CHAPTER 1: INTRODUCTION

1.1 Statement of the Problem

Consumption of vegetables has been associated with a reduction in the incidences of chronic diseases of lifestyle (CDL) such as cardiovascular diseases (CVD), coronary heart diseases (CHD) and various types of cancer. With increasing urbanization and westernization of dietary and other socio-behavioural attitudes in most sub-Saharan African countries, it is estimated that the burden of these diseases will increase to epidemic proportions in the region (Addo *et al*., 2007; Parkin *et al*., 2008). In sub-Saharan Africa as a whole, the magnitude of these diseases has been under-recognized and under-prioritized because of competing health priorities such as HIV/AIDS, tuberculosis and malaria (Parkin *et al*., 2008).

African green leafy vegetables (GLVs), also known generically as African spinach, contribute significantly to household food security and add variety to cereal-based staple diets in sub-Saharan Africa (van den Heever, 1997). The consumption pattern of these vegetables across Africa is however highly variable and depends on factors such as poverty status, degree of urbanization, distance to fresh produce markets and season of year (Jansen van Rensburg et *al*., 2007). Over the years, the frequency of consumption of African GLVs in sub-Saharan Africa has decreased. A possible reason for the decrease is that in this increasingly urbanized region of Africa, African GLVs remain seasonal in rural areas and, unlike the exotic vegetables, are not readily available in the urban areas. Furthermore, these vegetables are often considered to be inferior in their taste and nutritional value compared to exotic vegetables such as spinach (*Spinacea oleracea* L.) and cabbage (*Brassica oleracea* subsp. *capitata*) (Weinberger and Msuya, 2004). This perception is prevalent despite the fact that several studies have indicated that African GLVs contain micronutrient levels as high as or even higher than those found in most of their exotic counterparts (Kruger *et al*., 1998; Odhav *et al*., 2007; Steyn *et al*., 2001; Weinberger and Msuya, 2004). For example, Jansen van Rensberg *et al*. (2007) found that in South Africa, poor households consume more African GLVs than their

wealthier counterparts, while in Uganda, consumption is limited to casual encounters (Tabuti *et al*., 2004).

African GLVs contain phenolic compounds that have been shown to have antioxidant properties (Salawu *et al*., 2008 and 2009). In sub-Saharan Africa, the vegetables are usually cooked and less commonly stirfried and steamed before consumption. The level of antioxidant and radical scavenging activity after cooking is dependent on a number of factors including the type of vegetable, type and duration of boiling, boiling temperature, bioavailability of phenolics, localization and stability at high temperatures (Jimenez-Monreal *et al*., 2009). Differences in tissue hardness and phenolic profile of each vegetable are also major contributors to antioxidant activity (Yamaguchi *et al*., 2001). Published data on the effect of processing on phenolic contents, antioxidant activity of GLVs in general are inconsistent and seem to depend on the plant species, as well as the type of assay used for analysis. Although studies on the effect of thermal processing on the phenolic composition and antioxidant activity of African GLVs are limited, the trends are probably similar to those reported for GLVs originating elsewhere. Salawu *et al*. (2009) found that almost all phenolic constituents were stable after 10 min of boiling four African GLVs, while Salawu *et al*. (2008) found a decreased total phenolic content in *Corchorus olitorius* after boiling for 15 min. For Japanese vegetables, Yamaguchi *et al*. (2001) found both increases and decreases in polyphenol content after cooking. A decrease in polyphenol content could be due to leaching or heat lability of specific phenolics. An increase could be due to release of phenolics from large cellular components such as the cell wall material to which the phenolics may be bound and/or the inhibition of oxidative enzymes (Yamaguchi *et al*., 2001).

In a study based on secondary intake data, Louwrens *et al*. (2009) reported that South Africans only consumed about half of their total antioxidant requirement. Understanding the antioxidant properties of African GLVs is therefore crucial for reducing pathogenesis related to CDL in sub-Saharan Africa. Currently, the available data on the phenolic composition and antioxidant activity of some African GLVs (Akindahunsi and Salawu, 2005; Lindsey *et al*., 2002; Oboh *et al*., 2008; Odhav *et al*., 2007; Odukoya *et al*., 2007; Salawu *et al*., 2008 and 2009; Stangeland *et al*., 2009; van der Walt *et al*., 2009) is fragmented and incomplete. Different methods of

analysis and standards have been used to analyze the antioxidant content and activity of these vegetables, therefore in most cases, the results cannot be compared. The effect of boiling on these parameters in African GLVs has also not yet been well established. Knowledge on the above stated issues is required so that the potential of African GLVs as a source of antioxidant phenolics can be evaluated. The need for cost effective dietary chemopreventive compounds as well as the diverse biological activities of phenolics makes it even more necessary to investigate the ability of African GLVs phenolics to show antioxidative properties and health-promoting effects. The results of such investigation may form the basis for promoting the utilization of these vegetables in managing CDL, as well as the cultivation and commercialization of these vegetables in sub-Saharan Africa.

1.2 Literature Review

The potential contribution of African GLVs to household nutritional well-being is reviewed. Information regarding polyphenolic content and antioxidant activity of GLVs, as well as the effects of thermal processing thereon has been reviewed. The health-promoting effects associated with phenolic compounds found in these GLVs are discussed. The chemical and biological assays used for determining *in vitro* antioxidant activity of GLVs are also reviewed.

1.2.1 African green leafy vegetables

The term "African GLVs" refers to those vegetables largely consumed by Africans at a household level. This term includes GLVs that are endemic, indigenous, naturalized, traditional and local to sub-Saharan Africa. These vegetables are commonly harvested in the wild or may occur as spontaneous plants or weeds in cultivated fields. They are well adapted to the local environmental conditions and grow well with minimal water and poor soil fertility. To determine the antioxidant content and activity of African GLVs, four types regularly consumed within this region were chosen based on their popularity in South Africa (Fig. 1.2.1). The origin, characteristics and growth requirements of these African GLVs (*Amaranthus cruentus* L. (amaranth), *Corchorus olitorius* L. (jute mallow), *Cucurbita maxima* Duchesne (pumpkin) and *Vigna unguiculata* (L.) Walp. (cowpea)) are discussed below.

Amaranthus cruentus L.

Amaranthus cruentus L. belongs to the Amaranthaceae family and is an erect herb with oblong green leaves. Although it originates from Southern Mexico and Central America (van Wyk, 2005), it is now widely distributed throughout Africa. It is a C4 plant that grows optimally under warm conditions (above 25 °C during the day and not lower than 15 °C at night) (Jansen van Rensburg *et al*., 2007). The young leaves, growth points and whole seedlings of amaranth are harvested and cooked for use as a vegetable (Jansen van Rensburg *et al*., 2007).

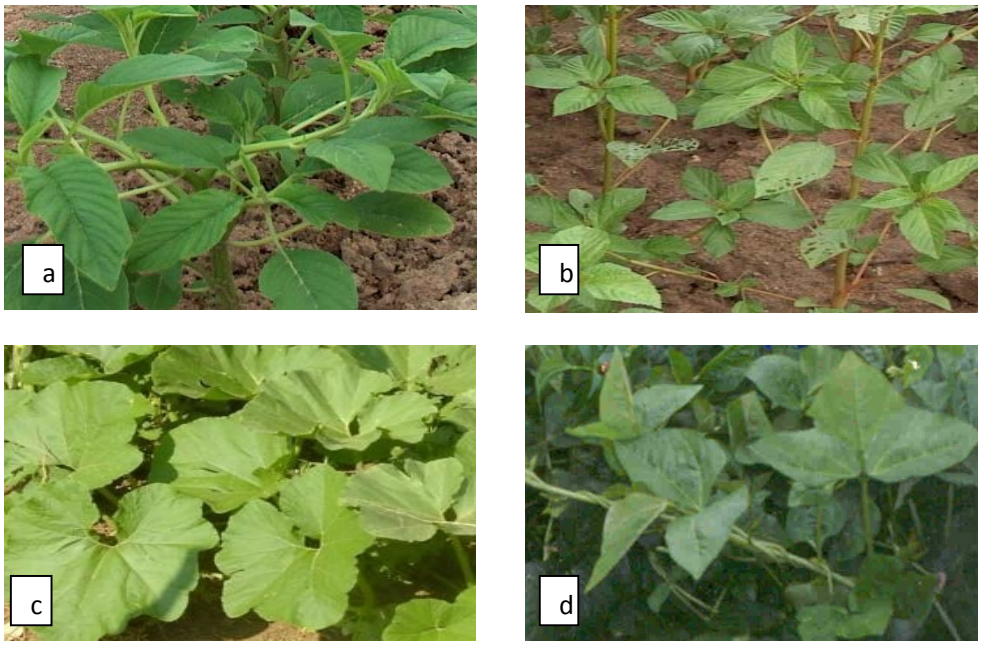


Figure 1.2.1 Photographs of African green leafy vegetables (a) *Amaranthus cruentus* L., (b) *Corchorus olitorius* L., (c) *Cucurbita maxima* Duchesne, and (d) *Vigna unguiculata* (L.) Walp. (photos obtained from W. Jansen van Rensberg).

Corchorus olitorius L.

Also known as jute mallow, *Corchorus olitorius* L. belongs to the Tiliaceae family and is an erect annual herb with oblong leaves that have serrated margins, distinct hair-like teeth at the base and small yellow flowers (van Wyk and Gericke, 2000). It originates from India (van Wyk, 2005), but it is now naturalized in Africa (Woomer and Imbumi, 2003). Jute mallow prefers warm, humid conditions and performs well in areas with high rainfall and high temperature (30 °C during the day and 25 °C at night) (Jansen van Rensburg *et al*., 2007). Cooked jute mallow has a mucilaginous texture. In South Africa, bicarbonate of soda is added to the cooking water to reduce the sliminess (Jansen van Rensburg *et al*., 2007), while in East Africa, jute mallow is usually combined with other African GLVs such as cowpea leaves because it is slimy when prepared on its own (Woomer and Imbumi, 2003).

Cucurbita maxima Duchesne

Also known as pumpkin, *C. maxima* Duchesne is a member of the Cucurbitaceae family and is almost vine-like, annual and herbaceous plant. Pumpkins are native to South America (Peru)

(van Wyk, 2005), but the leaves are widely used as vegetables in Africa. Pumpkins are characterized by long-running bristle stems with large deeply fine-lobed leaves, which have serrated margins. The leaves and the stem are covered in sharp and stiff translucent hairs (Jansen van Rensburg *et al*., 2007). The leaves are harvested on a regular basis as the plant grows (Onyeike *et al*., 2003). However, only young leaves are harvested for consumption, and can be consumed on their own.

Vigna unguiculata (L.) Walp

Commonly known as cowpea, *V. unguiculata* (L.) Walp. belongs to the Leguminosae family and are annual or perennial herbaceous plants with tri-foliate leaves (Jansen van Rensburg *et al*., 2007). Cowpea is indigenous to Africa where it was domesticated over 4000 years ago (van Wyk, 2005; Woomer and Imbumi, 2003). The varieties of this crop vary from prostate, indeterminate, erect, determinate, to low-branching types (Jansen van Rensburg *et al*., 2004). The varieties mainly used as leafy vegetables are the spreading, prostate types. The leaves are picked at about 4 weeks after planting, and this continues until the plants start to flower. These leaves are cooked as spinach and can be dried for later use.

1.2.2 Nutritional composition of African GLVs

African GLVs are a rich source of dietary fibre, vitamins, minerals and other components that have bioactive properties for good health (Gupta and Bains, 2006). However, no single vegetable provides all the nutrient requirements, therefore a diversified diet is needed to meet household daily micronutrient requirements (Grusak and Dellapenna, 1999). The proximate composition of 22 species of African GLVs is presented in Table 1.2.1.

Table 1.2.1 Proximate composition of some African green leafy vegetables (values per 100 g edible portion, fresh weight (fw) basis) (Uusiku *et al*., 2010).

African GLVs	Energy kJ (kcal)	Moisture (%)	Protein (g)	Fibre (g)	Fat (g)	Carbohydrates (g)
Adansonia digitata	289 (69)[a]	77[a]	4[a]	3[§a]	0.3[a]	16[†a]
***Amaranthus* sp.**	**113 - 222 (27- 53)**[b]	**83 - 91**[b]	**4 - 6**[b]	**3**[§b]	**0.2 - 0.6**[b]	**4 - 8**[†b]
Arachis hypogea	297 (71)[c]	82[c]	4[c]	8[§c]	0.5[c]	13[†c]
Bidens pilosa	163 - 222 (39 - 53)[a,b,d]	85 - 88[a,b,d]	3 - 5[a,b,d]	3 - 6[§a,b,d]	0.4 - 0.6[a,b,d]	2[‡d], 8[†ab]
Brassica sp.	100 - 142 (24 - 34)[c]	92 - 94[c]	1 - 2[c]	2 - 4[§c]	0.1 - 0.3[b]	5 - 6[†c]
Ceratotheca triloba	259 (62)[b]	85[b]	2[b]	2[§b]	2.1[b]	8[†b]
Chenopodium album	212 - 247 (44 - 59)[b,d]	83 - 85[b,d]	4 - 5[b,d]	2[§b,d]	0.8[b]	2[‡d], 8[†b]
Cleome sp.	142 - 218 (34 - 52)[a,b,d]	85 - 88[a,b,d]	5[a,b,d]	1 - 5[§a,b,d]	0.3 - 0.9[a,b,d]	2[‡d], 5[†ab]
Corchorus olitorius	**n.d.a.**	**n.d.a.**	**16**[δh]	**2**[δh]	**1.7**[δh]	**70**[δh]
***Cucurbita* sp.**	**109 (26)**[d]	**93**[d]	**3**[d]	**2**[§d]	**0.7**[d]	**0.4**[‡d]
Emex australis	151 (36)[b]	89[b]	5[b]	2[§b]	0.6[b]	3[†b]
Galinsoga parviflora	171 (41)[b]	89[b]	4[b]	1[§b]	0.5[b]	5[†b]
Ipomoea batatas	188 - 276 (45 - 66)[a,c,d]	83 - 88[a,c,d]	4 - 5[a,c,d]	2 - 5[§a,c,d]	0.2 - 1.1[a,c,d]	4[‡d], 10[†c]
Justicia flava	213 (51)[b]	84[b]	3[b]	1[§b]	0.4[b]	9[†b]
Lesianthera africana	305 (73)[e]	77[e]	3[e]	4[**e]	1.1[e]	n.d.a.
Manihot esculenta	381 (91)[b]	72[b]	7[b]	4[§b]	1.0[a]	18[†b]
Momordica sp.	222 (53)[a]	85[a]	5[a]	3[§a]	5.0[b]	7[†a]
Portulaca oleracea	96 (23)[b]	93[b]	3[b]	1[§b]	0.3[b]	3[†b]
Senna occidentalis	351 (84)[b]	77[b]	7[b]	3[§b]	2.2[b]	9[†b]
Solanum sp.	230 - 243 (55 - 58)[b,d,g]	83 - 90[b,d,g]	3 - 5[b,d,g]	1[**g], 2 - 6[§b,d]	0.6[b]	2[‡d], 9[†b]
Spinacea oleracea	**125 (30)**[d]	**92**[d]	**3**[d]	**3**[§d]	**0.4**[d]	**1**[‡d]
Vernonia sp.	167 - 343 (40 - 82)[a,f]	79 - 89[a,f]	3 - 5[a,f]	2 - 5[§a,f]	n.d.a.	n.d.a.
Vigna unguiculata	**180 (43)**[d]	**86**[d]	**5**[d]	**4**[§d]	**0.4**[d]	**2**[‡d]

[§] dietary fibre, [**] crude fibre, [†] carbohydrate value by difference, [‡] available carbohydrate, [δ] values on dry weight basis, n.d.a.: no data available, names and values in bold are of those GLVs currently being studied. [a] FAO, 1990; [b] Odhav *et al*., 2007; [c] Mosha and Gaga, 1999; [d] Kruger *et al*., 1998; [e] Isong and Idiong, 1997; [f] Ejoh *et al*., 2007; [g] Oboh *et al*., 2005; [h] Ndlovu and Afolayan, 2008.

1.2.2.1 Proximate composition of African GLVs

The energy values of selected African GLVs range from 23 - 91 kcal (96 - 381 kJ) for *Portulaca oleracea* and *Manihot esculenta*, respectively per 100 g edible portion. The carbohydrate content is highest (18 g/100g) for *Manihot esculenta* and lowest (0.4 g/100 g) for *Cucurbita pepo*. While the fat content range from 0.1 to 5.0 g per 100 g in *Brassica oleracea* subsp. *capitata* and *Momordica balsamina*, respectively. Compared to cereals and legumes, African GLVs are not very good sources of energy, carbohydrates and fat (Table 1.2.1). However, these vegetables may contribute to the total energy intake in individuals, especially in the populations whose diets normally are of marginal nutrient density. In 1999, the National Food Consumption Survey conducted in South Africa found that a significant majority of children aged 1-9 years consumed a diet deficient in energy and of poor nutrient density (MacIntyre and Labadarios, 2000).

The protein content of African GLVs range between 1 and 7 g per 100 g edible portion, and some African GLVs have higher protein content than their exotic counterparts (Kruger *et al*., 1998). FAO (1990) and Odhav *et al*. (2007) reported that the crude protein content of both *Senna occidentalis* and *Manihot esculenta*, was 7 g/100 g (fresh basis), which is greater than that reported for cabbage (*Brassica oleracea* subsp. *Capitata*) (Mosha and Gaga, 1999) and spinach (*Spinacea oleracea*) (Kruger *et al*., 1998) with values of 1 g/100 g and 3 g/100 g, respectively. Differences in the agro-climatic conditions may account for the variation in protein content for *Bidens pilosa* observed by FAO (1990), Kruger *et al*. (1998) and Odhav *et al*. (2007). Compared to legumes, GLVs are not very good sources of protein, which necessitates supplementation of the diet with animal protein or proteins from legumes to effectively contribute to good health.

As shown in Table 1.2.1 the crude fibre content of African GLVs varies from 1 g/100 g in *Galinsoga parviflora*, *Justicia flava*, *Portulaca oleracea* and *Solanum macrocarpon* to 8 g/100 g in *Arachis hypogea*. The total dietary fibre content of these African GLVs may have varied due to differences in stages of plant maturity, seasonal variation, fertilizers or chemicals used, variety of plant, geographical location and the method used for analysis (Aletor *et al*., 2002;

Punna and Parachuri, 2004). It is reported that dietary fibre has protective effects against colorectal cancer (Eastwood, 1999; Ferguson and Harris, 1999; Key *et al*., 2002). Increased migration of communities from rural areas to cities in sub-Saharan Africa is often associated with significant changes in diet, and an increase in diseases associated with consumption of a diet high in sugar and fat and low fibre contents such as diabetes, cardiovascular disease and cancer such as colorectal cancer (Walker *et al*., 2002). Inclusion of African GLVs in the household diet can potentially increase dietary fibre intake.

1.2.2.2 Micronutrient content of African GLVs

Several types of vitamins such as Vitamin A and C as well as folate and riboflavin are found in African GLVs (Table 1.2.2). African GLVs are a rich source of Vitamin A and C compared to folate and riboflavin. The presence of these vitamins in African GLVs can address their deficiencies in human populations. Worldwide, it is estimated that 33.3%, or 190 million children younger than 5 years are at risk of vitamin A deficiency, with Africa having the second highest prevalence of vitamin A deficiency, at 44.4% (WHO, 2009). Beta-carotene is the most important of the provitamin carotenoids in terms of its relative provitamin A activity and quantitative contribution to the diet (SACN, 2005). Lately, emphasis has been put on increasing the intake of dark-green leafy vegetables and yellow-orange fruits and vegetables to improve vitamin A intake. African GLVs can therefore play a significant role in this regard. The β-carotene content of African GLVs is highly species dependent and varies from 99 µg RE/100 g in *Vigna unguiculata* to 1970 µg RE/100 g for *Manihot esculenta* (Table 1.2.2). The effectiveness of GLVs in improving vitamin A status has however been questioned because a study by De Pee *et al*. (1995) showed that the bioavailability of β-carotene from GLVs is less than previously thought. A recent study, however, showed that daily consumption of cooked, pureed GLVs improved vitamin A status in populations at risk of vitamin A deficiency (Haskell *et al*., 2004). Takyi (1999) showed that an increased intake of African GLVs, with fat added, contributed significantly to improving the vitamin A status in children.

Table 1.2.2 Vitamin and mineral content of African green leafy vegetables (values per 100 g edible portion, fw basis) (Uusiku *et al.*, 2010).

African GLVs	Vitamin A (µg RE)	Vitamin C (mg)	Folate (µg)	Riboflavin (mg)	Calcium (mg)	Iron (mg)	Magnesium (mg)	Zinc (mg)
Adansonia digitata	n.d.a.	52[a]	n.d.a.	n.d.a.	410[a]	n.d.a.	n.d.a.	n.d.a.
***Amaranthus* sp.**	**327**[c]	**46 - 126**[c,j]	**64**[c]	**0.1 - 0.4**[a,c]	**253 - 425**[a,b,c]	**0.3 - 3.8**[b,d]	**105 - 224**[b,c]	**0.02 - 8.4**[b,d]
Arachis hypogea	n.d.a.	87[e]	n.d.a.	n.d.a.	n.d.a.	1.0[e]	n.d.a.	2.9[e]
Aystasia gangetica	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	0.6 - 3.7[b,f]	n.d.a.	0.1 - 1.1[b,d]
Bidens pilosa	301 - 985[a,c]	23[c,j]	351[c]	0.2[c]	162 - 340[a,b,c]	2.0 - 6.0[b,c]	79 - 135[b,c]	0.9 - 2.6[b,c]
Brassica sp.	n.d.a.	30 - 113[c,e]	16[c]	0.0 - 0.2[c,e]	27 - 31[c,d]	0.5 - 3.5[e]	13[c]	0.9 - 1.3[e]
Ceratotheca triloba	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	2.9[b]	n.d.a.	0.5[b]
Chenopodium album	917[c]	31[c]	30[c]	0.3[c]	15 - 226[c,d]	2.2 - 6.1[b,c]	155 - 211[b,c]	1.4 - 18.5[b,c]
Cleome sp.	1200[c]	13 - 50[a,c,e]	346[c]	0.1[c]	31 - 288[a,b,c]	2.6 - 2.9[b,c]	44 - 76[b,c]	0.6 - 0.8[b,c]
Colocasia esculenta	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	0.4 - 0.5[d,f]	n.d.a.	0.06 - 0.6[d,f]
Corchorus olitorius	**n.d.a.**	**n.d.a.**	**n.d.a.**	**n.d.a.**	**0.03**[δk]	**2.0**[d]	**0.06**[δk]	**0.05**[d]
Crotalaria sp.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	0.5[d]	n.d.a.	0.05[d]
***Cucurbita* sp.**	**194**[c]	**11**[c]	**36**[c]	**0.1**[c]	**39**[c]	**1.5**[d]	**38**[c]	**0.06 - 0.2**[c,d]
Emex australis	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	1.7[b]	n.d.a.	2.2[b]
Galinsoga parviflora	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	3.0[b]	n.d.a.	1.5[b]
Ipomoea batatas	103 - 980[a,c]	11 - 70[a,c]	80[c]	0.3 - 0.4[c,e]	37 - 158[a,c,d]	0.6 - 1.0[c,e]	61[c]	0.03 - 3.1[c,e]
Justicia flava	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	2.6[b]	225[b]	1.8[b]
Lesianthera africana	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	0.2[g]	n.d.a.	0.1[g]
Manihot esculenta	1970[a,c]	311[a]	n.d.a.	0.6[a]	30 - 303[a,d]	n.d.a.	n.d.a.	n.d.a.
Momordica sp.	n.d.a.	4[j]	n.d.a.	n.d.a.	n.d.a.	3.5[b]	n.d.a.	1.8[b]
Portulaca oleracea	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.	2.9[b]	n.d.a.	2.4[b]
Senna occidentalis	n.d.a.	n.d.a.	n.d.a.	n.d.a.	513[b]	2.5[b]	n.d.a.	2.1[b]
Solanum nigrum	1070[c]	2[c]	404[c]	0.3[c]	278 - 310[b,c]	8.5 -12.8[b,c]	84[c]	0.8 - 3.5[b,c,i]
Spinacea oleracea	**669**[c]	**28**[c]	**194**[c]	**0.2**[c]	**99**[c]	**2.7**[c]	**79**[c]	**0.5**[c]
Urica urens	n.d.a.	n.d.a.	n.d.a.	n.d.a.	668[c]	n.d.a.	133[c]	n.d.a.
Vernonia sp.	n.d.a.	51 - 198[a,h]	457[c]	0.3[c]	145[a]	0.8 - 3.2[h]	n.d.a.	0.08[d]
Vigna unguiculata	**99**[c]	**50**[c]	**141**[c]	**0.2**[c]	**188**[c]	**0.3 - 3.0**[c,d]	**60**[c]	**0.23**[d]

n.d.a: no data available, names and values in bold are of those GLVs currently being studied. [a] FAO, 1990; [b] Odhav *et al.*, 2007; [c] Kruger *et al.*, 1998; [d] Orech *et al.*, 2007; [e] Mosha and Gaga, 1999; [f] Mepba *et al.*, 2007; [g] Isong and Idiong, 1997; [h] Ejoh *et al.*, 2007; [i] Oboh *et al.*, 2005; [j] Steyn *et al.*, 2001; [k] Ndlovu and Afolayan, 2008.

Ascorbic acid is the active form of vitamin C and is an essential vitamin that must be obtained from the diet. From Table 1.2.2 it can be seen that ascorbic acid in the selected African GLVs varies from 2 to 311 mg/100 g in *Solanum nigrum* and *Manihot esculenta*, respectively. In sub-Saharan Africa and other developing countries, the supply of ascorbic acid in the diet is often determined by seasonal factors (FAO, 2001). Ascorbic acid promotes absorption of soluble non-haem iron by chelation or by maintaining the iron in the reduced form (FAO, 2001). Besides its ability to scavenge free radicals, ascorbic acid can also regenerate other antioxidants such as tocopheroxyl and the carotene radical cation from their radical species (Halliwell and Gutteridge, 1999).

African GLVs also contain the minerals calcium, iron, magnesium and zinc of which the levels of iron and zinc in some species can significantly contribute to deficiencies of these minerals. The range of dietary iron content of African GLVs varies from 0.2 to 12.8 mg/100 g for *Lesianthera africana* and *Solanum nigrum*, respectively (Table 1.2.2). Variations in mineral composition of GLVs may occur within the same species, between geographical locations, as well as across varieties. For zinc, the levels are variable and range from 0.02 to 8.4 mg/100 g for several *Amaranthus* species, from 1.4 to 18.5 mg/100 g for *Chenopodium album* and 0.03 to 3.1 mg/100 g for *Ipomoea batatas*. The mineral content of GLVs is a function of soil type and pH, water availability to the plant, climatic conditions, plant variety (Khader and Rama, 2003), plant age (Gupta *et al*., 1989) and the use of fertilizers (Guil Guerrero *et al*., 1998).

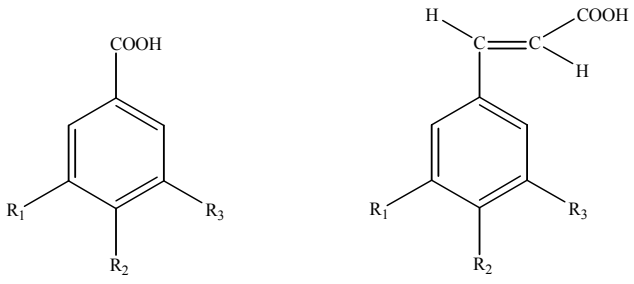
1.2.3 Chemistry of plant phenolics

Phenolic compounds are secondary metabolites that are synthesized by plants during normal development and in response to stress conditions such as infection, wound and UV radiation, amongst others (Naczk and Shahidi, 2004). Phenolic compounds possess a benzene ring bearing one or more hydroxyl substituents, including their functional derivatives (Manach *et al*., 2004; Waterman and Mole, 1994). These compounds can be classified into two categories, simple phenols and polyphenols, based on the number of phenol subunits present (Robbins, 2003). These compounds can further be categorized into classes depending on the structure

and then subcategorized within each class according to the number and position of hydroxyl (OH) groups and the presence of other substituents in the basic skeleton (Podsędek, 2007). Plant phenolics include phenolic acids, flavonoids, stilbenes, tannins, lignans and lignins (Manach *et al*., 2004; Naczk and Shahidi, 2004). Phenolic compounds of different types usually occur together in the same plant tissue.

1.2.3.1 Phenolic acids

Phenolic acids, in general, describe phenols that possess carboxylic acid functionality (Robbins, 2003). Phenolic acids are derivatives of benzoic (C6-C1) and cinnamic (C6-C3) acids, which contain hydroxyl and methoxyl groups substituted at sites on the benzene ring (Fig. 1.2.2) (Rice-Evans *et al*., 1996). The hydroxycinnamic acids are more common than the hydroxybenzoic acids. Examples of hydroxybenzoic acid are gallic, vanillic, syringic and protocatechuic acids. While, hydroxycinnamic acids consist mainly of *p*-coumaric, caffeic, ferulic and sinapic acids. These acids are rarely found in the free form, except in processed foods that have undergone freezing, fermentation or sterilization (El Gharras, 2009). The bound forms are glycosylated derivatives or esters of quinic acid, shikimic acid and tartaric acid.



(A) benzoic acid (B) cinnamic acid

Figure 1.2.2 Basic structures of common phenolic acids (Fukumoto and Mazza, 2000; Manach et al 2004).

Table 1.2.3 Functional groups of common phenolic acids (Fukumoto and Mazza, 2000).

