas-Barberan, 1999). Other proven functions include delay in age-related loss of brain function, reduce the extent of post-ischaemic stroke damage to the brain, and protection against cancer through various mechanisms (Hedges and Lister, 2007).

Figure 2.5: The LGV, *Spinacia olerencia*

2.2 Contribution of green leafy vegetables to the nutritional status of individuals

According to Bhupathiraju *et al.*, (2013), 4.4% of diseases, disability and death can be attributed to the low intake of fruits and vegetables. The ratio of the number of people and the amount of food available has impacted the nutritional status of people in many countries. As reported by FAO (2004), the world's population is increasing at an alarming rate, thus the challenge is to provide food for everybody. Due to the challenge of food availability, the majority of children in developing countries suffer from malnutrition in the first five years of life. Malnutrition can be subdivided into over and under-nutrition. In developed countries the major health problems are from non-communicable diseases, especially heart diseases, cancer and diabetes, and that has been aggravated by poor diets, due to consumption of highly refined foods. Problems of malnutrition, especially protein-energy malnutrition (PEM) and micronutrient malnutrition (iron deficiency anaemia, vitamin A deficiency (VAD), iodine deficiency disorders (IDD) and zinc deficiency) have contributed to the major health problems of developing countries (WHO, 2003). However, recent reports have identified the co-existence of under- and over nutrition in developing countries. For both conditions the

consumed fruits and vegetables can provide vital micronutrients, vitamins and antioxidants that can prevent associated disease.

Fruits and vegetables contribute to human intake of vitamin C, vitamin E, carotenoids, dietary fibre, folic acid, potassium and magnesium (Nambiar, 2004). Smith and Eyzaguirre (2007), suggested a minimal daily intake of 400g of fruits and vegetables, is required to meet the recommended daily allowance (RDA) of vitamins and minerals and this will ensure that children are protected from micronutrients deficiencies. African LGV do not only supply micronutrients, but also provide non-nutrient antioxidants which are associated with the prevention of the non-communicable diseases. Antioxidants not only protect the body from chronic diseases, but antioxidant vitamins such as Vitamin C and E play a vital role in protecting children from childhood diseases. Jack (2016) reported that certain studies suggest that an increment of one daily serving of LGV may lower the risk of CVD by 11%. According to Greger (2013), daily antioxidants intake is dependent on daily food intake. An example used is that for a man in the United States who consumes about 2500 kJ a day, the recommended intake related to antioxidant is 11000µM of Trolox equivalents (TE) a day, whereas for women who consumes 1800 calories should get at least 8000 µM TE a day. Daily intake in this population is generally too little to prevent CVD. However, the amount and the type of LGV consumed may address this dietary requirement and specifically in the South African context, little is known regarding the effects of digestion on the bioavailability of antioxidant molecules in LGV.

Investigating the relationship between dietary antioxidants and pathologies induced by the oxidative stress, measurement of the total antioxidant potential is a relevant tool (Pisoschi and Negulescu, 2011). LGV contain a variety of carotenoids and flavonoids and other antioxidants that have many beneficial effects. According to Yan (2013), because of their high antioxidant content, LGVs may be one of the best heart disease and cancer-preventing foods and 2 to 3 servings of LGV per week may lower the risk of stomach, breast and skin cancer. Serafini *et al.*, (2002) reported that an increase in the intake of antioxidants equivalents, from fruits and vegetables, was inversely associated with risk of gastric cancer. Louwrens, Rautenbach and Venter (2009), recommended the dietary total antioxidant capacity (TAC) to range between 19500 µm and 21500 µm TE/person/day. This TAC range was based on secondary intake data in relation to dietary recommendations. The TAC for an average adult South African dietary was 11433 TE/person/day, with beverages and tea the

main contributor as opposed to LGV. This study confirmed that LGV are an underutilized source of antioxidant molecules.

2.3 Antioxidants in vegetables

An inverse relationship between the intake of fruits, vegetables and grains and the incidence of CVD and certain cancers has been proven by a number of epidemiological surveys (Rice-Evans, Miller & Paganga, 1997 and Halliwell, Rafter & Jenner, 2005). Dietary components found in these fruits, vegetables and grains that contribute to antioxidant activity include, vitamin C and E, selenium, carotenoids, phytoestrogens, allium compounds, glucosinolates, fibre and folic acid. Phytochemical components or non-nutrient antioxidants include flavonoids, phenylpropanoids, and phenolic acids (Rice–Evans, *et al*., 1997). The activity of an antioxidant is determined by its reactivity as a hydrogen or electron donating agent and also the potential of the resulting antioxidant-derived radical to stabilise and delocalize an unpaired electron (Fang, Yang and Wu, 2002).

In a study by Serafini *et al*., (2002), the total antioxidant potential of fruits and vegetables was determined. Of the vegetable evaluated garlic and kale had the highest potential followed by spinach, broccoli and oranges. Common antioxidants found in LGV are flavonoids, phenolic acids and ß-carotenoids and these will be discussed further in greater detail.

2.3.1 Flavonoids

Flavonoids as described by Subasree, Baskar, Keerthana, Susan and Rajasekaran (2009), are large compounds occurring in plant-based foods that contain a number of phenolic hydroxyl groups on their ring structure and often occur as glycosides. Concentrations of flavonoids are 15 – 30mg/kg of fresh weight and the best sources include onions, leeks, broccoli and blueberries. The average daily intake of flavonoids ranges from 2.6mg/d in Finland, to about 68.2mg/d in Japan (Nijveldt, *et al.*, 2001) and this was mainly from the consumption of apples and onions.

According to Nijveldt, van Nood, van Hoorn, Boelens, van Norren and van Leeuwen (2001), many flavonoids are found to be strong antioxidants capable of successfully scavenging reactive oxygen species (ROS) because of their phenolic hydroxyl groups. Flavonoids can be

sub-divided into several families and these are the flavonols e.g. quecertin, the flavones e.g. luteolin, the flavanols e.g. catechin and the isoflavones e.g. genistein, (Subasree, *et al*. (2009); Nijveldt, *et al.* (2001)). Flavonols are the most abundant flavonoids in foods and the most common are quercetin and kaempferol (Manach, *et al.*, 2004).

Within the different classes of flavonoids there are many structural variations related to the degree of hydrogenation and hydroxylation of the three ring system as indicated in Figure 2.6. Due to the high reactivity of the hydroxyl group of flavonoids, flavonoids scavenge radicals resulting in the formation of more stable and less reactive radicals as shown in the following reaction.

F-OH + $R^{\cdot}$ → F-$O^{\cdot}$ + RH

Where $R^{\cdot}$ is a free radical, $O^{\cdot}$ is the more stable generated oxygen free radical and F is the flavonoid.

In a study by Subhasree *et al*. (2009), the antioxidant potential of LGV was evaluated and there was a significant positive correlation between flavonoid content and antioxidant activity of the plant extracts, whereas no correlation was found between antioxidant activity and phenol content. The beneficial effects of flavonoids, is not limited to the ability of these molecules to scavenge radicals. An additional beneficial effect is due to the anticancer properties of flavonoids which include the induction of detoxification enzymes, inhibition cancer cell proliferation and the promotion cell differentiation (Hedges and Lister, 2007). According to Ismail *et al*, (2012), they used 28 extracts from medicinal plants and evaluated for their in vitro anticancer properties on four human cancer cell lines, namely colon, breast, prostate and lung cancers. Their results showed that all extracts had some anticancer activity, although some showed outstanding results depending on the cancer cell used.

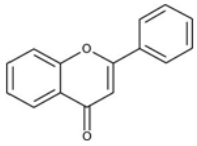
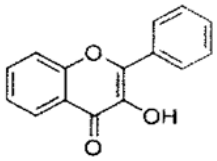
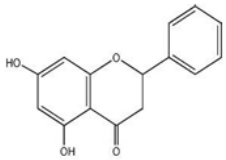
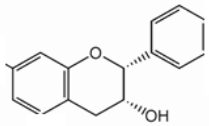
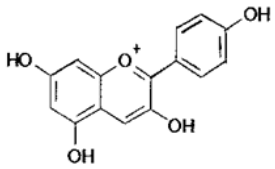
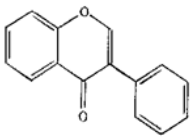
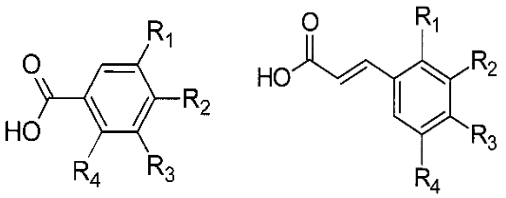
Flavonoid	Basic structure
Flavones	
Flavonols	
Flavanones	
Flavanols	
Anthocyanidins	
Isoflavones	

Figure 2.6: The basic chemical structures of the main classes of flavonoids (Reis Giada, 2013)

2.3.2 Phenolic acids

Phenolic acids are secondary metabolites in plants. Rice-Evans *et al.* (1996) and Manach *et al*., (2004), describe phenolic acids as derivatives of benzoic and cinnamic acids which contain hydroxyl and methoxyl groups substituted on site on the benzene ring. The benzoic acids have seven carbon atoms (C6-C1) and are the simplest phenolic acids found in nature. Cinnamic acids on the other hand, have nine carbon atoms (C6-C3) and are the most common. Figure 2.7 are the core structures of both the benzoic and cinnamic acid derivatives (Castellano, 2012).



Benzoic acidsCinnamic acids

Figure 2.7: Core structure of benzoic and cinnamic acids (Castellano, 2012**)**

The scavenging capability of phenolic acids is mainly due to the presence of hydroxyl groups. Phenolic acids have reducing properties as hydrogen or electron giving agents and therefore are potential free radical scavengers (Rice-Evans, *et al*., 1997) as shown below.

R+POH → RH +PO$^{\cdot}$

Where R$^{\cdot}$ is a free radical, PO$^{\cdot}$ is the more stable generated oxygen free radical and P is the phenolic acid. The phenoxy radical intermediates (PO·) are relatively stable due to resonance and therefore a new chain reaction is not easily initiated. Gallic, syringic, vanillic and protocatechuic acid are examples of hydrobenzoic acids with strong radical scavenging ability.

2.3.3 β –Carotene

The yellow-orange and red pigments, found in fruits and vegetables are called carotenoids. These colours are masked by chlorophyll, which is the green pigment found in plants. The largest amounts of carotenoids are found in dark LGV such as spinach and kale (Serano, Goni and Saura-Calixto, 2005). Carotenes and xanthophylls are classes of carotenoids and lutein is one of the major xanthophylls found in LGV and act as antioxidants. These molecules have been found to have anti-allergic, anti-cancer and anti-obesity activity (Kotake-Nara and Nagao, 2011).

α-Carotene, β-carotene and β-cryptoxanthin can be converted to retinol, or vitamin A, thus these molecules are often referred to as having provitamin A activity. Malnutrition, especially vitamin A deficiency, is a major public health problem in developing countries. According to Serano, Goni and Saura-Calixto (2005), the effectiveness of LGV as

contributors to carotenoids is questionable as the bioavailability of the carotenoids in vegetables is low, with 3 to 6% bioavailability compared to purified carotene. ß-Carotene is anti-carcinogenic and is beneficial in the prevention of heart disease (Kao, 2006). *In vitro* cell experiments have indicated that carotenoids also have other properties consistent with anticancer activity. ß-Carotene help prevent the formations of lesions that lead to cancer (Hedges and Lister, 2007). Carotenoids are more effective in quenching singlet oxygen and peroxyl radicals and often act synergistically with other antioxidants. According to Kao (2006), there are three possible mechanisms whereby carotenoids react with radical species as shown below:

$$ROO^{\cdot} + CAR \rightarrow ROO^{-} + CAR^{\cdot +}$$
$$ROO^{\cdot} + CAR \rightarrow ROOH + CAR^{\cdot}$$
$$ROO^{\cdot} + CAR \rightarrow (ROO\text{-}CAR)^{\cdot}$$

Where ROO˙is a peroxyl radical and CAR is a carotenoid.

The total phenolic and flavonoid, ß-carotene content as well as the antioxidant activity of raw extracts of African LGV and spinach used in this study has been determined by different authors and is presented in Table 2.1. However, to have a health benefit, these molecules must be bioavailable.

2.4 Bio-availability of antioxidants

According to Fernández-García, Carvajal-Lérida and Pérez-Gálvez (2009) and Cardoso *et al*. (2015), bioavailability is the fraction of the nutrient or bioactive compound ingested that is available for use in physiologic functions. Figure 2.8 shows the definition of bioavailability is the sum of bioaccessibility and bioactivity where bioaccessibility being a fraction of a compound that is released from its matrix in the gut, available for intestinal absorption, while bioactivity include events linked to how the bioactive compound is transported and reaches the target tissue or how it interacts with biomolecules (Fernández-García, Carvajal-Lérida and Pérez-Gálvez, 2009).

Table 2.1: Summary of antioxidants content and activity of different raw African LGV.

LGV	TPC (mg GAE/g)	TFC (mg/g CE)	β-carotene (mg/g)	ABTS (µmol TE/g)	DPPH (µmol TE/g)	ORAC (µmol TE/g)	References
Amaranth	2.1 – 186.7	1.5 – 69.7	3.3-23.0	44.7	24.2 – 32.7	20	Kruger *et al*., (1998); van der Walt *et al.* (2009); Mavhungu (2011); FAO (2003); Alajire and Azeez (2011)
Jute mallow	3 – 200	2.7 – 81	7.3	58.7	45.1 – 63	59.5	Kruger *et al*., (1998); Mavhungu (2011); FAO (2003); Alajire and Azeez (2011)
Pumpkin	2.7 – 251.9	1.6 – 117.3	1.9 -10.0	62.1	77.02 – 83.6	33	Kruger *et al.*, (1998); Mavhungu (2011); FAO (2003); Alajire and Azeez (2011)
Cowpea	2.4 – 29.1	1.5	1.0 – 7.0	75.3	81.5	36	Kruger *et al*., (1998); van der Walt *et al.* (2009); Mavhungu (2011); FAO (2003)
Spinach	0.33 – 204.7	0.5 – 139.6	1.0 – 6.7	0.102- 35.8	3.74 – 57.3	4.2 – 84.4	Kruger *et al.*, (1998); Yang (2006); Mavhungu (2011); Ismail, Marjan and Foomg (2004); Ahamad *et al.* (2007); Alajire and Azeez (2011)

Bioaccessibility is used in studies of nutritional efficiency of food and food formula developed with the aim of improving human health, because it provides an important information to select the appropriate dosage and source of food matrices to ensure nutritional efficacy of food products.

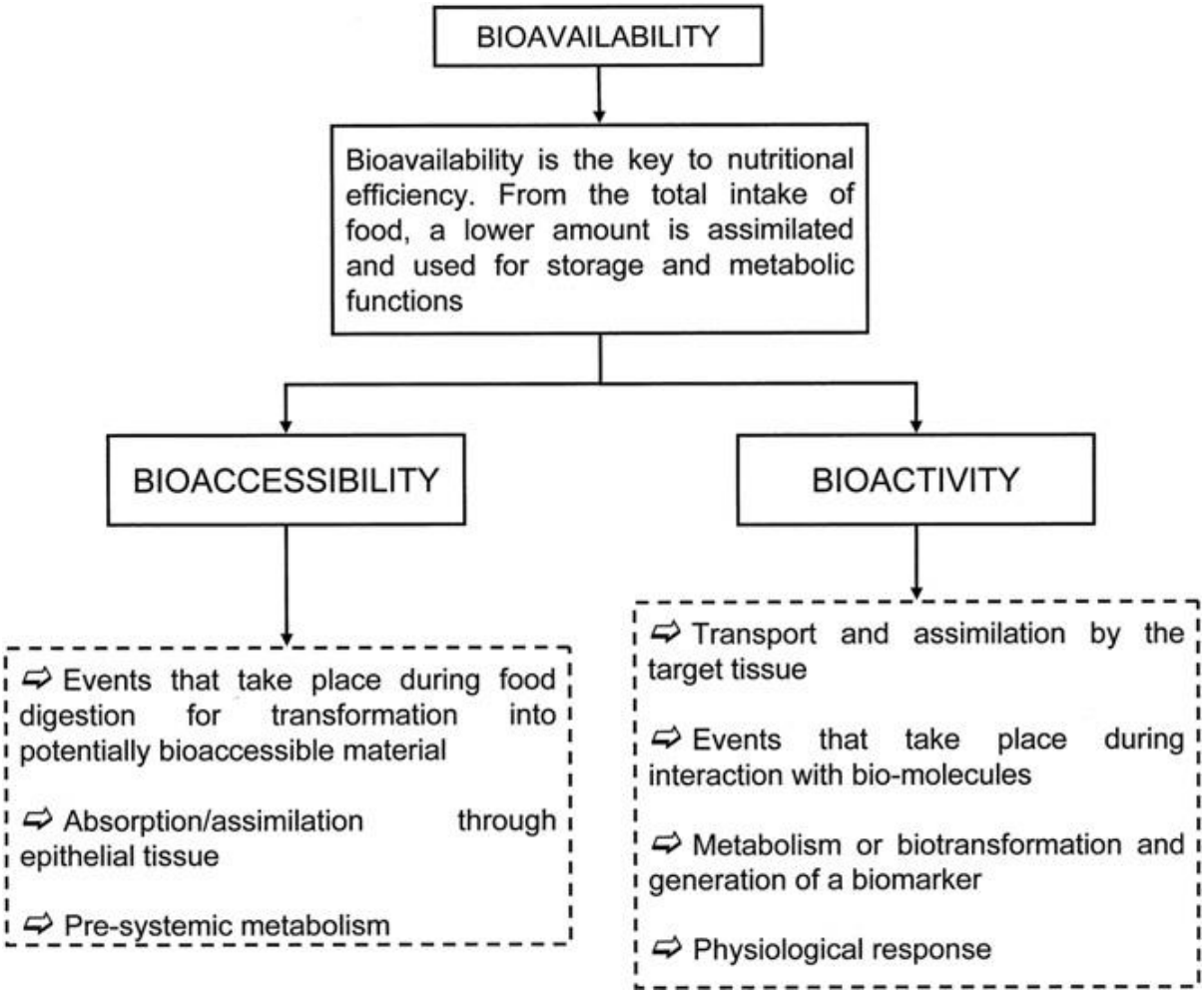


Figure 2.8: Definition of bioavailability as a sum of bioaccessibility and bioactivity (Fernández-García, Carvajal-Lérida and Pérez-Gálvez, 20

However, these studies provide little information on the effects of processing or cooking and digestion on the antioxidant properties of LGV. These processes may either reduce or may increase via extraction the antioxidant activity of LGV. Although many antioxidants such as flavonoids and phenolic acids are not bioavailable, within the gastrointestinal tract (GIT), bioactive molecules can protect the GIT against gastric and colonic cancer (Halliwell, Rafter and Jenner, 2005)

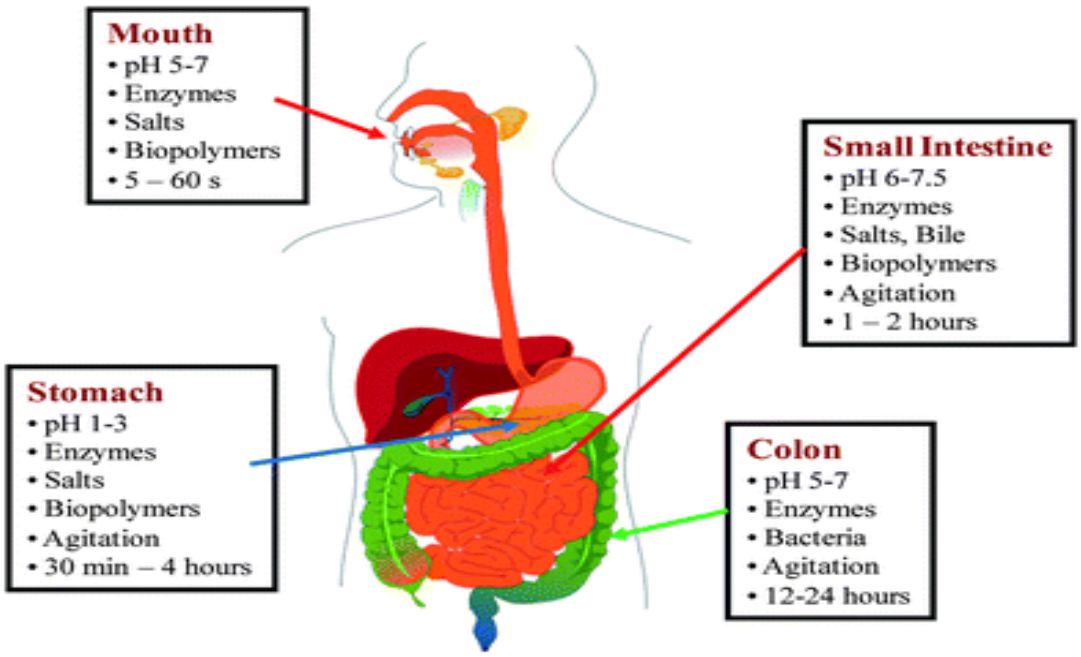


Figure 2.9: Schematic diagram of physicochemical conditions in the different regions of the human GI tract (McClements and Li, 2010).

The GI tract is constantly exposed to reactive oxygen, chlorine and nitrogen species of which most is from the diet and the rest is the result of phagocyte activation in the gut, as illustrated in table 2.2 (Halliwell, Rafter and Jenner, 2005). Exposure to saliva for 1 hour had little or no effect on the stability of catechins found in green tea (Spencer, 2003). This implies that after drinking green tea, the antioxidant properties of these polyphenols are retained with a beneficial effect the on the mucosa of the mouth and oesophagus. In contrast, a decrease in quercetin, was observed when mixed with saliva. This could be due to the polyphenol-protein interactions due to adsorption of quercetin by high molecular weight salivary protein, bacterial cells and mucous material (Spencer, 2003). These complexes when entering the stomach may be subjected to gastric digestion with the release of the complexed polyphenols and subsequently will have a benefical effect in the stomach.

Bermudez-Soto, Tomas-Barbaran and Garcia-Conesa (2007), investigated the stability of polyphenols in chokeberry subjected to *in vitro* gastric and pancreatic digestion. No substantial effect on any of the major phenolic compounds in chokeberry which included anthocyanins, flavan-3-ols, flavonols and caffeic acid derivatives occurred with gastric digestion. In contrast, effects of intestinal digestion on the stability of several polyphenols was variable.

Spencer (2003) reported that flavan-3-ols were stable in the stomach whereas flavanols were unstable under gastric conditions of low pH. pH rises from about 2 to 7 after transfer from the stomach to the jejunum. Due to the complexity of food matrices, the pH is likely to be buffered for long periods of time and therefore, flavanol oxidation may only occur to a limited extent during intestinal transit. It has been suggested that ascorbate increases the stability of flavanols incubated in intestinal fluid, and consequently can stabilize *in vitro* polyphenols in the neutral or alkaline environment of the small intestine (Spencer, 2003).

Odukoya *et al.,* (2007), reported that β-carotene and phenolic compounds are more bio-available from cooked vegetables compared with raw, as cooking breaks down the tough cell walls, releasing the nutrient content for easier absorption from the small intestine. Example maybe the carotenoids present in chloroplast in the dark leaves of vegetables that are released. In this study the effect of digestion on jute mallow was evaluated and findings were that jute mallow had high antioxidant activity and was amongst the best African LGV. The gastric pH has been shown to have no effect or increase the yield of antioxidants in LGV. With acidification, associated with gastric digestion the extractability of phenolic compounds increased and also polyphenols esterified to carbohydrate were also released (Liyana-Pathirana and Shadihi, 2005). The stability of fruit and vegetable juices with digestion was evaluated by Wootton-Beard, Moran and Ryan (2011). Findings were that antioxidant activity determined with the DPPH and TEAC assay was slightly increased with gastric and decreased following the intestinal phases of digestion. A similar trend was also observed for the polyphenol content (Wootton-Beard, Moran and Ryan, 2011).

Hedren, Diaz and Svanberg (2002) found that the presence of bile salts in the intestinal digests was essential for the maximal extraction and subsequent absorption of β-carotene. Gastric digestion and intestinal digestion in the absence of bile salts had little effect on β-

carotene release while intestinal digestion and the associated presence of bile salts ensured effective β-carotene release.

Pavan, Sancho and Pastore (2014), in a study evaluating the effects of digestion on the antioxidant activity of several fruit and found varying results, with a reduction in polyphenols being attributed to the instability of polyphenols at neutral pH, or an increase in polyphenol content being due to the release of polyphenols during the digestion process. For papaya extracts there was an increase in total flavonoid content after digestion, and this was attributed to the proteolytic cleavage of the β-linkage between carbohydrates and aglycone, not by the proteolytic activity of the digestive enzymes but rather due to fermentation by colonic bacteria.

Table 2.2: Available, absorption and role of antioxidants in the stomach, small intestines and colon/rectum (Halliwell, Rafter and Jenner, 2005)

	Stomach	Small intestine	Colon/Rectum
Availability	High concentration of ascorbate in food and gastric juice Carotenoids, tocopherols, tocotreinols, flavonoids and other phenolics in food (if fruit/ vegetable/ grain – rich diets consumed)	Some carotenoids cleaved by eccentric and excentric mechanism, eventually to yield vitamin A	Considerable amounts of unabsorbed flavonoids, other phenolics, carotenoids (if diet rich in these), tocotrienols, β, γ, δ-tocopherols Extensive metabolism by colonic flora to generate different phenolics
Absorption	Nothing is absorbed in the stomach	Vitamin C completely absorbed Vitamin E largely absorbed Some tocotrienols and β, γ, δ-tocopherols returned to GIT in bile Some carotenoids, flavonoids and other phenolic compounds are absorbed but many are not.	Limited α-tocopherol present
Function	Scavenges • RNS from HNO_2 • OH from Fe/ascorbate interactions • RO and RO_2 from dietary lipid peroxides • Some phenolics bind Fe^2, to decrease its ability to cause free radical generation • Ferryl species from haem protein/peroxide interactions • H_2O_2 in consumed beverages	Nothing of significance happens in the small intestine. Neutral pH can lead to polyphenol degradation	Scavenging or metal binding/ other actions of phenols may help to delay colon/rectal cancer development by exerting external protective effects on the colonic/rectal epithelium Inhibitors of LOX, COX-2, angiogenesis, matrix metalloproteinases etc by phenols may help to limit cancer development

2.5 Effect of processing on antioxidant content and activity

According to Fernández-García, Carvajal-Lérida and Pérez-Gálvez (2009), processing can impact on the nutritional quality of food, both positively and negatively. To determine the impact of processing, physiochemical changes on key nutrients content can be measured.