Benzoic acid derivatives	Functional groups		
	R_1	R_2	R_3
p-hydroxybenzoic	H	OH	H
protocatechuic	H	OH	OH
vannilic	CH_3O	OH	H
syringic	CH_3O	OH	CH_3O
gallic	OH	OH	OH
Cinnamic acid derivatives	**Functional groups**		
	R_1	R_2	R_3
p-coumaric	H	OH	H
caffeic	OH	OH	H
ferulic	CH_3O	OH	H
sinapic	CH_3O	OH	CH_3O

1.2.3.2 Flavonoids

Flavonoids are the most widespread and diverse group of the polyphenols (Podsędek, 2007). Flavonoids are built upon a diphenylpropane (C6-C3-C6) skeleton and consist of two phenyl rings, A and B, connected by a three-carbon bridge to form the heterocyclic ring C (Fig. 1.2.3). Flavonoids exist as aglycones or in a glycoside form bound to various sugars such as glucose, arabinose, galactose, rhamnose and xylose. Most flavonoids occur as glycosides. Figure 1.2.3 shows the structural variations within the rings that subdivide them into several families such as: flavonols, with the 3-hydroxypyran-4-one C ring; flavones, lacking the 3-hydroxyl group; flavanols, lacking the C2-C3 double bond and the 4-one structure; as well as isoflavones, in which the B ring is located in the C3 position on the C ring (Rice-Evans *et al.*, 1997). The multitude of substitution patterns with phenolic hydroxyls, O-sugars, methoxy groups, sulfates and glucuronides occur in the phenyl rings A and B. Flavonoids are the most important natural phenolics because of their broad spectrum of chemical and biological activities, including antioxidant and free radical scavenging properties (Kahkonen *et al.*, 1999).

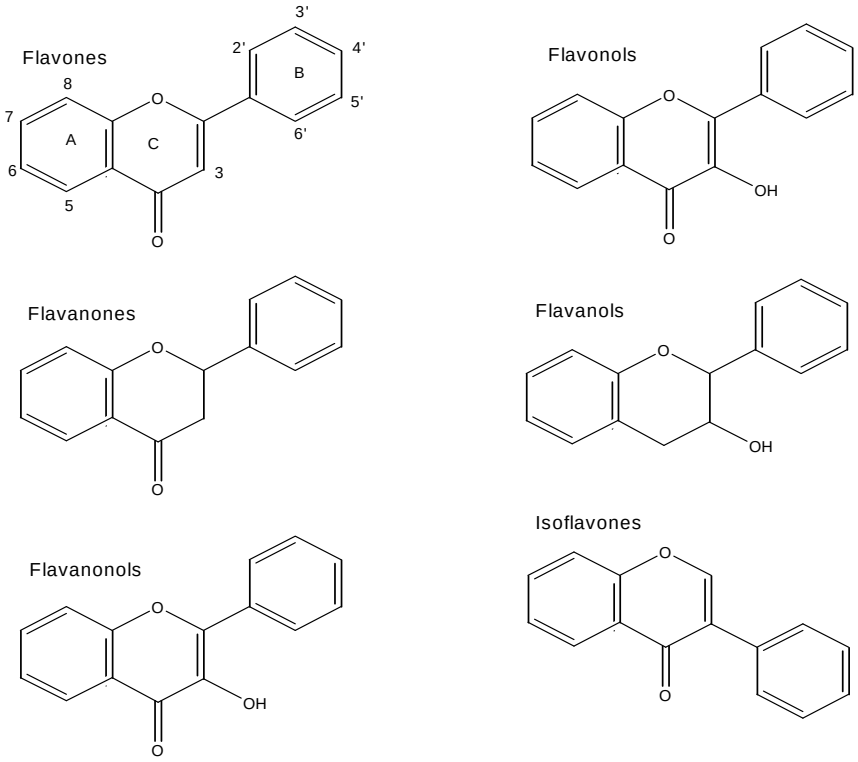
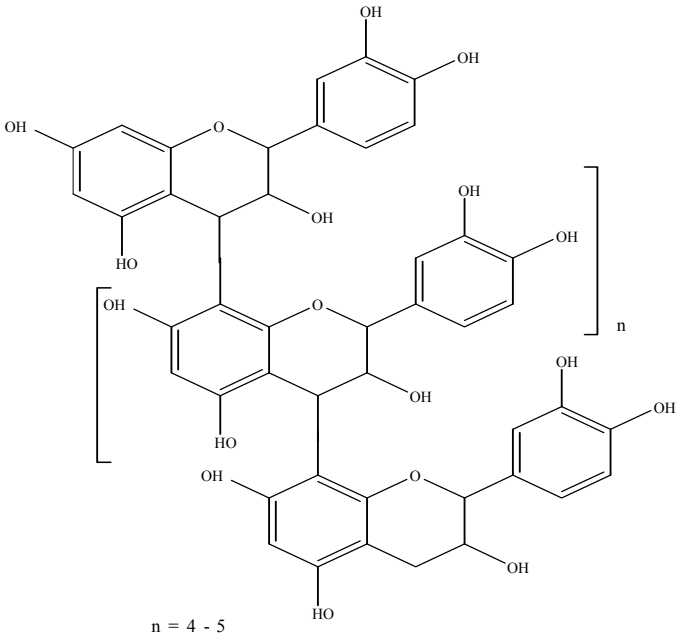


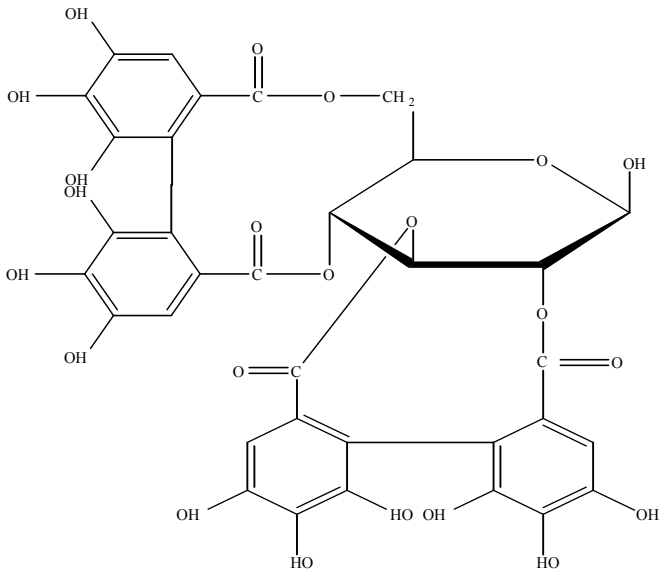
Figure 1.2.3 Chemical structures of the flavonoid family (Ren *et al*., 2003).

1.2.3.3 Tannins

Tannins are a unique group of phenolic metabolites of relatively high molecular weight, which have the ability to complex strongly with carbohydrates and proteins (Porter, 1989) and can be divided into hydrolysable and condensed tannins, also referred to as proanthocyanidins. Hydrolysable tannins are based on gallic acid usually as multiple esters with D-glucose, while condensed tannins are derived from flavanol monomers (Fig. 1.2.4) (Cowan, 1998). Tannins are formed by condensations of flavan derivatives, or alternatively by polymerization of quinine. Condensed tannins contribute to astrigency in plants and are considered as antinutrients as these compounds interfere with protein absorption and reduce availability of other nutrients (Bravo *et al*., 1994). They complex with proteins, starches and digestive enzymes, thereby reducing the nutritional value of foods (Chung *et al*., 1998; Serrano *et al*., 2009).



(A) condensed tannin



(B) hydrolysable tannin

Figure 1.2.4 Types of tannins (Hagerman *et al*., 1992).

1.2.4 Phenolic compounds present in GLVs

Substantial amounts of phenolic compounds have been reported in African GLVs (Table 1.2.4). Total phenolic content (TPC) values of up to 29.06 mg gallic acid equivalents (GAE)/g, dw have been reported (van der Walt *et al*., 2009). With regards to total flavonoid content (TFC), Oboh *et al*. (2008) reported a range of 2.31 to 5.61 mg 2,4-dihydroxyl flavone/g, dw in *Talinium triangulare* and *Ocimum gratissimum*, respectively. Phenolic compounds which have been reported in GLVs are mainly phenolic acids and flavonoids (Andarwulan *et al*., 2010; Azuma *et al*., 1999; Salawu *et al*., 2008 and 2009). Although there has been an increase in consumption of exotic GLVs such as spinach in sub-Saharan Africa, no study has yet determined the phenolic composition of spinach grown in this region. While, the total phenolics and flavonoids content of spinach grown elsewhere has been well studied and the values are variable (Table 1.2.4).

1.2.5 Antioxidant properties of GLV phenolics

The antioxidant activity of plant extracts mainly depends on the phenolic content of the extract (Salah *et al*., 1995). Generally, plant extracts that contain a high amount of polyphenols exhibit high antioxidant activity (Wong *et al*., 2006). Different assays such as 2,2-azobis-(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing ability of plasma (FRAP) and oxygen radical antioxidant capacity (ORAC) have been used to determine the radical scavenging capacity and antioxidant content of African GLVs and/or spinach grown elsewhere (Table 1.2.4).

Theoretically a high polyphenol of flavonoid content is associated with high antioxidant activity. This correlation depends on the methodology used as well as type of vegetables, where variations could be due to different antioxidant components contributing to antioxidant activity (Ismail *et al*., 2004). Maisuthisakul *et al*. (2007) found a distinct correlation between total phenolic content, total flavonoid content and antiradical activity in several Thai indigenous plant parts, perhaps suggesting that the phenolics could be the ones contributing to antiradical activity of plant extracts.

Table 1.2.4 Values of phenolic composition and antioxidant activity reported in African green leafy vegetables and spinach.

Type of GLVs	Range of values	References
	TPC	
Talinum triangulare - *Ocimum gratissimum*	4.37 - 20.69 mg GAE/g, dw	Oboh *et al*., 2008
Amaranthus hybridus - *Vigna unguiculata*	10.57 - 29.06 mg GAE/g, dw	van der Walt *et al*., 2009
Manihot utilissima Pohl. - *Corchorus olitorius* L.	8.0 - 42.0 mg TPC/g, dw	Salawu *et al*., 2008
Talinium triangulare (Jacq.) Wild. - *Gnetum bucholzianum* Welw.	0.22 - 5.47 mg TAE/g, dw	Odukoya *et al*., 2007
*spinach	0.33 - 2.17 mg GAE/g, fw 12.74 - 46.6 mg GAE/g, dw	Chun *et al*., 2005; Wu *et al*., 2004 Turkmen *et al*., 2005; Pandjaitan *et al*., 2003
	TFC	
Talinium triangulare - *Ocimum gratissimum*	2.31 - 5.61 mg 2,4-dihydroxyl flavone/g, dw	Oboh *et al*., 2008
	TEAC	
Talinium triangulare - *Ocimum gratissimum*	0.9 - 4.6 mmol TE/g, dw	Oboh *et al*., 2008
*spinach	0.085 mmol TE/g, fw 0.102 mmol TE/g, dw	Sun and Tanumihardjo, 2007 Yang *et al*., 2006b
	DPPH	
Galinsoga parviflora - *Portulaca oleracea*	76 - 96% free radical scavenging capacity	Odhav *et al*., 2007
Chenopodium album - *Nasturtium aquatic*	42 - 100% inhibition of linoleic acid oxidation	Lindsey *et al*., 2002
Amaranthus hybridus - *Xanthosom mafaffa*	4 - 99% inhibition of lipid peroxidation	Akindahunsi and Salawu, 2005, Odukoya *et al*., 2007
*spinach	3.74 - 7.90 µmol TE/g, fw	Yamaguchi *et al*., 2001;Yang *et al*., 2006b
	FRAP	
Solanum macrocarpon - *Cleome gynandra*	0.87 - 1.56 mmol Fe^{2+}/100g	Stangeland *et al*., 2009
	ORAC	
*spinach	4.20 - 84.4 µmol TE/g, fw 152 µmol TE/g, dw	Wu *et al*., 2004; Cho *et al*., 2008 Ou *et al*., 2002

*spinach was grown elsewhere, GAE: gallic acid equivalents, TPC: total phenolic content, TAE: tannic acid equivalents, TFC: total flavonoid content, TEAC: Trolox equivalent antioxidant capacity, TE: Trolox equivalents, DPPH: 2,2-diphenyl-2-picrylhydrazyl, FRAP: ferric reducing ability of plasma, ORAC: oxygen radical antioxidant capacity.

Mai *et al*. (2007) also found a correlation between antioxidant activity and the polyphenol contents of several Vietnamese plants. Meanwhile, no correlation between antioxidant activity and total phenolic content was found for some Indian leafy vegetables (Dasgupta and De, 2007) and commonly consumed vegetables in Malaysia (Ismail *et al*., 2004).

1.2.6 Antioxidant mechanisms and structure-activity relationship of plant phenolics

The antioxidant properties of plant phenolics is mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al*., 1997). Plant phenolics have been shown to exhibit higher *in vitro* antioxidant activity than other natural antioxidants such as vitamins E and C (Gardner *et al*., 2000). The efficiency of phenolics as antioxidants is diverse and depends on many factors such as the number of hydroxyl groups bonded to the aromatic ring, the site of bonding and mutual position of hydroxyl groups in the aromatic ring (Sroka and Cisowski, 2003).

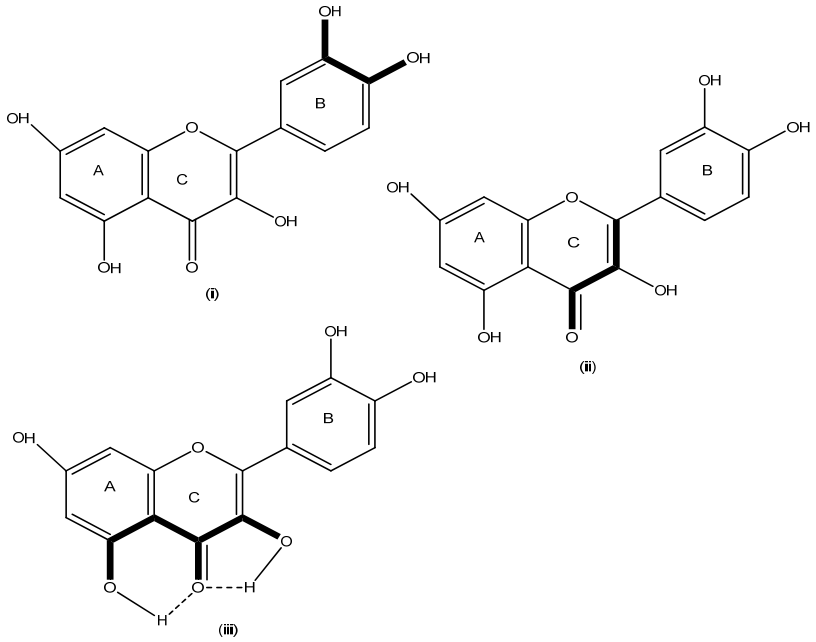


Figure 1.2.5 Structural groups responsible for radical scavenging (Ammar *et al*., 2009).

In phenolic acids, the antioxidant activity increases with the number of hydroxyl groups and the presence of conjugated double bonds (Rice-Evans *et al*., 1996). Hydroxycinnamic acids have been reported to exhibit higher antioxidant activity than their benzoic counterparts because of the electron-withdrawing property of the carboxyl group in the latter (Rice-Evans *et al*., 1996).

In flavonoids, the predominant mode of antioxidant activity is radical scavenging (Robbins, 2003). Figure 1.2.5 shows the major determinant for radical scavenging capabilities, which is characterized by (i) the presence of a hydroxyl group at the *ortho*-position, to give a catechol ring (the B ring) that lowers the O-H bond dissociation enthalpy and increases the rate of H-atom transfer to peroxyl radicals (e.g. in catechin, quercetin, luteolin, baicalein and taxifolin) (Shahidi and Wanasundara, 1992). Secondly, (ii) a 2,3-double bond conjugated with the 4-*oxo* function (carbonyl group) enhances electron delocalization (e.g. in quercetin) (Pietta, 2000), and finally (iii) the additional presence of both 3-, 5- and 7-hydroxyl groups (e.g. in kaempferol, apigenin and chrysin) (Ammar *et al*., 2009). An increase in the number of hydroxyl groups in the phenolic ring has been shown to further increase the antioxidant capacity (Fukumoto and Mazza, 2000; Ranalli *et al*., 2003). The combined actions of all these functional groups results in an increased antioxidant activity. Alterations in the arrangement of the hydroxyl groups and substitution of contributing hydroxyl group by glycosylation decreases antioxidant activity.

1.2.7 Free radicals and oxidative stress

Free radicals are natural by-products of human metabolism. They are unstable highly reactive and energized molecules that have unpaired electrons (Kaur and Kapoor, 2001). Examples of oxygen free radicals are superoxide (O_2^-), hydroxyl (OH^-), hydroperoxyl (HOO^-), peroxyl (ROO^-) and alkoxyl (RO^-) radicals, while nitrogen free radicals include nitric oxide (NO^-) and nitrogen dioxide (NO_2^-) (Fang *et al*., 2002; Prior and Cao, 2000). Oxygen and nitrogen free radicals can be converted to non-radical reactive species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr) and peroxynitrite anion (ONOO) (Fang *et al*., 2002). These radical and non-radical species are associated with cellular and metabolic

injury, as well as pathological conditions such as atherosclerosis, cancer and chronic inflammation (Halliwell, 1994) and even cell death. Among the reactive oxygen species (ROS), the hydroxyl radicals are the most reactive and induce severe damage to adjacent biomolecules (Dasgupta and De, 2007). Sites of intense oxidant challenge in the human body include the eye, the skin, gastrointestinal tract, areas of inflammation, or post-ischaemic reperfusion (Benzie, 2003).

Potential endogenous source of ROS include mitochondria, cytochrome P450 metabolism, peroxisomes and inflammatory cell activation (Inoue *et al.*, 2003). ROS also occur due to environmental factors such as pollution, radiation and cigarette smoking (Kaur and Kapoor, 2001). The electrons of the closest molecule are attacked; in turn these molecules also become free radicals, beginning a chain reaction (Kaur and Kapoor, 2001). This process can initiate lipid peroxidation that results in destabilization and disintegration of the cell membranes or oxidation of other cellular components like DNA and proteins, resulting in either disruption of normal cellular processes or cell death (Halliwell, 1996). However, the body is equipped with an effective defence system consisting of various enzymes and antioxidants that are capable of dealing with these free radicals. Among the endogenous defenses are antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, catalase, as well as non-enzymatic antioxidants such as glutathione and vitamins C and E (Scandalios, 1990). Superoxide dismutase is important for the elimination of superoxide radicals ($2O_2^{\bullet-} + 2H \rightarrow H_2O_2 + O_2$), while catalases and glutathione peroxidases are for the elimination of hydrogen peroxide and organic peroxides (Demmig-Adams and Adams, 2002). These enzymes are generally referred to as phase 2 enzymes, due to their ability to catalyze the conversion of toxic metabolites to compounds that are more readily excreted.

Oxidative stress refers to a condition characterized by the accumulation of non-enzymatic oxidative damage to molecules that threaten the normal functioning of the cell or the organism (Blomhoff, 2005). Cells normally deal with mild oxidative stress by upregulating the synthesis of antioxidant defense mechanisms through changes in gene expression. However, at higher levels of oxidative stress, cell injury may occur when adaptation is not adequate for the build-up of oxidation products (Klaunig and Kamendulis, 2004). This leads to oxidative damage to

all types of biomolecules including DNA, proteins and lipids that have been associated with aging and many chronic diseases of lifestyle (CDL). During oxidative stress, the ability of endogenous antioxidants to eliminate ROS is often exceeded and, therefore, dietary sources of antioxidants are required (Chew and Park, 2004). Dietary antioxidants provide bioactive mechanisms to prevent or reduce free radical induced oxidative stress (Kaur and Kapoor, 2001). These compounds neutralize free radicals by donating hydrogen from the phenolic hydroxyl groups, forming stable free radicals that do not initiate or propagate further oxidation.

DNA damage is believed to be mediated by transition metal ions, mainly iron and/or copper, which are able to catalyze the formation of hydroxyl radicals through a Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$) (Imlay *et al.*, 1988). Due to their extreme reactivity, hydroxyl radicals interact exclusively in the vicinity of the bound metal (Chevion, 1988). The formation of $^{\bullet}OH$ close to DNA (due to bound Fe^{2+} or Cu^{+} ions) results in its damage, including cell membrane damage, single- and double-DNA strand breaks, cross-linking, as well as chromosomal breakage and rearrangements (Liu, 2002). It has been proposed that the extent of DNA strand breaking by $^{\bullet}OH$ is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone (Valko *et al.*, 2006). Individuals with increased DNA damage and defective or deficient DNA repair may develop mutations of critical cellular targets, such as tumour suppressor genes and oncogenes, which are related to human carcinogenesis (Weinburg, 1989). Consumption of plant foods rich in phenolics may reduce free radical induced oxidative stress. However, In contrast to the cultured cells which are often antioxidant-deficient and respond well to supplementation of antioxidants, the beneficial effect of antioxidants is difficult to observe in normal healthy subjects with sufficient amounts of antioxidants (Polidori *et al.*, 2009).

Contrary to their antioxidant properties, dietary phenolics have also been shown to act as pro-oxidants in systems containing redox-active metals (Galati and O'Brien, 2004). Johnson and Loo (2000) found that at 10 µM, epigallocatechin gallate or quercetin each inhibited H_2O_2- and SIN-1-induced DNA damage, while at 100 µM, these compounds assumed the role of pro-oxidants. It has also been reported that in the presence of O_2, transition metals such as Cu and

Fe catalyze the redox cycling of phenolics, leading to the formation of ROS and phenoxyl radicals that can damage DNA, lipids and other biological molecules (Decker, 1997; Li and Trush, 1994; Yamanaka *et al*., 1997). Exposure of DNA to dihydrocaffeic acid in the presence of Cu resulted in more DNA single- and double-strand breaks than caffeic, whereas chlorogenic acid caused only minimal damage even though these phenolics have similar structures and redox potential (Sakihama *et al*., 2002). The authors proposed that the initial oxidation of the catechols by Cu^{2+} generated a semiquinone that reacted with O_2 to form $O_2^{\bullet-}$, which then oxidized the catechol to regenerate the semiquinone and H_2O_2. H_2O_2 was then rapidly converted by Cu^{1+} to the OH radical in a Fenton-type reaction.

1.2.8 Evidence for health-promoting effects of fruits and vegetables

Consumption of plant foods has been associated with a decrease in incidences of CVD, CHD and various types of cancer (Stan *et al*., 2008; Visioli *et al*., 2000). However, there is no study available yet showing evidence for health-promoting effects specifically of GLVs. Evidence for health-promoting effects that is available in the literature is for vegetables in general and fruits. The health effects have been attributed to the antioxidative activity of these plant foods against oxygen reactive compounds in biological systems. While epidemiological studies appear to demonstrate clear associations between consumption of both fruits and vegetables and a reduced risk of CVD, clinical studies have not yet established which specific phenolics or components are responsible. The most convincing evidence of antioxidant effect on cancer prevention involves consumption of fruits and vegetables rather than individual antioxidants, suggesting that other food components contained in vegetables may be equally involved.

The Nurses' Health Study and the Health Professionals' Follow-up Study conducted by Bazzano *et al*. (2002) indicated an equivalence of 4% reduction in CHD for every 1 serving/day increase in the intake of fruits and vegetables (Joshipura *et al*., 2001). GLVs showed the strongest inverse association with cardiovascular disease (Hung *et al*., 2004). Similar results indicating a decrease have been reported from the Women's Health Study (Liu *et al*., 2000) and the Physicians' Health Study (Liu *et al*., 2001).

The relationship between the consumption of vegetables and risk of various cancers has been studied but the results are not yet clear. A multiethnic case-control study by Kolonel *et al*. (2000) found that both cruciferous and yellow-orange vegetable intake were inversely related to prostate cancer among all the various ethnic groups studied (African American, white, Japanese and Chinese men). Data from the Nurses' Health Study and the Health Professionals' Follow-up Study found a 21% reduction in risk of lung cancer in women with increased intake of fruits and vegetables, while, among men, a lower risk of lung cancer was not observed with increased consumption of vegetables (Feskanich *et al*., 2000). Differences in relationships between antioxidant status and risk for gender-specific cancers may have contributed to the observed gender differences in total risk. Low plasma selenium (Waters *et al*., 2004) and vitamin C (Khaw *et al*., 2001) concentrations are associated with increased total cancer risk in men but not in women. Michels *et al*. (2000) found no association between vegetables consumption and the incidences of colon and rectal cancers. A meta-analysis of 26 studies by Gandini *et al*. (2000) found an association between risk of breast cancer and lower intake of vegetables. However, consumption of vegetables was not significantly associated with a reduced risk of breast cancer in a study by Smith-Warner *et al*. (2001) using pool data from 8 cohort studies.

1.2.9 Health-promoting effects of some flavonoid-rich foods

Several studies have reported an inverse association between flavonoid intake and risk of CHD. The Zutphen study of elderly men in The Netherlands (Hertog *et al*., 1993a) found that a high intake of flavonoids (approx. 30 mg per day) reduced CHD mortality rate by about 50% compared with individuals who had a low flavonoid intake (< 19 mg per day). Similar results were reported in a cohort study conducted in Finland by Knekt *et al*. (1996) and Knekt *et al*. (2002) among men and women consuming onions and apples, which are rich sources of dietary flavonoids. In a prospective study of postmenopausal women in Iowa, USA, Yochum *et al*. (1999), associated a decreased risk of CHD mortality with the group with highest flavonoid intake (32.2 mg per day) from tea, apples and broccoli, while the Health Professionals' Study also done in the USA (Rimm *et al*., 1996) did not find an association between new diagnosis of non-fatal myocardial infarction in men and flavonol and flavones

intake from tea, apples, broccoli and onions. The latter study, however, found a significant association between flavonoid intake and subsequent CHD mortality in men health professionals with existing CHD.

1.2.10 Health-promoting effects of plant phenolics with particular reference to GLVs

The health-promoting effects of phenolics is due to free radical scavenging activity of flavonoids which is highly dependent on the presence of a free 3-OH. Soobratte *et al*. (2005) found that flavones exhibited lower antioxidant activity than flavonol. Both flavones and flavonols have identical hydroxyl configuration, except the absence of the 3-OH functional group in the flavones, which may explain the results. While studying the antioxidant and free radical scavenging properties of three flavonoids isolated from leaves of *Rhamnus alaternus* L., Ammar *et al*. (2009) found that rhamnetin-3-O-ß-isorhamninoside was the most potent $DPPH^{\bullet}$ scavenger. This compound not only possesses the C2-C3 double bond in conjugation with 4-*oxo* function in the C ring, but also has 3′, 4′-dihydroxyl groups in the B ring and another 5-OH in the A ring, which are amongst the essential structural elements for potent radical scavenging activities of antioxidants (reviewed in section 1.2.6).

In the cell, flavonoids may not act as conventional hydrogen-donating antioxidants but may exert modulatory actions through actions at protein kinase and lipid kinase signaling pathways (Williams *et al*., 2004). Flavonoids and their metabolites are not likely to act as major antioxidants *in vivo* because endogenous antioxidants such as ascorbic acid are at much higher concentrations. However, concentrations are high enough to mediate receptor or enzyme activity (Galati and O'Brien, 2004). The protective effects of phenolics against DNA damage and ROS production have been conducted. Bergman *et al*. (2003) found that the presence of the hydroxyl group at the 5′ position in the B ring was responsible for the superior antioxidant activity of spinach in protecting against ROS formation in fibroblasts over epigallocatechin gallate (EGCG). EGCG has the gallate group at the 3 position of the catechins and an additional insertion of the hydroxyl group at the 5′ position of the B ring. Flavonoids have also been shown to offer protection against H_2O_2-induced DNA damage, Melidou *et al*. (2005) found that with an exception of catechin, flavonoids containing dihydroxyl groups in the *ortho*

position (e.g. 7,8-dihydroxyflavone, baicalein, luteolin, fisetin, quercetin, myricetin and taxifolin) exhibited relatively stronger DNA-protecting capacities than those without. Kumar and Chattopadhyay (2007) also found profound protective effects of pudina extract against oxidative DNA damage, free radical scavenging and inhibition of lipid peroxidation. These benefits were attributed to the absence of 3-OH in the B ring of the flavones, the 3-*oxo* and C2-C3 double bond in the B ring, OH in 5 and 7 position of the A ring and the presence of catechol moiety in the C ring (e.g. in luteolin).

In general, studies that have reported an association between consumption of phenolics-rich foods and risk of CDL indicate that putative mechanisms responsible for protective effects of plant foods against CVD mortality include inhibition of low density lipoprotein oxidation (Frankel *et al*., 1993), inhibition of platelet aggregation and adhesion (Demrow *et al*., 1995) and reduction of thromboxane synthesis (Tzeng *et al*., 1991). On the other hand, the mechanisms responsible for inverse relationship between consumption of flavonoid-rich foods and risks for certain types of cancer include direct scavenging of radical species, suppression of lipid peroxidation by regenerating antioxidants, as well as chelation of pro-oxidant metal ions such as iron and copper (McAnlis *et al*., 1999). With regards to tannins, Haslam (1996) suggested that tannins may exert their biological properties in three different ways, (i) by complexation with metal ions, (ii) through antioxidant and radical scavenging activities, or (iii) through their ability to complex with other molecules including protein and polysaccharides. All these activities lead to protection of DNA from oxidative damage, deactivation of carcinogens, inhibition of the expression of mutated genes and activity of enzymes that promote carcinogenesis, as well as promotion of xenobiotics detoxification (Yang *et al*., 2001), all which can exert health effects.

1.2.11 Dietary intake of flavonoids

An estimation of the total flavonoid intake is difficult, because only limited data on contents of food are available. Reliable data on flavonoid contents of GLVs are needed to be able to study the potential role of vegetable flavonoids in the prevention of CDL. So far, only data on flavonols and flavones are available (Table 1.2.5). Hertog *et al*. (1993b) estimated the total

average intake of flavonols (quercetin, myricetin and kaempferol) and flavones (luteolin and apigenin) in The Netherlands at 23 mg per day, of which quercetin contribute about 70%, kaempferol 17%, myricetin 6%, leteolin 4% and apigenin 3%. Kuhnau (1976) estimated the total intake of flavonoids in the United States at 1 g per day (expressed as glycosides) or 650 mg per day (expressed as aglycones), depending on the season. This amount is equivalent to about 115 mg flavonol and flavone aglycones per day, which is more than what Hertog *et al*. (1993b) estimated. These studies only contemplate the intake of some types of flavonoids and do not consider other phenolic compounds.

Table 1.2.5 Flavonol and flavone contents of exotic vegetables (Hollman and Katan, 1999a)

*Flavonol and flavone content	Vegetables
Low (< 10 mg/kg or < 10 mg/L)	Cabbage, spinach, carrots, peas, mushrooms
Medium (< 50 mg/kg or < 50 mg/L)	Lettuce, red pepper
High (> 50 mg/kg or 50 mg/L)	Broccoli, onions, kale, celery

*sum of quercetin, kaempferol, myricetin, luteolin and apigenin

1.2.12 Bioavailability of phenolics

Although little is known about the absorption and metabolism of leafy vegetable phenolics, only small amounts of polyphenols estimated at ≤ 30 mg per day are believed to be absorbed (Bravo, 1998), due to factors such as unfavourable physico-chemical properties, extensive first-pass metabolism and active efflux (Yang *et al*., 2008). Nevertheless such low levels have a potent antioxidant effect *in vivo*. Even at much diluted concentrations (10 µmol/L) *in vitro*, phenolics have been shown to contain antioxidant effects in human subjects (Frankel *et al*., 1993; Hollman *et al*., 1997; Yang *et al*., 2008). Blood concentrations of total catechins of 0.17 µmol/L after ingestion of black tea and up to 0.55 µmol/L after green tea were reported by van het Hof *et al*. (1998).

Intestinal absorption of phenolics depends on their chemical structures. Glycosides interact with proteins, while aglycones appear to undergo passive diffusion only (Meneth *et al*., 2003). An important factor determining the efficiency of the absorption of flavonoid glycosides from the intestine is the sugar moiety. Most phytochemicals are present in the food as precursors,

for example as glycosides, but are predominantly absorbed as aglycones. The cleavage of the glucose moiety is catalysed in the gut lumen by the brush border enzyme lactase phloridzin hydrolase (LPH) (Day *et al*., 2000). For phytochemicals with different sugar moieties, for example hesperidin (occurs naturally as a rhamnoside), LPH does not catalyse the hydrolysis of the sugar moiety. For these phytochemicals, the site and kinetics of absorption and finally their bioavailability can be modulated by enzymatic release of the attached sugar, for example rhamnose during processing (Nielsen *et al*., 2006). Hollman and Katan (1999b) and Hollman *et al*. (1999) showed that quercetin glycosides from onions were absorbed better (52%) than the pure aglycone (24%). Bound phenolics that cannot be digested by human enzymes may survive stomach and small intestine digestion, and therefore possibly reach the colon. The colonic flora may then release these bound phenolics through fermentation, and thus provide site-specific health benefits in colon and other tissues after absorption (Liu, 2007).

1.2.13 Effects of cooking on phenolic content and antioxidant activity

The effects of food processing on the overall antioxidant activity of foods are due to different events, which take place consecutively or simultaneously (Nicoli *et al*., 1999). The trend of antioxidant and radical scavenging activity after boiling is dependent on a number of factors including the type of vegetable, type and duration of boiling, boiling temperature, bioavailability of phenolics, localization and the stability of high temperatures (Jimenez-Monreal *et al*., 2009). Differences in tissue hardness and phenolic profile of each vegetable are also major contributors to antioxidant activity (Yamaguchi *et al*., 2001). The results of studies on the effect of processing on phenolic contents, antioxidant and radical-scavenging activity of GLVs are inconsistent. For example, a study by Turkmen *et al*. (2005) showed that antioxidant activity of cooked vegetables depended on the type of vegetable and not on the method of cooking. In contrast, Yamaguchi *et al*. (2001) found that the radical scavenging activity of vegetables cooked in a microwave oven was generally higher than those cooked by boiling. Roy *et al*. (2007) found that normal cooking temperatures detrimentally affected phenolic content as well as antiradical and antiproliferative activities of common vegetables juice, while mild heating preserved 80 - 100% of phenolic content and both antioxidant activity and cell proliferation inhibition activities.

During boiling, flavonoid content decreases due to leaching, and new products are formed from the breakdown of the flavonoids (Ruiz-Rodriguez *et al*., 2008). An increase in antioxidant activity after thermal processing could be attributed to many factors (Yamaguchi *et al*., 2001 and 2003). As explained by these authors, thermal processing may liberate a great amount of antioxidant components as a result of destruction of vegetable cell walls and subcellular compartments. Secondly, it could result from the production of stronger radical-scavenging antioxidants by thermal chemical reactions. Thirdly, thermal inactivation of oxidative enzymes such as polyphenol oxidase and ascorbate oxidase may also suppress oxidation of antioxidants. Absence of change in antioxidant activity following heat processing would indicate the thermal stability of the antioxidants.

1.2.14 Analytical methodology for the determination of antioxidant content and activity

Rationale for the evaluation of antioxidant activity usually involves the detemination of either total polyphenol content (TPC) and total flavonoid content (TFC) of which assays are based on the structural composition (e.g. polyphenol structure) thereof, and the determination of antioxidant activity assays which are based on protection of (i) another molecule (e.g. flourescein), (ii) cellular macromolecule (e.g. DNA) and (iii) cells (e.g. erythrocytes) against oxidative damage. Whether to evaluate the entire GLVs as such or to isolate and identify the individual constituent antioxidants is dependent on the research questions. It is recommended to use more than one method for the evaluation of antioxidant activity as GLVs extracts are a complex mixture of molecules, with different structures, solubilities and reactivity. Furthermore the reactions themselves that measure antioxidant activity are complex and are a function of the physico-chemical parameters of the assay reagents as well as the substrates (Soobrattee *et al*., 2005). In addition, the synergistic effects and concentration may bring effects that are not observed when individual constituents are tested (Kaur and Kapoor, 2001).

The analytical methods for determining and quantifying the biologically active compounds in GLVs involve extraction. In the extraction process of these compounds, a single extraction procedure is not sufficient (Rusak *et al*., 2008). Possible reasons for using different extraction

procedures and solvents could be because phenolic compounds exist in multiple forms and therefore their polarity may vary significantly, leading to difficulty in developing a uniform extraction method for different phenolic compounds from varying matrices (Xu and Chang, 2007). The available information about extraction solvents demonstrates that no one solvent is best for extracting total phenolics and evaluating the antioxidant activity in plant foods. Different solvent systems have been used to extract antioxidants from plant materials such as vegetables, fruits, legumes, tea and other foodstuffs. Water, aqueous or acidified mixtures of ethanol, methanol and acetone have been used to extract antioxidants from plant foods (Amin and Lee, 2005; Arabshahi-D *et al*., 2007; Chu *et al*., 2000; Chun *et al*., 2005; Harbaum *et al*., 2008; Ismail *et al*., 2004; Mai *et al*., 2007; Maisuthisakul *et al*., 2007; Myojin *et al*., 2008; Ninfali and Bacchiocca, 2003; Ou *et al*., 2002; Salawu *et al*., 2009; Silva *et al*., 2007; Wachtel-Galor *et al*., 2008; Xu and Chang, 2007; Zhang and Hamauzu, 2004; Zhao and Hall III, 2008).

The yield of chemical extraction depends on the type of solvents with varying polarities and pH, extraction procedure and time, temperature, physical characteristics of the sample, the ratio of sample to extraction solvent, as well as chemical properties of phenolics of interest (including polarity, acidity and hydrogen-bonding capacity of the OH groups to the aromatic ring) (Lee, 2000; Xu and Chang, 2007). Under the same condition of extraction time and temperature, the solvent used and the chemical properties of the food sample are the two most important factors. Glycoside flavonoids are more soluble in water, while the aglycones are more soluble in methanol (Lee, 2000). In general, organic solvents containing some water are more efficient in the extraction of polyphenolic compounds than water or pure organic solvents (Katsube *et al*., 2004; Xu and Chang, 2007; Zhao and Hall III, 2008). This may be attributed to water causing the plant tissues to swell allowing the solvent to better penetrate the sample matrix (Zhao and Hall III, 2008). Water only as a solvent yields an extract with high content of impurities (e.g. organis acids, sugars, soluble proteins) which could interfere in the phenolic extraction, identification and quantification (Chirinos *et al*., 2007). An increase in extraction temperature has been reported to increase analyte recoveries.

1.2.14.1 Determination of total polyphenol and flavonoid content

The most commonly used analytical method for the determination of TPC and TFC is the Folin-Ciocalteu (F-C) and the aluminium chloride assays, respectively. The principles of measurement and quantification as well as the main disadvantages of each method are summarized in Table 1.2.6. The F-C assay is based on a reduction-oxidation reaction during which the phenolate ion is oxidized under alkaline conditions while reducing the phosphotungstic-phospho-molybdic complex in the reagent to a blue coloured solution (Waterman and Mole, 1994). The disadvantage of this method is that it is not specific and, therefore, detects all phenolic groups found in the extract plus some amino acids such as tyrosine. With this assay, there is also interference of reducing substances such as ascorbic acid (Naczk and Shahidi, 2004) and sugar (Waterhouse, 2002). Waterhouse (2002) suggested that reducing sugar will only be a problem in this assay when analyzing fresh fruit samples. The presence of these substances may create the possibility of false TPC positive results.

Aluminium chloride assay is used to evaluate total flavonoids, and it is based on the formation of a red aluminum complex where the flavonoid acts as a bidental ligand (Amaral et al., 2009), forming acid labile complexes with the C-4 keto group and either the C-3 or C-5 OH group of flavones and flavonols (Chang et al., 2002). The disadvantage of this method is that it is only specific for flavones and flavonols. The advantages of using these methods are that both assays are rapid, reproducible and give a relatively accurate indication of antioxidant potential.

1.2.14.2 Measurement of antioxidant activity

High TPC and TFC indicate in most cases high antioxidant activity. Antioxidant activity can be measured using many assays which include the ABTS, DPPH, ORAC, hydroxyl (HO) radicals averting capacity (HORAC), total radical-trapping antioxidant parameter (TRAP), ferric reducing antioxidant power (FRAP), *N*,*N*-dimethyl-*p*-phenylelendiamine (DMPD) and photochemiluminescence (PCL) assays (Roginsky and Lissi, 2005; Schlesier *et al*., 2002; Sun and Tanumihardjo, 2007). Mechanistically, these methods are based on either a single electron transfer (SET) reaction or a hydrogen atom transfer (HAT) reaction between an oxidant and a free radical (Prior *et al*., 2005).

Table 1.2.6 Assays for determination of total phenolic and flavonoid contents

Assay	Principle of assay	Quantification	Disadvantages	References
Folin-Ciocalteu assay	Detection of phenolic hydroxyl groups or other potential oxidisable groups in the sample	Concentration dependent, increase in OD	Not specific to phenolics, react with e.g. amino acids	Singleton *et al*., 1999 ; Stratil *et al*., 2006
Aluminium chloride assay	Aluminium complex forms due to C-4 keto group and either the flavonoid C-3 or C-5 hydroxyl groups	Concentration dependent, increase in OD	Only specific for flavones and flavonols	Amaral *et al*., 2009 ; Chang *et al*., 2002

OD: Optical density

Table 1.2.7 Commonly used antioxidant assays

Assay	Mechanism	Oxidant	Principle of measurement	Disadvantages	References
ABTS	SET	$ABTS^{\bullet+}$	ABTS radical reduced by antioxidants, Decrease in OD	$ABTS^{\bullet}$ reagent is very unstable	Ou *et al*., 2002; Re *et al*., 1999
DPPH	SET	$DPPH^{\bullet}$	DPPH radical reduced by antioxidants, Decrease in OD	DPPH only soluble in organic solvent	Brand-Williams *et al*., 1995; Bondet *et al*., 1997
ORAC	HAT	Peroxyl radical	Oxidation of fluorescein causes fluorescence decay that can be delayed by antioxidants, Increase in AUC	Primarily measures hydrophilic antioxidant activity	Cao and Prior, 1999; Ou *et al*., 2001

ABTS: 2,2′-Azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt, DPPH: 2,2-diphenyl-2-picrylhydrazyl, ORAC: oxygen radical absorption capacity, SET: single electron transfer, HAT: hydrogen atom transfer, OD: Optical density, AUC: area under the fluorescence decay curve.

Due to the variety of these assays, it is difficult to compare the results of the different assays because of the different probes, reaction conditions and quantification methods used (Huang *et al*., 2005). The three most commonly used assays namely the ABTS, DPPH and ORAC assays are summarized in Table 1.2.7. Usually, two or more of these assays are used and although the reaction mechanisms and the values obtained differ, a strong correlation between assays is usually obtained (Maisuthisakul *et al*., 2007; Stratil *et al*., 2006; Sun and Tanumihardjo, 2007). Disadvantages include the instability of the $ABTS^{\bullet+}$ reagent and the fact that it reacts with any hydroxylated aromatics independently of their real antioxidative potential (Roginsky and Lissi, 2005), Whereas DPPH can only be dissolved in organic media, which is a limitation when interpreting the role of hydrophilic antioxidants in an extract (Arnao, 2000) and finally, the limitation to the ORAC assay is that it is performed in aqueous solution, therefore it primarily measures hydrophilic antioxidant activity against peroxyl radicals (Ou *et al*., 2001).

1.2.14.3 Biological and cellular assays

The ability of GLVs or antioxidant extracts to protect cellular macromolecules (plasma membrane and DNA) and cells (cell lines and primary cell cultures) aginst oxidative damage is evaluated in these assays. A summary including the advantages and disadvantages of the most commonly used methods are presented in Table 1.2.8.

Biological assays

Biological assays usually involve the use of a cellular macromolecule that is often a target of oxidative damage e.g. DNA and the cell membrane. The plasmid DNA assay is used to measure the extent of DNA damage induced by peroxyl radicals and the level of protection offered by the plant or antioxidant extracts. Oxidative damage causes either single or double stranded DNA breaks in the nuclear or mitochondrial DNA (Aronovitch *et al*., 2007). Plasmid DNA is circular and supercoiled, in contrast oxidative damage that causes strand breaks causes the plasmid DNA to unwind and become linear and as result moves slower through an agarose gel than the supercoiled form (Jeong *et al*., 2009; Zhang and Omaye, 2001). Therefore, the extent to which antioxidants can protect supercoiled plasmid DNA against oxidative damage can be measured (Wei *et al*., 2006). This method has been used by Gião *et al*. (2008), Jeong *et*

Table 1.2.8 Biological and cellular assays used to measure antioxidant effects

Assay	Oxidant	Principle measurement	Indicators	Advantages/Disadvantages	References
BIOLOGICAL ASSAYS					
Plasmid DNA	Peroxyl radical	Conversion of supercoiled plasmid to linear forms	Differences in electrophoretic mobility	Rapid, reproducible, single effect/ Weaker binding of EtBr to supercoiled DNA	Jeong *et al.*, 2009; Milligan *et al.*, 1992
Erythrocytes/ ghosts	Peroxyl radical	Oxidative damage to plasma membrane	Haemolysis or diene formation	Rapid, many runs with a single sample/ Problems with reproducibility due to different sources of blood, blood can only be stored for limited time	Blasa *et al.*, 2007
CELLULAR ASSAY: Short-term effects					
Dichlorofluores-cein (DCF) assay	Peroxyl radical	Formation of dichlorofluorescein (DCF) due to oxidative breakdown of DCFH-DA	Oxidation of DCFH-DA leads to increase in fluorescence that can be delayed by antioxidants	Rapid, reproducible, used with many cell types (erythrocytes, cell lines, primary cell cultures)/ Probe only functional in cytosol, only measure short term effects	Youdim *et al.*, 2000
CELLULAR ASSAYS: Long-term effects					
3,(4,5-dimethyl thiazol-2-yl)2,5-diphenyl tetra-zolium bromide (MTT) assay	Peroxyl radical	Conversion of MTT to insoluble formazan by mitochondrial succinate dehydrogenase	Oxidative damage causes a decrease in cell viability that can be prevented by antioxidants	Reproducible, used with many cell types (cell lines, primary cell cultures), measures sum of cellular ADME and toxicity/ Time-consuming (several days), measures only mitochondrial function, interference of reducing agents and respiratory chain inhibitors, low bioavailability.	Chiba *et al.*, 1998 ; Hansen *et al.*, 1989; Sgambato *et al.*, 2001
Neutral Red (NR) assay	Peroxyl radical	Uptake of NR by cells with intact lysosomal membrane	Oxidative damage causes a decrease in lysosomal membrane integrity that can be prevented by antioxidants	Reproducible, used with many cell types (cell lines, primary cell cultures), measures sum of cellular ADME and toxicity/ Time-consuming, measures only effect on lysosomal membrane, low bioavailability.	Chiba *et al.*, 1998 ; Ishiyama *et al.*, 1996
Crystal Violet (CV) assay	Peroxyl radical	Staining of cellular protein, measure of cell number	Oxidative damage causes cell death, decrease in cell number that can be prevented by antioxidants	Reproducible, used with many cell types (cell lines and primary cell cultures), measures sum of cellular ADME and toxicity/ Time-consuming, measures only cell number, low bioavailability.	Chiba *et al.*, 1998

EtBr: ethidium bromide, ADME: absorption, distribution, metabolism and excretion.

al. (2009) and Wang *et al*. (2007) to obtain level of protection of plant extracts against oxidative DNA degradation.

The erythrocytes haemolysis assay is used to measure the ability of plant extracts to prevent haemolysis through protection of the cell membrane against oxidative damage. Erythrocytes lack DNA and are therefore an ideal source of a typical lipid bilayer (Honzel *et al*., 2009). Oxidative damage can either result in diene formation or cause the loss of membrane integrity resulting in the leakage of cellular content (Grinberg *et al*., 1997). Furthermore, erythrocytes can also be used to determine the extent in which antioxidants may cross the cell membrane and mediate their effect intracellularly (Deng *et al*., 2006). Antioxidants that are lipid soluble (i.e. extracted using organic solvents) are either partitioned within the lipid bilayer or cross the cell membrane and enter the intracellular compartment. Whereas, the water soluble antioxidants are found extracellularly and may serve as a barrier protecting the cell membrane against oxidative damage (Blasa *et al*., 2007). Using this method, Arbos *et al*. (2008), Loganayaki *et al*. (2010), Wang *et al*. (2007) and Yang *et al*. (2006a) found appreciable protective effects of vegetable extracts on the membrane integrity of human erythrocytes.

In vitro cellular assays

In vitro cellular assays can be used to determine the short term (i.e. few hours) and long term (i.e. few days) effects of antioxidants. Furthermore, these cell cultures sytem can also be used to determine whether any cytotoxic molecules are present in the plant extracts. In these assays, the protective effects of antioxidants take place predominantly through radical scavenging activity and metal ion chelation either extracellularly or intracellularly depending on the solubility of the extracts (Melidou *et al*., 2005). The dichlorofluorescein (DCF) assay is used to measure the ability of plant extracts to protect cells against oxidative damage. In this assay, treated cells are exposed to a non-fluorescent probe, dichlorofluorescein diacetate (DCFH-DA) that readily crosses the cell membrane into cytosol (Honzel *et al*., 2008), where it is metabolized by intracellular esterases to dichlorofluorescin (DCFH). In the presence of reactive species, the DCFH is then converted to a fluorescent DCF (Myhre *et al*., 2003; Tarpey *et al*., 2004; Youdim *et al*., 2000). In the presence of antioxidants, the peroxyl radicals are scavenged and the DCF formation is reduced. Evaluation of protective effects of antioxidant-rich extracts in reducing oxidative damage has been studied in rats red blood cells (Youdim *et al*., 2000),

human adenocarcinoma colon cancer (Caco-2) cells (Elisia and Kitts, 2008), human umbilical vein endothelial cells (Hempel *et al*., 1999), PC 12 cells (Wang and Joseph, 1999) and HepG2 cells (Eberhardt *et al*., 2005; Tarpey *et al*., 2004), using this assay.

In the long term assays, the cells are exposed for anything from 24 - 96 h to both the peroxyl radicals and antioxidant extracts added to the cell culture medium. Following exposure, different endpoints can be measured such as the extent of DNA laddering, lactate dehydrogenase (LDH) leakage, diene formation, expression of specific proteins e.g. p53, mitochondrial activity, cell viability, lysosomal and plasma membrane integrity and cell number. The advantages and disadvantages of most widely used methods are presented in Table 1.2.8. The observed effect is the total sum of absorption, distribution, metabolism and excretion (ADME), as well as the ability of the cell to repair any oxidative damage (Noguchi *et al*., 2000). Individual or combinations of long term assays may be used to study the viability of the same cells and cytotoxicity (Arechabala *et al*., 1999; Chiba *et al*., 1998; Fotakis and Timbrell, 2006; Phillips, 1996).

1.2.15 Conclusions

African GLVs are widely consumed in sub-Saharan Africa. In this region, changes in diet and environmental factors, particularly among urban dwellers have caused a significant increase in CDL (Walker *et al*., 2002). GLVs are an excellent source of vitamins, minerals and antioxidants that can be used to prevent CDL. Little is known regarding the antioxidant activity of African GLVs and the effect of cooking on African GLVs such as *Amaranthus cruentus*, *Corchorus olitorius, Curcubita maxima* and *Vigna unguiculata* that are commonly consumed within sub-Saharan Africa. Likewise, little is known regarding the effect of extracts from African GLVs on the structure of cellular macromolecules and the functions of cell *in vitro.*

1.3 Hypotheses

1. Selected African GLVs (*Amaranthus cruentus*, *Corchorus olitorius, Curcubita maxima* and *Vigna unguiculata*) will contain higher total phenolic content,

flavonoid content and antioxidant activity than those of spinach (*Spinacea oleracea*). African GLVs are well adapted to local harsh environmental conditions and grow well with minimal water and poor soil fertility (Jansen van Rensburg *et al*., 2007). Phenolic compounds are secondary metabolites that are synthesized by plants during normal development and in response to stress conditions (Naczk and Shahidi, 2004) such as nutrient stress, lack of water, as well as defence against pathogens (Spina *et al*., 2008). All these conditions may induce the production of phenolic compounds. African GLVs would therefore exhibit higher total phenolics to respond to these stress conditions than spinach. Generally, plant extracts with high total phenolic content have high antioxidant activity (Maisuthisakul *et al*., 2007; Turkmen *et al*., 2006).

2. Boiling will reduce the total phenolic content, flavonoid content and antioxidant activity of all selected GLVs due to enhanced enzymatic and chemical oxidation of phenolic constituents (Yamaguchi *et al*., 2003). Thermal destruction of GLVs cell wall structures and sub-cellular compartments will also result in leaching of antioxidant components into the boiling water. Cooking has been found to detrimentally affect the phenolic content and antioxidant activity of vegetables (Roy *et al*., 2007).

3. Aqueous acetone extracts of GLVs will exhibit higher phenolic contents and antioxidant activity than extracts of water. Organic solvents containing some water have been found to be more efficient in extracting polyphenolic compounds than water or pure organic solvents (Turkmen *et al*., 2006; Xu and Chang, 2007). Water in the aqueous organic solvents causes the plant tissues to swell thereby allowing the solvent to better penetrate the sample matrix (Zhao and Hall III, 2008).

4. Extracts of raw and boiled GLVs will exhibit protective effects against AAPH-induced oxidative damage in erythrocytes, plasmid DNA, SC-1 fibroblast cells and human adenocarcinoma colon cancer (Caco-2) cells, due to their phenolic content and antioxidant activity. Oxidative damage of these substances is associated with free radicals (Blomhoff, 2005). Plant phenolics are able to stabilize free radicals by donating hydrogen atoms from their phenolic hydroxyl

groups, and form stable free radicals that do not initiate or propagate further oxidation (Salah *et al*., 1995).

5. Extracts of raw and boiled GLVs will exhibit cytotoxicity to cell cultures due to the presence of antinutritional factors such as oxalic acid, phytates, alkaloids, saponins, tannins and trysin inhibitors (Aletor and Adeogun, 1995; Ejoh *et al*., 2007; Radek and Savage, 2008, Wallace *et al*., 1998; Vanderjagt *et al*., 2000). Vegetables with high levels of antinutritional factors such as alkaloids are more cytotoxic than those with low levels of antinutritional factors (Costa-Lotufo *et al*., 2005; Phillips, 1996).

   The levels of cytotoxicities of extracts of GLVs will be dependent on the concentration and kind of antinutritional factors present in each extract as well as the type of cell line used. Different cytotoxicity assays yield different results depending on the type of assay used, the test agent used, endpoints (Weyermann *et al*., 2005) and cytotoxic substances found in the extracts (Morshed *et al*., 2011).

6. Cytotoxicity of extracts from raw GLVs will be higher than that of extracts from boiled GLVs. Boiling reduces the levels of antinutritional factors in GLVs (Vanderjagt *et al*., 2000), which will lead to lower levels of cytotoxicity in extracts of boiled samples.

1.4 Objectives

1. (a) To determine the total phenolic content, total flavonoid content and total antioxidant activity of selected African GLVs, in comparison to spinach. (Section 2.1)

   (b) To identify and quantify selected flavonoids in extracts of African GLVs and spinach using high-performance liquid chromatography (HPLC). (Section 2.2)

2. To determine the effect of boiling and extraction solvent on the total phenolic content, total flavonoid content and total antioxidant activity of selected GLVs. (Section 2.1)

3. To determine the ability of extracts of raw and boiled GLVs to protect against AAPH-induced oxidative damage in biological molecules (erythrocytes and plasmid DNA) and cell cultures (SC-1 fibroblast and Caco-2 cell lines). (Section 2.3)
4. To determine the levels of cytotoxicity in extracts of African GLVs in comparison to spinach and the effect of boiling on the cytotoxicity levels. (Section 2.3)

CHAPTER 2: RESEARCH

The research, which tested the hypotheses stated in Section 1.3, was done in three parts:

2.1 Raw and cooked African green leafy vegetables have greater antioxidant content and activity than spinach.

2.2 Comparative determination of flavonoids of African green leafy vegetables and spinach by high-performance liquid chromatography.

2.3 Protective effects of African green leafy vegetables against AAPH-induced oxidative damage.

2.1 Raw and cooked African green leafy vegetables have greater antioxidant content and activity than spinach [1]

2.1.1 Abstract

Leaves of *Amaranthus cruentus* L. (amaranth), *Corchorus olitorius* L. (jute mallow), *Cucurbita maxima* Duchesne (pumpkin) and *Vigna unguiculata* (L.) Walp. (cowpea) are widely consumed in sub-Saharan Africa as green leafy vegetables (GLVs). In this study, the total phenolic (TPC) and flavonoid content (TFC) of these African GLVs, as well as the total antioxidant activity (TAA) were determined and compared to *Spinacea oleracea* L. (spinach), also cultivated and consumed in this region. The TAA was measured using ABTS (2,2'-Azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt), DPPH (2,2-diphenyl-2-picrylhydrazyl) and ORAC (oxygen radical antioxidant capacity) assays. These GLVs were boiled for 0, 10, 30 and 60 min, drained and freeze-dried. Water and 75% acetone extracts from the freeze-dried samples were prepared for analysis. All raw African GLVs had phenolic content and antioxidant activity greater than spinach in both water and 75% acetone extracts. Boiling reduced the antioxidant content and activity of GLVs. The highest antioxidant content and activity was found in acetone extracts of jute mallow even after boiling for 60 min. A high degree of correlation was obtained between the different assays for the 75% acetone extracts and to a lesser degree for the water extracts. African GLVs may be considered important sources of antioxidant compounds with potential health benefits.

2.1.2 Introduction

Green leafy vegetables (GLVs) contribute significantly to household food security and add variety to cereal-based staple diets throughout rural sub-Saharan Africa (van den Heever, 1997). They are a rich dietary source of antioxidant components that are believed to protect the human body from diseases such as cardiovascular disease, cancer and neurodegenerative diseases that are associated with increased reactive oxygen species (ROS) (Govindarajan *et al*., 2005). The components responsible for antioxidative effects

[1] Part of this work is being prepared for publication.

in GLVs may include polyphenols, carotenoids and antioxidant vitamins such as vitamin C and vitamin E.

In a study of secondary data intake, Louwrens *et al*. (2009) reported that South Africans only consumed about half of their total antioxidant requirement. African GLVs could be an excellent source of antioxidants and could effectively address this dietary shortage. However there has been a decrease in the consumption of African GLVs even though several studies have indicated that these vegetables contain micronutrient levels as high or even more than those found in most of their exotic counterparts (Kruger *et al*., 1998; Steyn *et al*., 2001). Concurrently there has been an increase in consumption of exotic vegetables such as spinach. This is probably because African GLVs are often perceived to be inferior in their taste and nutritional value compared to exotic vegetables such as spinach (*Spinacea oleracea* L.) (Weinberger and Msuya, 2004). Another reason is that African GLVs are seasonal in rural areas and unlike the exotic vegetables, are not readily available in the urban areas.

Studies on antioxidant and radical scavenging activities of African GLVs are limited, while those on spinach are widely available. Appreciable levels of antioxidant content and free radical scavenging activities have been reported on African GLVs consumed in Nigeria (Akindanhusi and Salawu, 2005; Oboh *et al*., 2008; Odukoya *et al*., 2007 and Salawu *et al*., 2008 and 2009), South Africa (Lindsey *et al*., 2002; Odhav *et al*., 2007 and van der Walt *et al*., 2009) and Uganda (Stangeland *et al*., 2009). Because of the differences in GLVs growth and climatic conditions, as well as in analytical methods and standards used in these studies, it is difficult to compare the results obtained from these studies with spinach grown elsewhere. In this study, a comparative assessment of the antioxidant content and activity of African GLVs and that of spinach, grown under similar climatic environment is reported. Cooking has been shown to alter polyphenolic composition of various African GLVs (Salawu *et al*., 2008). In sub-Saharan Africa, cooking methods differ with different communities. Method and period of cooking and amount of water used for cooking is mostly dependent on the type of vegetable and the cultural background of the community. Some communities discard cooking water while others do not. Therefore, the second aim of this study was to determine the effect of cooking on antioxidant levels and activity by replicating cooking methods in the laboratory. The cooking methods used in this study are based on the traditional methods

obtained from the Bushbuckridge and Allandale communities in Mpumalanga province of South Africa.