2.5.1 Peeling and chopping

The way the vegetable is prepared may affect its nutrient content and according to Uusiku, *et al*., (2010), processing may also affect the antioxidant capacity of LGVs. Simple peeling of fruits and vegetables can decrease the polyphenol content, as most of the compounds are concentrated in the outer parts (Manach, Scalbert, Morand, Remesy & Jimenez, 2004; Mitic *et al*., 2013; Simopoulus, 2004). Traditionally the skin of vegetables is removed in order to reduce exposure to contaminants and pathogens.

2.5.2 Cooking

African LGV are mostly cooked (Odukoya *et al.,* 2007) and the method of cooking is based on tradition and taste preference. LGV may be cooked in water (boiling), microwaved, steamed or fried and these processes bringing about a number of changes in the physical and chemical characteristics of these LGV (Mirzaei *et al.*, 2014). Various studies have shown that β-carotene is poorly absorbed from certain vegetables, and that with cooking, carotenoid release is enhanced as cooking softens cell walls and the carotenoids become dissociated from the protein complex (Hedren, Diaz and Svanberg, 2002). Steiner-Asiedu *et al*, 2012 reported that the carotenoid levels of Corchorus *olitorius* was reduced after cooking for 30 minutes.

According to Manach, *et al*. (2004), onions and tomatoes lose up to 80% of their initial quercetin after boiling for 15 minutes, 65% after cooking in a microwave and 30% after frying. Hunter and Fletcher, (2002), also reported that boiling at above 95°C causes the loss of antioxidants. In contrast, a study by Turkmen *et al.,* (2005), showed that cooking has little effect on the total phenolics and antioxidant activity of vegetables. For spinach the TPC was slightly increased and the antioxidant activity of broccoli and spinach increased.

With cooking heat sensitive antioxidants like vitamin C are destroyed. Lycopene in tomatoes becomes more available and β-carotene levels in carrots are also increase with moderate heat

(Subramanian, 2009). Cooking according to Chandrika, Svanberg and Jansz (2006), causes a reduction in β-carotene, however it does in some instances increases the bio-availability of carotenoids in vegetables.

Amin *et al.*, (2006), reported that the antioxidant activity of the vegetables decreased after blanching and about 82% of the phenolic compounds were lost into the cooking water which is usually discarded (Mavhungu, 2011). In a study by Sreeramulu, *et al.*, (2013), some LGV showed either an increase or decrease in phenolic content after cooking with different methods. An increase was possibly due to the release during thermal processing of phenolics stored in pectin or cellulose networks of plants. Also, the levels of individual phenolics may sometimes be increased because heat can breakdown larger complex phenolic structures releasing the smaller constituent phenolic acids (Bunea *et al*., 2008).

Steaming has shown to increase the phenolic acid and antioxidant activity of vegetables due to the release the phenolic and/or flavonoid compounds (Adefegha and Oboh, 2011). Phenolic compounds and β –carotene are actually more bio-available from cooked vegetables than raw. This is attributed to the cooking process breaking down the tough cell walls, releasing the nutrient for easier absorption from the small intestines (Odukoya *et al*., 2007). Flavonoids, as water-soluble nutrients, can be lost through water contact, and in some cases, up to 80% of specific flavonoids can be lost into cooking water during the boiling of foods. Using the DPPH assay, Kunyanga *et al.*, (2011), found that antioxidant activity of LGV, pumpkin and amaranth leaves, was different after processing. The antioxidant activity of pumpkin leaves was increased with cooking and blanching, while the antioxidant activity of amaranth leaves remained unchanged with cooking but some loss of activity was found with blanching.

These studies have identified that the method of cooking, the LGV matrix, the type, complexity of structure and concentration of the polyphenols and carotenoids in LGV all contribute to the measured antioxidant activity.

2.5.3 Drying

Vegetables and fruits can be processed and preserved by drying. Drying concentrates a food's nutrients and preserves them for times when fresh food is not available. Improved technologies, such as solar dryers, retain higher quantities of vitamins in food better than traditional methods of sun drying (FAO, 2003). Oboh (2004), in a study determined the change in ascorbic acid, TPC and antioxidant activity of sun-dried commonly consumed Nigerian LGV and found that sun-drying of LGV caused a significant decrease in the vitamin C content and an increase in TPC. As vitamin C is also an antioxidant, the final total antioxidant capacity was unchanged. Medoua and Oldewage-Theron (2014), also found that drying caused no significant effect on the levels of most nutrients and antioxidants however vitamin C and β-carotene levels were significantly lowered.

2.6 STUDY AIM AND OBJECTIVES

2.6.1 STUDY AIM

The aim of this study is to evaluate the effect of cooking and digestion factors on the polyphenols, flavonoids and β-carotene content and associated antioxidant activity of a selection of African LGVs compared to spinach which is commercially and widely cultivated.

2.6.2 STUDY OBJECTIVES

The aim of this study will be achieved with the following objectives,

1. To determine the polyphenol, flavonoids and β-carotene content and associated antioxidant activity in selected LGV (spinach, pumpkin, cowpeas, jute mallow and amaranth), before and after cooking for 30 min.
2. To determine the effect of gastric and intestinal associated changes in pH during digestion on the polyphenol, flavonoids and β-carotene content and associated antioxidant activity of selected LGV.
3. To determine the effect of a simulated GIT digestion model digestion involving gastric and intestinal phases of digestion on the polyphenol, flavonoids and β-carotene content and associated antioxidant activity of water extracts of cooked LGV.
4. To determine the effect of a simulated GIT digestion model involving the oral, gastric and intestinal phases of digestion on the polyphenol, flavonoids and β-carotene content and associated antioxidant activity of cooked selected LGV.
5. Lastly to determine if the antioxidant properties of African LGV are better than commercially cultivated spinach.

CHAPTER 3: MATERIALS AND METHODS

3.1 MATERIALS

Hydrochloric acid (HCl), sodium hydrogen carbonate ($NaHCO_3$), were of analytical quality and were obtained from Merck Chemicals, Modderfontein South Africa (SA). Porcine pepsin, pancreatin, α-amylase, lipase and bile were obtained from the Sigma-Aldrich Company, Atlasville, SA. Folin-Ciocalteu's reagent, sodium carbonate anhydrous (Na_2CO_3), gallic acid and catechin, aluminium chloride ($AlCl_3$), sodium nitrite ($NaNO_3$), sodium hydroxide (NaOH), β-carotene, tert-butyl-methyl-2-picryhydrazl, 2,2-azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diaminium salt (ABTS), potassium peroxodisulfate, fluorescein, DPPH, and AAPH were also purchased from Sigma-Aldrich Company, Atlasville, SA.

Disposable plasticware including pipette tips and 25, 50ml plastic tubes were obtained from Greiner Bio-one and supplied by LASEC, Cape Town, SA.

3.2 METHODS

3.2.1 Samples

The samples used in this study were *Amaranthus cruentus L.*, *Corchorus oritorius*, *Curcumbita maxima*, *Vigna uunguiculata (L.),* and *Spinacia olerancia*, commonly known as amaranth (A), jute mallow (JM), pumpkin (P), cowpea (C) and spinach (S), respectively. The African LGVs were collected from the Vegetable and Ornamental Plant Institution of Agricultural Research Council, Gauteng Province, South Africa. The spinach was bought in a local supermarket in Pretoria.

3.2.2 Sample preparation

The LGVs were washed and divided into two fractions. The first was not processed and represents the raw LGV (0) and then the second fraction was cooked for 30 minutes (30) which mostly represents the length of cooking time for LGVs. A mass of 750g of the LGVs was added to 1800 ml of boiling water and was cooked for 30 minutes. As done traditionally, the cooked samples were then drained, and the excess water was discarded. Whether the water is discarded depends on household, region and country, therefore evaluating the

polyphenol content of the remaining solid material will provide information on the sensitivity of the polyphenols and flavonoids in the plant matrix to the effects of cooking. Both the uncooked and cooked samples were then freeze-dried, ground, homogenized in a blender before being sieved using a 500µm mesh sieve. The LGV samples were then packed in Ziploc plastic bags and stored at -20°C until used (Mavhungu, 2011).

3.2.3 Sample extraction

Several different solvents can be used to extract the polyphenol, flavonoids and β-carotene from LGV and the best solvent is dependent on the chemical composition and complexity of the polyphenols which can be simple to highly polymerized polyphenols (Sreeramulu, *et al.,* 2013). Some researchers have used, acetone-water, ethanol-water, methanol-water, and only water (Mavhungu, 2011). As this study is a nutritional study and the aim is to evaluate the effect of digestion on polyphenolics, flavonoids and β-carotene content as well as antioxidant activity, water extraction was used. LGV samples were extracted using a method by Sumazian *et al.* (2010), which was slightly modified. Briefly, 0.2g of the freeze-dried samples were mixed with 20ml distilled water and put on a shaker (130 rpm) to mix for 1 hour. The extract was then further centrifuged, using a Hermle Z300 centrifuge, supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, SA, at 965.95 xg for 2 minutes, and then the supernatant was stored at -20ºC until required. The polyphenol, flavonoids and β-carotene content and associated antioxidant activity of the cooked (30) were compared to the uncooked (0) samples determined as described in Section 3.3 and 3.4.

3.2.4 Simulated digestion processes

Two different digestion models were used. The first involved only the gastric and intestinal phases of digestion (GI) and was according to the method described by Liyana-Pathirana and Shahidi (2005). The second model was more complex and included the oral, gastric and intestinal phases of digestion (OGI) which included the presence of bile acids and lipase. The method used was adapted from a protocol kindly provided by Ferruzzi Laboratory, of Purdue University, USA.

3.2.4.1 Solutions and enzymes

Preparation of 1M HCl

Using a 99% hydrochloric acid, 36.46 ml of the acid was mixed with 1L double distilled, deionized water ($dddH_2O$) to prepare 1M HCl.

Preparation of 1M $NaHCO_3$

To prepare the 1M $NaHCO_3$, 84g of sodium hydrogen carbonate was dissolved in $dddH_2O$ and mixed well and made to a final volume of 1L.

Preparation of the oral base solution

A mass of 1.792 g potassium chloride, 1.776 g sodium phosphate, 1.140 g sodium sulphate, 0.596 g sodium chloride and 3.388 g sodium hydrogen carbonate were dissolved in a final volume of 1L $dddH_2O$ and the final pH was adjusted to 6.5.

Preparation of pepsin solution

To make 1 ml of the pepsin solution, 20 mg of the porcine pepsin was dissolved in one ml of 1M HCl and was shaken gently until dissolved.

Preparation of pancreatin solution

To make 1 ml of the pancreatic solution, 4 mg of the pancreatin was dissolved in one ml of 1M $NaHCO_3$ and was shaken gently until dissolved.

Preparation of the lipase solution

Lipase solution was made by dissolving 2 mg of the lipase in 1 ml of 1M $NaHCO_3$.

Preparation of the bile solution

The bile solution was made by dissolving 0.045 mg of the bile in one ml of 1M $NaHCO_3$.

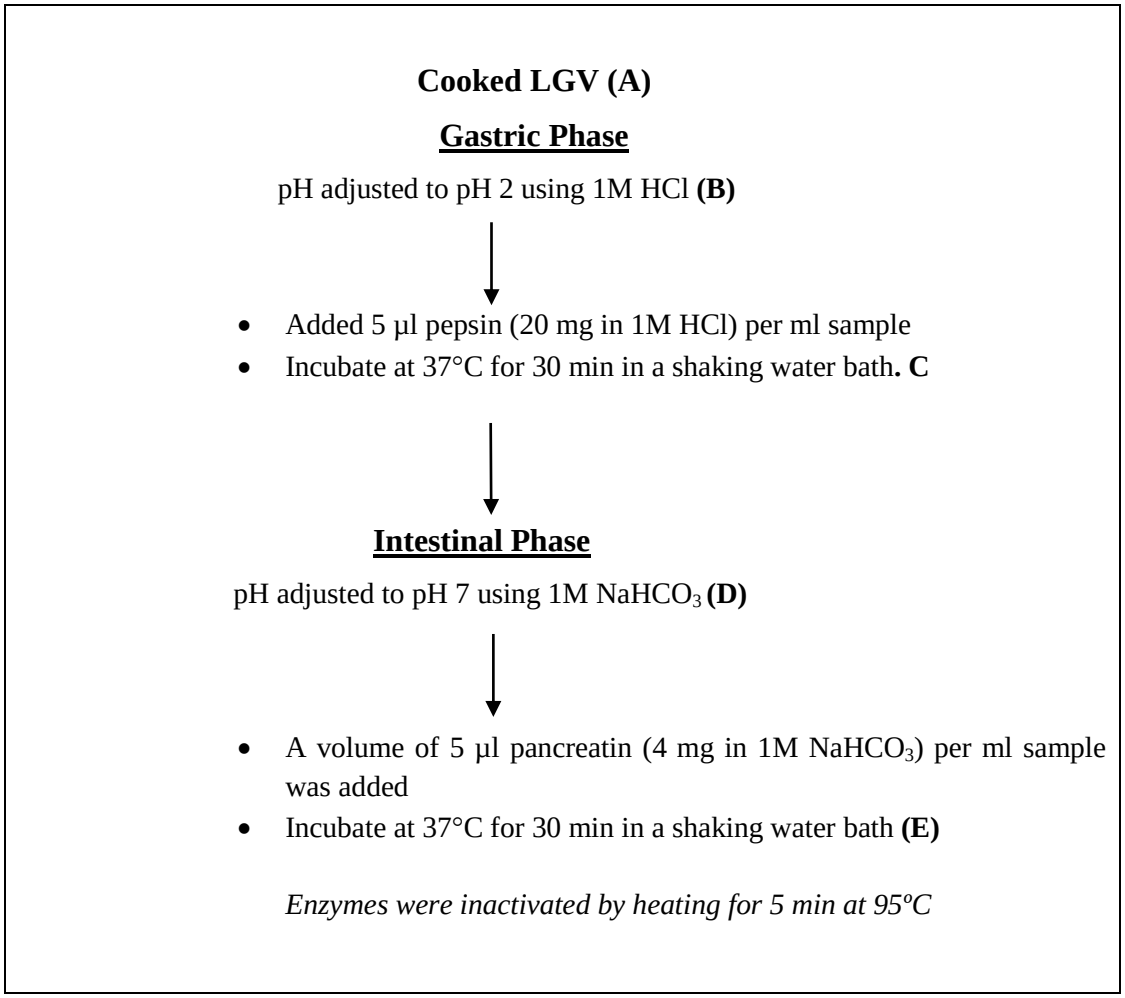


Figure 3.1: Flow diagram of the simulated GI digestion procedure, involving only the gastric and intestinal phases of digestion.

Samples A, B, C, D and E were collected. Each fraction was evaluated for polyphenol, flavonoids and β-carotene content and associated antioxidant activity as described in Section 3.3 and 3.4. To determine the effect of pH, B and D were compared. To determine the effect of the digestive enzymes, C was compared to B and E was compared to D. Lastly the bio-availability was determined by comparing A and E

Cooked LGV

Oral Phase

- Added 6 ml oral base solution
- Added 0.190 g α-amylase
- Then 10 ml distilled water was added to prepare a slurry
- Mixture was vortexed for 5 min, horizontally on a shaker and then was placed in a water bath at 37°C for 10 min **(A)**

↓

Gastric Phase

- Volume was increased to 30 ml with distilled water
- pH was adjusted to pH 4 using 1M HCl
- A volume of 5 µl pepsin (20 mg/ml) per ml sample was added
- pH was adjusted to pH 2.5 using 1M HCl
- Volume was increased to 30 ml with distilled water
- The sample placed horizontally on shaking water bath and incubated at 37°C for 1 hr **(B)**

↓

Intestinal Phase

- pH was adjusted to pH 4 using 1M $NaHCO_3$
- A volume of 5 µl pancreatin (4 mg/ml) and lipase (2 mg/ml) per ml sample was added
- Then 7.5 µl /ml of the bile solution was added
- Incubated at 37°C for 2 hour in a shaking water bath
- pH was adjusted to pH 6.5 with 1M NIO_3 **(C)**

Samples were heated after each phase for 5 min at 95ºC

Figure 3.2: Flow diagram of simulated OGI digestion procedure, involving the oral, gastric and intestinal phases of digestion.

Samples (A-C, Figure 3.2) were collected. Each fraction was evaluated for polyphenol, flavonoids and β-carotene content and associated antioxidant activity as described in sections 3.3 and 3.4. Activities of A was compared to B and C as well as B was compared to C.

3.3 Determination of the polyphenol, flavonoid and β-carotene content of LGV

3.3.1 Total polyphenol content (TPC)

The total polyphenol content (TPC) was determined using a method by Amin, Norazaidah and Hainida (2006). The method was modified for a 96 well format. A sample of 10 µl, vegetable extract was pipetted to each well of a 96 well microplate and mixed with 50 µl of Folin-Ciocalteau (F-C) reagent. Then 50 µl of a 7.5% of sodium carbonate solution was added and mixed well. The solution was allowed to stand in the dark for 90 min and the absorbance was read at 630 nm. The TPC of each sample was calculated from the standard calibration curve, which was prepared using gallic acid (0 – 0.046 mg/ml) and TPC was expressed as mg gallic acid equivalent (GAE)/g dry weight (DW).

3.3.2 Total flavonoid content (TFC)

The total flavonoids content (TFC) was determined using a method by Amin, Norazaidah and Emmy Hainida (2006) as described by Serem and Bester (2012). A 10 µl vegetable extract was pipetted to each well of a 96 well microplate. Then 30 µl of 2.5% sodium nitrite was added, followed by 20 µl of 2.5% aluminium chloride and lastly 100 µl of 2% sodium hydroxide and mixed well. The absorbance was read at 450 nm. The TFC of all samples was calculated from the standard calibration curve, which was prepared using catechin (0 – 0.042 mg/ml) and TFC was expressed as mg catechin (CE)/g DW.

3.3.3 β-carotene determination

The standard preparation of beta-carotene was as described by Ahamad *et al.* (2007). ß-Carotene (1 mg) was mixed with 10 ml tert-butyl-methyl ether (MTBE) (concentration equal to 100 ppm). The standard solution was diluted to different concentrations and these were 1, 5, 10, 15 and 20 ppm.

For the extracts, 5 ml was mixed with 2.5 ml MTBE. Extraction was done with gentle inversion for a few times being careful not to mix the aqueous and organic phase into an emulsion. The organic phase was aspirated, and the process was repeated with another 2.5 ml MTBE. The extraction was centrifuged (Rotanta 460R Hettiech, Labotec, SA) at 1509 *x* g for 5 min and filtered through 0.2 µm PTFE syringe directly into amber vials. For powder samples, 20 mg of sample was mixed with 2 ml MTBE and vortex mixed for an hour. It was

then centrifuged as above and then the supernatant was filtered through a 0.2 µm PTFE syringe directly into amber vials.

A Prominence Ultra-Fast Liquid Chromatograph equipped with a SIL – 20A Prominence auto-sampler, a DGU – $20A_3$ Prominence degasser, a CTO – 10AS VP Shimadzu column oven and a SPD – M20A Prominence diode array detector installed in the department of Food Science, University of Pretoria was used. The HPLC was calibrated by running mobile phase (methanol: MTBE), ratio 80:20 respectively at a rate of 0.8ml per minute. The concentration of the β- carotene standards was plotted against the peak area to obtain a straight line. β-carotene of the samples was identified based on retention time and the concentration was determined based on the peak area using the β-carotene standard curve and the concentration was expressed as mg/100g DW.

3.4 Determination of total antioxidant activity in LGV

Antioxidant activity determination using the electron transfer methods, the 1, 1-diphenyl –2-picrylhydrazyl (DPPH) and Trolox equivalent antioxidant capacity (TEAC) assays as well as the hydrogen atom transfer method, the oxygen radical absorbance capacity (ORAC) assay.

3.4.1 ABTS assay

The TEAC assay is based on the neutralization of radical cations formed by a single electron oxidation of a synthetic ABTS chromophrone to a strongly absorbing $ABTS^{\cdot+}$ radical (Stratil, *et al.,* 2006). A decrease in $ABTS^{\cdot+}$ concentration is linearly dependent on the antioxidant concentration. The reaction, is pH independent and according to Thaipong, *et al*, (2006) it is difficult to compare findings between studies as the concentration of ABTS solutions as well as the incubation times differ. 2,2'-Azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diaminium salt ($ABTS^{\cdot+}$) solution was generated by adding 3 mM of potassium peroxodisulfate solution ($K_2S_2O_8$) to 8 mM ABTS and the mixture was left to react in the dark for 12 hours. The working solution was prepared by diluting ABTS stock solution with 0.2 M phosphate buffer, pH 7.4. The concentration range of the Trolox standard was 0 – 1000 µM. A 10µl volume of each sample was added to 290 µl of ABTS solution and the absorbance was measured at 630 nm after an incubation time of 30 and 15 min for the samples and standards respectively. Results were expressed in µM TE/g DW.

3.4.2 DPPH assay

DPPH is one of the widely used methods for screening antioxidant activity of plant extracts (Subasree, *et al*., 2009). The DPPH assay provides a measure of free radical scavenging antioxidant activity, when DPPH reacts with a hydrogen donor and the reduced form of DPPH is generated (Sumazian, Syahida, Hakiman and Maziah, 2010). In this reaction the purple coloured stable free radical decolourises to a yellow colour when it is reduced to a diphenylpicrylhdrazine complex. According to Sharma and Bhat (2009), the reaction is pH dependent, influenced by the polarity of the reaction medium, the chemical structure of the radical scavenger and is light sensitive.

A stock solution of 24 mg DPPH dissolved in 100 ml methanol was prepared and stored at -20°C until needed. The working solution was prepared by diluting 20 ml of stock solution with 80 ml methanol. A Trolox, 25 mg/ml stock solution was prepared which was diluted to a final concentration of 0.1 to 1 µg/ml in methanol. A volume of 285 µl of DPPH was added to 15 µl of a sample and after 15 min in the dark the absorbance was read at 570 nm using a Biotek ELX 800 plate reader. Results were expressed in µM TE/g DW.

3.4.3 ORAC assay

The antioxidant activity of phenolic compounds is mainly due to their redox properties which play an important role in absorbing and neutralising free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Zheng and Wang, 2001). The ORAC assay is based on the ability of antioxidant compounds in samples to inhibit the decline in the fluorescence of fluorescein induced by a peroxyl radical generator, AAPH.

The procedure used is based on a method by Ou *et al.,* (2002). AAPH was used as a peroxyl radical generator, Trolox as standard (0 – 1000µM) and fluorescein as a fluorescent probe. PBS was used as a blank. To 5 µl volume of the sample, 160 µl volume of fluorescein working solution and 40 µl of a 0.11 µM AAPH solution was added. Samples were mixed well, and the microplate placed into the plate reader and incubated at 37°C. The fluorescence was measured every 5 min for 2h. Trolox 0 -1000 µM was used to generate a standard curve. For the standards and samples, the area under curve was calculated and all activity was expressed as µM TE/g DW.

3.5 Data management and statistical analysis

All assays consisting of triplicate data points were done in triplicate giving 9 data points. Quantification of β-carotene levels was the average of duplicate determinations. Results were recorded as an average of a mean ± SEM. A one-way analysis of variance (ANOVA) was carried out using GraphPad Prism 6 to determine correlation between antioxidant activity assays using Fischer's LSD ($p \leq 0.05$).

CHAPTER 4: RESULTS AND DISCUSSION

A: Effect of cooking on the polyphenol, flavonoid, B-carotene and antioxidant activity of LGV

INTRODUCTION

The different methods of cooking, as well as the time taken to cook the vegetables is solely dependent on individual preference in different communities. The cooking fluid maybe discarded and, in some instances, retained (Mavhungu, 2011) and this makes comparisons between different studies difficult. Plant based factors are differences in the genetic type as well as the geographical areas where these LGVs are grown as rainfall and soil factors will have an impact on the measured levels of nutrients and polyphenols (Hayouni *et al.,* 2007). Laboratory based experimental factors that further makes comparisons difficult and these include the methods used for sample preparation, extraction and quantification. Alcohol (methanol/ethanol) extraction methods are generally used to quantify the total polyphenol content and antioxidant activity of plants, however it provides little information specifically on polyphenols that are water soluble and physiologically relevant i.e. those nutrients, such as polyphenols and β-carotene that are bio-available and/or bioaccessible would have health benefits.

Many studies evaluate the antioxidant content and activity of raw LGV extracts, however many of these LGV are cooked and this can result in a decrease in the nutritional, antioxidant content and activity of LGV, as observed in studies by Mirzaei *et al*, (2014; Oboh (2004), Kao *et al*, (2014), Jimenez-Monreal *et al*, (2009) and Medoua and Oldewage-Theron, (2014). Cooking changes the physical characteristics and chemical composition of vegetables and is a function of temperature, time and type of cooking. Mavhungu (2011) evaluated the effects of cooking on the antioxidant activity of African LGV and found that cooking had variable effects on antioxidant content and activity. Turkmen *et al*., (2005), also observed variable effects where cooking significantly decreased the total antioxidant compounds of certain vegetables, although for spinach the compounds did not change significantly.

In this first part of this study the effect of cooking on the polyphenol and β-carotene content and antioxidant activity of a selection of African LGV was determined.