2.1.3 Materials and Methods

2.1.3.1 Green leafy vegetable samples and their preparation

The selected African GLVs: amaranth (accession collected from Arusha, Tanzania), jute mallow (accession collected from Arusha, Tanzania), pumpkin (cultivar obtained from traditional land race in KwaZulu-Natal, South Africa), and cowpea (cultivar obtained from traditional land race in KwaZulu-Natal, South Africa), were grown, harvested and collected from the Vegetable and Ornamental Plant Institute of the Agricultural Research Council (ARC) at Roodeplaat, Gauteng Province, South Africa. The vegetables were covered in black plastic bags and transported in a cooler box to the University of Pretoria. Spinach, although exotic, is commonly cultivated and consumed within this region. Information is available regarding the antioxidant content and activity of spinach and for this reason, it was included in this study to compare with the other GLVs. Spinach was purchased fresh locally and was transported to the University of Pretoria.

The vegetables were washed with plenty of water to remove any soil or dirt. The consignment of each type of vegetable was divided into four, one was kept raw and the other three were cooked in boiling water (750 g to 1800 ml water) for 10, 30 and 60 min. Cooking times were chosen to replicate the times that these African GLVs are traditionally cooked, usually in water from 10 to 60 min. After boiling, samples were drained, freeze-dried, ground and homogenized in a blender before passing through a 500 µm mesh sieve. The ground vegetables were finally packaged in air-tight Ziploc plastic bags and stored at -20˚C in the dark until analysis. For each type of vegetable, three independent samples were collected and processed immediately.

2.1.3.2 Crude plant extracts

Crude extracts were prepared for analysis by adding ground vegetable samples to distilled water or 75% acetone at 0.01 g/ml concentration. The extraction was carried out for 1 h at room temperature using a magnetic stirrer. The extraction mixture was filtered through

Whatman No. 4 filter paper, and the filtrate used for analysis. Analyses were determined using three independent samples, each one analysed in triplicate after the crude extracts were obtained.

2.1.3.3 Analyses

2.1.3.3.1 Total phenolics

The total phenolic content of each vegetable sample was determined using the Folin-Ciocalteu method as described by Amin *et al*. (2006). To water- or 75% aqueous acetone-extracts (0.1 ml), 0.75 ml of Folin-Ciocalteu phenol reagent was added and mixed thoroughly. The mixture was allowed to stand for 5 min at room temperature before addition of 0.75 ml of sodium carbonate solution (60 g/L). Ten millilitres of distilled water was then added. The contents were thoroughly mixed and allowed to stand at room temperature for 90 min. The absorbance of the mixture was read at 760 nm, using a lambda EZ150 spectrophotometer (Perkin Elmer, USA). Gallic acid was used as a standard and all data was expressed as gallic acid equivalents (GAE) per g sample.

2.1.3.3.2 Total flavonoids

The total flavonoid content was measured using a modified form of the aluminium chloride colorimetric assay (Zhishen *et al*., 1999). Catechin was used to make the standard curve. A 30 µl aliquot of vegetable extracts or catechin solutions (0 – 0.167 mg/ml) were placed in 96-well microplates. To each well, 30 µl 2.5% sodium nitrite, 20 µl 2.5% aluminium chloride and 100 µl 2% sodium hydroxide were added. The solution was mixed well and the absorbance was measured at 450 nm with a BioTek ELx800 plate reader (Analytical & Diagnostic Products, Weltevreden Park, South Africa). For each assay run, a blank with 30 µl phosphate buffer solution (PBS) (pH 7.4) or 75% aqueous acetone, respectively, was included to correct the absorbance values. Total flavonoid content of vegetables was expressed as mg catechin equivalents (CE) per g of sample.

2.1.3.3.3 Antioxidant activity

ABTS radical scavenging assay

The ABTS radical scavenging assay used in this study followed the method of Awika *et al.* (2003). The $ABTS^{\bullet+}$ was freshly generated by adding 3 mM of potassium peroxodisulfate ($K_2S_2O_8$) solution to 8 mM ABTS and the mixture was left to react in the dark for at least 12 h at room temperature. The working solution was prepared by diluting

$ABTS^{\bullet+}$ stock solution with 0.2 M PBS (pH 7.4). Trolox (water-soluble vitamin E analogue) was used as a standard, working at a concentration range of 0 to 1000 µM. The working solution (2.9 ml) was added to 0.1 ml of water- or the 75% aqueous acetone vegetable extracts or trolox. The reaction mixtures were left to stand at room temperature and the absorbance readings were taken at 734 nm after 30 min, using the Lambda EZ150 spectrophotometer (Perkin Elmer, USA). The antioxidant activity was expressed as µmol Trolox Equivalents (TE) per g of sample.

DPPH radical scavenging assay

The scavenging activity of vegetable extracts on DPPH radicals was measured according to the method of Awika *et al*. (2003) with some modifications. The stock solution was freshly prepared by dissolving 24 mg of DPPH in 100 ml methanol. The solution was shaken in a sonicator for about 20 min to ensure that all DPPH particles were dissolved. The working solution was obtained by diluting 10 ml stock solution with 50 ml methanol. The extracts (15 µl) were allowed to react with 285 µl working solution using a 96-well microplate, for 15 min in the dark. The absorbance was taken at 570 nm, using the BioTek ELx800 plate reader (Analytical & Diagnostic Products, Weltevreden Park, South Africa). The standard curve was linear between 0 and 800 µM trolox. For each assay, a blank with PBS or 75% aqueous acetone, respectively, was included to correct sample and standard absorbance values. The results were expressed as µmol TE per g of sample.

ORAC assay

ORAC assays were carried out on a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Offenburg, Germany). Procedures were based on a modified method of Ou *et al*. (2002). AAPH was used as a peroxyl radical generator, trolox as standard and fluorescein as a fluorescent probe. PBS or 75% aqueous acetone was used as blank. Briefly, vegetable extracts were diluted 15-fold with PBS or 75% aqueous acetone. Fluorescein working solution (0.139 M) and AAPH (0.11 µM) were added to diluted extracts or Trolox serial dilutions. The prepared microplate was placed into the plate reader and incubated at 37˚C. The fluorescence was measured every 5 min for 4 h. The assay protocol included the following basic parameters: a position delay of 0.5 s, a measurement start time of 0.0 s, 10 flashes per cycle, 300 s cycle time, 485 nm for the excitation filter and 520 nm for the emission filter. The ORAC values of the samples

were calculated by integrating the net area under the decay curves (AUC), using the Origin software Version 6.0 (Microcal, TM). The results were expressed as µmol TE per g sample.

2.1.3.4 Statistical analysis

The data was subjected to analysis of variance (ANOVA), using samples and cooking time as independent variables and the values determined as dependent variables. Fisher's least significant difference (LSD) test was used for comparison of means using Statistica software Version 9.0 (StatSoft, Tulsa, OK). Correlation analysis was also run with the same statistical package.

2.1.4 Results and Discussion

2.1.4.1 Antioxidant content and activity of African GLVs compared to spinach

Table 2.1.1 shows the TPC, TFC and TAA of water and 75% acetone extracts of the raw African GLVs compared to spinach. These are reported on a fresh weight basis to provide an indication of the TPC, TFC and antioxidant activity of the GLVs as is. Water and 75% acetone extracts from raw jute mallow, amaranth, pumpkin and cowpea had higher levels of TPC, TFC and TAA than extracts from spinach. Jute mallow had highest levels of TPC, TFC and TAA in both water and 75% acetone extracts among all the GLV samples.

It is difficult to conduct a direct comparison of TPC levels reported in GLVs in the literature due to variations in factors such as sample preparation, extraction methods (Hayouni *et al*., 2007), assay types, maturity factors (Pandjaitan *et al*., 2005) and genetics (Cho *et al*., 2008). In this work, TPC levels in spinach were 0.9 GAE/g, fw (10.7 GAE /g, dw) for water extracts and 1.2 mg GAE/g, fw (14.4 mg GAE/g, dw) for 75% acetone extracts. These values fall within the wide range of TPC levels reported for spinach in the literature although in some instances there are differences in sample preparation and extraction methods used. TPC levels reported for spinach include 0.3 mg GAE/g, fw (Chun *et al*., 2005), 2.2 mg GAE/g, fw (Wu *et al*., 2004), 12.7 mg GAE/g, dw (Turkmen *et al*., 2005) and 17.6 - 46.6 mg GAE/g, dw (Pandjaitan *et al*., 2005).

Table 2.1.1 Total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant activity of water and 75% acetone extracts of raw African green leafy vegetables (GLVs) compared to spinach.

GLVs	TPC (mg GAE/g, fw)		TFC (mg CE/g, fw)		ABTS (µmol TE/g, fw)		DPPH (µmol TE/g, fw)		ORAC (µmol TE/g, fw)	
	water	75% acetone	water	75% acetone	water	75% acetone	water	75% acetone	water	75% acetone
Amaranth	2.1 b[1] (0.0)[2]	2.3 b (0.1)	1.5 b (0.0)	1.8 b (0.1)	44.7 b (1.3)	98.5 b (5.0)	24.2 b (2.2)	97.7 b (9.1)	20.0 b (1.9)	16.7 b (1.6)
Jute mallow	3.0 c (0.0)	6.1 c (0.5)	2.7 c (0.0)	5.5 c (0.5)	58.7 c (1.1)	192.5 c (8.9)	45.1 c (5.4)	686.8 d (82.0)	59.5 d (7.1)	121.5 c (14.6)
Cowpea	2.4 b (0.1)	2.6 b (0.1)	1.5 b (0.1)	1.4 b (0.1)	75.3 d (5.2)	103.7 b (4.1)	81.5 d (8.3)	93.8 b (9.5)	33.0 c (3.4)	30.6 b (3.1)
Pumpkin	2.7 bc (0.0)	2.2 b (0.1)	1.6 b (0.0)	1.6 b (0.1)	62.1 c (2.5)	96.2 b (5.9)	83.6 d (9.5)	183.5 c (21.0)	36.0 c (4.1)	34.5 b (3.9)
Spinach	0.9 a (0.0)	1.2 a (0.1)	0.5 a (0.0)	0.7 (0.1)	35.8 a (1.7)	63.6 a (4.3)	8.2 a (0.7)	16.3 a (1.3)	15.6 a (1.2)	10.6 a (0.8)

GAE: gallic acid equivalent; CE: catechin equivalents; TE: trolox equivalents, [1] Means with different letters in the same column are significantly different ($p < 0.05$), [2] Standard deviation in parentheses.

Among the African GLVs, the TPC values found in the literature are 9.6 mg GAE/g, dw for the water extracts (Oboh *et al*., 2008) and a range of 10.6 – 21.8 mg GAE/g, dw for the 80% methanol extracts (van der Walt *et al*., 2009) of *Amaranthus* species, which are less than the 22.7 mg GAE/g, dw for the water extracts and 24.2 mg GAE/g, dw for the acetone extracts of amaranth found in this study. Van der Walt *et al*. (2009) also reported TPC value of 29.1 mg GAE/g, dw for 80% methanol extracts of cowpea leaves, which is higher than the 23.7 mg GAE/g, dw for the water extracts and 25.7 mg GAE/g for the 75% acetone extracts of cowpea reported in this study. Salawu *et al*. (2008) reported TPC values of 42.3 mg GAE/g, dw for the 70% ethanol extracts of jute mallow, which is more than the 25.0 mg GAE/g for water extracts and less than the 50.7 mg GAE/g for the 75% acetone extracts of jute mallow found in this study.

In the present study, the observed total flavonoid contents of 0.5 mg CE/g, fw (6.0 mg CE/g, dw) for the water extract and 0.7 mg CE/g, fw (8.9 mg CE/g, dw) for the 75% acetone extract of spinach are higher than that obtained by Chun *et al*. (2005) in aqueous methanol extract of spinach (0.014 mg CE/g, fw) using a similar assay. The assay used in this study is based on the formation of a red aluminum complex where the flavonoid acts as a bidental ligand (Amaral *et al*., 2009), forming complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols (Chang *et al*., 2002).

Antioxidant activity for both water and acetone extracts from the GLVs ranged between 35.8 - 192.5, 8.2 - 686.8 and 10.6 - 121.5 µmol TE/g, fw for the ABTS, DPPH and ORAC assays, respectively (Table 2.1.1). As observed with the TPC and TFC results, water and 75% acetone extracts from raw jute mallow, amaranth, pumpkin and cowpea had higher antioxidant activity than extracts from spinach as measured by the three antioxidant activity assays. Comparing the 75% acetone extracts, jute mallow had highest antioxidant activity for each antioxidant assay compared to the other GLVs. However, for the water extracts, cowpea had highest antioxidant activity according to the ABTS assay, pumpkin based on the DPPH assay and jute mallow had highest antioxidant activity according to the ORAC assay.

The antioxidant activity using the ABTS assay for spinach was 35.8 µmol TE/g, fw (447.6 µmol TE/g, dw) for water extracts and 63.6 µmol TE/g, fw (795.2 µmol TE/g, dw) for acetone extracts, which is greater than 8.5 µmol TE/g, fw reported by Sun and

Tanumihardjo (2007). Compared to this study, Yang *et al*. (2006) also reported lower ABTS radical scavenging values of 102, 79, 65 and 147 µmol TE/g, dw for *Spinacea oleracea*, *Cucurbita* spp., *Corchorus olitorius* and *Amaranthus* spp., respectively. Nevertheless, the antioxidant activity of spinach and other GLVs used in this study is within the range reported for Mauritian endemic plants (Soobrattee *et al*., 2008). All these results are however lower than the approx. 2 mmol TE/g, dw reported for water extracts of *Amaranthus cruentus* by Oboh *et al*. (2008).

Using the DPPH assay, the values determined in spinach were 8.2 and 16.3 µmol TE/g, fw in water and acetone extracts, respectively. The value of water extract is of the same order of 3.7 µmol TE/g, fw and 7.9 µmol TE/g, fw reported by Yamaguchi *et al*. (2001) and Yang *et al*. (2006), respectively. In the latter study, the antioxidant activity of *Amaranthus sp.* was also determined in water extracts and was found to be 4.0 µmol TE/g, fw less than the 24.2 µmol TE/g, fw found in the present study.

ORAC values for spinach reported in literature include 11.6 µmol TE/g, fw (USDA 2007), 16.2 µmol TE/g, fw (Sun and Tanumihardjo, 2007), 48.7 - 84.4 µmol/g, fw (different genotypes, 50% acetone extract) (Cho *et al*., 2008), 4.2 µmol TE/g, fw (Wu *et al*., 2004) and 152 µmol TE/g, dw (Ou *et al*., 2002), while this study found values of 15.6 µmol TE/g, fw (195.1 µmol TE/g, dw) for the water extracts and 10.6 µmol TE/g, fw (132.4 µmol TE/g, dw) for the acetone extracts. Overall, this data shows that the African GLVs under study have appreciable levels of phenolics and antioxidant activity and in higher quantities compared to spinach.

2.1.4.2 Effect of boiling on antioxidant content and activity of GLVs

African GLVs are usually cooked in water, the water is then discarded and the remaining solid material is consumed. Tables 2.1.2 and 2.1.3 show the effect of boiling on TPC, TFC and antioxidant activity of water and 75% acetone extracts of this material, respectively. Boiling for 10 min reduced TPC and TFC of all GLVs in both extracts, with the greatest effect observed in water extracts of pumpkin with an 82% and 75% decrease in TPC and TFC, respectively. Generally, TPC and TFC contents of water and 75% acetone extracts from GLVs boiled for 30 and 60 min were lower than for those of raw samples. Water extract of jute mallow and 75% acetone extract of spinach were

exceptions where there were no significant differences in TFC content between raw and boiled samples. Water and 75% acetone extracts from pumpkin boiled for 60 min had higher TPC and TFC than pumpkin boiled for 10 and 30 min. Although there was a general decrease in levels of TPC and TFC after boiling, the GLVs retained appreciable levels of antioxidants. Overall, after cooking, water and acetone extracts from jute mallow, cowpea and amaranth had higher TPC and TFC than for spinach.

In general, the trends in antioxidant activity were similar to those observed for TPC and TFC (Tables 2.1.2 and 2.1.3). The three assays (ABTS, DPPH and ORAC) showed that there was a decrease in antioxidant activity of water and 75% acetone extracts of the GLVs after boiling. Jute mallow appeared to deviate from this trend where its water extracts from boiled leaves had higher antioxidant activity than the uncooked leaves according to the three assays. Water extracts from boiled amaranth also had higher antioxidant activity than the uncooked amaranth leaves according to the DPPH assay (Table 2.1.2). As observed for TPC and TFC, water and 75% acetone extracts from pumpkin boiled for 60 min had higher antioxidant activity than pumpkin boiled for 10 and 30 min according to the three assays.

Boiling decreases the phenolic and antioxidant content of vegetables, which may have resulted from leaching of vegetable antioxidants into the cooking medium (Roy et al., 2007; Wachtel-Galor et al., 2008; Zhang and Hamauzu, 2004) or due to oxidation of polyphenol components by polyphenol oxidase in vegetables (Yamaguchi et al., 2003). Amin et al. (2006) found that different varieties of the same Amaranthus species differed significantly regarding TPC content and the effect of blanching on TPC. Salawu *et al*. (2008) reported both decrease and increase in phenolic content of several African GLVs after boiling. The trend of antioxidant and radical scavenging activity after boiling is dependent on a number of factors including the type of vegetable, type and duration of boiling, boiling temperature, bioavailability of phenolics, localization and the stability of high temperatures (Jimenez-Monreal *et al*., 2009). Differences in tissue hardness and phenolic profile of each vegetable are also major contributors to antioxidant activity (Yamaguchi *et al*., 2001).

Table 2.1.2 Effect of boiling on total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant activity of water extracts of green leafy vegetables (GLVs).

		Boiling time		
	Raw	10 min	30 min	60 min
Amaranth				
TPC[a]	22.7 d[1] (0.2)[2]	14.2 c (0.2)	11.3 b (0.1)	10.6 a (0.4)
TFC[b]	16.3 c (0.7)	10.2 b (1.0)	6.9 a (0.7)	7.2 a (0.8)
ABTS[c]	481.2 b (14.4)	446.1 ab (15.8)	416.7 a (13.9)	412.6 a (15.8)
DPPH[d]	260.4 a (9.0)	560.0 b (67.8)	525.2 b (64.9)	465.1 b (57.1)
ORAC[e]	215.5 b (8.3)	140.3 a (9.6)	110.1 a (8.4)	124.9 a (8.1)
Jute mallow				
TPC[a]	25.0 c (0.4)	23.7 b (0.2)	27.3 a (0.4)	20.0 a (0.2)
TFC[b]	22.5 a (0.7)	23.6 a (1.5)	24.7 a (2.4)	23.0 a (3.7)
ABTS[c]	489.3 a (9.0)	588.0 b (24.3)	578.7 b (18.6)	555.3 b (18.1)
DPPH[d]	376.5 a (9.0)	689.6 b (67.8)	887.0 c (65.0)	785.0 bc (57.1)
ORAC[e]	495.7 a (14.6)	676.7 c (29.6)	612.0 b (24.3)	596.4 b (30.5)
Cowpea				
TPC[a]	23.7 c (0.9)	15.6 b (0.5)	13.4 a (0.3)	13.52 a (0.1)
TFC[b]	14.7 b (1.2)	7.0 a (0.7)	6.8 a (0.7)	6.9 a (0.9)
ABTS[c]	737.8 b (50.8)	443.5 a (15.3)	471.4 a (16.4)	484.0 a (26.0)
DPPH[d]	799.9 b (97.7)	375.1 a (22.0)	351.4 a (32.8)	356.9 a (29.4)
ORAC[e]	323.8 b (14.2)	221.3 a (13.6)	253.2 a (11.7)	255.4 b (12.8)
Pumpkin				
TPC[a]	23.8 d (0.1)	4.2 a (0.1)	4.9 b (0.0)	13.3 c (0.3)
TFC[b]	14.3 c (0.7)	3.5 a (0.8)	3.5 a (0.6)	10.4 b (1.0)
ABTS[c]	545.1 c (22.0)	335.0 a (18.6)	342.9 a (19.2)	461.9 b (11.3)
DPPH[d]	733.1 c (85.3)	139.8 a (9.6)	171.1 a (10.2)	563.6 b (53.1)
ORAC[e]	315.5 c (16.4)	94.8 a (7.6)	82.7 a (8.1)	228.8 b (11.4)
Spinach				
TPC[a]	10.7 c (0.5)	5.7 b (0.2)	5.0 a (0.2)	5.6 b (0.3)
TFC[b]	6.0 c (0.1)	3.8 ab (0.3)	3.0 a (0.6)	4.2 b (0.8)
ABTS[c]	447.6 b (20.9)	377.6 a (22.0)	363.9 a (20.9)	386.4 a (24.3)
DPPH[d]	102.4 a (19.6)	87.5 a (7.3)	84.9 a (6.2)	139.6 b (5.1)
ORAC[e]	195.1 c (10.6)	181.9 b (10.4)	163.2 a (10.9)	177.8 b (10.5)

[a] Expressed as mg gallic acid equivalents/g, dry weight basis, [b] Expressed as mg catechin equivalents/g, dry weight basis, [c] Expressed as µmol trolox equivalents/g, dry weight basis, [d] Expressed as µmol trolox equivalents/g, dry weight basis, [e] Expressed as µmol trolox equivalents/g, dry weight basis, [1] Means with different letters in the same row are significantly different ($p < 0.05$), [2] Standard deviation in parentheses.

Table 2.1.3 Effect of boiling on total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant activity of 75% acetone extracts of green leafy vegetables (GLVs).

		Boiling time		
	Raw	10 min	30 min	60 min
Amaranth				
TPC[a]	24.2 c[1] (0.8)[2]	13.3 b (0.1)	11.2 a (0.3)	10.5 a (0.4)
TFC[b]	19.5 c (2.4)	12.8 b (0.7)	10.0 a (0.7)	7.2 a (0.8)
ABTS[c]	1059.2 b (54.2)	663.6 a (47.5)	708.3 a (46.3)	708.1 a (53.1)
DPPH[d]	1050.2 b (66.7)	551.6 a (50.3)	482.7 a (32.8)	517.6 a (23.7)
ORAC[e]	179.5 c (10.8)	112.4 b (8.3)	76.2 a (6.5)	79.5 a (6.7)
Jute mallow				
TPC[a]	50.7 c (0.1)	34.1 b (0.3)	32.4 a (0.3)	32.2 a (0.4)
TFC[b]	46.0 b (6.7)	30.8 a (4.2)	27.8 a (3.5)	27.1 a (4.4)
ABTS[c]	1604.3 c (74.0)	1371.8 b (29.9)	1303.3 b (40.7)	1116.1 a (40.1)
DPPH[d]	5723.7 b (238.4)	3842.3 a (194.4)	3638.6 a (218.6)	3690.4 a (158.7)
ORAC[e]	1012.4 b (47.2)	519.9 a (15.5)	543.0 a (17.0)	505.8 a (25.6)
Cowpea				
TPC[a]	25.7 d (0.5)	16.0 a (0.5)	17.2 a (0.3)	18.4 c (0.6)
TFC[b]	13.6 b (1.3)	10.1 a (1.0)	11.5 a (0.5)	11.6 a (0.8)
ABTS[c]	1017.8 b (40.7)	758.3 a (31.6)	901.8 b (48.6)	759.7 a (35.0)
DPPH[d]	920.5 b (37.3)	463.5 a (17.5)	502.7 a (27.1)	530.9 a (18.6)
ORAC[e]	300.1 b (14.6)	152.9 a (9.3)	149.8 a (8.3)	151.8 a (8.3)
Pumpkin				
TPC[a]	19.4 d (0.5)	5.4 a (0.1)	6.1 b (0.2)	13.2 c (0.3)
TFC[b]	13.7 c (1.3)	4.3 a (0.7)	3.8 a (0.9)	7.4 b (1.2)
ABTS[c]	843.8 b (51.4)	623.9 a (38.4)	594.0 a (45.8)	799.1 b (50.3)
DPPH[d]	1610.5 c (75.1)	310.2 a (9.0)	315.3 a (8.5)	1023.5 b (32.2)
ORAC[e]	302.3 c (14.6)	76.6 a (6.7)	77.7 a (6.0)	163.0 b (9.2)
Spinach				
TPC[a]	14.4 c (0.7)	10.5 b (0.7)	8.1 a (0.6)	9.5 ab (0.7)
TFC[b]	8.9 a (1.3)	8.3 a (1.0)	7.4 a (0.8)	8.1 a (0.8)
ABTS[c]	795.2 b (54.2)	655.8 a (40.7)	669.6 a (39.5)	674.9 a (54.8)
DPPH[d]	203.1 b (25.4)	98.3 a (14.1)	98.7 a (13.0)	175.6 b (24.9)
ORAC[e]	132.4 c (8.4)	82.7 ab (6.9)	66.9 a (5.0)	93.3 b (6.8)

[a] Expressed as mg gallic acid equivalents/g, dry weight basis, [b] Expressed as mg catechin equivalents/g, dry weight basis, [c] Expressed as µmol trolox equivalents/g, dry weight basis, [d] Expressed as µmol trolox equivalents/g, dry weight basis, [e] Expressed as µmol trolox equivalents/g, dry weight basis, [1] Means with different letters in the same row are significantly different ($p < 0.05$), [2] Standard deviation in parentheses.

Yamaguchi *et al*. (2001) found both decreases and increases in flavonoid content after cooking. An increase in antioxidant activity as was observed for pumpkin at 60 min may have resulted from liberation of high amounts of antioxidant components due to thermal destruction of vegetable cell wall structures and sub-cellular compartments; the production of redox active secondary plant metabolites or breakdown products from intracellular compartments, matrix modifications and more efficient release of antioxidants during homogenization of samples (Wachtel-Galor *et al*., 2008); as well as suppression of the oxidation of antioxidants by thermal inactivation of polyphenol oxidase and ascorbate oxidase resulting in exhibition of higher radical scavenging activity in boiled vegetables (Yamaguchi *et al*., 2003). Overall, all the antioxidant assays showed that both uncooked and boiled jute mallow (in particular, its 75% acetone extracts) had the highest antioxidant activity of all the GLV samples.

2.1.4.3 Effect of extraction solvent

Extractability of polyphenols is dependent on the solvent and extraction time. It is therefore impossible to find a single solvent for the extraction of all polyphenols. In this study, the TPC, TFC, ABTS and DPPH values of 75% acetone extracts were higher than those of water extracts, while the ORAC values of 75% acetone extracts, with exception of raw extract of jute mallow, were unexpectedly lower than those of water extracts. Water extract of raw jute mallow was very viscous and therefore water may not have been efficient in extraction of antioxidants from such plant material. Thus, in almost all antioxidant assays, water extracts of raw jute mallow had lower values than boiled samples, in contrast to the values of 75% acetone extracts. In general, 75% acetone seems to have mediated a more effective extraction of antioxidants from the leafy vegetables than water. Several researchers found optimal phenolic content and antioxidant activity from plant extracts using a range (50 - 80%) of aqueous acetone solutions (Tabart *et al*., 2007; Turkmen *et al*., 2007; Vatai *et al*., 2009; Zhao *et al*., 2008).

Table 2.1.4 shows correlations between water and 75% acetone extracts for each assay and for all GLVs. For amaranth, cowpea, pumpkin and spinach, high positive correlations ($r \geq 0.6$) were obtained in all assays. This result suggests that the values determined from water extracts were in agreement with those achieved using the 75% acetone extracts. Water extracts of cowpea, pumpkin, spinach and amaranth (except for DPPH) exhibited a

similar trend with the 75% acetone extracts of each vegetable. Lower and negative correlations ($r \leq 0.2$) were obtained for jute mallow (TPC, TFC, ABTS, DPPH and ORAC) and amaranth (DPPH), demonstrating that a single extraction solvent and assay is not sufficient to evaluate the total antioxidant activity of jute mallow and amaranth.

Table 2.1.4 Correlation coefficients (r) between water and 75% acetone extracts for each assay per green leafy vegetable.

GLVs	TPC	TFC	ABTS	DPPH	ORAC
Amaranth	0.99	0.97	0.89	- 0.94	0.99
Jute mallow	0.23	- 0.66	- 0.64	- 0.96	- 0.88
Cowpea	0.91	0.87	0.85	0.99	0.93
Pumpkin	0.99	0.95	0.97	0.99	0.97
Spinach	0.97	0.93	0.96	0.66	0.92

Table 2.1.5 shows correlation coefficients between different assays for water and 75% acetone extracts of GLVs. TPC, TFC and antioxidant assays showed a high degree of correlation for both water and 75% acetone extracts of cowpea and pumpkin. For jute mallow, correlations between the assays for the water extract contrasted considerably from the 75% acetone extracts, i.e. poor correlation for water extracts (possibly related to the viscous nature of the water extract resulting in relatively poorer antioxidant extraction efficiency) and strong correlation for acetone extracts. For amaranth and spinach, a strong correlation was found between TPC and TFC or ABTS or ORAC for both water and acetone extracts. Generally, a strong correlation was found between TPC or TFC and antioxidant assays, confirming that phenolic compounds are likely to have contributed to radical scavenging activity of these vegetable extracts, although the degree of contribution of individual phytochemicals to the radical scavenging activity varies in each extract of the GLVs species.

Table 2.1.5 Correlation coefficients (r) between TPC, TFC, ABTS, DPPH and ORAC for water and 75% acetone extracts.