4.1 RESULTS

African LGVs were cooked for 30 min, at 100^0C, the water was discarded, the LGVs were then freeze-dried and extracted with water. The colour, antioxidant and β-carotene content as well as antioxidant activity of water extracts of each cooked African LGV was determined.

LGV	Raw/uncooked	Cooked
Spinach		
Pumpkin		
Amaranth		
Cowpea		
Jute mallow		

Figure 4.1: The effect of 30 min cooking on the colour of raw LGV

4.1.1 Effect of cooking on the colour of cooked African LGV

Cooking had variable effects on the colour of the African LGV. The colour of spinach and pumpkin leaves became more intense while the colour of amaranth, cowpea and jute mallow changed from green to a green brown colour, Figure 4.1.

4.1.2 Total phenolic content of raw and cooked African LGV

The TPC of all raw LGV ranged from 194.4 to 357.4 GAE/g, and 43.7 to 212.6 GAE/g, following cooking for 30 min. The highest raw TPC for LGV was for cowpea and the lowest was for amaranth. Following cooking, normally used for the preparation of LGV, the TPC was the highest for amaranth and the lowest for pumpkin leaves. For amaranth and for jute mallow there was no significant changes in TPC levels with cooking. For cooked spinach, pumpkin and cowpea only 30.41%, 17.93% and 38.37% was bio-avaliable. In contrast for amaranth and jute mallow the TPC was unchanged (Figure 4.2, Table 4.1). For all cooked LGV, per DW, amaranth, jute mallow and cowpea had higher TPC than spinach.

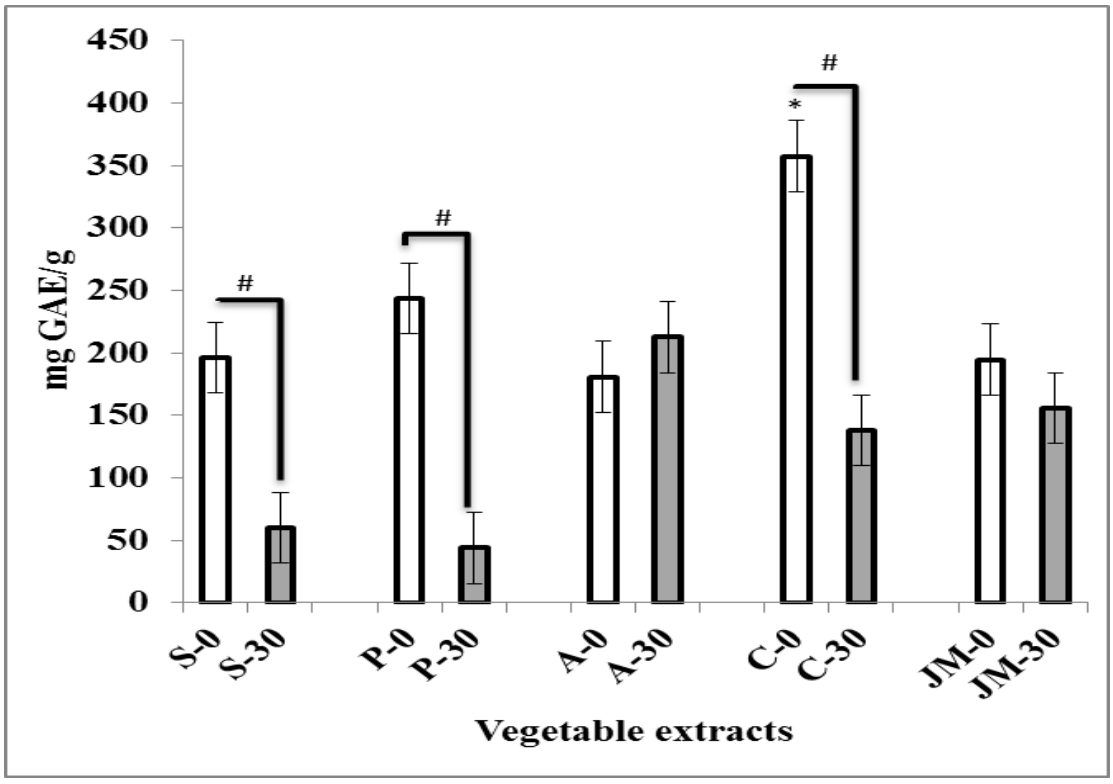


Figure 4.2: TPC of raw (0) and 30 min (30) cooked LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. * Indicates raw (0) LGV with the significantly highest TPC, # shows significant differences in TPC between raw (0) and cooked (30) LGV, $p<0.05$.

4.1.3 Total flavonoid content of raw and cooked LGV

The TFC of all raw LGV ranged from 61.4 to 120.1 mg/g CE, and 25.0 to 159.1 mg/g CE following cooking for 30 min. The highest TFC was found for cowpea and the lowest amaranth leaves. Following cooking jute mallow had the highest TFC and spinach and pumpkin leaves the lowest. A statistically significant loss in TFC was observed for spinach, pumpkin and cowpea leaves. In contrast the TFC of amaranth and jute mallow increased significantly. For spinach, pumpkin and cowpea, the bio-available was 39.00%, 30.63% and 33.83% respectively. The TFC of amaranth and jute mallow was increased by 1.66 and 1.06 fold respectively. (Figure 4.3, Table 4.1). After cooking, the TFC per gram DW of jute mallow and amaranth was greater than spinach.

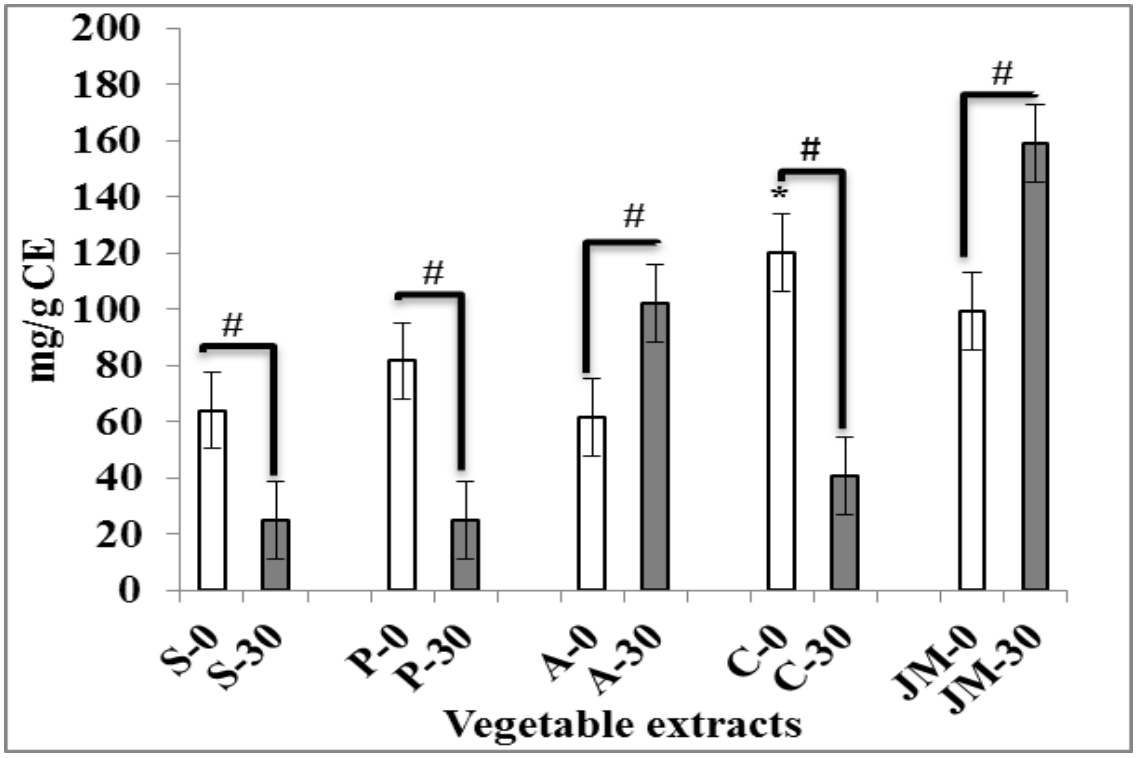


Figure 4.3: TFC of raw (0) and 30 min (30) cooked LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. * Indicates raw (0) LGV with the significantly highest TFC, # shows significant differences between the TFC of raw (0) and cooked (30) LGV, $p<0.05$.

4.1.4 β-carotene content of raw and cooked LGV

The β-carotene content of the raw LGV varied from 4.1 mg/100g – 46 mg/100g while following 30 min cooking levels varied from 15.5 to 42.9 mg/100g. Raw spinach had the highest levels of β-carotene and amaranth the lowest. Following cooking the highest levels

were measured for spinach and the lowest for amaranth and pumpkin. Cooking did not affect the β-carotene content of spinach, pumpkin or cowpea leaves while cooking caused β-carotene levels of cooked amaranth and jute mallow to increase significantly. For amaranth and jute mallow the fold increase in β-carotene was 3.78 and 1.58, respectively and for spinach, pumpkin and cowpea, it was unchanged (Figure 4.4). After cooking spinach had the highest β-carotene levels.

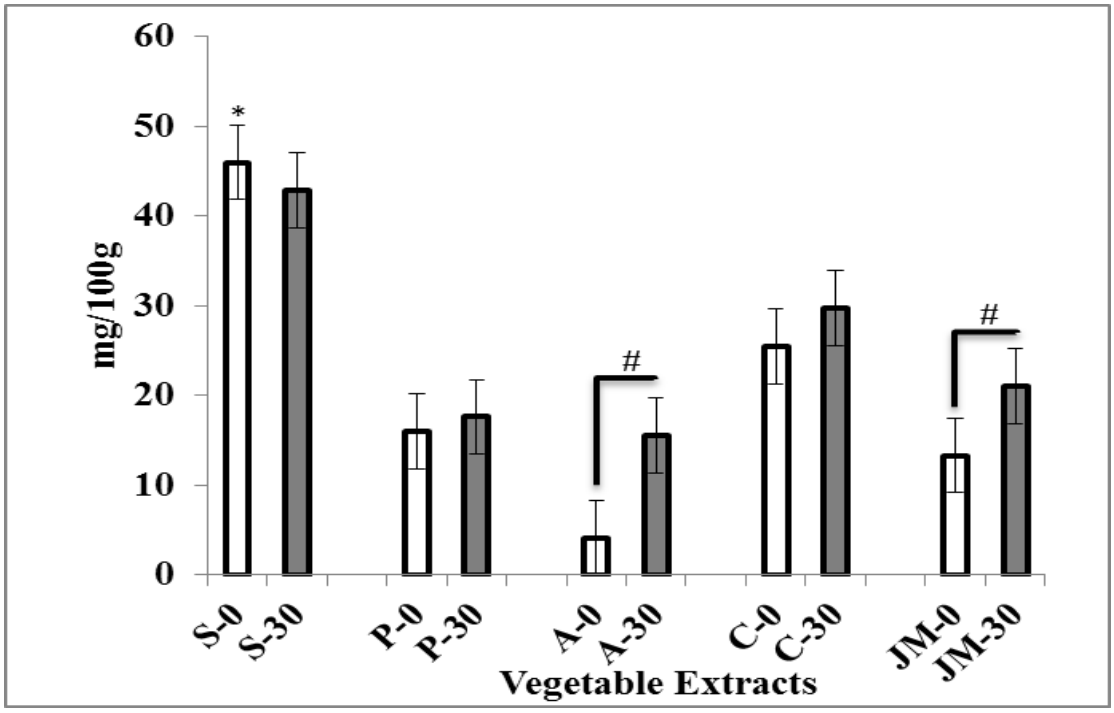


Figure 4.4: β-carotene content of raw (0) and 30 min (30) cooked LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. * Indicates raw (0) LGV with the significantly highest β-carotene content, # shows significant differences between raw (0) and cooked (30) LGV, $p<0.05$.

4.1.5 Antioxidant activity of raw and cooked LGV – TEAC assay

Antioxidant activity was measured with the TEAC assay and the range of antioxidant activity for the raw LGV was 283.4 – 336 mM TE/g and following cooking was 114.6 – 342.3 mM TE/g. Cooking for 30 min had variable effects on antioxidant activity and for amaranth and jute mallow antioxidant activity was unchanged while with spinach, cowpea and pumpkin leaves activity was significantly reduced. Pumpkin leaves were most sensitive to the effects of cooking where antioxidant activity measured with the TEAC assay decreased from 336 mM TE/g to 114.6 mM TE/g. Bio-availability was reduced to 56.86%, 34.10% and 84.34%

for spinach, pumpkin and cowpea respectively. Cooking did not affect the antioxidant activity of amaranth and jute mallow measured with the TEAC assay (Figure 4.5, Table 4.1). After cooking, amaranth, jute mallow and cowpea had better antioxidant activity than spinach.

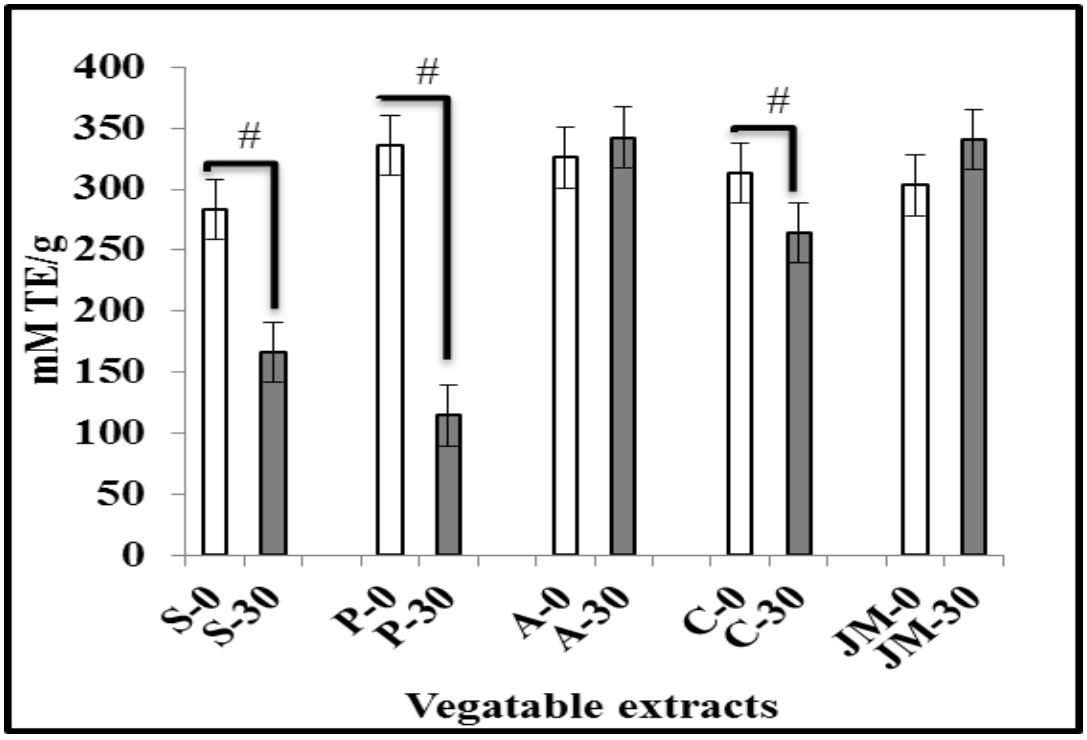


Figure 4.5: Antioxidant activity (TEAC assay) of raw (0) and 30 min (30) cooked LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. * Indicates raw (0) LGV with the significantly highest antioxidant activity, # shows significant differences between raw (0) and cooked (30) LGV, $p<0.05$.

4.1.6 Antioxidant activity of raw and cooked LGV – DPPH assay

The DPPH assay was also used to measure antioxidant activity and the range of antioxidant activity was -8.4 to 288.7 mM TE/g. The antioxidant activity of raw LGV was the highest for raw pumpkin and cowpea leaves and the lowest for spinach. With cooking the range of antioxidant activity was -6.6 to 266.9 mM TE/g. The highest antioxidant activity was measured for amaranth and the lowest for spinach and pumpkin leaves. Cooking for 30 min had variable effects on antioxidant activity. For spinach antioxidant activity remained low while there was a significant loss of antioxidant activity for pumpkin and cowpea leaves. In contrast cooking increased the antioxidant activity of amaranth and jute mallow from 18.7 to 266.9 mM TE/g and 26.5 to 204.1 mM TE/g respectively. Following cooking, the bio-availability for pumpkin and cowpea was 12.02% and 19.22% respectively. In contrast,

antioxidant activity following cooking was 14.27 and 7.70 fold increased for cowpea and jute mallow respectively. The antioxidant activity of spinach was low and remained unchanged with cooking. (Figure 4.6, Table 4.1). The antioxidant activity of amaranth, jute mallow and cowpea following cooking for 30 min was greater than spinach.

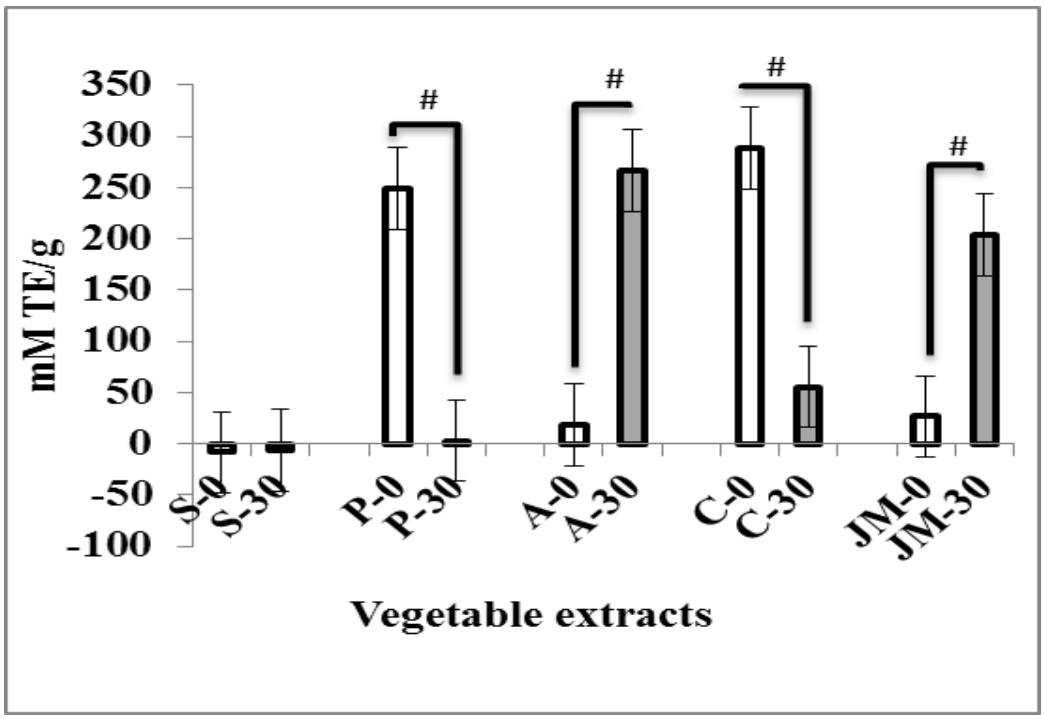


Figure 4.6: Antioxidant activity (DPPH assay) of raw (0) and 30 min (30) cooked LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. * Indicates raw (0) LGV with the significantly highest antioxidant activity, # shows significant differences between raw (0) and cooked (30) LGV, $p<0.05$.

4.1.7 Antioxidant activity of raw and cooked LGV – ORAC assay

Antioxidant activity was measured with the ORAC assay and the range of antioxidant activity for the raw LGV was 467.8 to 1288.5 mM TE/g. The antioxidant activity of the raw LGV was the highest for cowpea and the lowest for spinach with values of 1288.5 and 467.8 mM TE/g respectively. With cooking for 30 min the range of antioxidant activity was 226.7 to 763.1 mM TE/g. Variable effects was observed on antioxidant activity measured with the ORAC assay, antioxidant activity was reduced for all LGV, except for jute mallow where with cooking antioxidant activity was unchanged. The bio-availability of spinach, pumpkin, amaranth and cowpea was reduced to 53.96%, 34.40%, 41.97% and 46.55% respectively (Figure 4.7, Table 4.1). Antioxidant activity of jute mallow, cowpea and amaranth was greater than spinach, following cooking for 30 min.

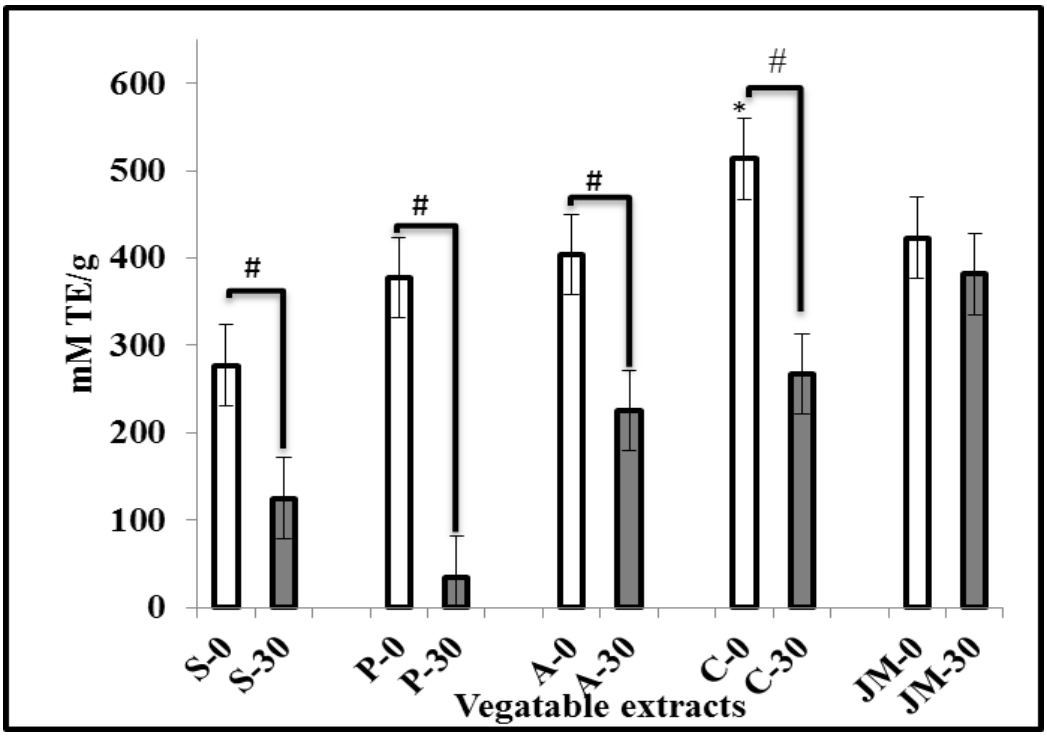


Figure 4.7: Antioxidant activity (ORAC assay) of raw (0) and 30 min (30) cooked LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. * Indicates raw (0) LGV with the significantly highest antioxidant activity, # shows significant differences between raw (0) and cooked (30) LGV, $p<0.05$.

The polyphenol, flavonoid and β-carotene content as well as the antioxidant activity of LGV is summarised in Table 4.1.

For spinach, the antioxidant content measured with the TPC and TFC assays and antioxidant activity measured with the TEAC and ORAC assays was reduced following cooking. In contrast antioxidant activity measured with the DPPH assay was unchanged. -Carotene levels were unchanged. Somewhat similar to spinach, the TPC and TFC of pumpkin was reduced as well as antioxidant activity measured with all assays. ß-Carotene levels were also unchanged.

For amaranth with cooking, the TFC, β-carotene and antioxidant activity measured with the DPPH assay was increased. Antioxidant content and activity measured with the TPC and TEAC assays were unchanged while antioxidant activity measured with the ORAC assay was reduced. For cowpea, with cooking, TPC and TFC as well as antioxidant activity measured with the TEAC, DPPH and ORAC assays were reduced. Only β-carotene levels were

unchanged. With cooking jute mallow, TFC, β-carotene levels and antioxidant activity measured with the DPPH was increased while TPC and antioxidant activity measured with the TEAC and ORAC assays was unchanged. Of the African LGV evaluated, following cooking jute mallow, amaranth and cowpea consistently had better antioxidant content and activity than spinach. In contrast spinach had the highest β-carotene content after cooking.

Table 4.1: Summary of polyphenol, flavonoid, β-carotene content and antioxidant activity of raw and cooked LGV

		TPC (mg GAE/g)	TFC (mg/g CE)	β-carotene (mg/100g)	TEAC (mM TE/g)	DPPH (mM TE/g)	ORAC (mM TE/g)
Spinach	0	196.3±9.7	64.1±5.9	46.0±0.2	283.4±15.6	-8.4±2.8	467.8±7.2
	30	59.7±5.0	25.0±2.5	42.9±0.1	166.3±10.4	-6.6±2.4	252.6±16.0
% Bio-availability		**30.41%**	**39.00%**	93.26%	**56.86%**	-	**53.96%**
Pumpkin	0	243.6±19.3	81.6±7.8	16.0±0.08	336.0±4.6	249.4±8.0	659.0±12.2
	30	43.7±2.8	25.0±3.2	17.6±0.02	114.6±7.6	3.0±5.4	226.7±5.3
% Bio- availability		**17.93%**	**30.63%**	110	**34.10%**	**12.02%**	**34.40%**
Amaranth	0	180.7±2.6	61.4±13.6	4.1±0.02	325.9±8.5	18.7±6.6	690.0±8.5
	30	212.6±8.2	102.2±4.0	15.5±0.3	342.3±7.9	266.9±9.8	289.6±2.4
% Bio- availability		117.65%	**166.44%**	**378.04%**	104.94%	**1427.27%**	**41.97%**
Cowpea	0	357.4±4.3	120.1±4.0	25.5±0.8	313.2±9.9	288.7±1.6	1288.5±2.3
	30	137.9±4.0	40.6±1.0	29.8±0.2	264.0±10.7	55.5±2.5	599.6±11.5
% Bio- availability		**38.37%**	**33.83%**	116.86	**84.34%**	**19.22%**	**46.55%**
Jute mallow	0	194.4±13.1	99.2±7.6	13.3±0.2	303±10.9	26.5±1.5	827.9±2.4
	30	155.7±7.7	159.1±7.7	21.0±0.04	340.7±7.5	204.1±6.5	763.1±1.3
% Bio- availability		80.09%	**160.38%**	**157.89%**	112.44%	**770.19%**	92.26%
Correlation 0 vs 30		0.167	**0.713**	-0.483	-0.044	-0.468	0.656

Data is an average of at least 3 experiments; Mean ± SD values. Bold indicates changes in bio-accessibility.