GLVs	Assays	Water extracts				75% Acetone extracts			
		TFC	ABTS	DPPH	ORAC	TFC	ABTS	DPPH	ORAC
Amaranth	TPC	0.99	0.98	- 0.84	0.98	0.96	0.95	0.99	0.98
	TFC		0.99	- 0.83	0.99		0.96	0.92	0.97
	ABTS			- 0.73	0.95			0.92	0.90
	DPPH				- 0.89				0.97
Jute mallow	TPC	0.57	0.00	- 0.02	- 0.11	0.99	0.88	0.99	0.99
	TFC		0.77	0.80	0.60		0.91	0.99	0.98
	ABTS			0.86	0.96			0.86	0.86
	DPPH				0.70				0.99
Cowpea	TPC	0.98	0.95	0.99	0.84	0.95	0.82	0.99	0.97
	TFC		0.99	0.99	0.93		0.86	0.93	0.87
	ABTS			0.99	0.97			0.84	0.83
	DPPH				0.92				0.99
Pumpkin	TPC	0.98	0.99	0.97	0.98	0.97	0.96	0.99	0.98
	TFC		0.99	0.99	0.99		0.90	0.98	0.99
	ABTS			0.99	0.99			0.97	0.91
	DPPH				0.99				0.99
Spinach	TPC	0.95	0.99	0.03	0.86	0.95	0.90	0.71	0.95
	TFC		0.99	0.29	0.93		0.74	0.70	0.92
	ABTS			0.19	0.90			0.79	0.92
	DPPH				0.21				0.89

2.1.5 Conclusion

This study indicates that these four GLVs consumed in sub-Saharan Africa have appreciable antioxidant content and activity even after boiling that is higher than that of spinach. Of these, jute mallow has the highest levels. The comparison of spinach that has well documented antioxidant content and activity and is cultivated under similar conditions to African GLVs provides information that is relevant and usable. The data obtained from this study can be used to encourage the consumption, cultivation and commercialization of these African GLVs. In turn this will assist in addressing the problem of low antioxidant status identified by Louwrens *et al*. (2009) eventually leading to the prevention of diseases associated with free radical formation.

2.1.6 References

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2.2 Comparative determination of flavonoids of African green leafy vegetables and spinach by high-performance liquid chromatography

2.2.1 Abstract

The aim of this study was to determine the flavonoid contents of crude extracts from raw and cooked African green leafy vegetables (GLVs) (*Amaranthus cruentus* L. (amaranth), *Corchorus olitorius* L. (jute mallow), *Cucurbita maxima* Duchesne (pumpkin) and *Vigna unguiculata* (L.) Walp. (cowpea)) in comparison to *Spinacea oleracea* L. (spinach) and their fate during boiling. Extracts were prepared using water and 75% acetone and analyzed by high performance liquid chromatography (HPLC) for the presence and content of epicatechin, rutin, myricetin, quercetin, kaempferol, luteolin and apigenin. The greatest amount of total flavonoids was detected in the water extracts of pumpkin and the 75% acetone extracts of cowpea. Epicatechin and rutin were the main flavonoids detected in water extracts, while higher values of rutin were observed in the 75% acetone extracts. After boiling, all flavonoids were fairly stable, but there were also reductions or increases in boiled samples, depending on the type of vegetable, specific flavonoids present in the vegetable and the extraction solvent.

2.2.2 Introduction

Vegetables contain bioactive compounds which are believed to be important in the maintenance of health and prevention of diseases (Scalbert *et al*., 2005; Rice-Evans *et al*., 1996). Leaves of plants harvested in the wild or weeds found in cultivated fields are widely consumed in sub-Saharan Africa as green leafy vegetables (GLVs). The African GLVs have been reported to possess antioxidant properties, even though only a small and incomplete portion of these vegetables has been scientifically investigated (Akindanhusi and Salawu, 2005; Lindsey *et al*., 2002; Oboh, 2005; Oboh *et al*., 2008; Odhav *et al*., 2007; Odukoya *et al*., 2007; Salawu *et al*., 2008 and 2009; Stangeland *et al*., 2009; van der Walt *et al*., 2009). The antioxidant properties of plant foods have been attributed to their phenolic content, mostly flavonoids and phenolic acids (Scalbert *et al*., 2005), which allow them to act as reducing agents, hydrogen or electron donating agents, free radical scavengers, singlet

oxygen quenchers (Rice-Evans *et al*., 1996 and 1997) and modulators of specific cellular proteins and enzyme activities (Rahman *et al*., 2006).

In order to understand the mechanisms underlying the antioxidant properties and beneficial effects of African GLV extracts, it is important to study the phenolic composition (e.g. flavonoids) of these plants. In the literature, estimates of the polyphenol constituents consider mostly the compositions of fresh African GLVs, and thus have not taken into account losses during cooking. Studies on the effect of cooking on phytochemicals of African GLVs is limited, the data available are fragmentary and incomplete, therefore making comparison among the studies difficult. Although different studies have been done on the total phenolic content and antioxidant properties of some African GLVs, there is very limited information on the phenolic profiles of leaves of GLVs such as amaranth, pumpkin, cowpea and spinach grown or cultivated in sub-Saharan Africa. A study by Salawu *et al*. (2009) characterized the phenolic composition of some African GLVs and found that the dicaffeoyl quinic acids and quercetin monoglycosides were the most dominant phenolic compounds in jute mallow.

The aim of the present study was to compare the levels of flavonoids (epicatechin, rutin, myricetin, quercetin, kaempferol, luteolin and apigenin) in water and 75% acetone extracts of selected African GLVs (amaranth, jute mallow, pumpkin, cowpea) with those of spinach, as well as the effect of boiling on these compounds.

2.2.3 Materials and Methods

2.2.3.1 Preparation of GLV samples and crude plant extracts

The procedures for acquiring the GLVs and the preparation of crude plant extracts have been described in Section 2.1, subsections 2.1.3.2 and 2.1.3.3.

2.2.3.2 Reversed-phase HPLC analysis

The reversed-phase HPLC analysis was conducted using the method of Kim *et al*. (2007). The sample extracts were filtered through 0.2 µm Millipore Millex filters prior to HPLC injection. The HPLC system consisted of a Waters 1525 binary HPLC pump and a Waters 2487 dual wavelength absorbance detector. The separation was accomplished by means of an

YMC-Pack ODS AM-303 (250 mm x 4.6 mm i.d., 5 µm particle size) column. Breeze™ software was used to monitor the separation process and after analysis a chromatogram was obtained for each sample extract.

The injection volume for all samples was 20 µl with the analysis conducted at a flow rate of 0.8 ml/min and monitored at 280 nm. The mobile phase consisted of 0.1% glacial acetic acid in distilled water (solvent A) and 0.1% glacial acetic acid in acetonitrile (solvent B). The linear gradient of the solvents was as follows: solvent B was increased from 8 to 10% in 2 min, then increased to 30% in 25 min, followed by an increase to 90% in 23 min, then increase to 100% in 2 min, kept at 100% of B for 4 min, and returned to the initial condition. Running time was 60 min and the column temperature was held at 25 °C during the run.

The flavonoid standards (myricetin, rutin, kaempferol, luteolin, apigenin, epicatechin and quercetin) were prepared in dimethylsulphoxide (DMSO) at concentrations of 150, 100, 50, 25, 20, 10 and 5 ppm (mg/L). Standards of 20 µl aliquots were chromatographed singly and as mixtures by injection into the HPLC system. Standard calibration curves were obtained for each compound by plotting peak areas versus concentrations. Regression equations that showed high degree of linearity ($R^2 \geq 0.984$) were obtained for each flavonoid from the calibration curves. Flavonoids in the samples were identified by comparing the retention time of the unknown with those of the standard flavonoids. The concentrations of the identified flavonoids were calculated using the regression equations obtained and expressed as mg/g of sample on dry basis.

2.2.3.3 Statistical analyses

The results are reported as means ± standard deviations. Chromatograms were drawn with the aid of the Origin software Version 6.0 (Microcal, TM). The data was subjected to analysis of variance (ANOVA), using samples and cooking time as independent variables and the values determined as dependent variables. Fisher's least significant difference (LSD) test was used for comparison of means using Statistica software Version 9.0 (StatSoft, Tulsa, OK).

2.2.4 Results and Discussion

2.2.4.1 Levels of flavonoids in raw GLVs

Almost all flavonoids were detected in water extracts of raw GLVs, except for luteolin and apigenin in extracts of jute mallow and spinach, as well as quercetin in jute mallow (Table 2.2.1). Among water extracts, epicatechin was the most abundant flavonoid in amaranth, while rutin was most abundant in spinach and pumpkin. Among water extracts, pumpkin had the highest amounts of total flavonoids (12.01 mg/g, dw or 1.36 mg/g, fw), while jute mallow had the least amounts with 0.18 mg/g, dw (0.02 mg/g, fw).

For 75% acetone extracts (Table 2.2.2), epicatechin and rutin were the main flavonoids detected in all raw GLV extracts, while kaempferol and luteolin and apigenin were not detected in any of the extracts. Mainly due to their rutin and to an extent epicatechin contents, cowpea had the highest amounts of total flavonoids (47.61 mg/g, dw or 3.86 mg/g, fw), while spinach had the least amount (8.14 mg/g, dw or 0.68 mg/g, fw). Studies of flavonoid composition of GLV species similar to the ones used in this study and grown in sub-Saharan Africa are limited, therefore it is difficult to make direct comparison of flavonoid levels found in this study with those in the literature from GLVs grown elsewhere. Factors such as variations in sample preparations and extraction methods, as well as differences in genotypes, agronomic, environmental and climatic growth conditions used are also critical in making comparisons (Cho *et al*., 2008; Howard *et al*., 2002; Kidmose *et al*., 2001; Luthria, 2006).

Flavonoid contents of acid extracts from Taiwanese GLV species similar to the ones being studied here have previously been reported by Yang *et al*. (2008). The researchers did not detect these flavonoids in amaranth and spinach extracts, however they reported 0.01, 0.60 and 1.05 mg/g, fw quercetin in pumpkin, jute mallow and cowpea, respectively, as well as 0.02, 0.04 and 0.11 mg/g, fw kaempferol in pumpkin, jute mallow and cowpea, respectively. These values are in the range of what the present study found. However, in contrast to the results observed in the present study, Yang *et al*. (2008) did not detect the flavones (luteolin and apigenin) in these GLVs.

Table 2.2.1 Effect of boiling on levels of flavonoids (mg/g, dry weight) in water extracts of selected green leafy vegetables (GLVs).

GLVs	Flavanol	Flavonols				Flavones		Total
extracts	Epicatechin	Rutin	Myricetin	Quercetin	Kaempferol	Luteolin	Apigenin	Flavonoids
amaranth								
raw	7.47 b[1] (0.56)[2]	1.64 a (0.78)	0.01 a (0.00)	0.01 a (0.00)	0.01 a (0.00)	0.01 a (0.00)	0.01 a (0.00)	9.16 a (2.78)
boiled	3.85 a (0.59)	7.50 b (0.11)	0.03 a (0.01)	0.01 a (0.00)	0.01 a (0.00)	0.01 a (0.00)	0.02 a (0.00)	11.44 b (2.96)
jute mallow								
raw	0.02 a (0.00)	0.13 a (0.04)	0.02 a (0.00)	n.d.	0.01 a (0.00)	n.d.	n.d.	0.18 a (0.06)
boiled	0.34 a (0.00)	1.59 b (0.02)	0.72 b (0.01)	n.d.	n.d.	n.d.	n.d	2.65 b (0.64)
cowpea								
raw	1.16 b (0.04)	0.86 a (0.37)	0.15 a (0.00)	0.01 a (0.00)	0.02 a (0.00)	0.02 a (0.00)	0.01 a (0.00)	2.23 b (0.48)
boiled	0.06 a (0.00)	0.21 a (0.04)	n.d.	0.01 a (0.00)	n.d.	n.d.	n.d.	0.28 a (0.10)
pumpkin								
raw	1.29 b (0.03)	3.64 b (0.63)	1.70 b (0.45)	0.76 b (0.08)	1.36 b (0.57)	1.64 b (0.09)	2.12 b (0.90)	12.01 b (0.95)
boiled	0.79 a (0.01)	1.14 a (0.50)	0.23 a (0.03)	0.07 a (0.02)	0.01 a (0.00)	0.06 a (0.01)	0.01 a (0.00)	2.31 a (0.45)
spinach								
raw	0.10 a (0.01)	5.03 b (0.04)	0.04 a (0.00)	0.01 a (0.00)	0.01 a (0.00)	n.d.	n.d.	5.19 b (2.23)
boiled	0.20 a (0.04)	2.43 a (0.05)	0.02 a (0.00)	0.01 a (0.00)	n.d.	n.d.	n.d.	2.61 a (1.18)

n.d.: not detected, [1] Means with different letters in the same column are significantly different ($p < 0.05$), [2] Standard deviation in parentheses.

Table 2.2.2 Effect of boiling on levels of flavonoids (mg/g, dry weight) in aqueous acetone extracts of selected green leafy vegetables (GLVs).

GLVs	Flavanol	Flavonols				Flavones		Total
extracts	Epicatechin	Rutin	Myricetin	Quercetin	Kaempferol	Luteolin	Apigenin	Flavonoids
amaranth								
raw	4.65 b[1] (1.50)[2]	6.36 b (0.02)	n.d.	n.d.	n.d.	n.d.	n.d.	11.01 b (1.21)
boiled	0.97 a (0.00)	2.63 a (0.01)	n.d.	n.d.	n.d.	n.d.	0.16 a (0.00)	3.77 a (1.26)
jute mallow								
raw	3.47 b (0.00)	40.07 b (1.37)	n.d.	0.50 a (0.00)	n.d.	n.d.	n.d.	44.04 b (22.04)
boiled	0.61 a (0.00)	9.28 a (0.37)	n.d.	n.d.	n.d.	n.d.	0.21 a (0.00)	10.10 a (5.13)
cowpea								
raw	4.20 b (0.60)	43.41 b (0.54)	n.d.	n.d.	n.d.	n.d.	n.d.	47.61 b (27.72)
boiled	0.38 a (0.03)	2.09 a (0.08)	n.d.	n.d.	n.d.	n.d.	n.d.	2.47 a (1.21)
pumpkin								
raw	0.70 a (0.09)	10.52 b (4.48)	0.58 a (0.09)	n.d.	n.d.	n.d.	n.d.	11.80 b (5.71)
boiled	n.d.	3.17 a (0.00)	n.d.	n.d.	n.d.	n.d.	n.d.	3.17 a (0.00)
spinach								
raw	0.46 a (0.00)	7.67 b (0.40)	n.d.	n.d.	n.d.	n.d.	n.d.	8.14 b (5.10)
boiled	n.d.	3.08 a (0.03)	n.d.	n.d.	n.d.	n.d.	n.d.	3.08 a (0.00)

n.d.: not detected, [1] Means with different letters in the same column are significantly different ($p < 0.05$), [2] Standard deviation in parentheses.

In the present study, water extracts of spinach contained 0.01 mg/g each of quercetin and kaempferol and 0.04 mg/g myricetin. The following ranges have been reported in various extracts of spinach in the literature: 5.6 x 10^{-4}, 4.2 x 10^{-4}, 0.02, mg/g of kaempferol, myricetin and quercetin, respectively (Chu *et al*., 2000) and 0.08, 1 x 10^{-4}, 0.04 and 7.40 x 10^{-3} mg/g, fw of kaempferol, myricetin, quercetin and luteolin (USDA, 2007). These flavonoids have also been previously detected in raw spinach by Chu *et al*. (2000), Franke *et al*. (2004), Gil *et al*. (1999) and Nuutila *et al*. (2000). Cho *et al*. (2008) did not find any of the flavonoids used in this study in extracts of spinach. Salawu *et al*. (2009) found that the dicaffeoyl quinic acids (12.1 mg/g, dw) and two quercetin monoglycosides (up to 5 mg/g, dw) were the dominant compounds in the 70% ethanol extracts of jute mallow grown in Nigeria.

Overall, the water extracts of African GLVs had comparable amounts of flavonoids to spinach, while the aqueous acetone extracts of African GLVs exhibited higher amounts of flavonoids than spinach. In the water extracts, there was no specific trend that differentiated the flavonoids found in African GLVs from the ones found in spinach. Each plant species seemed to possess a specific phenolic composition fingerprint based on its flavonoid compounds. The results of aqueous acetone extracts seem to correspond with those of total phenolics and antioxidant activity of the same extract as discussed in Section 2.1.

2.2.4.2 Effect of boiling on flavonoid contents of GLVs

Table 2.2.1 indicates that the contents of rutin in water extracts of amaranth and jute mallow, as well as myricetin in jute mallow increased significantly ($p < 0.05$) as a result of boiling. Water extracts of boiled amaranth and jute mallow also had higher total flavonoids than extracts of raw samples. Stewart *et al*. (2000) reported that processing increased flavonoid levels in tomato-based products. An increase in total flavonoid levels is attributed to new products with phenolic properties forming as a result of exposure to heat (Ruiz-Rodriguez *et al*., 2008), liberation of high amounts of individual flavonoids due to thermal destruction of vegetable cell wall structures and sub-cellular compartments (Yamaguchi *et al*., 2001), as well as suppression of the oxidation of phenolics by thermal inactivation of polyphenol oxidase in boiled vegetables (Yamaguchi *et al*., 2003). The accumulation of quercetin or release of the aglycone forms in processed foods may be attributed to enzymatic hydrolysis of quercetin conjugates that may occur during processing (Price and Rhodes, 1997; Stewart *et al*., 2000).

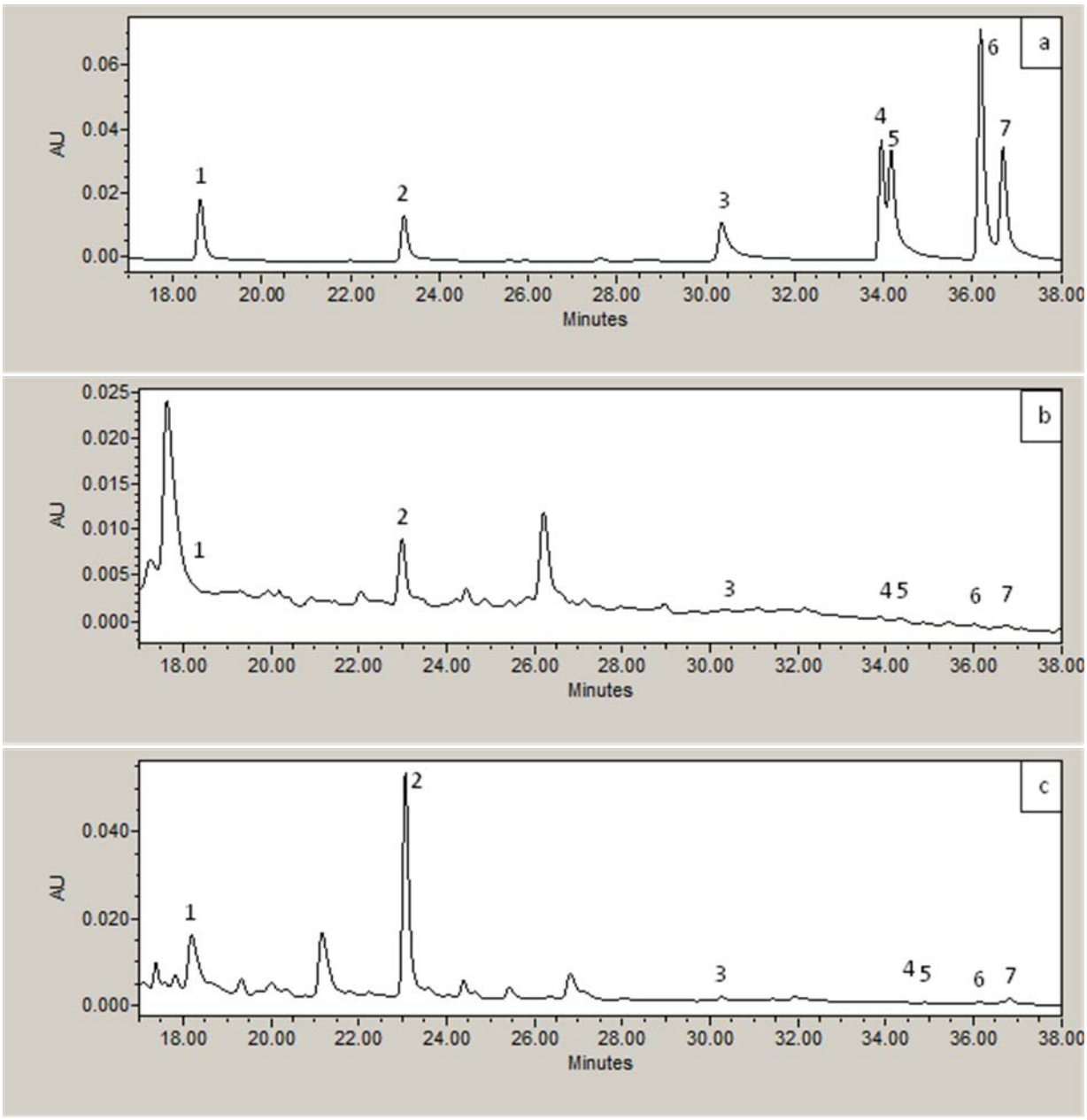

Figure 2.2.1 HPLC chromatograms of (a) standards and water extracts of (b) raw and (c) boiled amaranth. 1: epicatechin, 2: rutin, 3: myricetin, 4: luteolin, 5: quercetin, 6: apigenin, and 7: kaempferol.

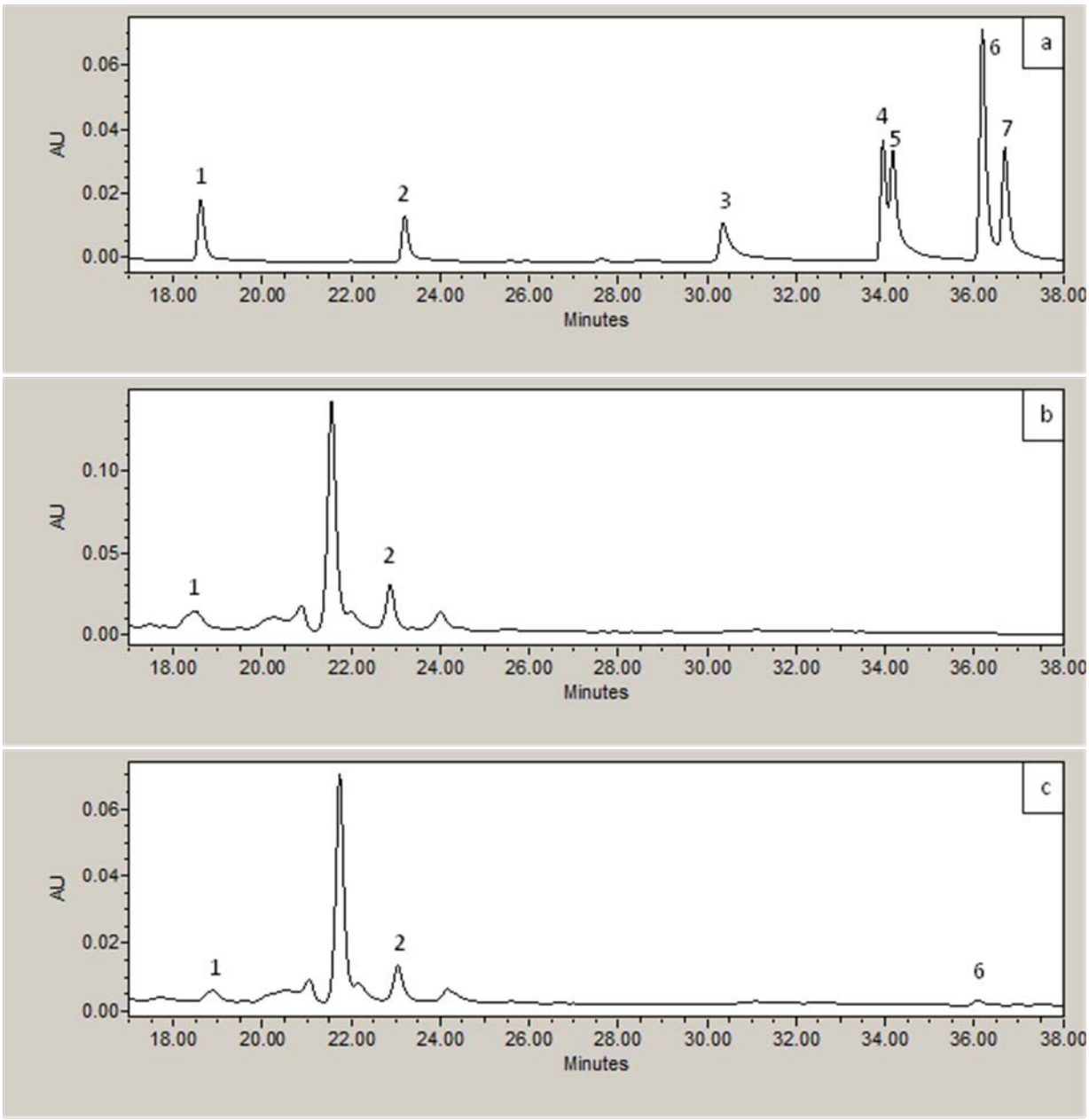

Figure 2.2.2 HPLC chromatograms of (a) standards and aqueous acetone extracts of (b) raw and (c) boiled amaranth. 1: epicatechin, 2: rutin, 3: myricetin, 4: luteolin, 5: quercetin, 6: apigenin, and 7: kaempferol.

Among the water extracts, the contents of epicatechin in amaranth, cowpea and pumpkin, rutin in pumpkin and spinach, as well as myricetin, quercetin, kaempferol, luteolin and apigenin in pumpkin decreased significantly ($p < 0.05$) after boiling. Among the 75% acetone extracts, epicatechin contents of amaranth, jute mallow and cowpea, as well as rutin contents of all GLVs also decreased after boiling. The content of total flavonoids of water extracts from boiled cowpea, pumpkin and spinach was less than that of raw samples. The 75% acetone extracts of all GLVs also exhibited a decrease in total flavonoids after boiling. A decrease in the flavonoid constituents of most GLVs after boiling may have resulted from leaching of vegetable flavonoids into the boiling water (Ruiz-Rodriguez *et al*., 2008). Boiling results in oxidation, thermal degradation, leaching and other events that led to lower levels of phenolics in processed food compared to fresh (Kalt, 2005). Salawu *et al*. (2009) found that extracts from blanched samples of jute mallow were highly viscous and showed very low concentration of phenolic constituents, forcing these researchers not to quantitatively evaluate the phenolic fractions in the extract. Gil *et al*. (1999) also reported leaching of approximately half of the flavonoids from spinach into boiling water. Boiling has also been reported to have induced flavonol losses of up to 20% and 40% in onions and asparagus, respectively (Makris and Rossiter, 2001). Price *et al*. (1998) reported significant leaching of flavonol glycosides into cooking water, which was also dependent on the conjugates. The decrease in phenolic compounds in broccoli inflorescences was also attributed to both leaching into the cooking water and thermal degradation by Vallejo *et al*. (2003).

The results of this study are in agreement with the study of Puupponen-Pimiä *et al*. (2003) who found that the effects of processing on flavonols were highly plant species-dependent. Because GLVs contain various kinds of polyphenol compounds, some polyphenols may protect others from decomposition during boiling. Buchner *et al*. (2006) studied the effect of thermal processing on rutin and quercetin and found that quercetin had the most intense degradation probably because these compounds split through different breakdown patterns, which could be explained by the protocatechuic acid being one of the cleavage reaction product of quercetin and the C-3 diglycosilation of rutin. In the present study, protocatechuic acid was not analysed and therefore the breakdown products of these flavonols in the extracts are not known. Murakami *et al*. (2004) reported that chlorogenic acid shielded rutin from decomposition when the two phenolic compounds were heated together. This was attributed to chlorogenic acid being an ester and therefore more easily decomposed than rutin. In contrast to the study of Murakami *et al*. (2004), chlorogenic acid decreased after boiling in

water extracts of amaranth, cowpea, pumpkin and spinach as well as acetone extracts of amaranth (results not shown), but only water extracts of amaranth exhibited an increase in rutin after boiling.

2.2.4.3 Effect of extraction solvent

Figures 2.2.1 and 2.2.2 indicate that the type of phenolic compounds found in GLVs and the effects of cooking on these compounds are also dependent on the type of extraction solvent. Water was efficient in extracting almost all of the flavonoids from plant materials (Fig. 2.2.1), while the 75% acetone was mostly efficient in extracting rutin and epicatechin (Fig. 2.2.2). This was possibly due to differences in polarities of rutin and epicatechin in comparison to other flavonoids present in the extracts. For example, rutin is diglycosilated on the C-3 position of the C-ring, while other flavonoids are pure aglycones. It seems that the presence of two sugar parts increased the solubility of this compound in 75% acetone extracts. In general, the contents of total flavonoid of 75% acetone extracts were also higher than those of water extracts, agreeing with the results of total antioxidant assays in Section 2.1. Kim *et al*. (2005) also found that 50% acetone extracts of buckwheat produced the highest yields of rutin than water extracts and attributed it to the solubility of rutin in the solvent. Many flavonoids that were detected in the water extracts (Fig. 2.2.1) of GLVs could not be detected in aqueous acetone extracts (Fig. 2.2.2). For example all flavonoids were detected in the water extracts of raw amaranth and pumpkin, but only epicatechin and rutin were detected in the 75% acetone extracts. In addition, although kaempferol was detected in water extracts of amaranth, cowpea and pumpkin, it could not be detected in the 75% acetone extracts of the same African GLVs. The solubility of flavonoids is reportedly affected by the nature of both the solvent and flavonoid structure, as well as their ability to form hydrogen bonds with the surrounding solvent (Chebil *et al*., 2007).

The variation in presence and content of flavonoids found in the two extraction solvents clearly show that it may not always be possible to extract multiple forms of phenolic compounds of interest with a single extraction solvent or solvent mixture. Luthria (2006) recommended the use of multiple solvent mixtures to extract different forms of compounds with varying polarities of conjugated mixtures. Polarity matching between the flavonoids of interest and extraction solvent is critical for optimum extraction as has been shown in this study.

2.2.5 Conclusion

The study indicates that these GLVs are sources of flavonoids. There are no major differences observed between the flavonoids found in African GLVs and those in spinach. However, aqueous acetone extracts of African GLVs exhibit higher amounts of total flavonoids than spinach. The contents of flavonoids are dependent on the type of vegetable and extraction solvents. There is therefore a need to equally promote the cultivation and consumption of African GLVs as has been done for spinach, because the levels of phenolic constituents are comparable and in some cases (such as extracts of aqueous acetone) higher than of spinach. Vegetables containing these compounds may offer potential health benefits such as reduction in chronic diseases of lifestyle in communities that consume them.