In Table 4.1, the correlation between the raw (0) and cooked (30) was 0.713 for TFC and low and negative for all other assays, which implies that cooking does not alter the flavonoid content and but does alter TPC and associated antioxidant activity.

In Table 4.2, data generated for cooked LGV for each assay (Table 4.2) was correlated. A strong correlation of 0.714 was found between TPC and TFC and antioxidant activity measured with the TEAC assays. A strong correlation was found between TFC and antioxidant activity measured with the DPPH and ORAC assays. This implies that although with cooking the antioxidant activity is altered it still correlates with polyphenol content and specifically the flavonoids.

Table 4.2: Correlation between assays for cooked LGV

	TFC	β-carotene	TEAC	DPPH	ORAC
TPC	**0.714**	-0.463	**0.955**	-0.159	0.178
TFC		-0.012	0.179	**0.860**	**0.657**
β-C			0.360	-0.614	-0.079
TEAC				**0.912**	**0.626**
DPPH					0.355

4.2 DISCUSSION

Following cooking a change in colour of the LGV was observed. The green colour of LGV is due to chlorophyll and a decrease in colour is due to the formation of pheophytin by the exchange of Mg^{2+} with H^{+} in the centre of the porphyrin ring of chlorophyll (Pellegrini *et al*, 2010). In Figure 4.1 spinach and pumpkin leaves increased their greenness after cooking and this could be due to altered surface reflecting properties and depth of light penetrating into tissues of the boiled LGV, caused by air loss and loss of other dissolved cellular gases and their replacement by cooking water and cell juices. An unaltered colour after cooking is often also as a result of using certain additives like baking soda, which reacts with chlorophyll and transform it into chlorophyllin, a light green coloured molecule. Jute mallow, amaranth and cowpeas lost their green colour (chlorophyll) after cooking and turned to a brownish colour (pheophytin). The extent of this conversion is directly proportion to time and temperature of the cooking water (Pellegrini *et al*, 2010). In addition, flavonoids are often coloured molecules and with cooking, leaching of these flavonoids results in dulling of the colour of

LGV. This may also be an effect observed with cowpea but not with jute mallow or amaranth (Kunyanga *et al.,* (2011)).

Many different methodologies can be used to determine antioxidant activity and these include the DPPH, ABTS the ferric reducing antioxidant power (FRAP), the ORAC, the hydroxyl radical averting capacity (HORAC) the total peroxyl radical trapping antioxidant (TRAP), the lipid peroxidation inhibition assay and the potassium ferricyanide reducing power (PFRAP) assays. Generally these assays can be divided into two major groups of assays and these are the electron transfer (ET) and the hydrogen atom transfer (HAT) assays (Apak *et al.*, 2007).

The ET assays involve measuring the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. ET assays include the ABTS/TEAC, CUPRAC, DPPH, Folin-Ciocalteu and FRAP assays, with each using different chromogenic redox reagents with different standard potentials (Apak *et al*., 2007). The most widely used ET assays are the TEAC and DPPH assays. The majority of HAT assays such as the ORAC assay is kinetics based, and involves a competitive reaction in which antioxidant and substrate compete for peroxyl radicals thermally generated through the decomposition of azo compounds.

Arnao (2000), describes $DPPH^{\cdot}$ as a free radical that is acquired directly without preparation, while $ABTS^{+}$ is generated by enzymatic or chemical reactions. The TEAC assay measures the antioxidant activity of both hydrophilic and lipophilic polyphenols (Arnao, 2000). As opposed to $ABTS^{+}$, $DPPH^{\cdot}$ can only be dissolved in organic media, which means that antioxidant activity measurement is only limited to hydrophobic antioxidants. The $ABTS^{+}$ radical is generated from its precursor and presents a spectrum with three absorbance maxima at 414, 713 and 873 nm while $DPPH^{\cdot}$ has a peak maximum at 515 nm.

The simplest way to measure antioxidant activity, is firstly, to dissolve the radical chromogen in an appropriate medium, then to add the antioxidant. The loss of the radical chromogen is determined using a spectrophotometer, by observing the decrease in absorbance at a fixed time, and then finally correlating the decreases in a dose-response curve with a standard antioxidant (Trolox or ascorbic acid) (Arnao, 2000; Pisoschi and Negulescu, 2011). Problems associated with these assays is interference due to the presence of coloured compounds in the samples or due to the formation of secondary reaction products between the chromogen and the sample. Most researchers advise that a wavelength that is far from the visible region

should be chosen to avoid interferences. Moniruzzaman *et al.,* (2012) noted that with the ABTS radical, the problem of interferences is not of concern, since the chromogen presents absorbance peaks at 730 and 842 nm, whereas with the DPPH the problem is more serious since it does not presents bands higher than 515 nm (Arnao, 2000).

When evaluating the antioxidant activity of plant extracts interference by plant material for example chlorophyll has strong absorbance– at 700 - 715 nm (Cendrero-Mateo *et al*., 2016). The wavelengths near the visible region the measured antioxidant activity is underestimated due to sample interferences, as occurs with ABTS at 414 nm and DPPH at 515 nm. However, in samples with a high antioxidant activity dilution of samples reduces interference.

4.2.1 Effect of cooking on the antioxidant and the β-carotene content as well as antioxidant activity of ALGVs

4.2.1.1 Effect of cooking on the polyphenol, flavonoids and β-carotene content of LGV

Results from different studies show that cooking has different effects on the antioxidant content and activity of vegetables (Kao, Chiu and Chiang, 2014) and this may be due to the concentration and type of polyphenols found in these vegetables and is a function of the geographical and climatic conditions as well as the age of the plant. Differences in polyphenol content have also been found between age of the leaves as well as the leaves and stalks (Shih *et al*., 2011). With cooking, polyphenols can leach from the plant material and when the water is discarded, these polyphenols are lost and consequently there is a measured decrease in polyphenol content and associated antioxidant activity. Alternatively, the polyphenols, remain as part of the LGV plant material and do not leach into the water used for cooking. An increase in antioxidant content/activity may be partly caused by heat induce release of polyphenols from the cell walls and subcellular compartments and/or due to the increased solubility of these polyphenols in water. Hydrophilic polyphenols will leach rapidly from the plant matrix. Adefegha and Oboh (2011), evaluated the effects of cooking on the antioxidant properties of some tropical LGVs such as (*Talinium triangulare, Ocimum gratissimum, Amaranthus hybridus, Telfairia occidentalis, Ipomea batata, Cnidoscolous aconitifolius, Baselia alba and Senecio biafrae leaves)*, found that most of the phenolic compounds were trapped in the fibre and with cooking of these LGVs became more available.

The plant matrix may play an important role on whether polyphenols leach from the plant material into the cooking water and are subsequently discarded. Therefore, alternative cooking methods such as pressure cooking, steaming and microwaving may reduce the loss of these important polyphenols (Zhang & Hamauzu, (2004); Mirzaei *et al*., 2014). Addition of starches that absorb the cooking fluid may be an alternative way to reduce the loss of these polyphenols. Examples of complex insoluble polyphenols are tannins, while gallic acids are smaller, highly water-soluble polyphenols (Cheynier, 2005).

With cooking, variable effects on TPC of African LGV was observed where with cooking the TPC of spinach, pumpkin, cowpea and jute mallow was reduced, while the TPC of amaranth was unchanged (Figure 4.2). The TPC of the studied LGVs was comparable to other studies. The levels of polyphenols in the cooked cowpea leaves extracts was several fold higher than reported by other researchers (137.9 – 357.4 mg GAE/g vs 2.4 – 29.1 mg GAE/g (Kruger *et al*., 1998; van der Walt *et al.,* 2009; Mavhungu, 2011; FAO (2003). For raw amaranth they reported TPC values of 2.1 to 186.7 mg GAE/g. The levels reported by Olajire and Azeez (2011) of 180.7 and 212.6 mg GAE/g for raw and cooked amaranth was similar to the findings of the present study. Bunea *et al.,* (2008) and Ko *et al.,* (2014), obtained 2.09 and 1.47 mg GAE/g for the TPC for raw spinach which was lower than the TPC found in the present study of 196.3 mg GAE/g. Chipurura (2010), in a study done in Zimbabwe concluded that indigenous LGV had higher TPC when compared to exotic vegetables. Likewise, in the present study after cooking the polyphenolic content of jute mallow, cowpea and amaranth was greater than spinach.

The TFC values ranged from 0.5 to 139.6 mg/g CE. These values fall within the same range as reported by Kruger *et al*., (1998), Mavhungu (2011); FAO (2003), Olajire and Azeez (2011). Application of heat during cooking causes changes in the structural integrity and cellular matrix of the vegetables and this changes the phytochemical properties of these vegetables (Mirzaei *et al*., 2014) An increase in the flavonoid content could be due to the breakdown of the cellular matrix, of pectin or cellulose and making the flavonoids more extractable. In contrast, cooking can lead to polyphenol decomposition and therefore a reduction in measured levels. Although the TPC and TFC of the LGV were altered, the degree changes varied. For example, amaranth and jute mallow's TPC remained the same and TFC increased after cooking. The TPC consists of phenolic acids and flavonoids and with

cooking the phenolic acids may degraded while there was an increase in the extraction of more stable flavonoids. As suggested by Mavhungu, (2011), the increase in the TPC and TFC of amaranth after cooking might be because when raw, the extract very viscous which might have made polyphenol extraction difficult and extractability improves after cooking.

β-Carotene content of all LGV are lower than reported by Kruger *et al.,* (1998), Mavhungu (2011) and Olaijire & Azeez (2011), which reported values between 100 to 1000 mg/100g. This variation in β-carotene content may be due to differences in sample preparation and/or methodologies used. Raw amaranth, cowpea and jute mallow contained 4.1, 25.5 and 13.3 mg/100g β-carotene respectively and the amount of β-carotene content increased after cooking for 30 minutes to 15.5, 29.8 and 21 mg/100g, respectively, as shown in figure 4.4. According to Kao, *et al* (2014), water cooking or boiling might be considered a suitable cooking method to preserve or enhance the nutritional qualities of vegetables although effects may be variable. An increase in total carotenoid content in boiled vegetables may be partly caused by cell wall and subcellular compartments upon boiling, hence the composition of the plant and cellular structural matrix is likely to be the determining factor whether β-carotenoids and other phytochemicals are extracted. Plant age will also be a determining factor where younger plants are more tender while the matrix of older plants is tougher and harder and consequently require a longer cooking time and this may also affect polyphenol extraction.

Dietz, Kantha and Erdman (1988), reported that the β-carotene content of spinach ranged from 1.5 mg/100g when raw to 112mg/100g when cooked, which is similar to the findings of the present study (table 4.1). In contrast to the present study, Simopoulos (2004), reported β-carotene content in spinach was as high as 63.5mg/100g. Amaranth and jute mallow had a significantly ($p \leq 0.05$) increased β-carotene levels after cooking. The β-carotene value of raw amaranth was low and contained 4.1 mg/100g compared to 8.7mg/100g reported by Veda *et al*., (2006).

Carotenoids are susceptible to oxidation, because of the presence of numerous double bonds (Hedren, Diaz and Svanberg, 2002), and the may account for the variability observed between studies especially if precautions are not taken to prevent oxidation. Additional sources of varaiation may rather be due to differences in experimental conditions, extraction

procedures, column length, diameter, temperature and solvents used as a mobile phase in HPLC (Ahamad *et al.*, 2007).

4.2.1.2. Effect of cooking on the antioxidant activity of LGV

In the TEAC assay, the ABTS cation radical which is a dark blue colour is formed by the loss of an electron by the nitrogen atom of ATBS. Addition of Trolox or of another hydrogen donating antioxidant, the nitrogen atom quenches the hydrogen atom, yielding a discoloured solution. The advantage of the TEAC assay is that it measures the antioxidant properties of both non-polar and polar antioxidant molecules.

A decrease in TEAC values is due to a decrease in polyphenols content after cooking, as there is a strong positive correlation between TPC and TEAC (table 4.3). These results were comparable to other studies such as that of Jimenez-Monreal, *et al.* (2009). In this study it was found that boiling cause a decrease in the antioxidant activity measured with the TEAC assay of vegetables when compared to their fresh counterparts. As shown in figure 4.5, pumpkin had the highest antioxidant activity when raw, whereas spinach had the least activity, however, when cooked, the antioxidant activity of pumpkin dropped drastically from 243.6 to 43.7 mM TE/g and was found to be the LGV with the lowest antioxidant activity while amaranth and jute mallow remained unchanged. This highlights that cooking has variable effects on antioxidant activity and that some African LGV has antioxidant activity greater than spinach.

In a study by Jimenez-Monreal, (2009), the percentage loss of ABTS radical anions scavenging by spinach when boiled was 11.1%, which is less than the 44% decrease in antioxidant activity measured with ABTS in the present study. This again highlights the variability between findings due to the plant-based factors and cooking methods.

According to Pisoschi and Negulescu (2011); Moniruzzaman *et al*., (2012), $DPPH^{\cdot}$ is a stable free radical, and its delocalisation determines the occurrence of a purple colour which it reacts with a hydrogen donor, the reduced form of DPPH is generated and this is associated with a loss of colour. Negative values (Table 4.1) which are dissimilar to the results found for the TEAC and ORAC assays may be due to the presence of coloured pigments that interfere or secondary reaction product formation between the chromogen and the vegetable extracts (Moniruzzaman *et al.*, 2012).

For the cooked samples there was a poor correlation between TPC and antioxidant activity measured with the DPPH assay, while in contrast there was a strong correlation between TFC and measured antioxidant activity. This implies that the flavonoids in LGV are resistant to the effects of cooking. For amaranth and jute mallow both TFC and β-carotene was increased following cooking and this was associated with an increase in antioxidant activity measured with the DPPH assay. A previous study has shown that the antioxidant activity measured with the DPPH assay was unaltered with cooking. This is in contrast ot the findings of the present study where antioxidant activity measured with the DPPH assay decreased significantly after cooking (from 288.7 to 55.5 mM TE/g).

The effects of cooking on the antioxidant activity are variable. Kunyanga *et al.* (2011), reported that the antioxidant activity of LGVs, pumpkin and amaranth, was different after processing. With cooking or blanching the antioxidant activity of pumpkin leaves were increased while that of amaranth leaves remained unchanged when cooked but decreased slightly with blanching. In the present study it was found that antioxidant activity for pumpkin leaves reduced considerably while antioxidant activity for amaranth was increased.

ORAC assay is a HAT method for the measurement of antioxidant activity. In this assay the sample to be tested (i.e. the antioxidant) is combined with a fluorescent compound (fluorescein) as well as a free radical generator such as 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH). As free radicals are being generated, the fluorescein is damaged and causing a loses in fluorescence (Moniruzzaman *et al.,* 2012). The ability of antioxidants such as polyphenols found in LGV to protect fluorescein against peroxyl radicals is measured. Compared to the TEAC and DPPH assay, the ORAC assay is considered to be physiologically relevant. The reason for this is that the peroxyl radicals generated from AAPH is a common source of cell and tissue damage in humans.

Of the LGV evaluated cowpea had the highest ORAC value which decreased significantly after cooking. Cooking caused a significant decrease in antioxidant activity for spinach, cowpea, pumpkin and amaranth, although the activity of jute mallow did not change. In contrast, Ko *et al.,* (2014) reported that there no significant difference in the ORAC values of raw and cooked spinach and values were between 252.5 and 467.8 µM TE/g. Ou *et al*.,

(2002) reported ORAC values of spinach between 12 and 300 µM TE/g. These values were similar to the ORAC values determined for spinach in the present study (Table 4.1).

Subramanian (2009), reported changes in ORAC values with cooking for pumpkin and amaranth of 76 to 84 µM TE/g and 78 to 88 µM TE/g, respectively. In the present study the ORAC values were several folds higher the bio-availability of pumpkin and amaranth was reduced to 34.40 and 41.97% respectively.

Differences between studies are not only due to LGV factors such as the composition of the food matrix, stability of polyphenols and the cooking method but also the design of the experiment. In an attempt to accurately mimic traditional cooking methods the cooking water was discarded. In this process water soluble vitamins and polyphenols are lost. Usually the water is discarded as the water and heating is generally seen as a method to kill bacteria, facilitate softening of the plant material and to remove any anti-nutrients. However, as shown in this study there is a loss of water soluble polyphenols. Besides water extraction of these polyphenols heat can contribute to the degradation of water soluble vitamins and polyphenols. The findings of this part of the study is that alternative methods of preparation should be investigated, that may involve addition of potatoes, rice and other starches that can absorb the water containing the water-soluble vitamins and polyphenols and that would also be beneficial.

4.3 SUMMARY

For spinach, cooking improved its colour to bright green. The polyphenol and flavonoid bio-availability was reduced was reduced to 30.41% and 39.00% respectively while the β-carotene levels were unchanged. Antioxidant activity of spinach measured with TEAC and ORAC assays was reduced to 56.86% and 53.96% respectively.

The colour of the pumpkin leaves also improved with cooking, to a brighter green, although the bio- availability of the polyphenols and flavonoids decreased to 17.93% and 30.63% respectively. This loss was reflected in the measured antioxidant activity which retained levels of 34.10%, 12.01% and 34.40% for the TEAC, DPPH and ORAC assays respectively.

Amaranth colour changed from green to a brown colour, whereas the polyphenol content was unchanged, while the flavonoid and ß-carotene levels were increased. The measured

antioxidant activity was different for each assay and was unchanged, increased and reduced when measured with the TEAC, DPPH and ORAC assays respectively. With the ORAC assay, 41.97% of the measured antioxidant activity was bio-available.

The colour of cowpea was altered with cooking, to an undesirable brown colour and the polyphenol and flavonoid content was reduced to 38.37% and 33.83% respectively. The ß-carotene levels were unchanged. Measured antioxidant activity was reduced and the bio-availability was 46.55% when measured with the ORAC assay.

For jute mallow, cooking changed the colour from green to a shade of brown, while the polyphenol content was unchanged, the flavonoid and ß-carotene levels were increased. Antioxidant activity measured with the TEAC and ORAC assays were unchanged while antioxidant activity measured with the DPPH assay was reduced.

4.4 CONCLUSION

Cooking had variable effects on the polyphenol content and antioxidant activity of African LGV. Following cooking the bio- availability of antioxidants were the highest for cowpea and jute mallow, when determined with the physiologically relevant ORAC assay.

B. Effect of gastric and intestinal on the polyphenolic, flavonoid, β-carotene and antioxidant activity of water extracts of LGV

In vitro digestion models are a useful alternative to human or animal studies and allow rapid screening of the nutritional value of meals. By systematic examining each in a standardized way various method of food processing methods and dietary factors that can affect bioactivity and the bio- availability of nutrients and other bioactive molecules can be identified. As discussed in section A, the method of cooking such can variably affect the bio- availability of polyphenols and β-carotene in LGV and this is related to solubility, physiochemical properties and the composition of the food matrix. Digestion factors include pH and the presence of digestive enzymes (Pavan, *et al*., 2014). For example, the hydrophilicity/lipophilicity balance is crucial in driving the solubilization of hydrophilic phenolic compounds into the aqueous phase of the intestinal digesta and the restructuring of lipophilic carotenoids into mixed micelles. Thus a thorough understanding of the changes that occur during digestion is crucial for the understanding and estimating the bio-availability as only bioavailable phytochemicals will exert fully their potential beneficial effects (Alminger *et al*., 2014).

In section A: the effect of cooking on the water extracted polyphenols, flavonoids, β-carotene content and antioxidant activity of each LGV was determined. In this part of the study the effect of gastric and intestinal digestion on the polyphenolic, flavonoid, β-carotene content and antioxidant activity of LGV was determined. The effect of pH will be evaluated as in general it is accepted that a low pH promotes extraction of polyphenols from the plant matrix while a high pH can cause polyphenol degradation (Bermudez-Soto *et al.*, 2007). Digestive proteolytic enzyme in digestion causes the degradation of protein and consequently the release of amino acids and peptides as well as polyphenols with bioactivity (Pihlanto and Korhonen, 2003).

The aim of this part of the study is to determine the effect of gastric and intestinal associated changes in pH during digestion on the polyphenol, flavonoids and β-carotene content and associated antioxidant activity of selected LGV. Then to determine the effect of each phase of digestion on the polyphenol, flavonoids and β-carotene content and associated antioxidant activity of water extracts of cooked LGV.

4.5 RESULTS

Vegetable extracts were digested following two stages of digestion, the gastric and intestinal stage. Samples were collected after each stage and evaluated for polyphenols, flavonoids, β-carotene and antioxidant activities (using the TEAC, DPPH and ORAC assay).

4.5.1 Effect of GI digestion on the polyphenolic content of cooked LGV

The TPC (Table 4.3a) of the LGV ranged between 43.7 to 212.6 mg GAE/g, at pH2, 47.6 – 235.7 mg GAE/g and at pH7 39.5 – 284.8 mg GAE/g. Change in pH, associated with digestion did not alter the TPC of spinach, pumpkin and cowpea. For amaranth change in pH caused a 54.6% (pH2) and 64.7 % (pH7) decrease in TPC compared to the pre-digested, cooked amaranth sample. In contrast for jute mallow there was statistically significant increase of 51.4% and 82.9% in TPC compared to the pre-digested, cooked jute mallow sample.

Table 4.3a: Effect of GI digestion associated pH change on TPC of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	59.7	43.7	212.6	137.9	155.7
pH 2	56.2	47.6	96.6#	121.8	235.7*
pH7	52.4	39.5	75.1#	133.5	284.8*
Fold	0.88	0.90	**0.35**	0.97	**1.83**

Values are mean of at least 3 experiments. # $P<0.05$, denotes a significant decrease from the TPC value after pH adjustment. * $P<0.05$, denotes a significant increase from the TPC value after pH adjustment. Fold change is compared to the cooked samples.

Compared to the pH controls with the addition of digestive enzymes (Table 4.3b), the TPC range following gastric digestion was 95.7 – 218.6 mg GAE/g and following intestinal digestion 102.3 – 179.2 mg GAE/g. Following gastric and intestinal digestion, TPC was the highest for cowpea. Compared to the pH control, the presence of pepsin increased the TPC of spinach, pumpkin, amaranth and cowpea by 144.3, 101.1, 68.3, and 79.5% respectively. The increase is also shown by the fold changes in table 4.3b. Following intestinal digestion this high TPC was retained for spinach, pumpkin, amaranth and cowpea. In contrast, TPC levels of digests were reduced compared to the pH controls for jute mallow, however these TPC levels were similar to spinach, pumpkin and amaranth. With digestion there is a significant increase in the TPC of spinach, pumpkin and cowpea, while the TPC of amaranth decreased and that of jute mallow was unchanged (Figure 4.8).

Table 4.3b: Effect of GI digestion vs pH control on the TPC of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	59.7	43.7	212.6	137.9	155.7
pH 2	56.2	47.6	96.6	121.8	235.7
Gastric digestion	137.3*$	95.7*$	162.6*$	218.6*$	176.9#
	2.44	**2.01**	**1.68**	**1.79**	0.75
pH7	52.4	39.5	75.1	133.5	284.8
Intestinal digestion	124.0*$	102.3*$	127.8*$	179.2*$	150.9#
	2.37	**2.59**	**1.70**	**1.34**	0.53

Values are mean of at least 3 experiments. # P<0.05, denotes a significant decrease from the TPC value after gastric digestion. * P<0.05, denotes a significant increase from the TPC value after gastric digestion. $ Indicates significant differences compared to the cooked sample. Fold change are digested samples compared to pH.

Compared to the cooked samples, following intestinal digestion (Figure 4.8), TPC was significantly increased for spinach, pumpkin and cowpea, while the TPC of jute mallow was unchanged. In contrast the TPC of amaranth was significantly reduced. The effect of pH was low whereas the digestive enzymes resulted in increased extraction of polyphenols. Following intestinal digestion, the TPC of pumpkin, amaranth and jute mallow was similar to spinach while the TPC of cowpea was higher than spinach.

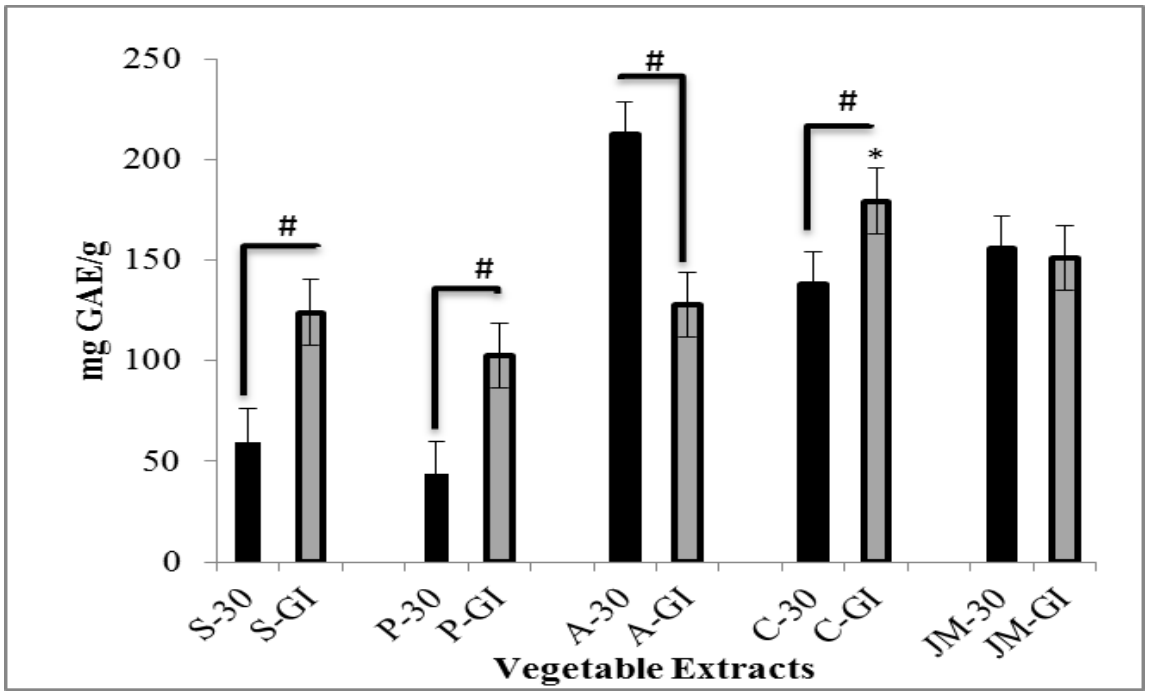


Figure 4.8: Summary showing the variable effects of GI digestion on the TPC of LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest TPC after ID. # Indicates significant differences between cooked and ID extracts, p<0.05.