2.2.6 References

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2.3 Protective effects of African green leafy vegetables against AAPH-induced oxidative damage [2]

2.3.1 Abstract

Consumption of vegetables is associated with lower risk of developing chronic diseases. In this study, extracts of four African green leafy vegetables (GLVs) ((*Amaranthus cruentus* L. (amaranth), *Corchorus olitorius L.* (jute mallow), *Cucurbita maxima* Duchesne (pumpkin), *Vigna unguiculata* (L.) Walp. (cowpea)) were evaluated for biological and cellular protective activities in comparison to *Spinacea oleracea* L. (spinach). The GLVs were boiled for 0, 10, 30 and 60 min, drained and freeze-dried. Water extracts from freeze-dried samples were prepared for analysis. The results of erythrocyte haemolysis assay indicated highest protection activity against oxidative damage by amaranth extracts, while extracts of raw jute mallow contributed to the damage of erythrocytes. The plasmid DNA assay showed that the highest antioxidant protection activity against oxidative damage was offered by extracts of jute mallow and lowest by spinach. All long-term cell viability assays (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), neutral red (NR) and crystal violet (CV)) showed good proliferations of SC-1 fibroblast cells after treatment with vegetable extracts, while the results of the short-term cell viability assay using dichlorofluorescein (DCF) indicated significant levels of cytotoxicity in all extracts of raw GLVs, with highest levels observed in spinach than in African GLVs. African GLVs offered higher cellular antioxidant protection against oxidative damage than spinach. Human adenocarcinoma colon cancer (Caco-2) cells exhibited higher sensitivity to treatments than the SC-1 fibroblast cells.

2.3.2 Introduction

Frequent consumption of vegetables has been associated with a reduced risk of developing chronic diseases such as cardiovascular disease (Frei, 1995; Visioli *et al*., 2000), stroke (Gillman *et al*., 1995), diabetes (Craig, 1997) and cancer (Ray, 2005; Stan *et al*., 2008). The beneficial effects of vegetable consumptions have been attributed to their antioxidant, radical

[2] Part of this work is being prepared for publication.

scavenging and chelating activities (Rice-Evans *et al*., 1996). Some studies in human models have also shown increased plasma antioxidant concentrations after consumption of phenolic rich foods.

Oxidative stress is an accumulation of non-enzymatic oxidative damage to molecules that threaten the normal functioning of the cell or the organism (Blomhoff, 2005). Higher levels of oxidative stress leads to damage to all types of biomolecules including DNA, proteins and lipids, which may lead to development of chronic diseases. During this period, the ability of endogenous antioxidants to eliminate the radical species is often exceeded and, therefore, dietary sources of antioxidants are required (Chew and Park, 2004). The antioxidant activity of plant extracts mainly depends on their phenolic content (Salah *et al*., 1995; Wong *et al*., 2006). Phenolics are able to stabilize free radicals by donating hydrogen atoms from their OH groups, and form stable free radicals that do not initiate or propagate further oxidation. Consumption of green leafy vegetables (GLVs) rich in phenolics may therefore reduce free radical-induced oxidative stress.

GLVs are traditionally used in sub-Saharan Africa as part of the diet. African GLVs have been shown to contain phenolic compounds that have antioxidant activity (Salawu *et al*., 2009). In Section 2.1, the antioxidant activity of extracts from African GLVs and spinach was determined using chemical assays. The results demonstrated that African GLVs have higher antioxidant activity than spinach. Wolfe and Liu (2007) argued that cellular antioxidant activity assays are more biologically relevant methods than the popular chemical antioxidant activity assays because they account for some aspects of uptake, metabolism, and location of antioxidant compounds within the cells. Little is known about the functional role of the African GLV extracts on oxidative damage as well as their toxicity level in cell cultures. The aim of the present study was to determine the level of cytotoxicity of GLV extracts and evaluate the ability of these extracts to protect against 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative damage in erythrocytes, plasmid DNA, as well as SC-1 mouse fibroblast and human adenocarcinoma colon cancer (Caco-2) cell lines. The effect of boiling on the efficiencies of these extracts to offer protection on cell cultures was also determined.

2.3.3 Materials and Methods

2.3.3.1 Green leafy vegetable samples and the preparation of crude plant extracts

The procedure for acquiring the GLVs and the preparation of crude plant extracts has been described in subsection 2.1.3.2. Only the water extracts were used for analysis in this Section. The water extracts provides an indication of the extractability of antioxidants in the gastrointestinal tract.

2.3.3.2 Analyses

2.3.3.2.1 Biological assays

Preparation of erythrocytes suspension and induction of oxidative stress

After informed consents were obtained, blood was obtained from student volunteers via vein-puncture into tubes containing ethylenediaminetetracetric acid (EDTA) as anticoagulant. Erythrocytes were isolated by centrifugation at 2750 x g for 3 min and washed at least four times with isotonic phosphate buffer solution (iso-PBS; 0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO_4 and 8.1 mM Na_2HPO_4, pH 7.4) to remove plasma and platelets.

Induction of haemolysis was carried out based on the modified method of Ko *et al*. (1997). Briefly, 0.04 mg/ml of GLV extracts was added to 10 µl of erythrocytes suspension and the volume was made up to 150 µl with iso-PBS before incubation at 37 °C for 20 min. In order to induce free radical chain oxidation in erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH. 4.4 x 10^{-3} µM AAPH solution was then added. The final mixture was incubated at 37 °C for 90 min before centrifugation at 2750 x g for 3 min. The extent of haemolysis corresponding to liberation of haemoglobin was determined by measuring the absorbance of the supernatant at 570 nm using the BioTek ELx800 plate reader (Analytical & Diagnostic Products, Weltevreden Park, South Africa). The reference values were determined using the same volume of erythrocytes in iso-PBS. The percentage protection against haemolysis was calculated using the formula:
100 – [(absorbance of sample/control) x 100].

pBR 322 plasmid DNA damage assay

Conversion of the supercoiled form of plasmid DNA to the open-circular and further linear forms has been used as an index of DNA damage (Jung and Surh, 2001). The three forms can

be separated by agarose gel electrophoresis due to their different electrophoretic mobility (Aronovitch *et al*., 2007). Procedures for this assay were based on a modified method of Wei *et al*. (2006). Briefly, the reaction mixtures containing 2.5 µg of pBR 322 plasmid DNA, 5 x 10^{-5} mg/ml of GLV extracts, 5.5 x 10^{-4} µM AAPH were made to 22.5 µl with phosphate buffer solution (BPS, pH 7.4) and incubated at 37 °C for 90 min. For the control samples, PBS replaced the GLV samples. After incubation, samples were mixed with an equal amount (22.5 µl) of gel loading buffer (0.13% bromophenol blue and 40% sucrose) and the reaction mixtures were immediately loaded into a 1% agarose gel containing 40 mM Tris, 20 mM glacial acetic acid and 0.5 M EDTA (pH 8.0) containing 0.5 µg/ml ethidium bromide. The gel was electrophoresed in a horizontal slab gel apparatus in tris acetate-EDTA buffer (pH 8.0) for 2 h (60 V/30 mA). The DNA bands were visualized by fluorescence under ultraviolet light.

Imaging and data analysis

Gel imaging and data analysis followed the method of Benherlal and Arumughan (2008). Stained gels were placed on a UV trans-illuminator at 254 nm and photographed to tagged image file format (TIFF) with a 4 mega-pixel digital camera (Canon power shot, Japan). The image captured in RGB format was imported into the image processing Gel Pro Analyzer, version 3.0 software (Media Cybernetics, Silver Spring, MD, USA) to quantify the density of the supercoiled DNA. The image was transformed to 8-bit grayscale format. The grayscale image was then normalized based on the blank lane by adjusting the brightness and contrast tool. The fluorescent region of each lane was then selected using free-hand selection tool and measured the average intensity of all pixels in the selected region. The numeric value of a pixel laid between 0 and 255 based on their signal strength. The values of all pixels in the selected area were computed and the average value per pixel was arrived at for each lane. Average fluorescence in the blank lane was considered 100% (0% DNA damage) and the difference between the blank and treated DNA was taken as the extent of damage in percentage.

2.3.3.2.2 In-vitro cellular assays

The SC-1 mouse fibroblast and Caco-2 cell lines obtained initially from the American Type Culture Collection (Rockville, MD, USA) were used in this study. Five hundred microlitres (500 µl) of DMEM medium supplemented with 5% FCS and 1% penicillin streptomycin was used to culture the cells in 24-well microplates. 1 x 10^4 of mouse fibroblast SC-1 or Caco-2

cell lines were added into each well and incubated at 37 °C for 24 h in a humidified CO_2 water-jacketed incubator (Forma Scientific, Ohio, USA). The cells were then treated with 0.25 mg/ml GLV extracts and the plates were further incubated for 72 h.

Long-term cytotoxicity assays

MTT assay

This assay followed a modified method of Sgambato *et al*. (2001). After the 72 h incubation, 25 µl of MTT (1 mg/ml) solution was added into each well and the plates were further incubated at 37 °C for 3 h in a humidified CO_2 water-jacketed incubator (Forma Scientific, Ohio, USA) to allow cleavage of the tetrazolium ring by dehydrogenase enzymes, which is able to convert MTT to an insoluble purple formazan in living cells. After that, the medium with excess MTT was removed from the wells and 100 µl of acid-isopropanol solution (0.04 N HCl in isopropanol) was added to extract the dye. The plates were then mixed well and the absorbance was measured at a wavelength of 570 nm using the BioTek ELx800 plate reader (Analytical & Diagnostic Products, Weltevreden Park, South Africa).

NR assay

This assay followed a modified method of Ishiyama *et al*. (1996). After 72 h incubation, 10 µl of NR (0.15%) solution was added into each well and the plates were further incubated at 37 °C for 90 min in a humidified CO_2 water-jacketed incubator (Forma Scientific, Ohio, USA) to allow for uptake of the NR dye into the lysosomes of viable and uninjured cells. After that, the medium with excess NR was removed from the wells and 200 µl of the fixative (1% acetic acid and 1% ethanol) was added. The fixative solution fixes and preserves the cells in their present state after treatment and removes any remaining unincorporated NR (Lasarow *et al*., 1992). The plates were mixed well and the absorbance was read at 570 nm by using the BioTek ELx800 plate reader (Analytical & Diagnostic Products, Weltevreden Park, South Africa).

CV assay

Using the same microplates that were used for NR assay, 500 µl of CV dye was added into each well containing the residual cells adhered on the microplates. The microplates were then incubated at 37 °C for 30 min in a humidified CO_2 water-jacketed incubator (Forma Scientific, Ohio, USA). After that, CV dye was removed from the wells and the plates were rinsed with water and dried completely before 200 µl of 10% acetic acid was added. The

plates were shaken well and the absorbance was read at 570 nm by using the BioTek ELx800 plate reader (Analytical & Diagnostic Products, Weltevreden Park, South Africa). For each assay, three replicate microplates were used to determine the cytotoxicity of each plant extracts. The % proliferation was determined using the following formula:

% proliferation = (Absorbance of sample)/Absorbance of control) x 100

Short-term (DCF) cytotoxicity and antioxidant assay

This assay followed a modified method of Elisia and Kitts (2008). 2 x 10^4 of SC-1 mouse fibroblast or Caco-2 cell lines was added to 96-well microplates and incubated at 37 °C for 24 h in a CO_2 water-jacketed incubator (Forma Scientific, Ohio, USA). DCFH-DA probe was initially dissolved in PBS to make 200 µmol aliquout of DCFH-DA stock solution. For *total cellular protection*, a working solution of 20 µmol DCFH-DA was then added to cells as the probe and incubated for 1 h. This was followed by addition of 0.2 mg/ml of GLV extracts together with 0.11 µM AAPH. To determine the *intracellular protection*, a working solution of 20 µmol DCFH-DA and the 0.2 mg/ml of GLV extracts were added together to the cells before incubation for 1 h. This was followed by a wash with PBS and the cells were then exposed to 0.11 µM AAPH before fluorescence readings were taken. Fluorescence readings were taken using a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Offenburg, Germany) immediately and every 2 min up to 1 h. The results were expressed as % cell protection. *Extracellular protection* was calculated by subtracting the percentage values of intracellular protection from total cellular protection.

Cytotoxicity:

Cells +DCFH-DA → (Uptake, 1hr) → Measure fluorescence

Total cellular antioxidant protection:

Cells + DCFH-DA → (Uptake, 1hr) → Add GLV extracts + AAPH (oxidant) → Immediately measure change in fluorescence, 1hr

Intracellular antioxidant protection:

Cells + DCFH-DA + GLV extracts → (Uptake, 1 hr) → Wash cells, add AAPH → Immediately measure change in fluorescence, 1 hr

2.3.3.3 Statistical Analysis

All experiments were repeated at least three times and the results were expressed as mean ± SEM. The data was subjected to analysis of variance (ANOVA), using samples and cooking time as independent variables and the values determined as dependent variables. Fisher's least significant difference (LSD) test was used for comparison of means using Statistica software Version 9.0 (StatSoft, Tulsa, OK). Correlation analysis was also run with the same statistical package.

2.3.4 Results and Discussion

2.3.4.1 Biological assays

2.3.4.1.1 Protection of erythrocytes by African GLVs against oxidative damage

Among raw samples, all GLV extracts with exception of raw jute mallow, significantly protected erythrocytes from AAPH-induced haemolysis (Figure 2.3.1). Extracts of raw jute mallow contributed to the damage of erythrocytes membrane and therefore offered no protection against haemolysis, while extracts from raw pumpkin offered the highest protection against haemolysis than the other extracts. GLVs are consumed after boiling, therefore it was appropriate to evaluate the effects of boiling on the efficiencies of these extracts to prevent haemolysis. Among extracts from boiled GLVs, amaranth offered highest protection against haemolysis, while jute mallow and pumpkin offered least protection. There was no difference in protection against haemolysis between raw and cooked amaranth extracts, although the results from the total antioxidant activity assays suggested that boiling reduced antioxidant activity in this vegetable (subsection 2.1.4.2). Furthermore, in agreement with the results of total antioxidant activity, extracts of raw pumpkin and spinach inhibited more haemolysis than when cooked.

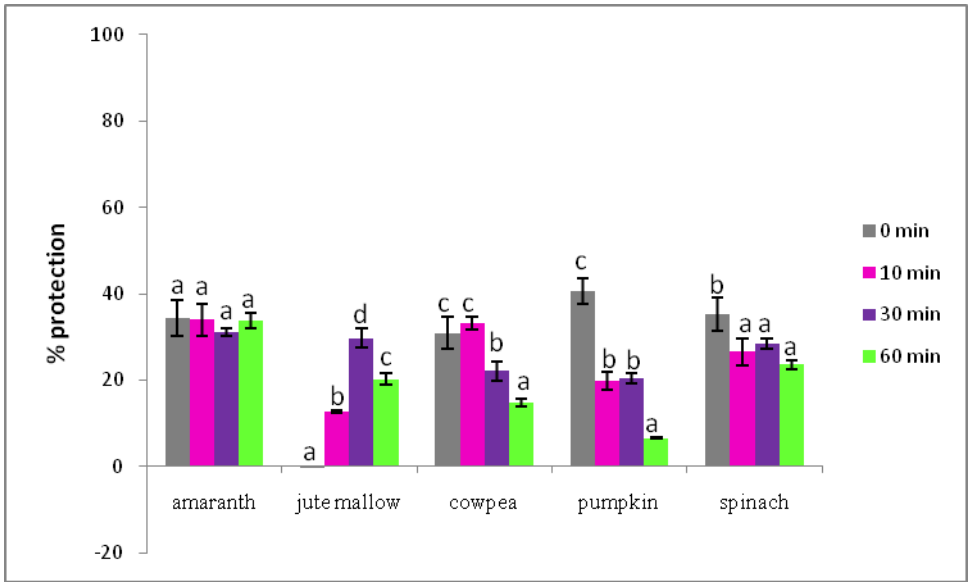


Figure 2.3.1 Protection against AAPH-induced damage of erythrocytes by green leafy vegetables. For each vegetable, means with different letters are significantly different ($p < 0.05$).

Studies on protective effects of African GLV extracts against oxidative damage in erythrocytes and/or evaluation of boiling effects on the efficiency of these GLV extracts to protect against haemolysis are limited. Effective protection of erythrocytes against free radical-induced oxidative haemolysis was reportedly offered by extracts of raw Indian *Solanum nigrum* L. and *Solanum torvum* L. (Loganayaki *et al*., 2010), Taiwanese *Bidens pilosa* (Yang *et al*., 2006), Taiwanese *BauYuan* vegetables and fruits concoction (Wang *et al*., 2007), Brazilian broccoli and kale (Arbos *et al*., 2008), and some Portuguese plants (Gião *et al*., 2010). It has been reported that in some plants, direct or inverse dependence of quantitative protection effect was based on the extract concentration, whereas in other plants no significant dependence was found (Dai *et al*., 2006; Gião *et al*., 2010; Grinberg *et al*., 1997; Wang *et al*., 2007). Inhibition of haemolysis by plant extracts has been associated with their respective antioxidant properties, especially the number and position of phenolic OH groups (Edenharder and Grunhage, 2003), binding of the flavonoids to the plasma membrane (Blasa *et al*., 2007), ability of flavonoids to penetrate lipid bilayers (Lopez-Revuelta *et al*., 2005), H-atom abstraction from the phenolic groups (Deng *et al*., 2006), as well as iron chelation by the polyphenols (Grinberg *et al*., 1997). Souza *et al*. (2008) reported limited ability of polyphenol-rich Amazonian methanolic plant extracts to delay AAPH-induced haemolysis and attributed this to the presence of glycosylated flavonoids in the extracts, which are known to have low diffusibility into erythrocytes (Kitagawa *et al*., 2004), which apparently lead to low ability to protect lipid targets in the erythrocyte membranes.

The damage to the cell membrane caused by extracts of raw jute mallow (Fig. 2.3.1) could be due to the presence of polysaccharides similar to a gel found in the extracts, which made it slimy. Polyphenols can create hydrogen bonds between their OH groups and H atoms of polysaccharides in the cell wall, leading to a gel-like structure that can encapsulate phenolic compounds and make their extraction difficult (Freitas *et al*., 2003). The increase in protection by boiled samples of jute mallow could be due to the break down of hydrogen bonds and rupture of cell walls as a result of boiling, which also might have made these extracts less slimy.

African GLVs and jute mallow in particular have appreciable levels of phenolics and antioxidant activity and in higher quantities compared to spinach (subsection 2.1.4.1). However, in this assay, % protection of spinach extracts against haemolysis was higher than that of jute mallow. These results indicate that the protection of cell membrane by GLV extracts may be dependent on the types of phenolics present in the extracts, as well as other constituents of the extracts including their synergistic effects, and not necessarily on the concentrations of phenolics and levels of total antioxidant activity. Souza *et al*. (2008) found that erythrocyte protection of Amazonian plant extracts showed no correlation with any of the phenolic content indicators (i.e. total phenolics, total flavanoids, total flavonols, ORAC and TRAP). Full identification and characterization of compounds present in these extracts may account for the respective damaging and protective properties of these GLV extracts on erythrocytes membrane.

2.3.4.1.2 Protection of plasmid DNA by African GLVs against oxidative damage

Figue 2.3.2 shows the electrophoretic pattern of pBR 322 plasmid DNA after AAPH-induced oxidative damage in the presence of GLV extracts. The absence of a band in Lane 2 is due to oxidative effects of AAPH that caused extensive damage and little of the original form of DNA. In other lanes (7 to 26), AAPH oxidation of plasmid DNA resulted in cleavage of supercoiled DNA to give the prominent open-circular form of DNA. Most of the plasmid DNA treated with African GLVs was retained in the supercoiled circular form (Lanes 8 to 22). This figure shows only two bands present in the agarose gel, indicating that the DNA was cut only once and converted to the open-circular form.

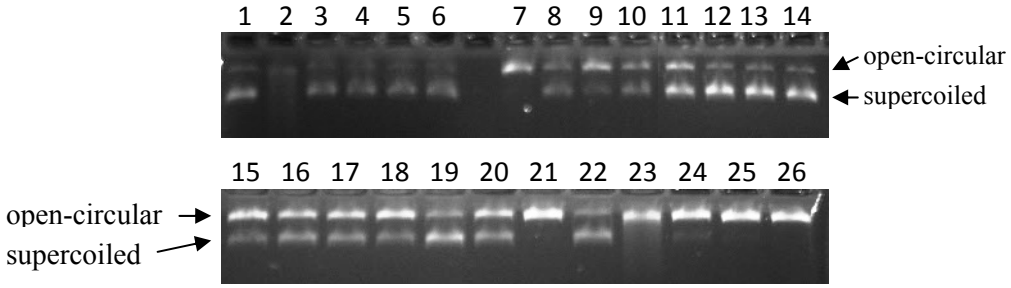


Figure 2.3.2 Effect of green leafy vegetable extracts on oxidatively damaged pBR 322 plasmid DNA. Lane 1: pBR 322 plasmid DNA; Lane 2: pBR 322 plasmid DNA + AAPH; Lane 3 - 6: Trolox at different concentrations; Lane 7: amaranth 0 min; Lane 8: amaranth 10 min; Lane 9: amaranth 30 min; Lane 10: amaranth 60 min; Lane 11: jute mallow 0 min; Lane 12: jute mallow 10 min; Lane 13: jute mallow 30 min; Lane 14: jute mallow 60 min; Lane 15: cowpea 0 min; Lane 16: cowpea 10 min; Lane 17: cowpea 30 min; Lane 18: cowpea 60 min; Lane 19: pumpkin 0 min; Lane 20: cowpea 10 min; Lane 21: cowpea 30 min; Lane 22: cowpea 60 min; Lane 23: spinach 0 min; Lane 24: spinach 10 min; Lane 25: spinach 30 min; Lane 26: spinach 60 min.

An increase in the concentration of AAPH would have led into a second DNA strand scission event that would convert open-circular DNA into the linear form, provided that this event occurs on the other uncut strand and probably within about 5 base pairs of the break in the first strand (Wei *et al*., 2006). With some extracts, the formation of open-circular DNA was less than the supercoiled (e.g. Lanes 12, 13 and 14), indicating an increased protection of the supercoiled DNA. This figure confirms that quenching of the radicals and the consequently inhibition of plasmid DNA degradation was dependent on the type of plant extract.

Fig. 2.3.3 shows the quantitative % protection of the GLV extracts against oxidative damage on pBR 322 plasmid DNA. Among the extracts of raw samples, cowpea offered highest protection, while amaranth offered least protection against AAPH-induced oxidative plasmid DNA. The protective effects of extracts of boiled samples were dependent on the type of vegetables. Boiling reduced the protective effects of cowpea, pumpkin and spinach and increased those of amaranth and jute mallow. Overall, jute mallow showed most protective effects, while spinach showed the least protection. These results seem to be in agreement with those of total antioxidant activity assay observed in Section 2.1, possibly indicating that the protection abilities of GLV extracts against plasmid DNA damage may be attributed to their antioxidant activity.

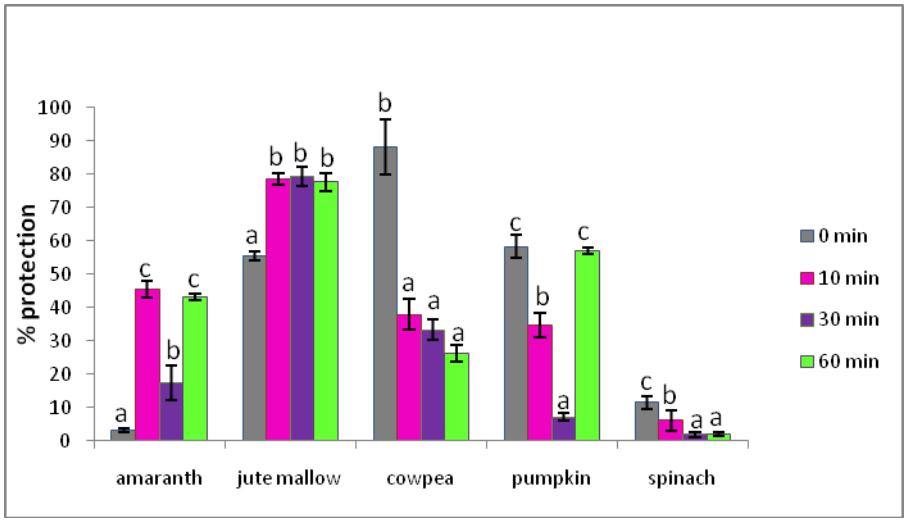


Figure 2.3.3 Protection of green leafy vegetable extracts against AAPH-induced damage on pBR 322 plasmid DNA. For each vegetable, means with different letters are significantly different ($p < 0.05$).

Significant protection of DNA against degradation initiated with reactive oxygen species has been reported using extracts of Portuguese wild plants (Gião *et al*., 2008), Korean *Cnidium officinale* (Jeong *et al*., 2009), Taiwanese *BauYuan* vegetables and fruits concoction (Wang *et al*., 2007), some Indian plants (Benherlal and Arumughan, 2008), Indian *Mentha spicata* Linn (Kumar and Chattopadhyay, 2007) and green tea polyphenols (Wei *et al*., 2006). The extent of protection of DNA from oxidative damage has been found to be dependent on the concentration of the oxidants (Jeong *et al*., 2009; Wang *et al*., 2007; Wei *et al*., 2006), and has been attributed to iron chelation properties of extracts (Benherlal and Arumughan, 2008; Melidou *et al*., 2005), as well as the number and position of the phenolic OH groups, presence of oxo group at proximal carbon positions, and the presence of C2-C3 double bond of individual flavonoids in the extracts (Johnson and Loo, 2000; Kumar and Chattopadhyay, 2007; Melidou *et al*., 2005; Oshima *et al*., 1998).

Overall, extracts of cowpea gave better protection among raw GLVs, while extracts of jute mallow offered highest protection among extracts of boiled samples. Extracts of African GLVs protected the pBR 322 plasmid DNA against oxidative damage better than extracts of spinach.

2.3.4.2 In-vitro cellular assays

2.3.4.2.1 Cell viability assays

Long-term cell viability effects of African GLVs

The MTT, NR and CV are well known dyes used in assays that develop colour in response to the viability of cells, allowing the colorimetric measurement of cell viability. These methods are usually carried out alone or in combination and on separate or same wells. The percentages of cell viability or cell proliferation after treatment with GLV extracts are shown in Fig. 2.3.4 (a - c). The results indicate that, in general, all GLV extracts had low or no toxicity to SC-1 fibroblasts cells at the concentration used.

The results of the MTT assay (a) show that the % viability of SC-1 fibroblast cells treated with extracts of raw and cooked GLVs were similar ($p < 0.05$). In general, all GLV extracts promoted good proliferation of cells, however highest proliferation was found using extracts of amaranth and jute mallow, while the lowest proliferation was observed in cells treated with pumpkin extracts. The results of the NR assay (b) also showed good proliferation of the SC-1 fibroblast cells after treatment with GLV extracts. Statistically, there was no difference between extracts of raw and cooked samples for all GLVs. Wells treated with extracts of amaranth had the least % viable cells in comparison to those treated with extracts of other GLVs. The results of the CV assay (c) indicate that statistically there was no difference in % viability between cells treated with extracts of raw and cooked amaranth, jute mallow and cowpea. Extracts of pumpkin boiled for 30 and 60 min improved proliferation significantly in comparison to those of 0 and 10 min, while for spinach, extracts of 10 and 30 min also exhibited higher proliferation than extracts of 0 and 60 min.

The MTT assay is based on the uptake and the reduction by mitochondrial succinic dehydrogenase of the soluble yellow MTT tetrazolium salt to an insoluble blue MTT formazan product. The NR assay is based on the uptake of NR dye which accumulates in the lysosomes of viable cells. While, the CV assay is based on the growth rate reduction of cells, which is reflected by the colorimetric determination of the stained cell membranes (Chiba *et al*., 1998; Ishiyama *et al*., 1996).

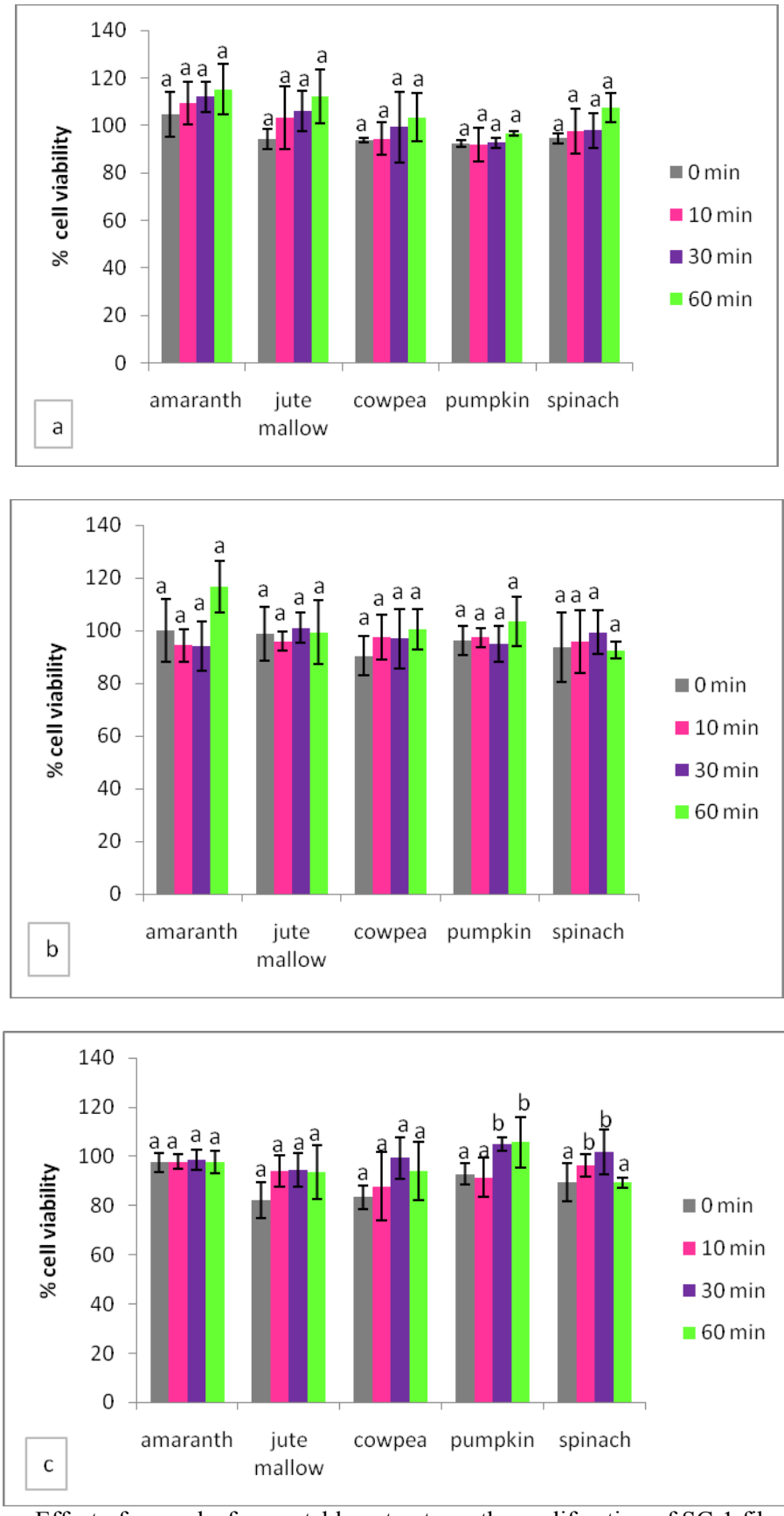


Figure 2.3.4 Effect of green leafy vegetable extracts on the proliferation of SC-1 fibroblast cells as determined with (a) MTT, (b) neutral red, and (c) crystal violet assays. For each vegetable, means with different letters are significantly different ($p < 0.05$).

Chiba *et al*. (1998) reported that although the specific mechanisms of action of these assays are different, the results of these assays are often comparable. This could be the reason why the MTT and NR assays gave similar results. Weyermann *et al*. (2005) also reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed. This could explain why the CV assay gave differences among cells treated with extracts of pumpkin and amaranth, while the other two assays (MTT and NR) did not give differences. Other possible reasons could be that the cultured cell type used in this study behaves differently under different cytotoxicity assays, as well as the choice of assay endpoints selected for these assays (Chiba *et al*., 1998). Vistica *et al*. (1991) reported that the reduction of MTT to MTT-formazan by cultured cells was dependent on the amount of MTT in the incubation medium, and that the concentration required to achieve maximal MTT-formazan production differed widely for various cell lines. The L929 fibroblasts could not be used by Vian *et al*. (1995) for the MTT test because these cells have weak succinate dehydrogenase activity.

Overall, all the cytotoxicity assays used in this study were useful for long-term cytotoxicity screening of GLV extracts and yielded similar results. The results from MTT and NR assays and some of the CV assay suggest validated promotion of cell growth and viability by extracts of both raw and boiled GLVs. There were no major differences in proliferation of SC-1 fibroblast cells treated with extracts of raw and cooked GLVs, as well as between cytotoxicity levels of extracts of African GLVs and spinach.

Short-term cell viability effects of African GLVs

The dichlorofluorescein (DCF) assay was used to determine the short-term viability effects of African GLV extracts on SC-1 fibroblast and Caco-2 cell lines.

SC-1 fibroblast cells

Fig. 2.3.5 indicates that extracts of raw GLVs were highly cytotoxic to SC-1 fibroblast cells than extracts of boiled samples, demonstrating the presence of cytotoxins or antinutritional factors in these extracts. Among extracts of raw samples, spinach caused the highest % cell damage, while cowpea caused the least damage. Boiling seems to have reduced the cytotoxicity levels of these extracts, with more protection offered by extracts of cowpea and pumpkin.

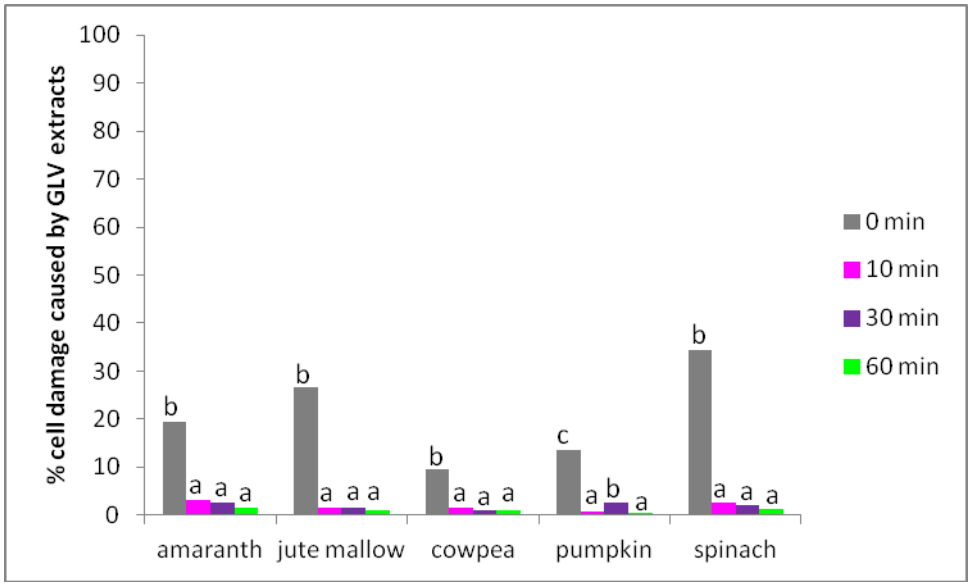


Figure 2.3.5 Effect of green leafy vegetable extracts on the viability of SC-1 fibroblast cells as determined with dichlorofluorescein assay. For each vegetable, means with different letters are significantly different ($p < 0.05$).

Caco-2 cells

Similar to the SC-1 fibroblast cells, extracts of raw GLVs were significantly more cytotoxic to Caco-2 cells than those of cooked samples (Fig. 2.3.6). Extracts of raw jute mallow were different because they exhibited less damage than extracts of samples boiled for 10 min. Generally, extracts of spinach caused highest damage to the cells than extracts of African GLVs. Elevated % damage observed in Caco-2 cells than in SC-1 fibroblast cells indicate higher sensitivity of the former cells to GLV extracts than their counterparts.

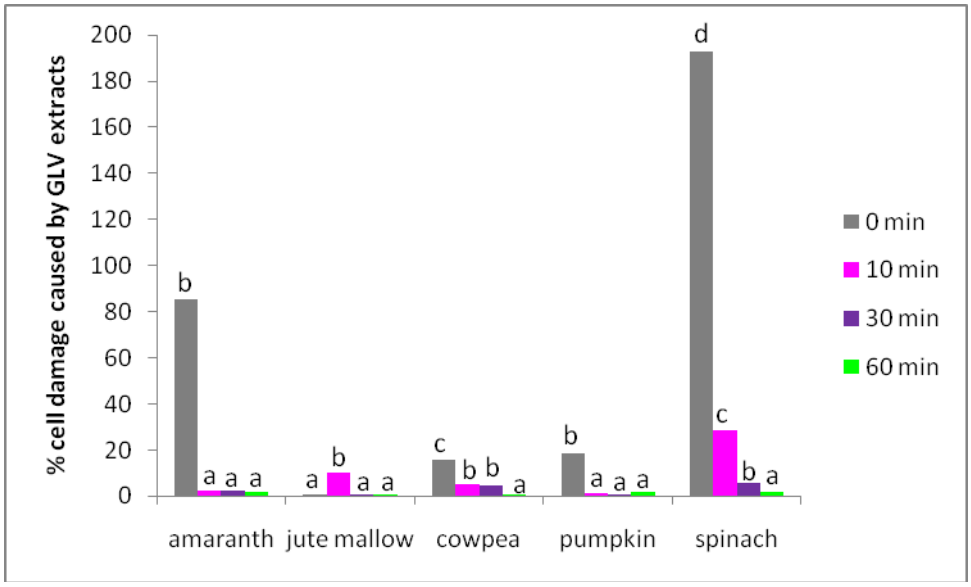


Figure 2.3.6 Effect of green leafy vegetable extracts on the viability of Caco-2 cells as determined with dichlorofluorescein assay. For each vegetable, means with different letters are significantly different ($p < 0.05$).

The short-term cell viability assay was a good method of complementing the long-term cell viability assays. Contrary to the results of short-term viability assays, differences between cells treated with extracts of raw and those treated with extracts of boiled GLVs were not observed using the long-term cell viability assays. The findings of both short- and long-term cell viability assays suggest that there was an initial cytotoxic effect as extracts of raw GLVs were added to the cells as observed with the short-term assay, however after about 72 h, these cells had a full recovery and started proliferating as usual, leading to the initial cytotoxic effect not to be observed in the long-term cell viability assays.

Determination of cell damage by extracts of GLVs in the presence of AAPH

SC-1 fibroblast cells

Figure 2.3.7 (a) indicates that in the presence of AAPH, the severity of total damage on SC-1 fibroblast cells was largely dependent on the type of GLV extracts and not only on whether the extracts were from raw or boiled samples. Extracts of raw amaranth, jute mallow and spinach caused more damage to SC-1 fibroblast cells than extracts of boiled samples, although the degree of damage was dependent on each type of GLV (Fig. 2.3.7 (a)). For cowpea and pumpkin, less damage was observed in cells treated with extracts of raw samples than those treated with extracts of samples boiled for 10 and 30 min. Overall, spinach caused highest total damage than African GLVs.

Due to exposure of the cells to AAPH, the efficacy of extracts of raw GLVs to protect SC-1 fibroblast cells was increased (Fig. 2.3.7 (b)), in comparison to when AAPH was not added (Fig. 2.3.5). It seems that the constituents of extracts of raw samples were able to scavenge most peroxyl radicals before they caused damage, better than those in extracts of cooked samples. This led to less damage observed in SC-1 fibroblast cells treated with extracts of raw samples in comparison to those treated with extracts of boiled samples.

In general, the SC-1 fibroblast cells treated with extracts of African GLVs, especially amaranth, had less damage than cells treated with extracts of spinach.

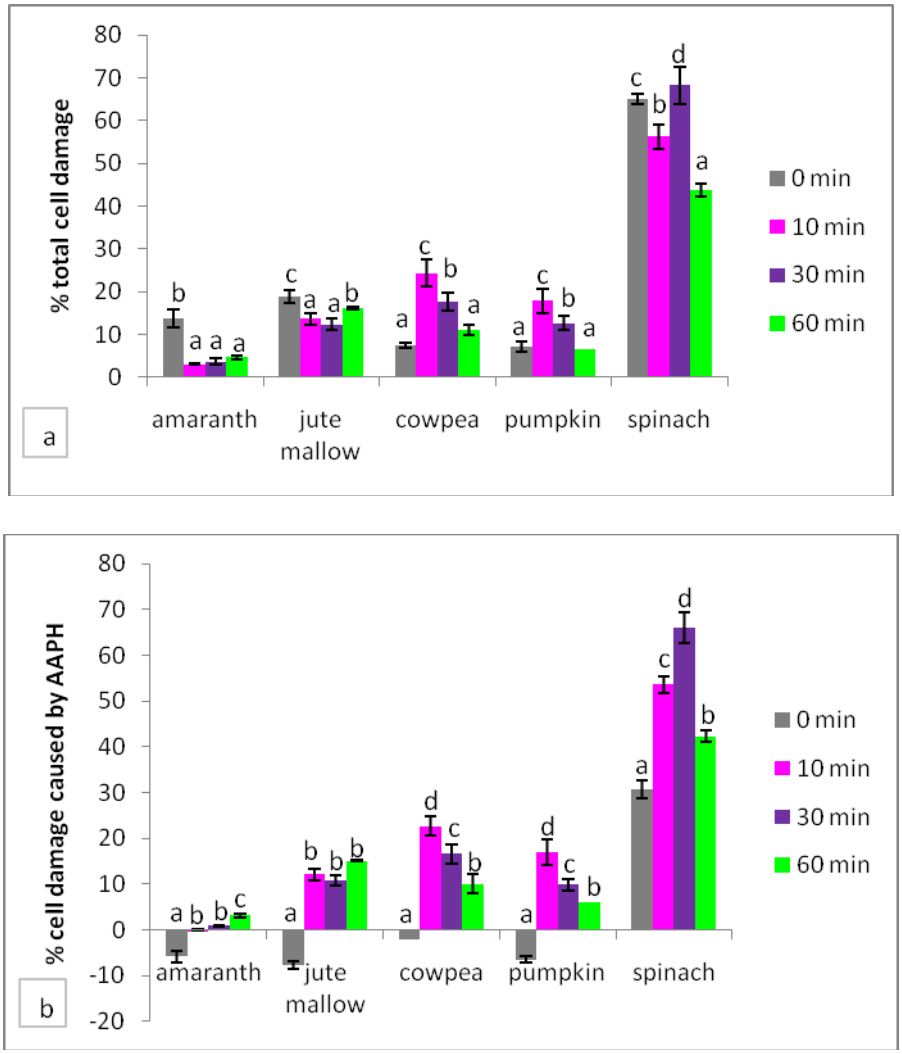


Figure 2.3.7 Percentage damage of SC-1 fibroblast cells due to (a) treatment with both green leafy vegetable extracts and AAPH, and (b) AAPH only, as determined with the dichlorofluorescein assay. For each vegetable, means with different letters are significantly different ($p < 0.05$).

Caco-2 cells

The % total cell damage of Caco-2 cells treated with spinach extracts was higher than that of cells treated with extracts of African GLVs (Fig. 2.3.8 (a)). The % cell damage contributed by AAPH alone is shown in Fig. 2.3.8 (b). Caco-2 cells treated with extracts of raw cowpea, jute mallow and amaranth were more damaged than those treated with extracts of boiled samples. While, the % damage to Caco-2 cells treated with extracts of raw pumpkin were similar to those of samples boiled for 10 min but higher than those for 30 and 60 min. In this experiment of Caco-2 cells, it seems that extracts of raw spinach were able to scavenge most of the peroxyl radicals before they caused damage.

In general, among extracts of raw samples, spinach, cowpea and amaranth offered higher % total Caco-2 cell damage than pumpkin and jute mallow. Extracts of raw cowpea exhibited highest damage by AAPH alone, while raw spinach exhibited the least. For boiled samples, extracts of spinach showed the highest damage, while jute mallow showed the least damage as a result of exposure of Caco-2 cells to AAPH.

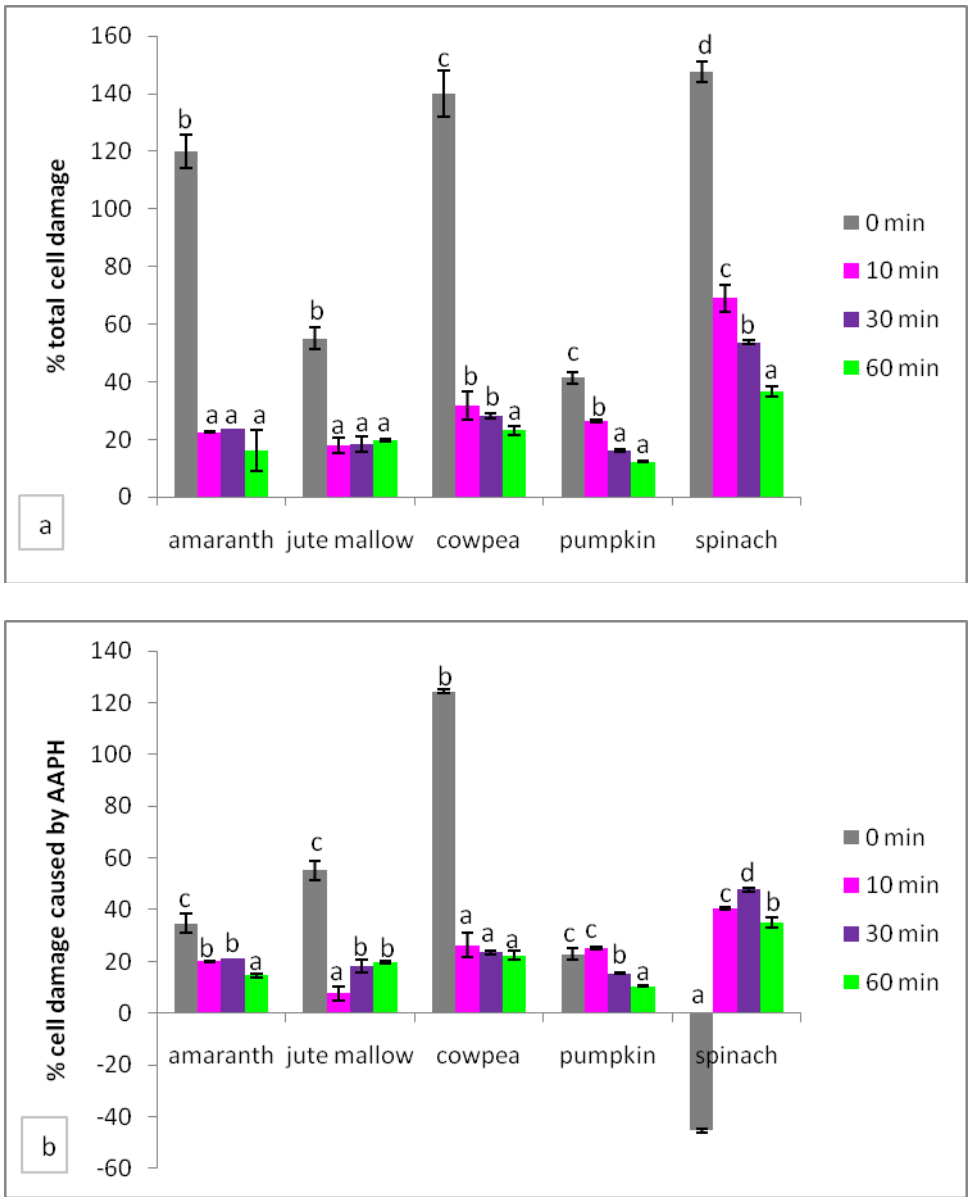


Figure 2.3.8 Percentage damage of Caco-2 cells due to (a) treatment with both green leafy vegetable extracts and AAPH, and (b) AAPH only, as determined with the dichlorofluorescein assay. For each vegetable, means with different letters are significantly different ($p < 0.05$).

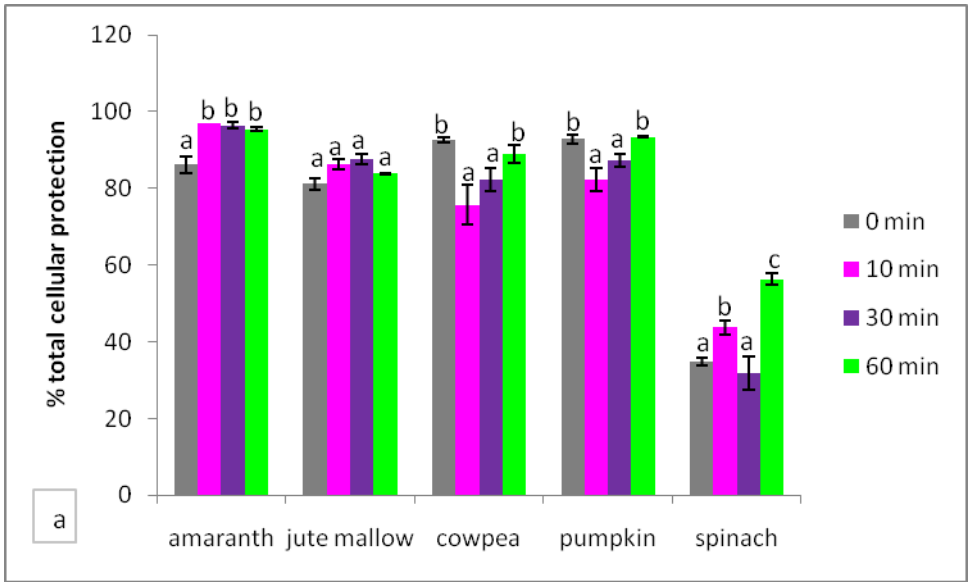
Generally, in the presence of AAPH, the damage to cell cultures was dependent on the type of cell cultures used, type of GLVs and the nature of extracts (i.e. from raw or boiled samples).

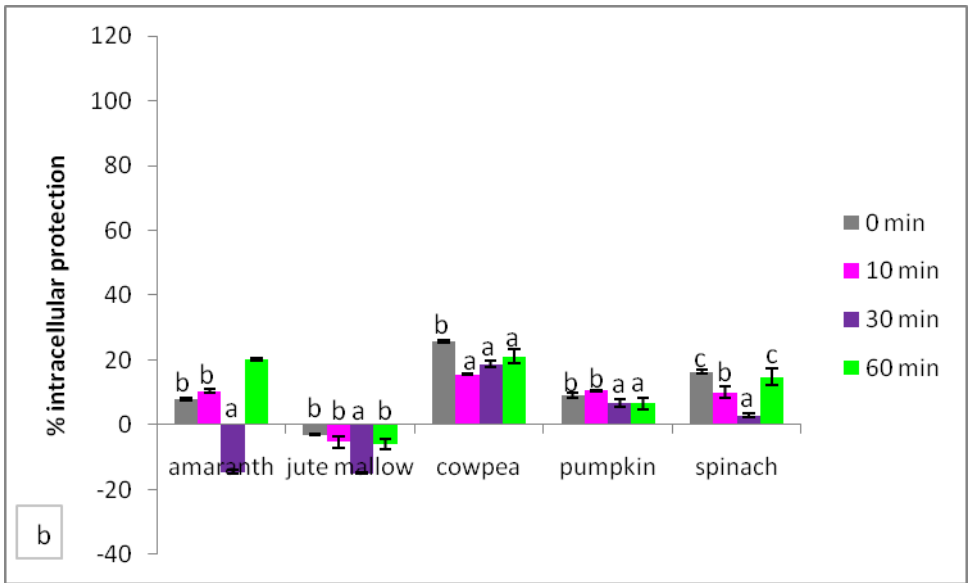
2.3.4.2.2 In vitro cellular antioxidant properties: Comparison of total, intra- and extracellular protection assays

Figures 2.3.9 and 2.3.10 show comparisons of results of total, intra- and extracellular protection of GLV extracts against AAPH-induced damage on SC-1 fibroblasts cells and Caco-2 cells, respectively. The results indicate that GLV extracts offered protection against oxidative damage to both cell lines, although the degree of protections varied. The total cellular protection assays gave overall, the best results in assessing % protection of GLV extracts. Extracts seem to have offered higher levels of protection extracellularly, probably by scavenging of radicals before they caused damage to the cells. For intracellular protection, it seems there were factors or mechanisms competing with the uptake of these extracts, resulting in less uptake of the extracts by the cells. Furthermore, it is possible that the incubation time for the cells to take up the GLV extracts was not enough to effect higher intracellular protection. The negative intracellular protection observed in Figures 2.3.9 (b) and 2.3.10 (b) probably indicates the inability of the extracts to penetrate the cell membrane for intracellular protection. For example, extracts of jute mallow are somehow slimy and may have possibly contained substances that hindered cellular uptake.

SC-1 fibroblast cells

The levels of total, intra- and extracellular protection of SC-1 fibroblast cells were dependent on the types of GLVs and their constituents. Using this assay, boiling seems to have had no specific effect or trend on the efficiency of extracts to protect against SC-1 fibroblast cells oxidative damage. In general, spinach offered the least total, intra- and extracellular protection of SC-1 fibroblast cells against AAPH-induced damage than African GLVs.


% total cellular protection
120
100
80
60
40
20
0
a b b b
a a a a
b a a b
b a a b
a b a c
amaranth
jute mallow
cowpea
pumpkin
spinach
0 min
10 min
30 min
60 min
a


% intracellular protection
120
100
80
60
40
20
0
-20
-40
b b a
b b a b
b a a a
b b a a
c b a c
amaranth
jute mallow
cowpea
pumpkin
spinach
0 min
10 min
30 min
60 min
b

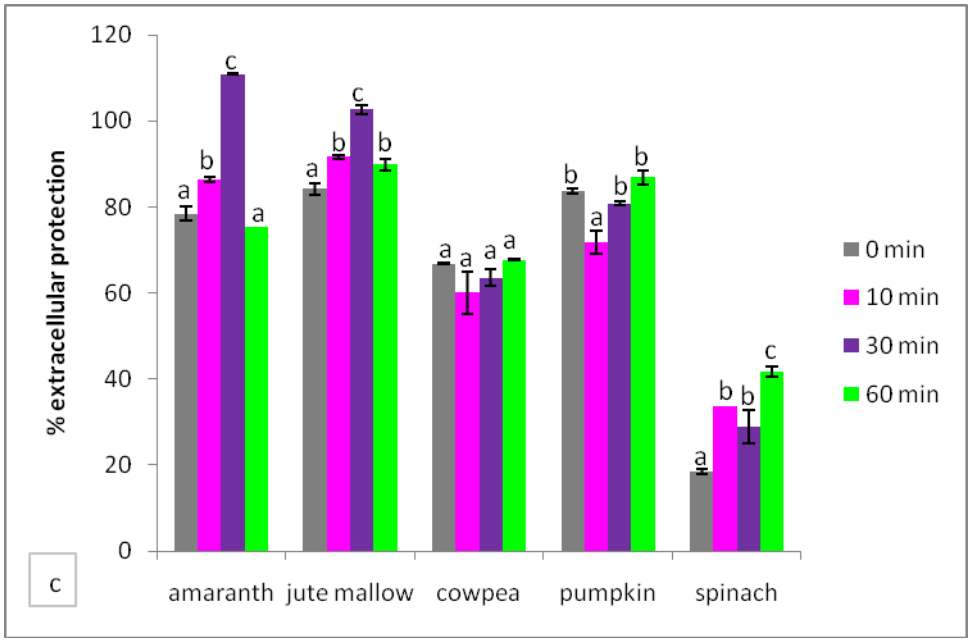
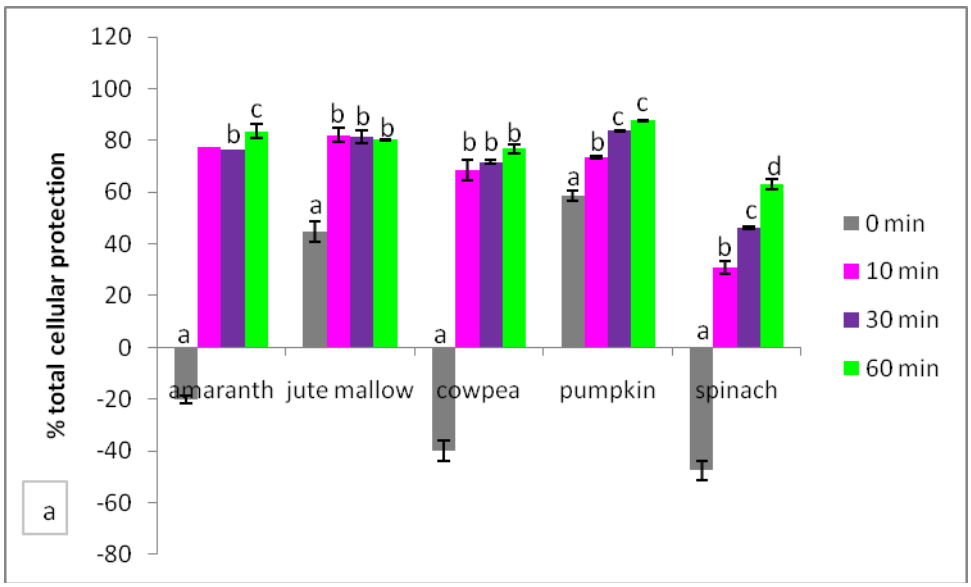


Figure 2.3.9 Percentage (a) total, (b) intra- and (c) extracellular protection of green leafy vegetable extracts against AAPH-induced oxidative damage on SC-1 fibroblast cells, as determined with the dichlorofluorescein assay. For each vegetable, means with different letters are significantly different ($p < 0.05$).

Caco-2 cells

The results of total and extracellular protection (Fig. 2.3.10 (a and c)) indicate that extracts of raw samples were highly cytotoxic to Caco-2 cells than those of boiled samples. Like with the experiment of SC-1 fibroblast cells, the intracellular protection of extracts against damage in Caco-2 cells followed no specific trend and seemed to be dependent on the type of GLVs (Fig. 2.3.10 (b)).