4.5.2 Effect of GI digestion on the flavonoid content of cooked LGV

For the LGV evaluated in this study, the TFC (Table 4.4a) of the LGV was 25 to 159.1 mg CE/g for the cooked samples, 23 – 106.2 mg CE/g at pH2 and 22.5 – 81.4 mg CE/g at pH7. Change in pH, associated with digestion did not alter the TFC of spinach, pumpkin and cowpea. A pH dependent decrease in TFC was observed for amaranth and jute mallow. Compared to the cooked LGV there was a 0.43 and 0.51-fold decrease in TFC respectively. This is in contrast to the measured TPC for jute mallow where an increase in TPC was observed.

Table 4.4a: Effect of GI digestion associated pH change on TFC of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	25.0	25.0	102.2	40.6	159.1
pH 2	23	26.6	48.5#	38.3	106.2#
pH7	22.5	24.4	44.1#	38.6	81.4#
Fold	0.90	0.98	**0.43**	0.95	**0.51**

Values are mean of at least 3 experiments. # $P<0.05$, denotes a significant decrease from the TFC value after pH adjustment. * $P<0.05$, denotes a significant increase from the TFC value after pH adjustment. Fold change is compared to the cooked samples.

Comparison between pH2 and gastric phase of digestion indicates that there is no change in TFC for spinach, pumpkin, amaranth and jute mallow, although for cowpea there was a significant increase in TFC. An increase to pH 7 caused no change in the TFC for spinach, pumpkin and cowpea while a pH dependent of 0.43 and 0.51 - fold decrease in TFC was measured for amaranth and jute mallow respectively.

Comparison between pH and phase of digestion resulted in no change in TFC for spinach, pumpkin, amaranth and jute mallow. The TFC of cowpea was 1.53 fold increased. With intestinal digestion there was not significant change in TFC for spinach, pumpkin, amaranth and cowpea and only the TFC of jute mallow was increased (Table 4.4b).

Compared to the cooked samples (Figure 4.9), the TFC of spinach, pumpkin and cowpea was unchanged while for amaranth TFC was significantly reduced. Following digestion the TFC of pumpkin, amaranth and cowpea was similar to spinach and that of jute mallow was greater than spinach. Although digestion caused increased extraction of flavonoids, changes in pH had the largest impact on the measured TFC of amaranth and jute mallow.

Table 4.4b: Effect of GI digestion vs pH control on the TFC of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	25.0	25.0	102.2	40.6	159.1
pH 2	23.0	26.6	48.5	38.3	106.2
Gastric digestion	24.7	31.0	57.7	58.7*	112.6
Fold	1.07	1.17	1.19	**1.53**	1.06
pH7	22.5	24.4	44.1	38.6	81.4
Intestinal digestion	25.3	33.0	45.1$^{\$}$	48.8	100.2*$^{\$}$
Fold	1.12	1.34	1.02	1.26	**1.23***

Values are mean of at least 3 experiments. # P<0.05, denotes a significant decrease from the TFC value after pH adjustment. * P<0.05, denotes a significant increase from the TFC value after pH adjustment. $^{\$}$ Indicates significant differences compared to the cooked sample. Fold change is digested compared to pH.

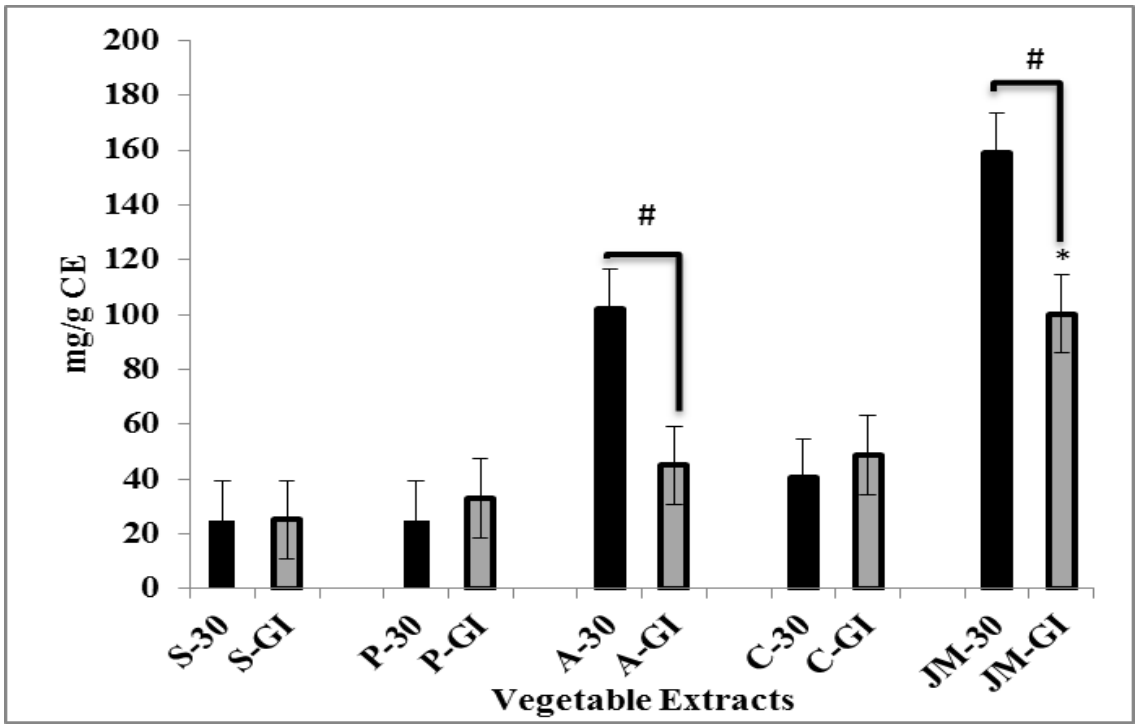


Figure 4.9: Summary showing the variable effects of GI digestion on the TFC of LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest TFC after ID. # Indicates significant differences between cooked and ID extracts, p<0.05.

For spinach and pumpkin leaves, the TPC was increased (Figure 4.8) and the TFC was unchanged (Figure 4.9). For amaranth both TPC and TFC of amaranth was reduced as shown in Figure 4.8 and 4.9, while the TPC of cowpea was increased and TFC was unchanged. In contrast for jute mallow, TPC was unchanged and TFC was reduced. In conclusion, gastric digestion had variable effects on the TPC and TFC of African LGV.

4.5.3 Effect of GI digestion on the β-carotene content of cooked LGV

After cooking, the β-carotene content of the LGV evaluated varied from 15.5 – 42.9 mg/100g. With gastric digestion the β-carotene levels were 7.1 – 67.8 mg/100g and then with subsequent intestinal digestion the β-carotene levels were 6.3 – 40.2 mg/100g (Table 4.5).

After cooking, spinach had the highest β-carotene content of 42.9 mg/100g, while amaranth had the lowest content of 15.5 mg/100g (Table 4.5). Gastric digestion increased the β-carotene levels of spinach to 67.8 mg/100g. With gastric digestion the ß-carotene content of pumpkin, amaranth, cowpea and jute mallow was reduced.

Intestinal digestion had variable effects, the β-carotene levels were reduced for spinach, amaranth and cowpea but increased for pumpkin and jute mallow. Compared to the cooked samples (Figure 4.10), spinach still had the highest ß-carotene content compared to all other LGV while for amaranth, cowpea and jute mallow with digestion the ß-carotene content was reduced.

Table 4.5: Effect of GI digestion on β-carotene content of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	42.9	17.6	15.5	29.8	21.0
Gastric digestion	67.8*	9.0#	10.2#	25.1#	7.1#
Intestinal digestion	40.2$^{\$}$	20.3$^{\$}$	6.3#$^{\$}$	16.8#$^{\$}$	10.3#$^{\$}$

Values are mean of at least 3 experiments. # $P<0.05$, denotes a significant decrease from the β-carotene value prior to *in vitro* digestion. * $P<0.05$, denotes a significant increase from the β-carotene value prior to *in vitro* digestion. $^{\$}$ Indicates significant differences compared to the cooked sample. Fold change is compared to the cooked samples.

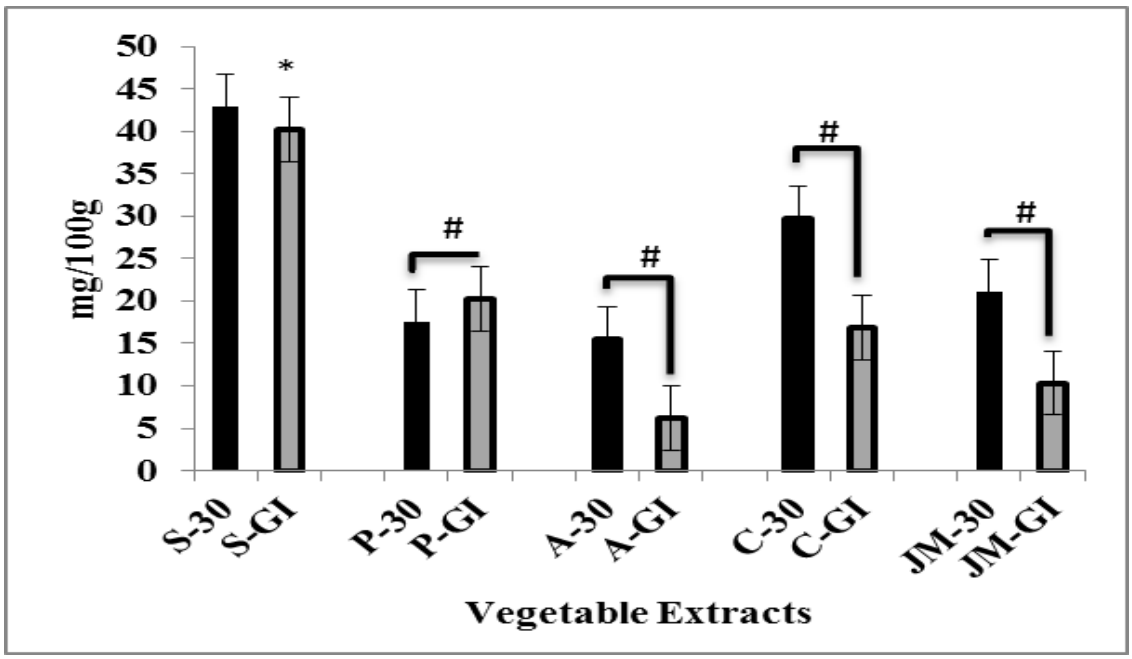


Figure 4.10: Summary showing the variable effects of GI digestion on the β-carotene content of LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest β-carotene after ID. # Indicates significant differences between cooked and ID extracts, $p<0.05$.

4.5.4 Antioxidant activity of GI digested cooked LGV – TEAC assay

The TEAC assay measures the antioxidant activity of both hydrophilic and hydrophobic antioxidant molecules. The antioxidant activity for the cooked LGV evaluated in this study with the TEAC assay was 114.6-340.7 mM TE/g (Table 4.6a). At pH2 associated with gastric digestion, antioxidant activity was significantly reduced for amaranth but not for any of the other LGV evaluated. Further adjustment of pH to pH7 resulted in a decrease in antioxidant activity for all LGV except cowpea.

Table 4.6a: Effect of GI digestion, pH change on antioxidant activity (TEAC assay) of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	166.3	114.6	342.3	264.0	340.7
pH 2	139.6	129.2	260.5#	235.1	343.4
pH7	112.6#	77.4#	139.1#	264.9	259.9#
Fold	**0.68**	**0.68**	**0.41**	1.00	**0.76**

Values are mean of at least 3 experiments. # $P<0.05$, denotes a significant decrease from the TEAC value after pH adjustment. * $P<0.05$, denotes a significant increase from the TEAC value after pH adjustment. Fold change is compared to the cooked samples.

Comparing the pH controls and digests, with gastric digestion the antioxidant activity of spinach, pumpkin and cowpea increased significantly by 2.24, 2.37 and 1.43 fold respectively. The antioxidant activity of amaranth and jute mallow remained unchanged. For all African LGV evaluated compared to pH7 alone, there was a significant increase in antioxidant activity The greatest increase in antioxidant activity was found for pumpkin and spinach with a 4.14 and 2.83 fold increase in antioxidant activity respectively (Table 4.6b) while a significant loss of antioxidant activity was observed for amaranth.

Compared to the cooked LGV, antioxidant activity was increased for spinach, pumpkin and cowpea, while levels were unchanged for amaranth and jute mallow (Figure 4.10 and Table 4.1). Although pH (Table 4.6a) and the presence of digestive enzymes (Table 4.6b) have variable effects on antioxidant activity, following gastric and intestinal digestion, antioxidant activity measured with the TEAC assay compared to the cooked LGV was either unchanged or increased.

Table 4.6b: Effect of GI digestion vs pH control on antioxidant activity (TEAC assay) of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	166.3	114.6	342.3	264.0	340.7
pH 2	139.6	129.2	260.5	235.1	343.4
Gastric digestion	313.1*	306.4*	338.1*	338.0*	343.5
Fold	**2.24**	**2.37**	0.98	**1.43**	1.00
pH7	112.6	77.4	139.1	264.9	259.9
Intestinal digestion	316.4*$	320.9*$	310.5*	329.9*$	331.7*
Fold	**2.83**	**4.14**	**2.23**	**1.25**	**1.2**

Values are mean of at least 3 experiments. # P<0.05, denotes a significant decrease from the TEAC value after pH adjustment. * P<0.05, denotes a significant increase from the TEAC value after pH adjustment. $ Indicates significant differences compared to the cooked sample. Fold change is digested compared to pH.

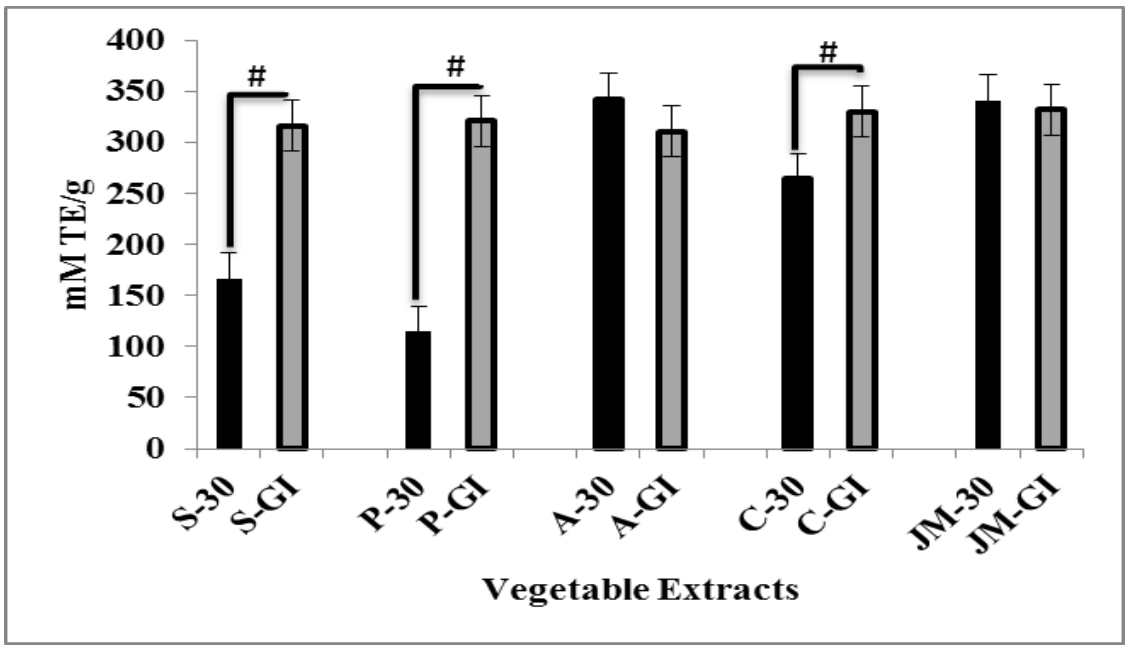


Figure 4.11: Summary showing the variable effects of GI digestion on the antioxidant activity (TEAC assay) of LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest TEAC value after ID.# Indicates significant differences between cooked and ID extracts, $p<0.05$.

4.5.5 Antioxidant activity of digested cooked LGV – DPPH assay

The effect of digestion, pH and enzymes was also determined with the DPPH assay. The antioxidant activity of the LGV evaluated in this study varied from -6.6 – 266.9 mM/g. The negative value for spinach implies that spinach evaluated with the DPPH assay may have a pro-oxidant effect or there is interference by pigmented dye molecules.

With adjustment of the pH to pH2 the antioxidant activity of spinach, pumpkin and cowpea increased with a range of 31.0 – 164.9 mM/g. For amaranth and jute mallow a decrease in antioxidant activity was measured. Further adjustment of pH, resulted in a decrease in antioxidant activity of spinach, pumpkin, amaranth and jute mallow by -1.88, -0.28, 0.0007 – and 0.62 - fold respectively. The antioxidant activity of cowpea was unchanged.

Table 4.7a: Effect of GI digestion, pH change on antioxidant activity (DPPH assay) of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	-6.6	3.0	266.9	55.5	204.1
pH 2	31.2*	31.0*	55.0#	97.4*	164.9#
pH7	-12.4	-6.5	0.2#	59.0	127.0#
Fold	**-1.88**	**-0.28**	**0.0007**	1.06	**0.62**

Values are mean of at least 3 experiments. # P<0.05, denotes a significant decrease from the DPPH value after pH adjustment. * P<0.05, denotes a significant increase from the DPPH value after pH adjustment. Fold change is compared to the cooked samples.

With digestion compared to the pH control, for gastric digestion, antioxidant activity was reduced for spinach, pumpkin, cowpea and jute mallow but not for amaranth. With intestinal digestion a loss of antioxidant activity was observed for all evaluated LGVs, expect for pumpkin where no significant change in antioxidant activity was observed.

Following digestion, antioxidant activity measured with the DPPH assay was the highest for jute mallow (Figure 4.11 and Table 4.7b). Only cowpea and jute mallow following digestion had better antioxidant activity than spinach.

Table 4.7b: Effect of GI digestion vs pH control on antioxidant activity (DPPH assay) of cooked

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	-6.6	3.0	266.9	55.5	204.1
pH 2	31.2	31.0	55.0	97.4	164.9
Gastric digestion	11.0	16.1	81.3*	$38.8^{\#}$	$107.7^{\#}$
	0.35	**0.52**	**1.45**	**0.40**	**0.65**
pH7	-12.4	-6.5	0.2	59.0	127.0
Intestinal digestion	-18.6	1.2	$-10.3^{\$}$	54.3	$90.4^{\#\$}$
	-1.46	***-0.2***	**-51.5**	**0.072**	**0.71**

Values are mean of at least 3 experiments. # P<0.05, denotes a significant decrease from the DPPH value after pH adjustment. * P<0.05, denotes a significant increase from the DPPH value after pH adjustment. $^{\$}$ Indicates significant differences compared to the cooked sample. Fold change is digested compared to pH.

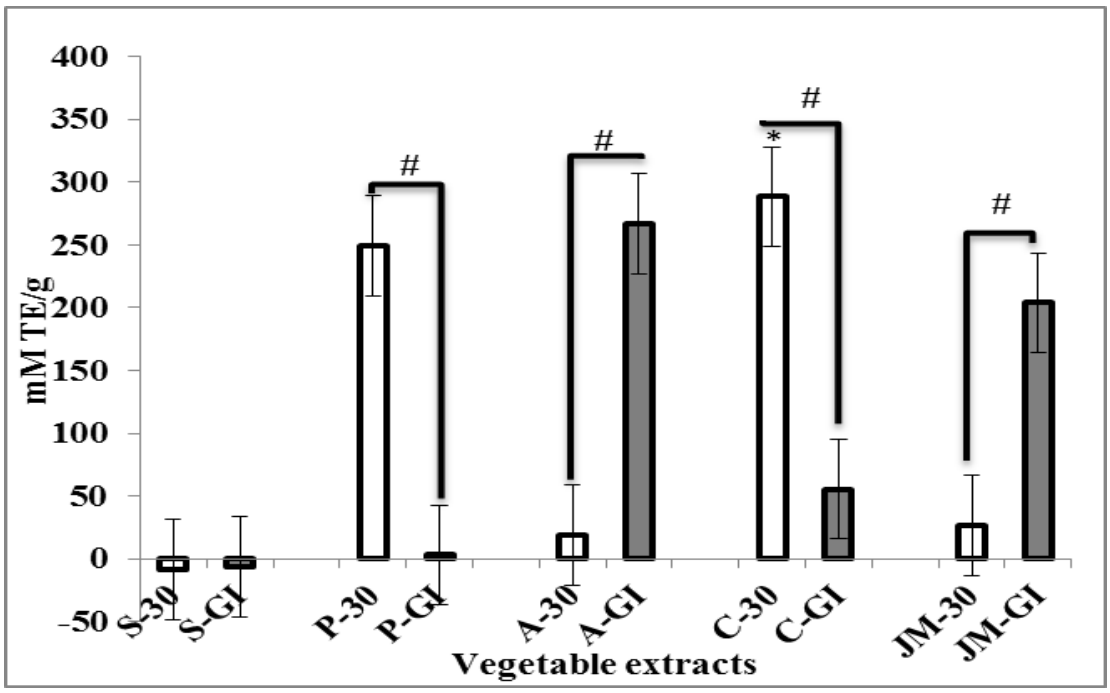


Figure 4.12: Summary showing the variable effects of GI digestion on the antioxidant activity (DPPH assay) of LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest DPPH value after ID.# Indicates significant differences between cooked and GI extracts, $p<0.05$.

Both the TEAC and DPPH assays are ET assays, however observed differences in antioxidant activity may be related to interfering pigments or the mechanisms involved. The TEAC assay measures the activity of both hydrophobic and hydrophilic antioxidants, the FRAP assay measure the antioxidant activity of hydrophilic antioxidants and the DPPH measures the activity of antioxidants that are soluble in organic solvents. Therefore due to the ability to measure the activity of both hydrophobic and hydrophilic antioxidants, (Apak *et al*., 2007), the generated data is the most reliable. The findings of the TEAC assay were confirmed with the ORAC assay.

4.5.6 Antioxidant activity of digested cooked LGV – ORAC assay

The ORAC assay measures the ability of antioxidants to prevent the lipid oxidation in food as well as biological systems (Apak *et al.*, 2007). It is the only antioxidant assay that combines inhibition time and degree of inhibition into one parameter using area under the curve (AUC). As for the TEAC assay, the ORAC assay also measures both hydrophilic and lipophilic chain-breaking antioxidant capacity.

The antioxidant activity of cooked African LGV was 35.16 – 381.59 mM/g with jute mallow with the highest antioxidant activity and pumpkin with the lowest activity (Table 4.8a). pH adjusted to 2, did not cause a significant change in antioxidant activity. Further adjustment to pH7, resulted in a significant increase in antioxidant activity for all LGV (Table 4.8a). At pH7 the highest antioxidant activity was observed for pumpkin with antioxidant activity of 1052.15 mM/g and a 29.92 - fold increase in activity compared to cooked pumpkin.

Table 4.8a: Effect of GI digestion, pH change on antioxidant activity (ORAC assay) of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	125.14	35.16	225.19	267.32	381.59
pH 2	198.06	115.93	261.81	180.96	413.35
pH 7	925.75*	1052.15*	962.07*	906.08*	845.07*
Fold	**7.39**	**29.92**	**4.27**	**3.39**	**2.21**

Values are mean of at least 3 experiments. # P<0.05, denotes a significant decrease compared to cooked. * P<0.05, denotes a significant increase compared to cooked. Fold change is compared to the cooked samples.

The effect of digestion was then determined. The range of antioxidant activity was 458.87 – 1043.35 mM/g and there was compared to the pH2 fractions there was a significant increase in antioxidant activity for spinach, pumpkin, amaranth and cowpea (Table 4.8b). No change in antioxidant activity was found for jute mallow. Following intestinal digestion, the antioxidant activity was 898.41 – 1199.32 mM/g and compared to the pH controls the antioxidant activity of cowpea was only significantly increased (Table 4.8b). Comparison between cooked and intestinal digestion found that with intestinal digestion there is a significant increase in antioxidant activity for all LGV, and a bio-availability of 132% was the highest for cowpea. With digestion the antioxidant activity of the LGV (Figure 4.13) evaluated in this study was increased. The antioxidant activity of pumpkin, pumpkin, amaranth and jute mallow measured with the ORAC assay was similar to spinach. In contrast the antioxidant activity of cowpea was greater 23% greater than spinach.

Table 4.8b: Effect of GI digestion vs pH control on antioxidant activity (ORAC assay) of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	125.14	35.16	225.19	267.32	381.59
pH 2	198.06	115.93	261.81	180.96	413.35
Gastric digestion	828.06*	458.87*	700.75*	1043.35*	566.77
	4.18	**3.96**	**2.68**	**5.77**	**1.37**
pH 7	925.75	1052.15	962.07	906.09	845.07
Intestinal digestion	981.89$^{\$}$	941.86$^{\$}$	955.46$^{\$}$	1199.32*$^{\$}$	898.41$^{\$}$
	1.06	0.89	0.99	**1.32**	1.06

Values are mean of at least 3 experiments. # P<0.05, denotes a significant decrease from the ORAC value after pH adjustment. * P<0.05, denotes a significant increase from the ORAC value after pH adjustment. $^{\$}$ Indicates significant differences compared to the cooked sample. Fold change is digested compared to pH.

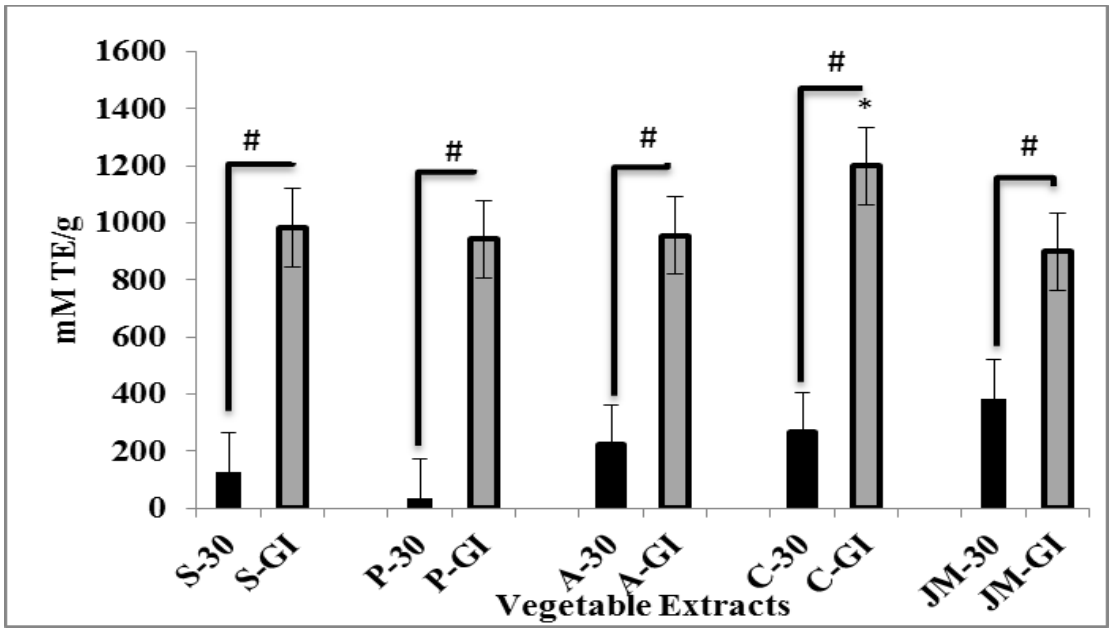


Figure 4.13: Summary showing the variable effects of GI digestion on the antioxidant activity (ORAC assay) of LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest ORAC value after ID. # indicates significant differences between cooked and ID extracts, p<0.05.

Correlation between the cooked and digested samples revealed a strong correlation between TFC and ß-carotene which implies that with digestion the effect on flavonoid and ß-carotene content is the same for all LGV. Correlations between assays for the digested samples were also determined and correlations were the highest for TFC and TEAC, TFC and the DPPH and the TEAC and DPPH assays (Table 4.9). Only between TPC and ORAC was there some degree of correlation.

Table 4.9: Summary of polyphenol, flavonoid, β-carotene content and antioxidant activity of cooked and intestinal digested LGV

		TPC (mg GAE/g)	TFC (mg CE/g)	β-carotene (mg/100g)	TEAC (mM TE/g)	DPPH (mM TE/g)	ORAC (mM TE/g)
Spinach	C	59.7±5.0	25.0±2.5	42.9±0.1	166.3±10.4	-6.6±2.4	125.1±16.0
	D	124.0±5.2	25.3±2.7	40.2±0.008	316.4±9.5	-18.6±4.2	981.9±3.7
% Bio- availability		**208**%	101%	94%	**190**%	-295%	**780**%
Pumpkin	C	43.7±2.8	25.0±3.2	17.6±0.02	114.6±7.6	3.0±5.4	35.1±5.3
	D	102.3±1.5	33.03.7	20.3±0.17	320.9±7.5	1.2±5.2	941.9±5.4
% Bio- availability		**234**%	132%	**115**%	**280**%	40%	**268%**
Amaranth	C	212.6±8.2	102.2±4.0	15.5±0.3	342.3±7.9	266.9±9.8	225.2±2.4
	D	127.8±4.6	45.1±3.2	6.3±0.07	310.5±8.7	-10.3±4.8	955.5±2.0
% Bio- availability		**60%**	**44**%	**41**%	91%	**-4**%	**424**%
Cowpea	C	137.9±4.0	40.6±1.0	29.8±0.2	264.0±10.7	55.5±2.5	267.3±11.5
	D	179.2±7.2	48.8±1.9	16.8±0.46	329.9±9.6	54.3±5.5	1199.33±8.6
% Bio- availability		**130%**	120%	**56%**	**125**%	98%	**449**%
Jute mallow	C	155.7±7.7	159.1±7.7	21.0±0.04	340.7±7.5	204.1±6.5	381.6±1.3
	D	150.9±9.1	100.2±1.6	10.3±0.06	331.7±9.3	90.4±4.3	898.4±15.8
% Bio- availability		97%	**63%**	**49**%	97%	**44**%	**235%**
Correlation C vs GI		0.47	**0.90**	**0.87**	-0.536	0.164	0.078

Data is an average of at least 3 experiments; Mean ± SD values. Bold indicates changes in bio-accessibility.

Table 4.10: Correlation between assays for GI digested LGV

	TFC	ß-carotene	TEAC	DPPH	ORAC
TPC	0.47	-0.27	0.21	0.70	**0.70**
TFC		0.60	**0.93**	**0.89**	0.27
ß-carotene			0.37	-0.46	0.09
TEAC				**0.74**	0.53
DPPH					0.13

To summarize for spinach, with digestion the polyphenol content was increased, and the flavonoid and ß-carotene content was unchanged. This was associated with an increase in antioxidant activity measured with the TEAC and ORAC assays. Following digestion, the polyphenol and flavonoid content of digested pumpkin leaves were increased while ß-carotene levels were unchanged, and this was associated with an increase in antioxidant activity measured with the TEAC and ORAC assays. The polyphenol, flavonoid and ß-carotene content of amaranth was reduced after digestion and antioxidant activity measured with the TEAC assay was unchanged but increased with the ORAC assay. For antioxidant measured with the DPPH assay variable results were obtained and possible reasons are provided below.

4.6 DISCUSSION

In this chapter the effects of digestion on the antioxidant properties of a selection of African LGVs was determined. In addition, the contribution of pH and digestive enzymes to the measured levels and activity was determined. pH had minimal effects on the measured TPC of spinach, pumpkin and cowpea. In contrast, the TPC of amaranth and jute mallow was decreased and increased respectively. Pavan *et al*., (2014), reported a decrease in polyphenolic content of fruit extracts (araticum and papaya) after pH adjustment and this effect was attributed to the instability of phenolic compounds at neutral and high pH. Yuwei et *al*. (2014) reported that low pH, released matrix bound phenolics in wheat and rice flour, as phenolics are generally esterified in the form of sugars or acids. Findings in the present study were that for spinach, pumpkin and cowpea pH changes associated with digestion did not cause major changes in TPC. The TPC of amaranth and jute mallow was reduced indicating that the extracted polyphenols were pH sensitive. With gastric and gastro-duodenal, TPC was increased for spinach, pumpkin, amaranth and cowpea and was reduced for jute mallow. Chiang, Kadouh and Zhou, (2013) reported that *in vitro* digestion improved the TPC of

gooseberries. Pavan *et al*., (2014) reported that an increase in TPC is associated with the gradual release of the polyphenols during the digestion process. The protein content of LGV is generally low, however protein is an important constituent in cell walls and with proteolytic digestion, the release of intracellular polyphenols is mediated. In addition, the pancreatic enzyme fraction also contains amylase that digests carbohydrates and associated release of polyphenols as described by Yuwei *et al*. (2014) can occur.

Bouayed, Hoffmann and Bohn, (2011) reported that polyphenol release was mainly achieved at the gastric phase (65% of phenolics and flavonoids), with a slight further release (10%) during intestinal digestion. Likewise, in the present study, gastric digestion resulted in an increase in TPC while with duodenal digestion a lower but further extraction occurred for most of the LGV. Pepsin digests protein and the release of polyphenols may be due to the digestion of the protein component of the plant matrix. Further trypsin digestion can further digest proteins with an additional further release of matrix associated polyphenols. Amylase also present in the pancreatic solution hydrolyses the β-linkages between carbohydrates and this can result in the release of free polyphenols or polyphenols bound to sugars (Wootton-Beard, 2011).

Both pH2 and pH7 caused a decrease in TFC of amaranth and jute mallow. Following gastric digestion, the TFC of cowpea and amaranth was increased and the TFC of the other LGV was unchanged. Compared to the cooked samples the TFC of amaranth and jute mallow was reduced. Any observed loss of TFC may be due to the instability of flavonoids such as glycosylated flavonols and anthocyanins at physiological pH, while increased levels is due to increased extraction as described for the polyphenols. For each LGV there is a dynamic equilibrium between extraction and degradation of polyphenols.

The β-carotene content of the African LGV evaluated in this study was increased for spinach and reduced for other LGV after gastric digestion. For all LGV, following duodenal digestion, the β-carotene content was further reduced. Hedren, Diaz and Svanberg, (2002) reported that carotenoids are susceptible to oxidation, owing to the numerous double bonds, and therefore may be susceptible to oxidation with digestion.

The effects of pH associated with digestion on antioxidant activity measured with the TEAC assay was variable. Gastric pH2, had a limited effect on the antioxidant activity of spinach,

pumpkin, cowpea and jute mallow. In contrast the antioxidant activity of amaranth was reduced. At pH7 associated with intestinal digestion there was a loss in the antioxidant activity of spinach, pumpkin, amaranth and jute mallow. Cowpea was stable at both pH 2 and 7.

With gastric digestion a significant increase in antioxidant activity of all LGV except jute mallow was observed. Likewise following intestinal digestion the antioxidant activity for all LGV was also increased. Compared to the cooked LGV antioxidant activity measured with the TEAC assay was increased for spinach, pumpkin and cowpea while the antioxidant activity was unchanged for amaranth and jute mallow. This confirms the findings a study by Yuwei Luo *et al*., 2014, where gastric digestion of cowpea resulted in an increase in antioxidant activity due to increase in the polyphenol solubility at low pH and digestion of proteins that reduces the density of the plant matrix facilitating the extraction of polyphenols. Further enzymatic carbohydrate and protein hydrolysis will also occur in the duodenum. In the present study, antioxidant activity measured with the TEAC assay, indicates that in the gastric phase for most LGV, antioxidants are released. With further digestion and at a physiological pH, these molecules, including polyphenols are stable.

Antioxidant activity was measured with the DPPH assay was different from antioxidant activity measured with the TEAC assay. Activity at pH2 was increased for spinach, pumpkin and cowpea and decreased for amaranth and jute mallow. (Table 4.7a). At pH 7, activity was absent or low for spinach, pumpkin and amaranth although some activity was observed for cowpea and jute mallow. With gastric digestion the antioxidant activity was increased for amaranth but was lost following intestinal digestion. In contrast with gastric digestion the antioxidant activity of cowpea and jute mallow was reduced. For all LGV following digestion compared to the cooked samples antioxidant activity was lost except for cowpea where no change was observed.

For several samples some results are negative, and this was observed for the cooked and intestinal digested samples and this may be due to the effect of pH on the DPPH assay. Pekal and Pyrzynska (2015), evaluated the effect of pH and metal ions on DPPH radical scavenging activity of tea and found higher radical quenching was observed in acidic media. A similar effect was observed by Ruenroengklin *et al*., (2008) when analysing anthocyanin from litchi fruit where pH changes associated with digestion significantly influenced the total antioxidant ability and scavenging activities against DPPH radicals. These authors concluded

that besides increased extraction of antioxidants at a low pH, the DPPH assay may also be pH dependent and the optimal pH for determining antioxidant activity with the DPPH was at pH 3-5. This may account for the negative values obtained for the cooked and the gastroduodenal digests. Although a good correlation was obtained between the TFC and the TEAC assays, such correlations reflect trends rather than actual values.

Wootton-Beard *et al.* (2011), determined the effect of *in vitro* digestion on the antioxidant properties of vegetable juices and found that majority of vegetable juices showed increased antioxidant activity after digestion. In the present study the effect of *in vitro* simulated digestion on cooked LGV was determined. Activity was determined using the TEAC, DPPH and ORAC assays. An increase in antioxidant activity measured with the DPPH assay was similar to that reported by Chan, *et al*., (2012) for the digestion of wheat and rice flours. In the latter study pH adjustment alone caused a significant increase by 2 fold, which implies that at an acidic pH, increased polyphenol extraction from carbohydrate rich plants occurred.

All the samples showed antioxidant protection against peroxide radicals with the ORAC assay. At pH 2 there was no significant change in antioxidant activity while at pH7 antioxidant activity increased by several folds (Table 4.8a). All solutions used for the ORAC assay were prepared in PBS with a pH7.4 To a volume of 5ul sample is added to 200 µl reagent phosphate buffered solution, therefore even for a sample at pH2, there is sufficient buffering capacity to ensure that pH does not adversely affect measured activity. In addition to the high sensitivity of the method being a fluorimetric assay, small volumes of samples are required and consequently possible interference effects are reduced.

The presence of pepsin in the gastric phase of digestion increased antioxidant content and activity of spinach, pumpkin, amaranth but not jute mallow as seen for the TPC and the TEAC assays. The effect of gastroduodenal digestion was less and further increase in antioxidant activity was only observed for cowpea. This study clearly shows that the gastric phase of digestion plays and important role in the extraction of polyphenols from the LGV plant matrix, where protein digestion causes the matrix to become accessible for the extraction polyphenols.

Spinach is widely grown in rural community schemes and it is considered an excellent source of essential nutrients such as vitamin K, vitamin A, folate and iron (Hedges and Lister, 2007). Due to the robustness, the low water requirements and being pest resistant, it is recommended

that the cultivation of LGV should be encouraged (Cernansky, 2015). However, little is known if the antioxidant properties of African LGV are comparable to exotic LGV such as spinach and cabbage. In this study it was found that following digestion the TPC of spinach was less than amaranth, cowpea and jute mallow while the TFC was less than all other LGV evaluated. In contrast, the β-carotene content of spinach was the highest. Antioxidant activity analysis with the TEAC and ORAC assays showed that the antioxidant activity of spinach was similar to all other LGV evaluated. With the DPPH assay, antioxidant activity of spinach was less than all other LGV evaluated. The ORAC assay is considered to be physiologically relevant and based on the findings using this assay the antioxidant activity of African LGV evaluated is similar to spinach.

4.7 CONCLUSION

With digestion, the presence especially of pepsin in the gastric phase of digestion increases the extraction of polyphenols and flavonoids. Using a model of digestion which includes only the gastric (pH2 and pepsin) and the duodenal (pH7 and pancreatic enzymes) phases of digestion β-carotene levels are reduced. Associated antioxidant activity with the TEAC and ORAC assays reveal that with digestion antioxidant activity was significantly increased especially after gastric digestion. In contrast, antioxidant activity evaluated with the DPPH assay was mostly reduced but this may be related to pH dependence of the assay. With digestion the antioxidant activity of African LGV is comparable to spinach.

C: The effect of a simulated GIT digestion model involving the oral, gastric and intestinal phases of digestion on the polyphenol, flavonoids and β-carotene content and antioxidant activity of selected LGV

INTRODUCTION

In this part of the study the effect of oral, gastric and intestinal digestion on the polyphenolic, flavonoid, β-carotene content and antioxidant activity of LGV was determined. In the previous chapter a simplified simulated digestion model was used and this model did not include the oral phase of digestion as well as lipase and bile salts in the intestinal phase of digestion.

α-Amylase in the oral phase of digestion hydrolyses the α-1,4 linked D-glucose units of polysaccharides with three of more α-1,4 linked D glucose units. Tea polyphenols and polyphenols from vegetables have been shown to inhibit α-amylase activity (Hara and Honda, 1990). However, α–amylase can hydrolyse the food matrix and specifically complex carbohydrates found in LGV resulting in the release of polyphenols (Alminger, *et al.*, 2014). Therefore, the inclusion of the oral phase of digestion may further have an effect on the matrix which with further gastric digestion increases the extraction of polyphenols and resulting theoretically in increased antioxidant activity (Changpraykaew and Petchlert, 2015). However, the inhibition of activity by polyphenols in LGV may result in poor extraction of the polyphenols. In addition, neutral pH present in the oral cavity can lead to the degradation of polyphenols (Lee, *et al*., 2004).

In the intestinal phase of digestion, bile salts aid in the digestion of lipophilic nutrients in the intestinal tract. Bile acids are responsible for the emulsification of lipid aggregates as well as the solubilisation and transport of lipids in an aqueous environment. In addition, bile acids are also critical for the transport and the absorption of fat-soluble enzymes as well as β-carotene. Carotenoids released from vegetable matrix must be well dispersed in the GIT, however due to the high hydrophobicity of the isoprenoid carbon skeleton this does not occur. The presence of dietary lipids facilitates carotenoid dispersion, where the presence of lipolytic enzymes such as lipase, bile fluid and carotenoids form a mixed micelle that is more accessible to the intestinal epithelial cells for digestion (Palafox-Carlos, Ayala-Zavala and Gonzalez-Aguilar, 2010). Although the lipid content of LGV is low, bile acids and lipase, may impact on measured activities and therefore these are included in the intestinal phase of digestion.

The aim of this part of the study is to determine the contribution of oral digestion on the polyphenol, flavonoids and β-carotene content and associated antioxidant activity of selected LGV. Then to determine the effect of each phase of digestion on the polyphenol, flavonoids and β-carotene content and associated antioxidant activity of water extracts of cooked LGV.

4.8 RESULTS

A selection of African LGV was subjected to simulated digestion as detailed in figure 3.2. Samples at each phase of digestion were analysed for polyphenol, flavonoid, ß-carotene content as well as antioxidant activity as described in sections 3.5 and 3.6. As the presence of digestive enzymes especially in the gastric phase of digestion and not pH was the major contributing factor to the released of polyphenols and associated increase in antioxidant activity, only the different phases of digestion were compared in this section of the study.

4.8.1 Effect of OGI digestion on the polyphenolic content of cooked LGV

The polyphenolic content of the OGI digested LGV was 74.0±3.0 - 105.7±1.3 mg/g GAE. Polyphenolics of spinach and pumpkin increased significantly after the oral phase of digestion to 78.9 ±2.9 and 78.3 ±7.6 mg/g GAE, respectively (table 4.11). Following the gastric and intestinal phases of digestion no change in TPC was observed for both LGVs. The TPC of amaranth, cowpea and jute mallow was reduced after the oral phase of digestion and remained low following gastric and intestinal digestion (Table 4.11). Compared to the cooked samples TPC was increased for spinach, pumpkin and reduced for amaranth, cowpea and jute mallow (Figure 4.14a). The TPC of jute mallow was the highest after digestion although the TPC of all other LGV was similar to spinach.

Comparison between GI and OGI digestion reveals that with OGI digestion TPC levels are lower for all LGV evaluated.

Table 4.11: Effect of OGI digestion on the polyphenolic content of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	59.7±5.04	43.7±2.8	212.6±8.2	137.9±4.0	155.7±7.7
Oral phase	78.9±2.9*	78.3±7.6*	94.8±4.7#	100.2±5.4#	110.2±3.5#
Gastric phase	79.8±4.3*	80.3±4.6*	97.3±4.3#	93.8±5.9#	95.65±2.8#
Intestinal phase	80.0±1.9*	74.0±3.0*	87.7±1.1#	93.3±2.5#	105.7±1.3#

Values are expressed as mean ± SD, # $P<0.05$, a significant decrease compared to cooked. * $P<0.05$, a significant increase compared to cooked.

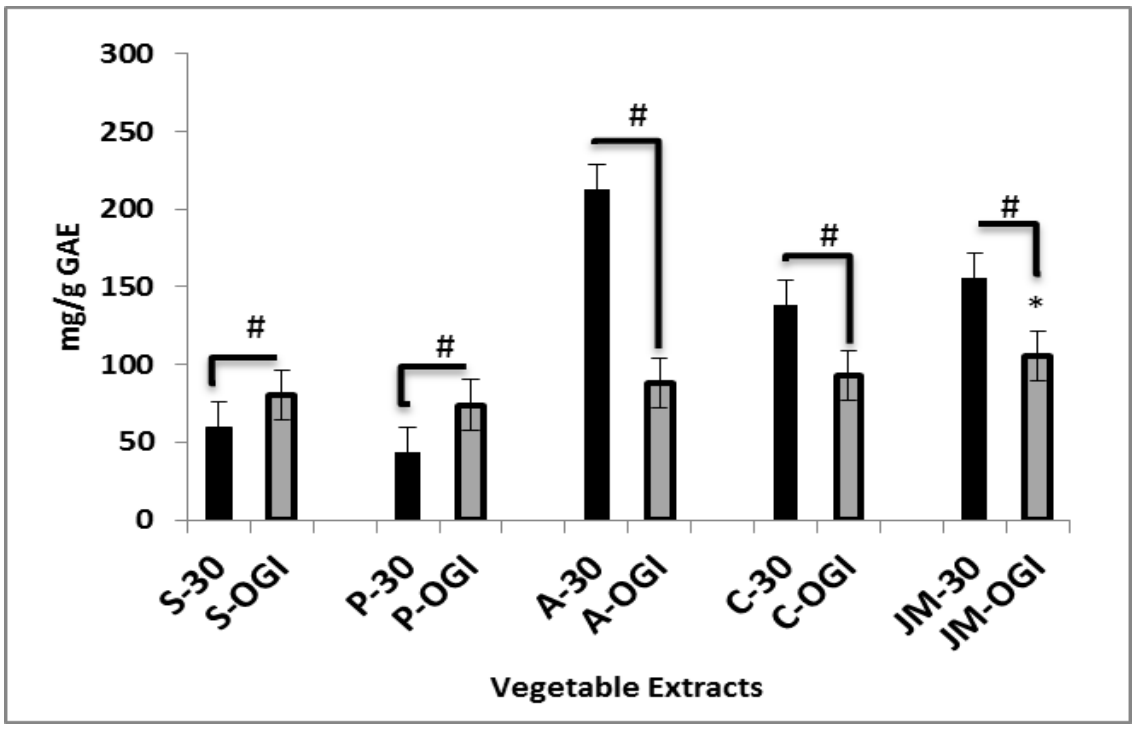


Figure 4.14a: Summary showing the variable TPC of cooked LGV compared with OGI digestion of spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest TPC after digestion. # Indicates a significant difference between cooked and digested extracts, $p<0.05$.

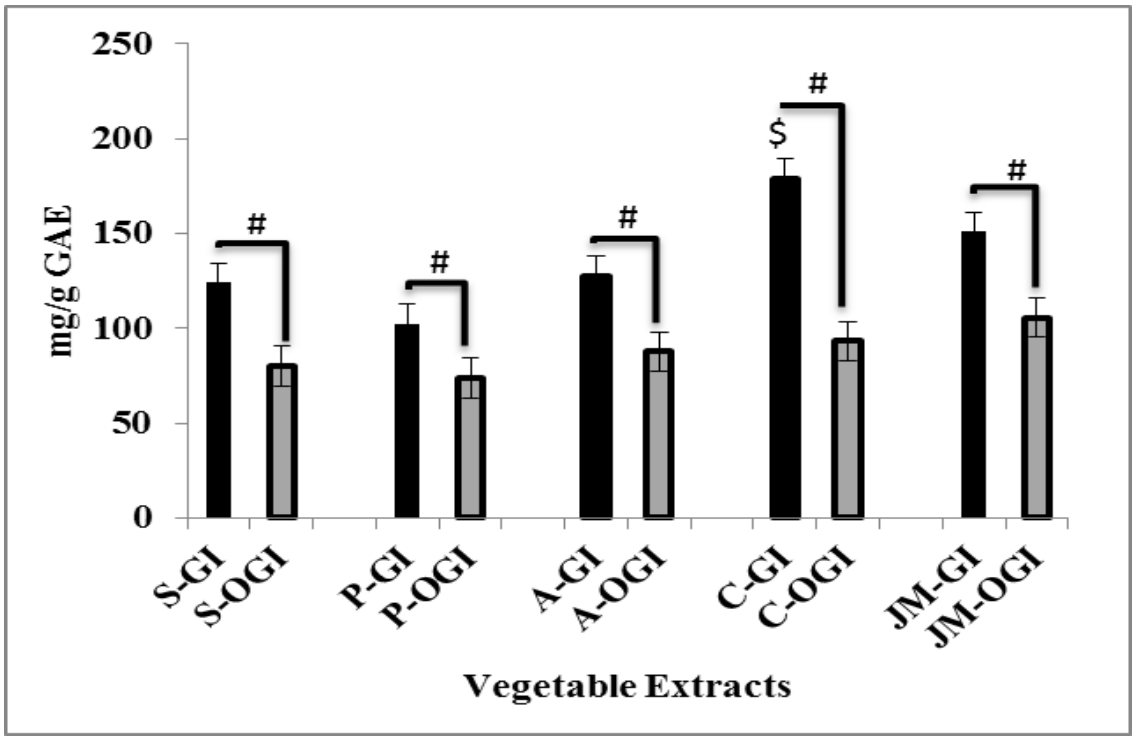


Figure 4.14b: Differences in the TPC of GI compared with OGI digested LGVs, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. $ indicate a LGV with the highest TPC after GI digestion and *Indicates a LGV with the highest TPC after OGI digestion. # indicate a significant difference between the GI vs OGI digestion, $p<0.05$.

4.8.2 Effect of OGI complex digestion on the flavonoid content of cooked LGV

For the cooked LGV following digestion the TFC of all LGV was 24.9±0.4 - 56.5±1.5mg/g CE after OGI digestion. For spinach, pumpkin and cowpea, following the oral phase of digestion there was no change in TFC. In contrast the TFC of amaranth and jute mallow was significantly reduced (Table 4.12). Generally following the gastric and intestinal phases of digestion the TFC remained unchanged. Comparison between the TFC of the cooked and OGI digested LGV, the TFC (Figure 4.15a) was unchanged for spinach, pumpkin and cowpea but was reduced for amaranth and jute mallow. Compared to spinach the TFC of all LGVs were similar. Comparison of TFC following GI and OGI digestion (Figure 4.15b) revealed that the TFC was similar for spinach, pumpkin and cowpea but reduced for cowpea and jute mallow.

Table 4.12: Effect of OGI digestion on the flavonoid content of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	25.0±2.4	25.0±3.2	102.2±4.0	40.6±1.0	159.1±7.8
Oral phase	27.6±2.7	25.8±1.5	44.8±1.9#	40.3±2.1	72.3±4.3#
Gastric phase	16.9±1.5#	28.4±1.6	38.5±1.3#	43.2±1.9	62.0±1.4#
Intestinal phase	24.9±0.4	27.4±0.96	39.7±1.2#	41.4±2.5	56.5±1.5#

Values are expressed as mean ± SD, # P<0.05, a significant decrease compared to cooked. * P<0.05, a significant increase compared to cooked.

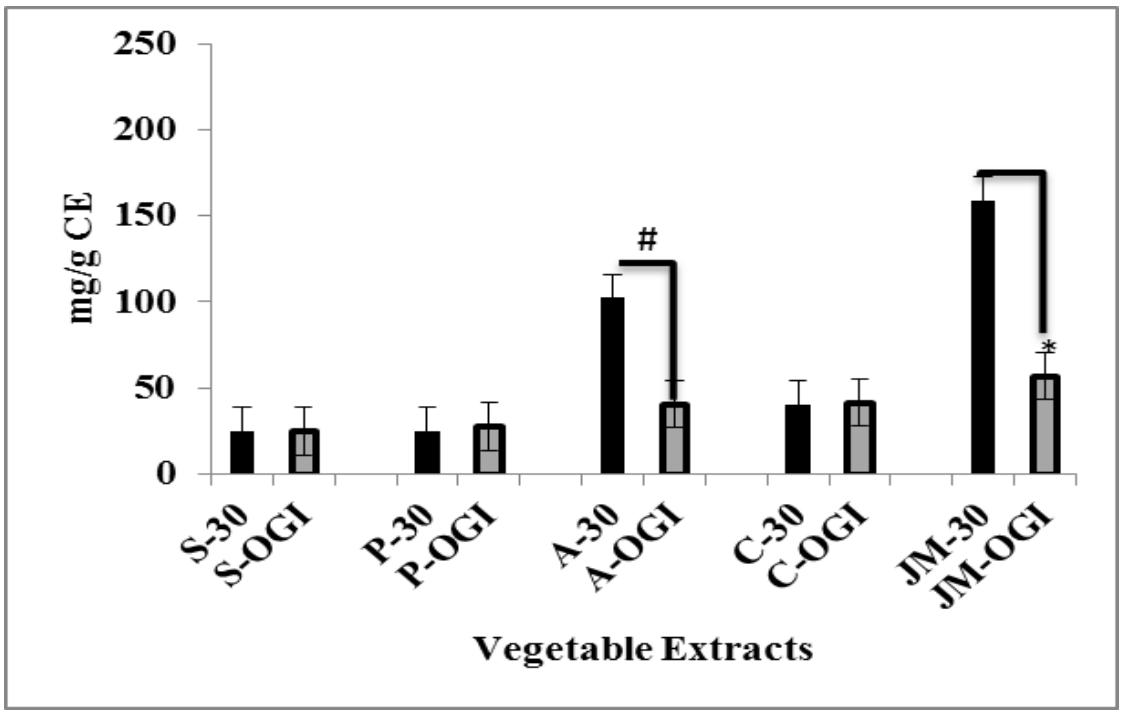


Figure 4.15a: Summary showing the variable TFC of cooked LGV compared with OGI digestion of LGV, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest TFC after digestion. # Indicates a significant difference between cooked and digested extracts, $p<0.05$.

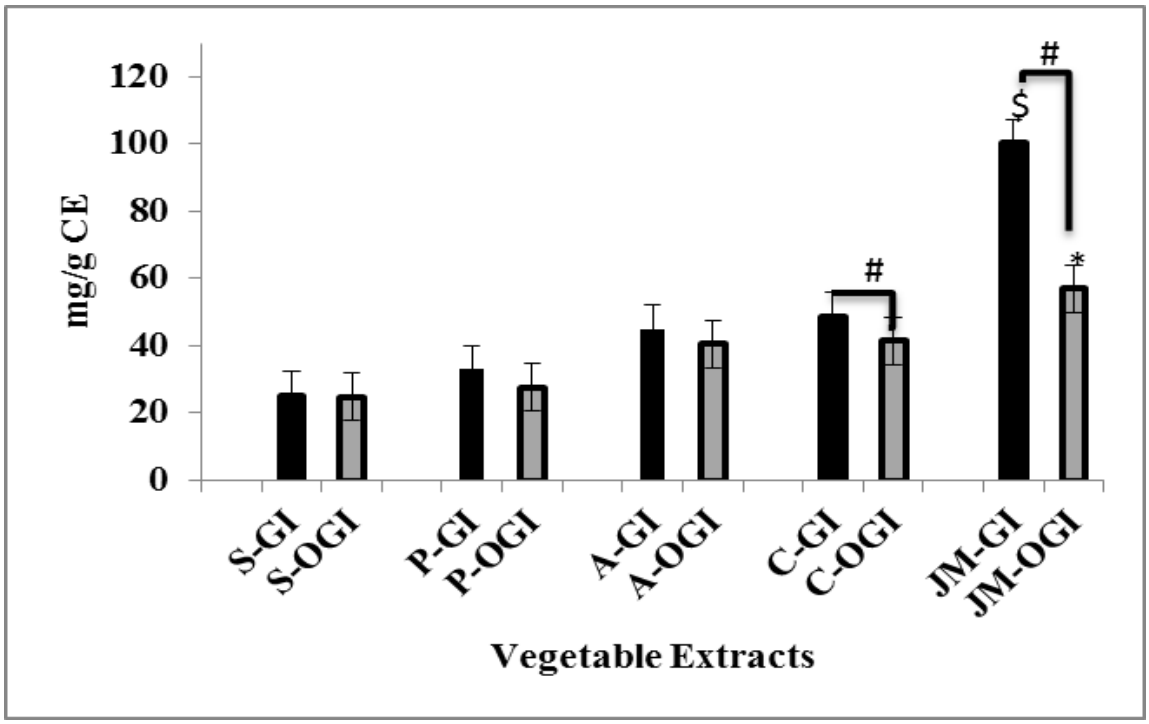


Figure 4.15b: Differences between GI and OGI digestion on the TFC of spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. $ indicate the LGV with the highest TPC after D and *indicates the LGV

4.8.3 Effect of OGI digestion on the β-carotene content of cooked LGV

Following the OGI of the selected African LGV, the β-carotene varied form 2.09±0.02 to 32.3±0.3 mg/100g (Table 4.13). With the oral phase of digestion, the β-carotene content was reduced for all LGVs evaluated. With further gastric and intestinal digestion levels were increased for spinach, pumpkin and cowpea. The β-carotene content was reduced for amaranth and jute mallow although the β-carotene content was still the highest for the LGV evaluated (Table 4.13 and Figure 4.16a). Variable differences were found when GI was compared to OGI digestion. The ß-carotene content of the spinach, amaranth and jute mallow was lower, while the ß-carotene content of pumpkin and cowpea was unchanged

Table 4.13: Effect of OGI digestion on the β-carotene content of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	42.6±0.14	17.6±0.02	15.5±0.3	29.8±0.2	21.0±0.04
Oral Phase	3.17±0.05#	12.8±0.05#	7.5±0.01#	6.4±0.01#	3.2±0.03#
Gastric Phase	101.2±0.38*	30.1±0.2*	6.9±0.05#	34.3±0.2*	13.2±0.08#
Intestinal Phase	14.24±0.07#	20.2±0.04*	2.09±0.02#	32.3±0.3*	5.1±0.03#

Values are expressed as mean ± SD, # P<0.05, a significant decrease compared to cooked. * P<0.05, a significant increase compared to cooked.

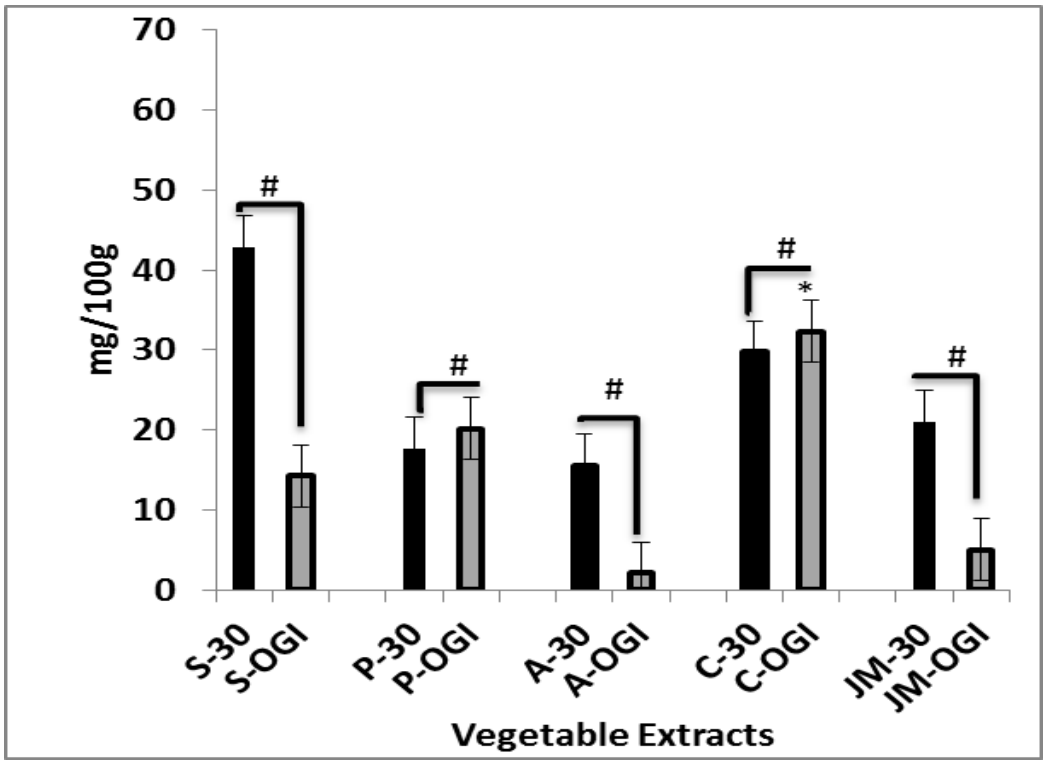


Figure 4.16a: Summary of the variable β-carotene levels of cooked LGV compared with OGI digestion of spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest β-carotene after digestion. # Indicates a significant difference between cooked and digested extracts, p<0.05.

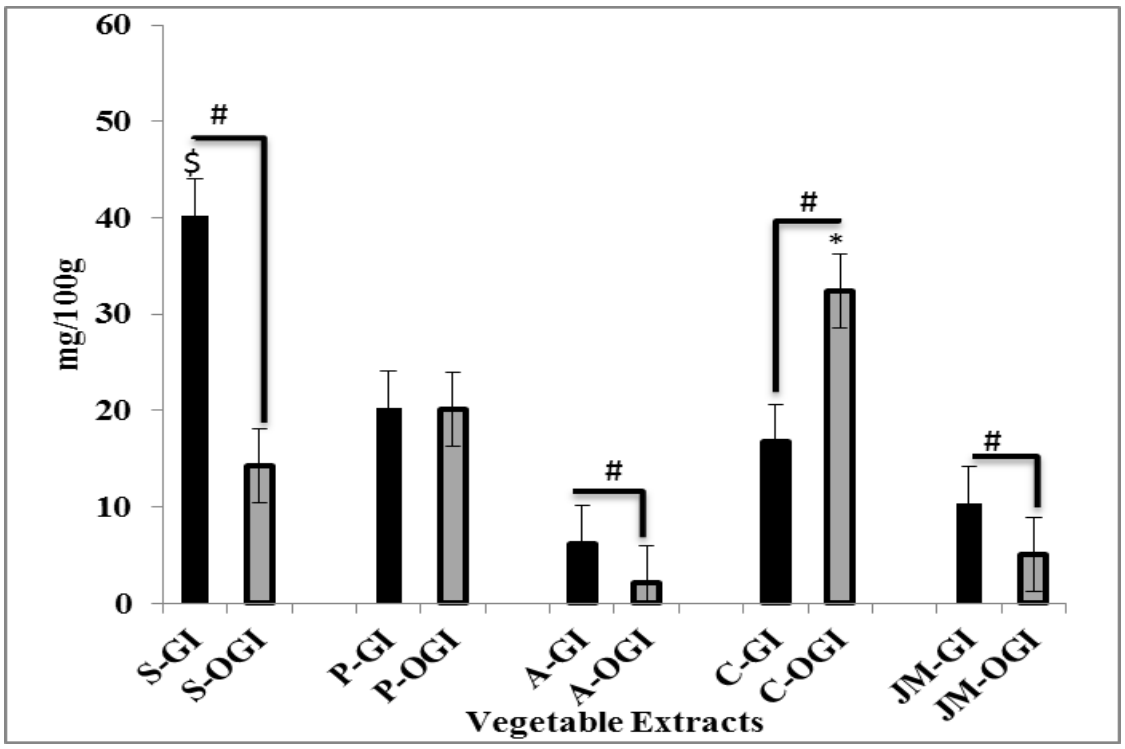


Figure 4.16b: Differences in the ß-carotene content of GI compared with OGI digested LGVs, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. $ indicate a LGV with the highest β-carotene after GI digestion. * indicate a LGV with the highest β-carotene after OGI digestion and # Indicates a significant difference between GI and OGI digestion, $p<0.05$.

OGI had variable effects on the antioxidant content of LGV. For spinach the TPC was increased and TFC was unchanged while the ß-carotene levels were reduced. The TPC for pumpkin was increased, TFC and ß-carotene levels were unchanged. For both amaranth and jute mallow all measured levels were reduced. For cowpea TPC was reduced, TFC was unchanged and ß-carotene levels were increased.

The TPC of cowpea and jute mallow, the TFC of amaranth, cowpea and jute mallow was higher than spinach. In contrast the β-carotene content of the LGV, cowpea was higher than spinach.

4.8.4 Effect of OGI digestion on the antioxidant activity (TEAC assay) of cooked LGV

The antioxidant activity measured with the TEAC assay (Table 4.14) was increased from 557.8±2.5 to 658.7±4.9 mM/g for all LGVs evaluated. The oral phase of digestion caused the largest increased in measured antioxidant activity while the contribution gastric and intestinal phases of digestion to the measured antioxidant activity was small (Table 4.14). Compared to

the cooked samples that antioxidant activity of all LGVs evaluated was increased (Figure 4.17a). Evaluation of the differences in antioxidant activity measured with the GI and OGI digestion models reveal that with OGI digestion antioxidant activity is increased for all samples (Figure 4.17b) and the antioxidant activity of all LGV is similar to spinach.

Table 4.14: Effect of OGI digestion on the antioxidant activity (TEAC assay) of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	166.3±13.5	114.6±12.2	342.3±14.4	264.0±6.5	340.7±5.3
Oral phase	639.9±14.5*	600.0±6.1*	649.8±22.4*	610.3±6.9*	568.9±12.3*
Gastric phase	702.6±22.2*	645.5±6.8*	693.9±26.1*	686.2±2.3*	657.7±5.0*
Intestinal phase	658.7±4.9*	602.2±23.5*	611.1±2.3*	600.1±11.1*	557.8±2.5*

Values are expressed as mean ± SD, # P<0.05, a significant decrease compared to cooked. * P<0.05, a significant increase compared to cooked.

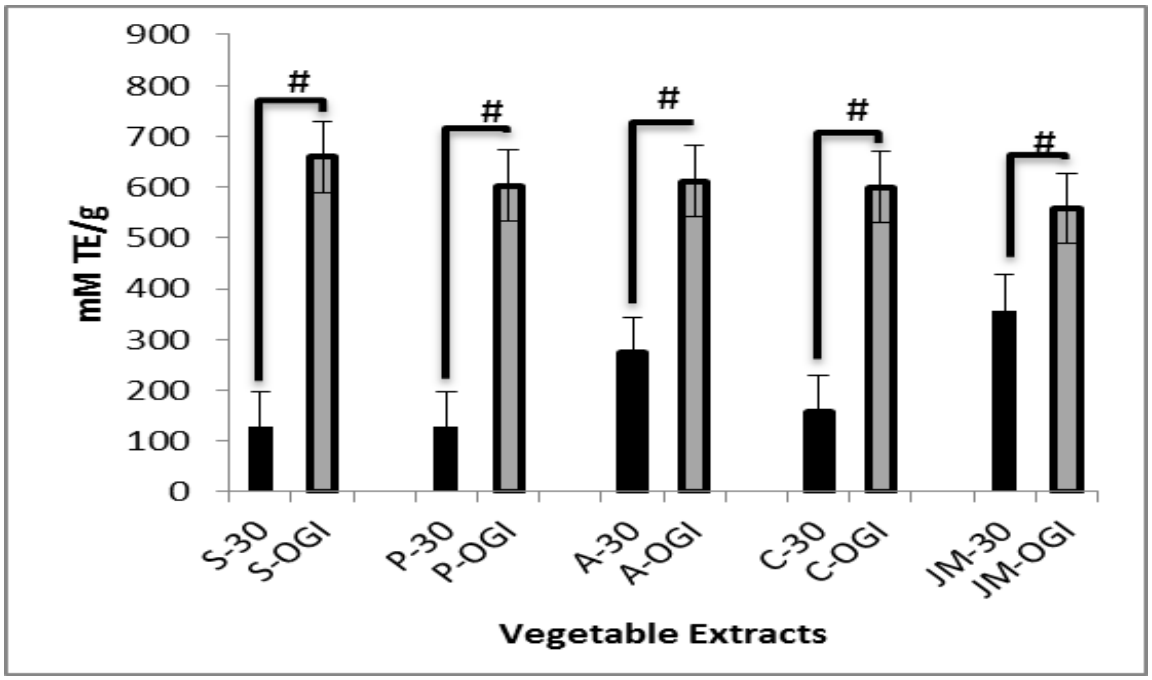


Figure 4.17a: Summary of the variable effects of digestion on the antioxidant activity (TEAC assay) of cooked LGV compared with OGI digests of spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest TEAC after OGI digestion. # Indicates a significant difference between cooked and OGI digested extracts, p<0.05.

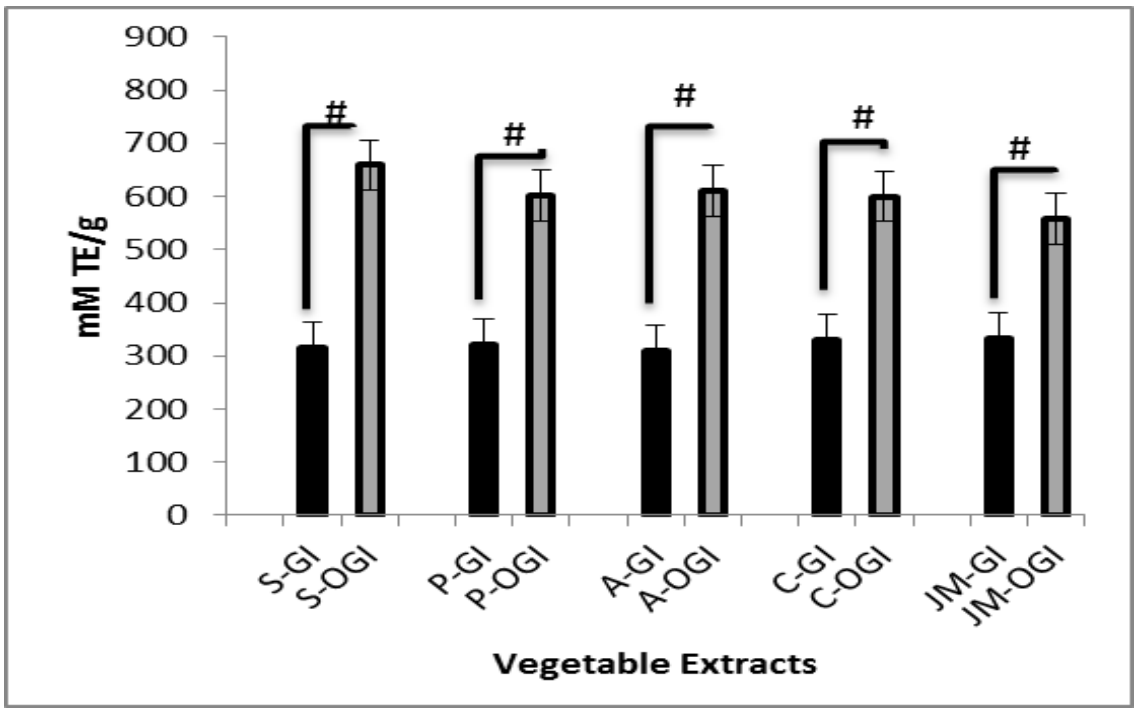


Figure 4.17b: Differences in the antioxidant activity (TEAC assay) of GI compared with OGI digested LGVs, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. $ indicate a LGV with the highest antioxidant activity after GI digestion. * indicate a LGV with the highest antioxidant activity after OGI digestion and # Indicates a significant difference between GI and OGI digestion, $p<0.05$.

4.8.5 Effect of OGI digestion on the antioxidant activity (DPPH assay) of cooked LGV

Antioxidant activity of the OGI digested LGVs as evaluated with the DPPH assay and the range of activity was -11.2±3.4 -48.6±4.3# mM/g (Table 4.15). Following the oral phase of digestion antioxidant activity was increased for spinach and pumpkin leaves but reduced for cowpea, amaranth and jute mallow. Gastric and intestinal digestion further caused an increase in antioxidant activity while for pumpkin gastric digestion caused a decrease and intestinal digestion an increase in antioxidant activity. For cowpea, amaranth and jute mallow levels remained low.

Table 4.15: Effect of OGI digestion on the antioxidant activity (DPPH assay) of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	-6.6±17.5	3.0±5.4	266.9±2.7	55.59±11.3	204.1±4.6
Oral phase	2.0±4.3*	40.1±6.8*	41.7±2.1#	30.0±3.1#	33.3±12.3#
Gastric phase	11.6±5.2*	20.5±11.3*	27.6±13.7#	28.7±8.4#	-1.5±3.3#
Intestinal phase	19.5±5.9*	42.7±9.6*	48.6±4.3#	29.7±9.9#	-11.2±3.4#

Values are expressed as mean ± SD, # P<0.05, a significant decrease compared to cooked. * P<0.05, a significant increase compared to cooked.

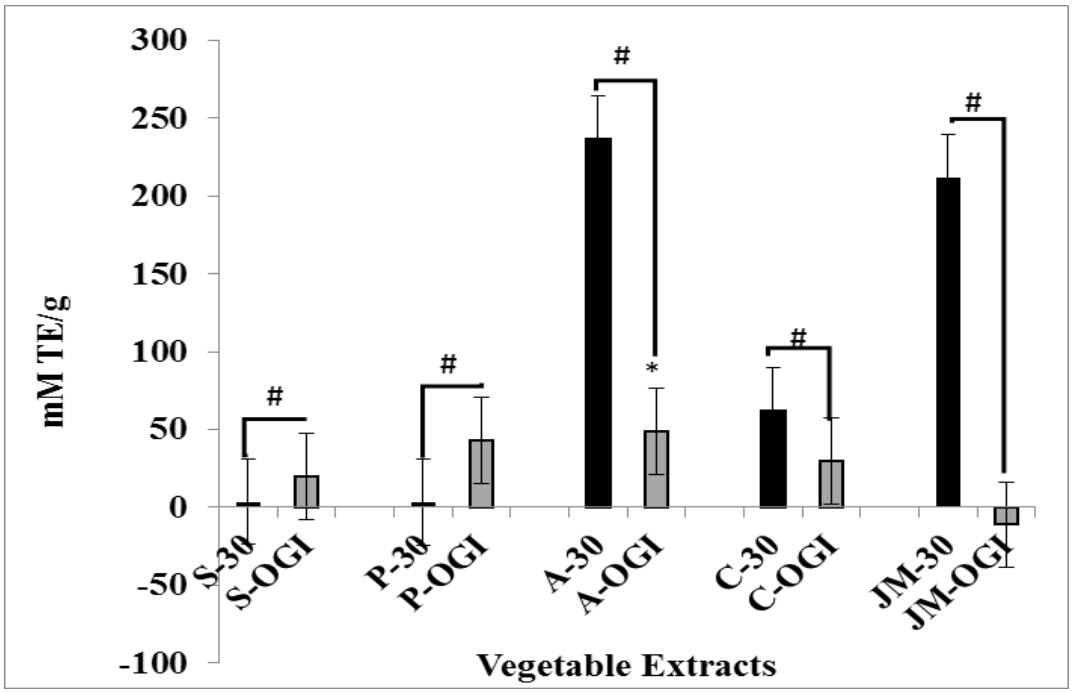


Figure 4.18a: Summary of the variable effects of digestion on the antioxidant activity (DPPH assay) of cooked LGV compared with OGI digests of spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest DPPH after OGI digestion. # Indicates a significant difference between cooked and OGI digested extracts, p<0.05.

In the simulated digestion model, the inclusion of the oral phase of digestion and bile acids in the intestinal phase resulted in an increase in antioxidant activity for spinach, pumpkin, amaranth and cowpea when measured with the DPPH assay.

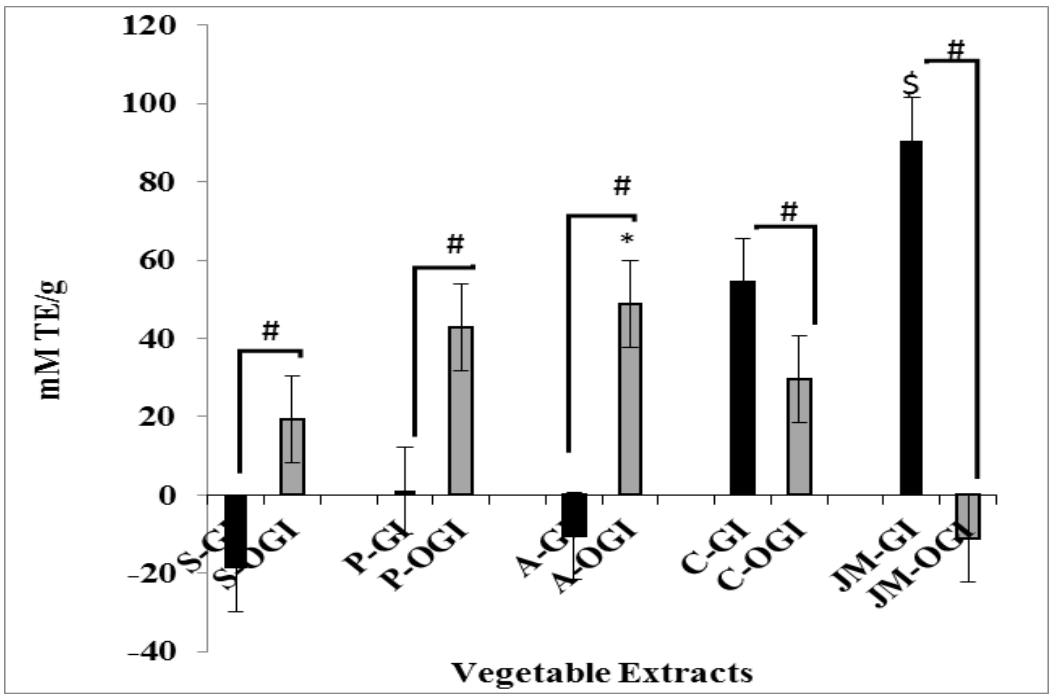


Figure 4.18b: Summary of the antioxidant activity (DPPH assay) of GI compared OGI digested LGVs, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. $ indicate a LGV with the highest antioxidant activity after GI digestion. * indicate a LGV with the highest antioxidant activity after OGI digestion and # Indicates a significant difference between GI and OGI digestion, $p<0.05$.

4.8.6 Effect of OGI digestion on the antioxidant activity (ORAC assay) of LGV

Determination of antioxidant activity using the ORAC assay increased after the oral phase of digestion of more than 100%, for all the LGVs. For spinach this was from 125.1 to 657.2 µM TE/g, for pumpkin, 35.1 to 853 µM TE/g, for amaranth, 225.2 to 746.8 µM TE/g, for cowpea, 267.3 to 759.1 µM TE/g and for jute mallow, 381.6 to 1140 µM TE/g. Following gastric digestion the antioxidant activity did not change, except for jute mallow which has a decreased antioxidant activity after gastric digestion to 487.8 µM TE/g and increased again after gastroduodenal to 869 µM TE/g (Table 4.16). Jute mallow had the highest antioxidant activity after OGI.

Table 4.16: Effect of OGI digestion on the antioxidant activity (ORAC assay) of cooked LGV

Digestion phase	Spinach	Pumpkin	Amaranth	Cowpea	Jute mallow
Cooked	125.1±9.1	35.1±17.3	225.2±24	267.3±2.0	381.6±3.9
Oral Phase	657.2±16.3*	853.0±4.5*	746.8±10.9*	759.1±6.9*	1140.0±11.8*
Gastric Phase	439.3±12.6*	655.9±11.9*	642.2±15.8*	651.4±21	487.8±22#
Intestinal Phase	538.2±3.1*	517.3±24.3*	609.0±4.4*	658.5±12.4	869.0±2.9*

Values are expressed as mean ± SD, # $P<0.05$, a significant decrease compared to cooked. * $P<0.05$, a significant increase compared to cooked.

OGI digestion increased the antioxidant activity of all the vegetable extracts except for the cowpea which showed a decrease in activity when measured by the ORAC assay (Figure 4.19a). Antioxidant activity measured by the ORAC assay was higher in GI digests than OGI digests, except for jute mallow, where both methods resulted in digests with the same antioxidant activity (Figure 4.19b). Cowpea had the highest antioxidant activity after GI, while jute mallow had the highest activity after the OGI.

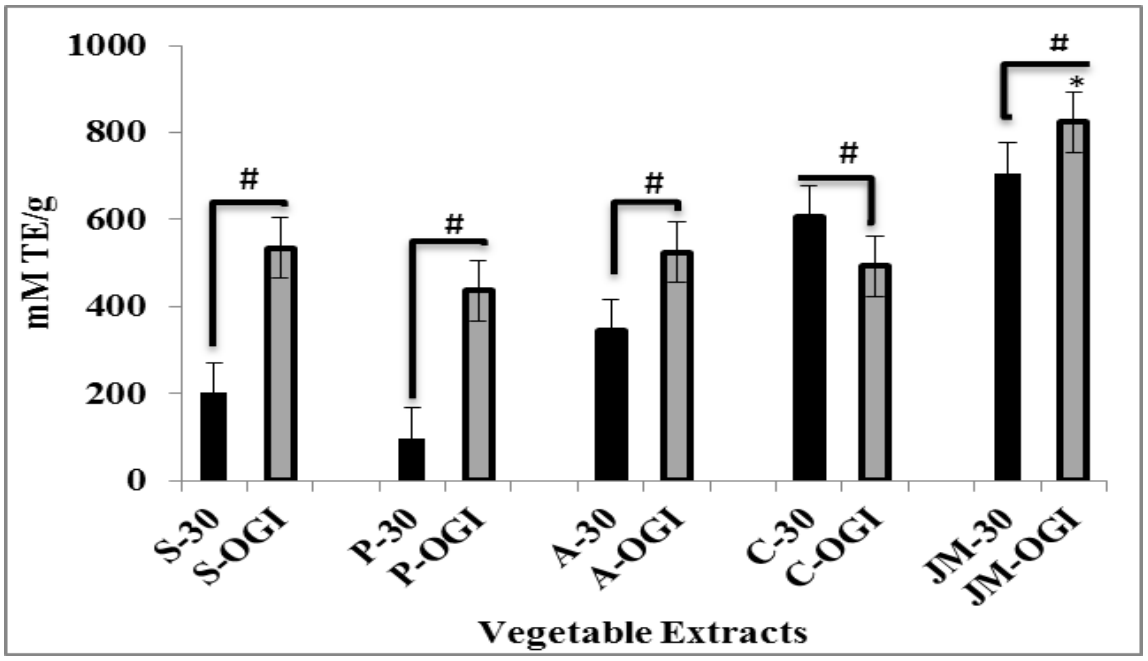


Figure 4.19a: Summary of the variable effects of digestion on the antioxidant activity (ORAC assay) of cooked LGV compared to OGI digests of spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM.* indicates LGV with the highest ORAC after OGI digestion. # Indicates a significant difference between cooked and OGI digested extracts, $p<0.05$.

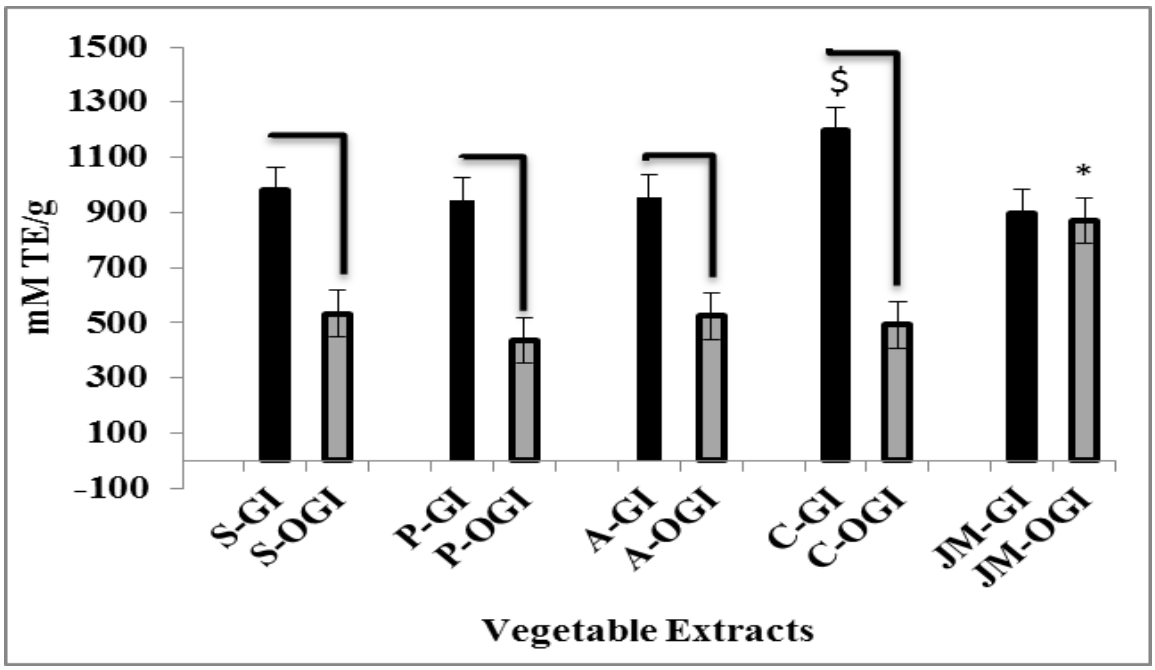


Figure 4.19b: Differences in the antioxidant activity (ORAC assay) of GI compared with OGI digested LGVs, spinach (S), pumpkin (P), amaranth (A), cowpea (C) and jute mallow (JM). Data is an average of at least 3 experiments ± SEM. $ indicate a LGV with the highest antioxidant activity after GI digestion. * indicate a LGV with the highest antioxidant activity after OGI digestion and # Indicates a significant difference between GI and OGI digestion, $p<0.05$.

The findings related to the effect of OGI digestion is summarised in Table 4.17. For spinach, TPC was increased, TFC unchanged and ß-carotene levels were reduced. Following digestion antioxidant activity measured with the TEAC and ORAC assays were increased. For pumpkin all measured parameters were increased following digestion. For cowpea, TPC was reduced, TFC unchanged and ß-carotene levels were increased. This translated in increased antioxidant activity measured by the TEAC and ORAC assays. In contrast antioxidant activity measured with the DPPH assay was reduced. The effects of digestion on the antioxidant properties of amaranth and jute mallow was similar. With digestion the TPC, TFC and ß-carotene levels were reduced although interestingly antioxidant activity was increased when measured with the TEAC and ORAC assays. With the DPPH assay measured antioxidant activity was unchanged. The latter results indicate that hydrophilic polyphenols and ß-carotene contribute significantly to measured activity.

Compared to spinach, the TPC and TFC of jute mallow was slightly higher. The ß-carotene levels of cowpea were greater than spinach. Antioxidant activity of all LGV evaluated was similar while the antioxidant activity of jute mallow measured with the ORAC assay was higher.

Correlation analysis between cooked and digested samples revealed a correlation between TPC and antioxidant activity measured with the ORAC assay, which implies with digestion there is an associated changed in TPC and this is reflected in the measured antioxidant activity.

Further correlation analysis showed a strong correlation between TPC and TFC as well as between TFC and the ORAC assay again indicating that polyphenols, including flavonoids contribute to the antioxidant activity of LGV, after OGI digestion.

Table 4.17: Summary of polyphenol, flavonoid, β-carotene content and antioxidant activity of cooked and OGI digested cooked LGV

		TPC (mg GAE/g)	TFC (mg/g CE)	β-carotene (mg/100g)	TEAC (mM TE/g)	DPPH (mM TE/g)	ORAC (mM TE/g)
Spinach	**C**	59.7±5.0	25.0±2.5	42.9±0.1	166.3±10.4	-6.6±2.4	125.1±16.0
	OGI	80.0±1.9	24.9±2.7	14.24±0.07	658.7±4.9	19.5±5.9	538.2±6.3
% Bio-availability		**134%**	99%	**33**%	**396**%	-295%	**430**%
Pumpkin	**C**	43.7±2.8	25.0±3.2	17.6±0.02	114.6±7.6	3.0±5.4	35.1±5.3
	OGI	74.0±3.0	27.4±0.96	20.2±0.04	602.2±3.5	42.7±9.6	517.3±4.3
% Bio-availability		**169%**	110%	**114**%	**530**%	**900**%	**1474↑**
Amaranth	**C**	212.6±8.2	102.2±4.0	15.5±0.3	342.3±7.9	266.9±9.8	225.2±2.4
	OGI	87.7±1.1	39.7±1.2	2.09±0.02	611.1±2.3	48.6±4.3	609±4.4
% Bio-availability		**41**%	**39**%	**13**%	**179%**	**18%**	**270%**
Cowpea	**C**	137.9±4.0	40.6±1.0	29.8±0.2	264.0±10.7	55.5±2.5	267.3±2.0
	OGI	93.3±2.5	41.4±2.5	32.3±0.3	600.1±11.1	29.7±9.9	658.5±12.4
% Bio-availability		**68%**	102%	**108%**	**227%**	54%	**246%**
Jute mallow	**C**	155.7±7.7	159.1±7.7	21.0±0.04	340.7±7.5	204.1±6.5	381.6±1.3
	OGI	105.7±1.3	56.5±1.5	5.1±0.03	557.8±2.5	-11.2±3.4	869±2.9
% Bio-availability		**67%**	**36%**	24**%**	**164%**	nd	**228**%
Correlation C vs OGI		0.65	**0.89**	0.35	-0.53	-0.10	**0.94**

Data is an average of at least 3 experiments; Mean ± SD values. Bold indicates changes in bio-accessibility.

Table 4.18: Correlation between assays for OGI digested LGV

	TFC	ß-carotene	TEAC	DPPH	ORAC
TPC	**0.964**	-0.245	-0.711	-0.714	**0.970**
TFC		-0.311	-0.853	0.610	**0.966**
ß-carotene			0.141	0.202	-0.308
TEAC				0.436	-0.800
DPPH					-0.793

4.9 DISCUSSION

The effect of the a more complex model of digestion which included the oral as well as lipase and bile acids in the intestinal phase on the content of molecules with antioxidant activity was determined. For African LGV variable effects were observed and the effect of digestion was different for each LGV. The TPC, TFC and ß-carotene content of amaranth and jute mallow following digestion was reduced, while for pumpkin the TPC and ß-carotene content was increased. In a more complex environment factors such as pH and the effects of proteolytic activity and micelle formation can affect the measured content.

The release of polyphenols from a food matrix is mostly determined by their chemical structure, such as its size, basic structure, degree of glycosylation, acetylation and polymerisation and solubility. In addition, the ability of these polyphenols to inhibit enzymatic activity can also play a role. Zheng, Hwand and Chung, (2009), reported that for apples, the addition of α-amylase and amyloglycan increased the extraction of polyphenols and this was due to the degradation of the cell wall polysaccharides. This increased extraction of polyphenols by α-amylase was found for spinach and pumpkin. Alvarez *et al.* (2016), reported that polyphenol content of the studied fruits and vegetables extracts obtained with enzymatic extraction was significantly high, implying that enzymes plays a major role in polyphenol extraction. The oral phase of digestion is at a neutral pH, and at this pH polyphenols degrade and such an effect was observed for amaranth, cowpea and jute mallow. The polyphenol fraction includes flavonoids and was less affected by α-amaylase and the TFC levels were unaltered for spinach, pumpkin and cowpea although TFC was reduced for amaranth and jute mallow.

Carotenoids rapidly degrade in an aqueous environment due to the presence of many double bonds. To reduce this effect Hedrén *et al.* (2002), added ascorbic acid to the digests to

prevent the oxidation of extracted carotenoids. LGV are a good source of vitamin C and the presence of vitamin C during the digestion process will ensure the stability of ß-carotene. The vitamin content of amaranth is 0.79 – 1.57 mg/100g (Funke, 2011), while that of spinach is 31.6 mg/100g (Favell, 1998), therefore due to the higher vitamin C content and associated antioxidant activity, the ß-carotene is to a greater degree protected against oxidation. Likewise the vitamin C content of cowpea leaves is 410mg/100g, in spite of a loss in vitamin C with cooking (Imungi and Potter, 2006), the raw levels are higher than that of spinach and therefore, still a greater protective effect would be observed as shown in Figure 4.3b.

Antioxidants in LGV include polyphenols, ß-carotene and vitamin C. Measurement of antioxidant activity is the sum of the effect of all these molecules. The antioxidant activity measured with the TEAC and ORAC assay showed a significant increase in antioxidant activity. As observed for GI digestion variable results were obtained with the DPPH assay. Floegel *et al.* (2011) in a study that evaluated the antioxidant activity of 50 antioxidant rich fruits, vegetables and beverages found that a strong positive correlation between the TEAC and ORAC assays. Also, antioxidant activity measured with the TEAC assay was higher than that measured with the DPPH assay. These authors recommended that the DPPH assay is not ideal for the evaluation of highly pigmented plant material such as LGV, however differences is related to the principle of the DPPH and TEAC assays.

In the present study a correlation was found between the cooked and digested samples for the TPC indicating that with digestion there is an associated change in TPC for all LGV and this is reflected in the measured antioxidant activity. For amaranth and jute mallow there was a decrease in TPC, TFC and ß-carotene content while the antioxidant activity was increased when measured with the TEAC and ORAC assays. This implies that other molecules found in the extracts are contributing to measured antioxidant activity and this may be the presence of peptides with antioxidant activity. Peptides with antioxidant activity have been found natural products (Zou, *et al.,* 2016) such as cereals, legumes and fermented products.

In general, the inclusion of the oral phase for digestion resulted in a reduction in antioxidant activity measured with the TEAC and ORAC assays. Therefore, the GI digestion model that is widely used results in an overestimation of the antioxidant properties of LGV. This may be due to amylase in the oral phase digesting the polysaccharides in the cell wall of LGV resulting in the release of polyphenols. These polyphenols, due to them not being associated

with the plant matrix undergo degradation during intestinal digestion. In a study by Neilson *et al*, (2007), when analyzing the bioavailability of catechins, these researchers concluded that these flavonoids underwent degradation with digestion. The greater the degree of digestion the more polyphenols are released and in a soluble environment are more vulnerable to neutral pH associated degradation.

4.10 CONCLUSION

OGI digestion like GI digestion gave variable results. For spinach, TPC was increased, TFC unchanged and ß-carotene levels were reduced and associated antioxidant activity was increased (TEAC and ORAC assays). For pumpkin all measured parameters were increased. For cowpea, TPC was reduced, TFC unchanged and ß-carotene levels were increased which translated in increased antioxidant activity measured by the TEAC and ORAC assays. In contrast antioxidant activity measured with the DPPH assay was reduced. The effects of digestion on the properties of amaranth and jute mallow were similar. With digestion the TPC, TFC and ß-carotene levels were reduced although interestingly antioxidant activity was increased (TEAC and ORAC assays). With the DPPH assay measured antioxidant activity was unchanged. This implies that other bioactive molecules besides polyphenols and ß-carotene are responsible for the increase in antioxidant activity.

CHAPTER 5: CONCLUDING DISCUSSION

5.1 SUMMARY OF RESULTS

The cooking of LGV, spinach, pumpkin, amaranth, cowpea and jute mallow was simulated and as traditionally done the cooking water was discarded. Water extracts of the uncooked and cooked LGV were prepared and the antioxidant properties of these extracts were compared. The cooked LGVs were then digested using a two laboratory based models that simulate digestion and these were the GI and OGI models. The GI model provided information on the effect of pH and the presence of proteolytic enzymes on the polyphenol, flavonoid and β-carotene content of LGV as well as the associated antioxidant activity determined with the TEAC, DPPH and ORAC assays. A more complex model that included the oral phase of digestion as well as bile salts and lipase in the intestinal phase of digestion was also used and the contribution of oral digestion and the presence of bile salts and lipase on the measured parameters was also determined.

Firstly, the effect of cooking on the measured parameters was variable. For amaranth and jute mallow the effect of cooking was similar. The TPC and antioxidant activity measured with the TEAC assay was not change. TFC and β –carotene content was increased and antioxidant activity (DPPH assay) was increased. Cooking had no effect of on the β-carotene content of spinach, pumpkin and cowpea. In contrast the polyphenol, flavonoid and antioxidant activity was reduced after cooking.

Using the GI model, gastric associated protein digestion of the plant matrix increased extraction of the polyphenols and flavonoids occurred, however the β-carotene content was reduced. Associated antioxidant activity (TEAC and ORAC assays) was significantly increased. In contrast, antioxidant activity evaluated with the DPPH assay was mostly reduced. Many studies have identified that pH is the major contributing factor to the extraction and at neutral pH the degradation of polyphenols including flavonoids. In the present study, the presence of proteolytic enzymes that caused the degradation of the food matrix and subsequent release of polyphenols was identified as the major contributing factor.

ODI digestion, which included the oral phase of digestion and bile salts and lipase required for micelle formation required for the absorption of ß-carotene was evaluated. For spinach, TPC was increased, TFC unchanged and ß-carotene levels were reduced and antioxidant

activity measured with the TEAC and ORAC assays was increased. All measured parameters for pumpkin were increased. For cowpea, TPC was reduced, TFC unchanged and ß-carotene levels were increased as well as antioxidant activity measured with the TEAC and ORAC assays. Antioxidant activity measured with the DPPH assay was reduced. The properties of amaranth and jute mallow following digestion was similar. With digestion the TPC, TFC and ß-carotene levels were reduced but antioxidant activity measured with the TEAC and ORAC assays was increased and antioxidant activity measured with the DPPH assay was unchanged. This implies that other molecules besides polyphenols and ß-carotene are responsible for the increase in antioxidant activity and these could be bioactive peptides that are generated following proteolytic digestion of protein.

The oral phase of digestion contributed significantly to the increased polyphenol and flavonoid content measured before gastric digestion. However, oral phase digestion caused a decrease in ß-carotene content. Comparison between cooked and complex digested samples revealed an increase in antioxidant activity measured with the TEAC, variable changes with the DPPH assay and a reduction in antioxidant activity measured with the ORAC assay. The ORAC assay is considered to be a physiologically relevant assay for the measurement of antioxidant activity and methods that exclude the oral phase of digestion may overestimate the bioactivity of LGV.

The bioactivity of several of the LGV evaluated were comparable to spinach, taking into account the benefits related to water consumption, resistance to drought and ease of cultivation (Cernansky, 2015), the cultivation and consumption of especially pumpkin and cowpea should be promoted.

5.2 LIMITATIONS AND RECOMMENDATIONS

Oxidative damage plays a central role in the development of diseases such cancers of the GIT. The incidence of these diseases are increasing in southern Africa. Several studies have shown that diets rich in LGV and fruit reduces the risk of developing these non-communicable diseases. In this study African LGVs and spinach have been identified as ideal LGV that can be cultivated and the consumption of these LGV can contribute to prevention of these diseases. Using a simulated, laboratory-based digestion it was found that significant levels of polyphenols and ß-carotene is retained as well as associated antioxidant activity.

A limitation of this study is that it does not provide information on whether these digests can protect physiologically relevant cells and tissue such as that found in the oesophagus, stomach and intestines against oxidative damage, thereby preventing the development of GIT associated cancer and inflammatory disorders.

Halliwell (2007) reported that it has been estimated that flavonoids can reach concentrations as high as 300 µM in the GIT and polyphenols, before absorption, do protect the GIT from oxidative damage, especially by directly preventing and quenching of oxygen radical species, thereby preventing and/or delaying the development of stomach, colon and rectal cancers. Cell lines that that can be used to evaluate these cellular effects include Suit-2 and the Caco-2 cell lines that represent the oesophagus, stomach and the colon, respectively (Youns and Abdel Halim Hegazy, 2017). Cellular antioxidant activity should be determined using the dichloroflourescein diacetate (DCFH-DA) assay as described by Serem and Bester (2012).

A major limitation of this study is that the polyphenols contributing to activity were not identified. It is important to also determine at each phase of digestion which polyphenols are sensitive to the effects of pH and proteolytic enzyme activity. This will identify, those polyphenols that are bioavailable following digestion. Polyphenols that are not absorbed in the stomach or small intestines are carried to the colon. Colonic microorganisms have catalytic and hydrolytic potential, therefore deconjugation reactions readily occur. Examples of polyphenols that are not hydrolysed by the digestive enzymes and that are readily hydrolysed by gut microflora such as *Bacteroids distasonis, B.uniformis and B. ovatus* are quercetin-3-0-rhamnoglucoside and quercetin-3-0-rhamnoside (Scalber and Williamson, 2000).

Each identified polyphenol may be re-evaluated for activity alone or in combination to determine if synergism occurs. Likewise, the ability of the identified polyphenols to protect ß-carotene against oxidative damage can also be determined. High performance liquid chromatography-mass spectrometry (HPLC-MS) can be used to firstly identify the polyphenols in the cooked samples and then the effect of digestion on the concentration of each individual polyphenol can be determined as described by Apea-Bah *et al*., (2014).

Although, simulated *in vitro* methods are meant to provide rapid testing of food ingredients *in vivo* animal studies can provide information on the bioavailability of these polyphenols. Absorption and the distribution of these polyphenols can be determined by measuring levels

as well as the total antioxidant capacity of the blood. Lastly dietary intervention studies can be undertaken to determine the effects of cooked LGV on the health and well-being of South African communities. (Faber and Wenhold, 2007; Faber *et al*, 2002)

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