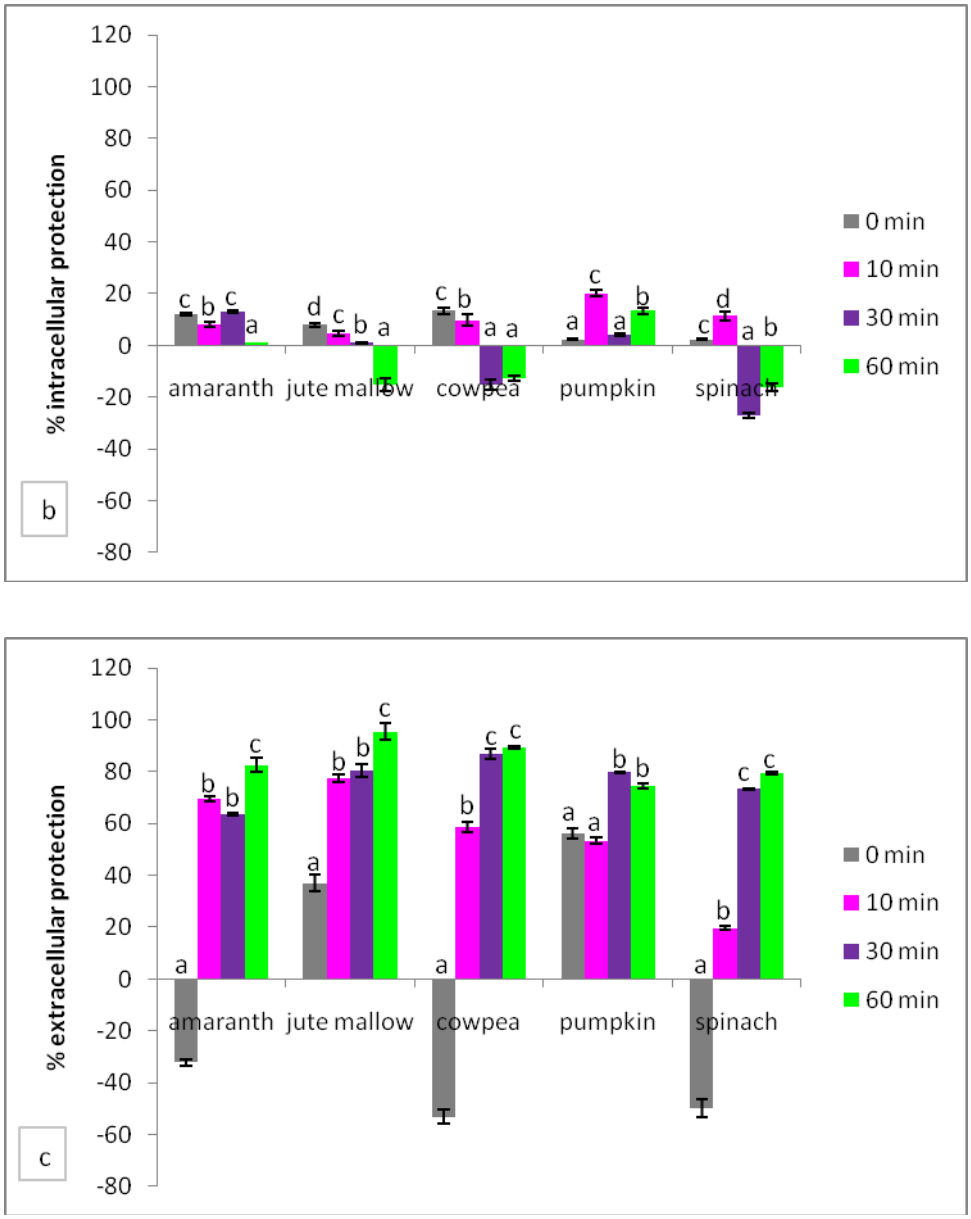


Figure 2.3.10 Percentage (a) total, (b) intra- and (c) extracellular protection of green leafy vegetable extracts against AAPH-induced oxidative damage on Caco-2 cells as determined with the dichlorofluorescein assay. For each vegetable, means with different letters are significantly different ($p < 0.05$).

Overall, extracts of African GLVs especially those of boiled jute mallow and amaranth exhibited highest total and extracellular protection, while spinach exhibited least protection against AAPH-induced damage on both cell cultures. The Caco-2 cell line was the most sensitive to the assays and the GLV extracts used, because it yielded consistently similar and clear results in comparison to the SC-1 fibroblast cell line. Caco-2 cells are physiologically relevant because they indicate possible cellular uptake and metabolism in humans.

Contrary to the results of chemical in vitro antioxidant assays (Section 2.1), it seems that the phytochemicals and other constituents of extracts from boiled GLVs are better able to promote growth and prevent damage in both cell cultures than those present in extracts of raw GLVs. The possible explanation could be that cooking destroyed most of the cytotoxins or antinutritional factors present in raw GLVs, leading to an effective growth and prevention of total cellular damage by extracts of cooked GLVs. Antinutritional factors such as oxalic acid (Aletor and Adeogun, 1995; Ejoh et al., 2007; Isong and Idiong, 1997; Wallace et al., 1998), phytate (Aletor and Adeogun, 1995; Oboh et al., 2005; Wallace et al., 1998), saponins (Ejoh et al., 2007; Wallace et al., 1998), tannins (Wallace et al., 1998), alkaloids (Orech et al., 2005) and trypsin inhibitors (Vanderjagt et al., 2000) have been reported in raw African GLVs and spinach (Radek and Savage, 2008). Costa-Lotufo *et al*. (2005) and Phillips (1996) found that vegetables with high levels of alkaloids are more cytotoxic than those with low levels of these antinutritional factors. However, boiling some African GLVs for 5 min has been found to significantly reduce trypsin inhibitors (Vanderjagt et al., 2000).

The physical properties of flavonoids and other classes of phytochemicals present in the GLV extracts determine their interactions with the cell membrane (Oteiza *et al*., 2005). Hydrophobic flavonoids may be deeply embedded in membranes where they can influence membrane fluidity and break oxidative chain reactions, while more polar compounds interact with membrane surfaces via hydrogen bonding, where they are able to protect membranes from external and internal oxidative stress (Wolfe and Liu, 2007). Youdim *et al*. (2000) have shown that polyphenols are able to localize both within the cell membrane and cytosol of vascular endothelial cells, within a short period of 30 min. The results of total cellular protection assay strongly indicate an affinity of phenolics in the extracts for extracellular protection of SC-1 fibroblast and Caco-2 cells by directly scavenging the AAPH-induced peroxyl radicals before they caused damage. Furthermore, it is also possible that GLV extracts could have protected the cell cultures against AAPH-induced cytotoxicity by improving the antioxidant status of these cells. While using the DCF assay, Elisia and Kitts (2008) attributed the protective effects of blackberry extracts against oxidative damage in Caco-2 cells to the presence of cyanidin-3-O-glucoside, which was the dominant anthocyanin identified in the extract.

The protective effects of blueberries against oxidative damage of erythrocytes has been attributed to localization of polyphenols within the different cellular milieus of the cells,

radical scavenging of other ROS that were induced following an introduction of H_2O_2, as well as possible stimulation of other biological mechanisms utilized by cells to protect themselves from ROS such as endogenous antioxidant enzyme systems (Aherne *et al*., 2007; Cadenas and Davies, 2000; Chiou and Tzeng, 2000; Youdim *et al*., 2000). Protection of cell cultures against oxidative damage by plant extracts has also been attributed to the ability of the extracts to act as reducing agent, free radical scavengers, and quenchers of singlet O_2 formation (Amarowicz *et al*., 2004; Moreno *et al*., 2006). Binding of redox-active iron from specific intracellular locations (such as endosomal and lysosomal cell compartments) (Melidou *et al*., 2005), as well as the number and structural positions of phenolic OH groups of individual flavonoids present in the extracts (Johnson and Loo, 2000; Melidou *et al*., 2005; Wang and Joseph, 1999) have also been attributed to protection of plant extracts against oxidative damage in cell cultures.

Overall, African GLVs exhibited better protection of the SC-1 fibroblast and Caco-2 cell lines than spinach.

2.3.4.3 Correlation coefficients between different assays

Table 2.3.1 shows correlation coefficients (r) between some of the chemical, biological and cellular antioxidant assays for all GLVs. The degree of correlation between these assays differs with the type of GLVs, with no specific trend. Strong correlations were obtained between TPC or ORAC and plasmid DNA in cowpea, pumpkin and spinach. TPC or ORAC and erythrocytes also had appreciable positive correlations. Correlations between DCF_{SC-1} and TPC or ORAC were strong for cowpea and pumpkin, while the DCF_{Caco-2} and TPC or ORAC had negative correlations for most GLVs. Positive r-values ($0.01 \leq r \leq 0.99$, $p < 0.05$) suggest that some of the protective effects observed in the cellular assays may be attributed to the presence of phenolics and antioxidant activity in the extracts. The positive r-values also indicate that these specific assays were suitable and reliable for assessing cellular antioxidant capacities of GLV extracts, although the degree of correlations differs. The negative r-values ($-0.99 \leq r \leq -0.09$, $p < 0.05$) observed especially for amaranth, jute mallow, pumpkin and spinach (TPC, ORAC and DCF_{Caco-2}) and amaranth, jute mallow and spinach (TPC, ORAC and DCF_{SC-1}) indicate no correlations between these respective assays, emphasizing the need for the use of more than one type of assay for determinations of antioxidant capacities and cellular effects of these GLVs. Negative correlations may also indicate that the protective

effects observed in the cell cultures may not be attributed to the phenolics and antioxidant activities but to other constituents in the extracts as well as the mechanisms involved. Each assay has a different mechanism of action and different reaction conditions. Chemical antioxidant assays measure specific aspects, while the cellular systems measure the total sum of all effects including uptake, metabolism and excretion.

Table 2.3.1 Correlation coefficients (r) between different antioxidant assays.

Assays	amaranth	jute mallow	cowpea	pumpkin	spinach
TPC vs ORAC	0.98	- 0.11	0.84	0.98	0.96
TPC vs plasmid DNA	- 0.68	- 0.16	0.99	0.77	0.93
ORAC vs plasmid DNA	- 0.61	0.89	0.87	0.86	0.90
TPC vs erythrocytes	0.55	0.11	0.60	0.59	0.88
ORAC vs erythrocytes	0.67	0.56	0.11	0.45	0.56
TPC vs DCF_{SC-1}	- 0.88	- 0.07	0.84	0.98	- 0.96
ORAC vs DCF_{SC-1}	- 0.91	- 0.82	0.85	0.92	0.86
TPC vs DCF_{Caco-2}	- 0.97	- 0.19	0.99	- 0.63	- 0.96
ORAC vs DCF_{Caco-2}	- 0.96	- 0.90	0.91	- 0.53	- 0.79

2.3.5 Conclusion

The present study found that African GLVs offered higher protection against AAPH-induced damage to plasmid DNA and cell cultures than spinach. Protection against erythrocytes damage is dependent on the type of vegetables. Using the long-term cytotoxicity assays, extracts of raw samples promotes cell viability while the DCF assay indicates cytotoxicity of these extracts probably due to the presence of antinutritional factors. The GLV extracts contain phytochemicals that are able to scavenge AAPH-induced peroxyl radicals. The extent of protections in cell culture was dependent on the type of vegetable and its constituents, boiling time and type of assay. African GLVs offered higher protection against oxidative damage than spinach. These findings suggest potential beneficial roles that may arise following dietary consumption of African GLVs and their possible abilities to reduce chronic diseases of lifestyle (CDL) associated with radical species. Consumption of African GLVs should therefore be promoted in sub-Saharan Africa.

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CHAPTER 3: GENERAL DISCUSSION

This chapter comprises three sections. The first section evaluates the methodologies used in the study. The second section discusses the main findings of the research, with emphasis on (1) the antioxidant properties, their fate during boiling and the effects of extraction solvent on these properties in comparison to spinach, (2) the specific phenolics found in these GLV extracts and their associated structure-activity functions, and (3) the cellular protective effects of African GLVs. The final section critically evaluates the possibility of using the African GLVs as a source of phenolics to manage CDL in sub-Saharan Africa.

3.1 Methodologies

This section discusses the principles, advantages and disadvantages of the methods used in this study. Justifications of why these methods were used in this study are provided. Suggestions of how the methodologies could be approached differently in order to give better results are also made.

Boiling was the method used to cook the GLVs. The vegetables were boiled for 10, 30 and 60 min. The cooking method was provided by women groups from the communities of Bushbuckridge and Allandale in the Mpumalanga province of South Africa. The boiling times covered all types of GLVs, because traditionally, boiling times may last for 10 to 60 min, depending on the type of vegetable. Generally, the amount of water used for cooking varies with communities, and some communities discard this cooking water while others do not. In this study, the amount of water used for boiling was dependent on the amount of water required to boil all the vegetable samples for 60 min on a hot plate, and cooking water was discarded afterwards. However, phenolic compounds may have leached into boiling water. Wachtel-Galor *et al*. (2008) found an increase in antioxidant content of cooking water used for microwaving and boiling vegetables. The boiling method used in this study reflected methods that are used by the communities, therefore it was appropriate to use it as such. However, it may be improved by not discarding the boiling water, but to analyze it for total phenolics and antioxidant activity in order to determine percentage leaching.

The type of solvent used for extractions plays an important role in ensuring maximum extraction of phenolics. The crude extracts were obtained using distilled water and 75% acetone. Different extraction procedures and solvents have been widely used in the literature, yielding conflicting results. Seemingly, the results are dependent on the food samples and water-to-solvent ratio rather than the type of solvent used. The solubility of phenolic compounds is mainly dependent on the polarity of the solvent, degree of polymerization of the phenolics, as well as the interaction of phenolics with other food components and formation of insoluble complexes (Lee, 2000; Naczk and Shahidi, 2004). Polar solvents are suitable for extracting polar phenolic compounds, while non-polar solvents are suitable for non-polar phenolic compounds. Some phenolic compounds may be lipid- or water-soluble and therefore activated in either the hydrophobic or hydrophilic layers of the cell, while others are ampiphilic and therefore activated in both water and to some extent in non-polar organic solvents.

Organic solvents containing some percentage of water have been found to be more efficient in the extraction of polyphenolic compounds than water or pure organic solvents in plant foods (Katsube *et al*., 2004; Xu and Chang, 2007; Zhao and Hall III, 2008). This may be attributed to water causing the plant tissues to swell, allowing the solvent to better penetrate the sample matrix (Zhao and Hall III, 2008). The extraction method used in this study could be improved by using the method that involves evaporation of solvent and reconstitution of the extracts in order to concentrate and increase the yield of phenolics for chemical and biological analysis. Increasing the extraction temperature and time may also increase analyte recoveries by promoting chemical reactions that yields certain forms of derivatives (Houghton and Raman, 1998). The yields would then be compared.

The TPC of the selected GLVs was determined using the F-C method (Section 2.1). This method is based on detection of the number of phenolic hydroxyl groups or other potential oxidisable groups present in the sample (Singleton *et al*., 1999). It quantifies the total concentration of phenolic hydroxyl groups present in the sample being assayed, including those in the extractable proteins (Waterman and Mole, 1994). The advantages and disadvantages of this assay have been discussed in Section 1.2, subsection 1.2.14. The F-C method seems to be the method of choice to estimate the TPC values of GLVs. In this study, it was therefore deemed appropriate to use the same method in order to make direct comparison of the TPC values, although not necessarily from the same vegetable species.

This method could be improved by determining the levels of ascorbic acid in the samples in order to rule out its interference.

The aluminium chloride assay was used to estimate the TFC of the GLVs (Section 2.1). This method determines the presence of flavonoids and it is very much dependent on their ability to donate hydrogen or electrons to a free radical. In this assay, acid labile complexes are formed with the C-4 keto group and either the C-3 or C-5 OH group of flavones and flavonols (Chang *et al*., 2002), making this method to be specific only for flavones and flavonols. For flavonols, kaempferol has C-3 and C-5 OH groups, while rutin, quercetin, quercitin and myricetin have extra ortho-dihydroxyl groups (Fig. 3.2). With regards to flavones, chrysin and apigenin have only the C-5 OH and C-4 keto groups, while luteolin has the C-5 OH group and the ortho-dihydroxyl group in the B ring forming a complex. Flavanols such as epicatechin do not possess the C-4 keto group (Fig. 3.2), while flavanones such as naringin, naringenin and hesperetin, as well as genistein (an isoflavone) have the C-5 OH group. The total flavonoid content should be the sum of flavonoid content, therefore this method could be combined with the 2,4-dinitrophenyllhydrazine method which is specific for flavanones in order to better represent the real content of total flavonoids (Chang *et al*., 2002).

The TAA of selected GLVs was measured by using the ABTS, DPPH and ORAC assays (Section 2.1). The ABTS assay is based on the neutralization of radical cation formed by a single-electron oxidation of a synthetic ABTS chromophore to a strongly absorbing $ABTS^{\bullet+}$. Addition of antioxidants to the pre-formed radical cation reduces it depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction (Re *et al*., 1999). The advantage of this assay is that the $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents (Arnao, 2000), therefore the antioxidant activity of both hydrophilic and lipophilic compounds in the extracts from the selected GLVs should have been detected. There are several disadvantages of using this assay: the $ABTS^{\bullet+}$ reagent is very unstable and it reacts with any hydroxylated aromatics. In fact, the ABTS assay essentially involves titration of aromatic OH groups including the OH groups which do not contribute to antioxidation (Roginsky and Lissi, 2005). Furthermore, this method is not fully standardized, and therefore comparison of results obtained from different laboratories is problematic (Awika *et al*., 2003). The use of $ABTS^{\bullet+}$, which is not biologically relevant *in vivo* is also a matter of concern.

The DPPH method is based on the ability of antioxidant to give hydrogen radicals to synthetic stable organic nitrogen DPPH$^{\bullet}$. The DPPH$^{\bullet}$ is less reactive than the ABTS radical cation (Gordon, 2003). The interaction of a potential antioxidant with DPPH$^{\bullet}$ depends on its structural conformation. Some compounds react very rapidly with the DPPH$^{\bullet}$, reducing a number of DPPH$^{\bullet}$ molecules corresponding to the number of available OH groups. The drawback of this method is that data is frequently reported as EC_{50}, which is the concentration of antioxidant required for 50% scavenging of DPPH$^{\bullet}$ in the specified time period (Maisuthisakul *et al*., 2007). This approach makes it difficult to compare data from different studies because it seems to be more rational to express the hydrogen-donating capacity as the amount of DPPH which may be scavenged by the sample tested (Roginsky and Rossi, 2005). Other disadvantages of this assay is that DPPH can only be dissolved in organic media, which is a limitation when interpreting the role of hydrophilic antioxidants (Arnao, 2000), as well as the use of DPPH$^{\bullet}$, which is not biologically relevant *in vivo*.

The ORAC assay measures the capacity of an antioxidant to directly quench peroxyl radicals (ROO$^{\bullet}$) (Cao and Prior, 1999). Thermal decomposition of an azo compound, AAPH, generates ROO$^{\bullet}$ that reacts with fluorescein, a fluorescent probe, and consequently leads to a loss of fluorescence intensity. In the presence of antioxidants, ROO$^{\bullet}$ removes a hydrogen atom from the antioxidant to form hydroperoxide and a stable antioxidant radical, resulting in a delay or inhibition of the reaction between ROO$^{\bullet}$ and fluorescein (Ou *et al*., 2001). The high molar ratio between the free radical generator and an antioxidant sample in the ORAC assay indicates high specificity of this assay compared to the other assays (Cao and Prior, 1999). There are several limitations to this assay; it requires the use of expensive equipment with large data variability (Awika *et al*., 2003), it is performed in aqueous solution, it primarily measures only hydrophilic antioxidant activity against peroxyl radicals (Ou *et al*., 2001 and 2002), and should therefore not have been regarded as a 'total antioxidant activity assay'. Biologically relevant ROS also include $O_2^{-\bullet}$. HO$^{\bullet}$, $ONOO^{-}$, and singlet oxygen. Different ROS have different reaction mechanisms, therefore in order to completely evaluate antioxidant activity a full profile of antioxidant activity against various ROS, comprehensive assays are needed. It is important that more than one method is used for the evaluation of antioxidant activity as was done in this study because GLV extracts are a complex mixture of substituents, with different structures, solubility and reactivity. The reactions themselves that

measure antioxidant activity are complex and are functions of the physico-chemical parameters of the assay reagents as well as the substrates (Soobrattee *et al*., 2005).

The HPLC method of analysis was used to detect and quantify flavonoids found in the water and acetone extracts of GLVs (Section 2.2). Each plant has a unique phenolic composition profile, which makes quantification of these compounds difficult. The flavonoids were quantified by comparison to the retention times of authentic standards following extraction. In order to improve this method, characterization of the phenolic constituents of the GLVs such as that used by Salawu *et al*. (2009) using HPLC/DAD/MS is needed to identify all compounds including those whose standards were not available in the laboratory, as well as compounds with similar retention times. The mass spectral results of the phenolic compounds would clearly show characteristic aglycone masses (M^+) or $[M-H]^-$ belonging to each of the flavonoids identified in this study and other phenolics, for a more specific identification.

To establish whether the results obtained from the *in vitro* chemical antioxidant assays would yield similar effects when subjected to *in vitro* biological and cellular assays, the erythrocyte haemolysis, plasmid DNA and cell culture assays were used (Section 2.3). Only the water extracts of GLVs were used for biological and cell culture assays, because these extracts are physiologically relevant. The 75% acetone extracts could not be used because of the possible toxic interactions with cellular contents which cannot be avoided while mimicking biological metabolites and reactions that happen after consumption of GLVs.

The erythrocytes haemolysis assay is a convenient test model for determining antioxidant extracts that are able to protect the cell membranes. The decomposition of AAPH produces peroxyl radicals which attack the susceptible polyunsaturated fatty acids in the erythrocyte membranes to induce lipid peroxidation (Deng *et al*., 2006). During oxidative stress, iron in erythrocytes may be released from haemoglobin or ferritin (Scott and Eaton, 1995) and bind to intracellular metabolites or membrane components where it may become vulnerable to iron-catalyzed $HO^{\bullet}$ attack and lipid peroxidation (Grinberg *et al*., 1997). The presence of phenolics with antioxidant activity scavenges the radicals before they damage the erythrocyte membranes, thus preventing haemolysis (Blasa *et al*., 2007). It has been established that the extent of the damage is in most cases proportional to the amount of AAPH (Miki *et al*., 1987) and the concentration of phenolics in the extract (Blasa *et al*., 2007). The advantage of using this method is that it is biologically relevant, because erythrocytes play critical roles in

antioxidant protection in the blood. Using erythrocytes as a cell model reduces the confounding contribution of cellular signaling. In addition, this assay is not affected by mitochondrial production of ROS, as erythrocytes do not have mitochondria but instead generate cellular energy by a glycolysis pathway (Honzel *et al*., 2008; Kabanova *et al*., 2009). It therefore provides a cleaner signal for antioxidant capacity of a test product than other cells (Honzel *et al*., 2008).

The plasmid DNA assay was used to determine the role of phenolics in protection against oxidative damage. This damage is dependent on the concentration of the oxidant, exposure time, as well as the type of DNA being investigated (Wei *et al*., 2006). During oxidative stress, supercoiled plasmid DNA is converted into a relaxed circular form due to single-strand breaks and into linear form due to double-strand breaks (Aronovitch *et al*., 2007; Lewis *et al*., 1988; Zhang and Omaye, 2001). Samples rich in phenolics quench the radicals and consequently inhibit plasmid DNA degradation. Those radicals not scavenged by phenolic components of the extracts attack the plasmid DNA, thus degrading it into a series of fragments (Gião *et al*., 2008) (Section 2.3). The advantage of this method is that it permits large amounts of DNA to be processed in one run. Although DNA migration assay is a sensitive biomarker of the DNA damage, the method of quantifying the relative DNA damage has low sensitivity and accuracy. However, the results obtained in this study are convincing due to the large difference between the protective and non-protective effects of different GLV extracts. Other disadvantages include (1) occurrence of other DNA lesions being introduced into the plasmid samples through the use of ethidium bromide dye for staining (Elliot *et al*., 2000), and (2) weaker binding of ethidium bromide dye to supercoiled DNA in comparison to the circular or linear forms (Milligan *et al*., 1992). This method can be improved by using the anion exchange HPLC method to measure the extent of damage, because this method employs mild conditions for purification of supercoiled DNA (Elliot *et al*., 2000).

In this study, SC-1 fibroblasts were used for cell culture work, because these cells were readily available, easy to grow in culture medium and are reasonably sensitive to injury in the systems used. Caco-2 cells were used as target cells to predict what could happen in the human colon after absorption. The usefulness of a cell type for an assay is determined by its ability to predict clinical cytotoxicity, rather than by an attempt to duplicate the *in vivo* situation. Different cytotoxicity assays may reveal different results depending on the assay employed, the test agent used and endpoints (Weyermann *et al*., 2005). Thus, it is

recommended to use multiple, mechanistically different endpoint parameters for the evaluation of cytotoxicity. Three long-term cell viability assays (MTT, NR and CV) were used to determine the effect of GLV extracts on the viability or proliferation of SC-1 fibroblast cells (Section 2.3). The advantages of these assays have been outlined in Section 1.2, Table 1.2.8.

The MTT assay was used to determine the cytotoxic effect of GLV extracts on uninjured viable cells, which is reflected by the uptake and reduction of an insoluble blue formazan from the soluble yellow MTT tetrazolium salt by succinic dehydrogenase in mitochondria (Hansen *et al*., 1989; Tan *et al*., 2008). The NR assay was used to estimate the viability of cells by measuring the amount of NR uptake into the lysosomes of viable cells (Scott-Fordsmand and Weeks, 2000). Only the viable, uninjured cells accumulate the NR in their lysosomes. An important disadvantage of this assay is that some bioactive compounds, especially those that inhibit cell proliferation may need longer time to exert their cytotoxicity. The NR assay is dependent on the growing number of the cells thus shorter incubation periods would result in failure to discriminate the potential cytotoxic activity of the extracts. The CV assay is used to measure cell density as an indicator of cytotoxicity by staining with crystal violet (Awika *et al*., 2009). The CV dye primarily stains the membrane of viable cells (Ishiyama *et al*., 1996). This assay was used as a complementary test after completion of the NR assay. The disadvantage of this assay is that the absorbance of stained cells does not always correspond to the viable cell numbers when the culture is continued after reaching confluence (Chiba *et al*., 1998). Furthermore, all adherent cells including dead cells may be regarded as viable cells because of nonspecific staining by the CV dye. All the above-mentioned long-term cytotoxicity assays were used to measure long-term effects of GLV extracts on SC-1 fibroblast cells. To obtain comprehensive pro- and/or anti-oxidative effects of GLV extracts on cells, these assays were combined with short-term DCF cytotoxicity assay.

The DCF method was used to measure both the short-term cytotoxicity effect and the antioxidant activity of GLV extracts on SC-1 fibroblast and Caco-2 cells (Section 2.3). In this assay, DCFH-DA is deacetylated by intracellular esterases to dichlorofluorescin (DCFH), which is nonfluorescent, and converted by reactive species into DCF, which is fluorescent (Halliwell and Whiteman, 2004). The presence of phenolics with antioxidant capacities scavenges the radical species and reduces the DCF formation. The efficiency of cellular

uptake and/or membrane binding of phenolic compounds combined with the radical scavenging activity dictates the efficacy of GLV extracts (Wolfe and Liu, 2007). The advantages and disadvantages of this assay have been outlined in Section 1.2, Table 1.2.8. To improve this method, it was combined with long-term cytotoxicity assays and other assays for measuring antioxidant activity, in order to obtain comprehensive effects of GLV extracts on cell cultures.

3.2 Research Findings

This section discusses the main findings of this study, which show that African GLVs contain more phenolic compounds that contribute to their antioxidant activities and cellular protective effects than spinach, and that the effect of boiling on the phenolics and antioxidant properties is dependent on the type of vegetables.

As described earlier (Section 2.1, Table 2.1.1), this study found that African GLVs contain higher TPC, TFC and TAA than spinach. These findings clearly indicate that genetic and environmental factors strongly affect the phenolic content and composition of GLVs. In comparison to spinach, African GLVs are well adapted to local harsh environmental conditions and grow well with minimal water and poor soil fertility (Jansen van Rensburg *et al*., 2007). Plant phenolics play an important role in an effective defense mechanism that renders plants resistant to numerous potential pathogens and stress (Inderjit *et al*., 1999). The accumulation of plant phenolics and their role in defense has been well documented (Vermerris and Nicholson, 2006). In addition to constitutive levels of phenolic compounds found in unstressed plants, accumulative levels may also occur in unstressed plants, as an early event during the induction process of defense against pathogens (Ebel and Grisebach, 1988, Inderjit *et al*., 1999). There is a complex set of interactions between plant growth and abiotic environmental factors such as nutrient stress, irrigation and lack of water, as well as other conditions that may enhance the production of phenolic compounds, thus inducing a stress factor. Plant resistance to biotic and abiotic stress shows marked inter- and intra-species differences, often related to aglycone synthesis or the hydrolysis of pre-formed glycosides (Spina *et al*., 2008), which could explain the difference in total phenolics and antioxidant activity of African GLVs and spinach found in this study.

Table 3.1 Summary of the effect of boiling (for 30 min) on antioxidant activity of African green leafy vegetables as found in this study.

In vitro chemical antioxidant assays

Assay	African GLVs	Effect of boiling	% effect	Comparison to boiled spinach
TPC	amaranth	decrease	53.7	higher
	jute mallow	decrease	36.1	higher
	cowpea	decrease	34.1	higher
	pumpkin	decrease	68.6	lower
TFC	amaranth	decrease	48.7	higher
	jute mallow	decrease	39.6	higher
	cowpea	decrease	15.4	higher
	pumpkin	decrease	72.3	lower
ABTS	amaranth	decrease	33.1	higher
	jute mallow	decrease	18.8	higher
	cowpea	decrease	11.4	higher
	pumpkin	decrease	29.6	lower
DPPH	amaranth	decrease	54.0	higher
	jute mallow	decrease	36.4	higher
	cowpea	decrease	45.4	higher
	pumpkin	decrease	80.4	higher
ORAC	amaranth	decrease	57.5	higher
	jute mallow	decrease	57.3	higher
	cowpea	decrease	50.1	higher
	pumpkin	decrease	74.3	higher

In vitro biological and cellular antioxidant assays

Assay	African GLVs	Effect of boiling	% effect	Comparison to boiled spinach
Erythrocytes	amaranth	no change	9.6	higher
	jute mallow	increase	122.2	similar
	cowpea	decrease	28.5	lower
	pumpkin	decrease	49.8	lower
DNA plasmid	amaranth	increase	453.0	higher
	jute mallow	increase	42.9	higher
	cowpea	decrease	62.1	higher
	pumpkin	decrease	87.8	higher
DCF_{SC-1}	amaranth	increase	11.8	higher
	jute mallow	no change	8.0	higher
	cowpea	decrease	11.1	higher
	pumpkin	decrease	5.8	higher
DCF_{Caco-2}	amaranth	increase	482.5	higher
	jute mallow	increase	82.5	higher
	cowpea	increase	279.7	higher
	pumpkin	increase	43.1	higher

Boiling resulted in a decrease of levels of total phenolics, flavonoids and antioxidant activity in GLVs (Table 3.1). Figure 3.1 shows an example of the different and simultaneous reactions that can happen in GLV matrix subjected to heat treatment. For short heat treatment, a reduction in overall antioxidant activity occurs due to loss of naturally occurring antioxidants. While for longer heat treatment, this loss can be minimized by a recovery or even an enhancement of the antioxidant activity due to the formation of new products with antioxidant properties. Boiling probably resulted in the destruction of vegetable cell walls and sub-cellular compartments which lead to leaching of phenolic components into boiling water. Phenolics are mainly found in the vacuoles of leaves and any damage may lead to their escape. Boiling has also been reported to cause oxidation of polyphenol components by polyphenol oxidase in GLVs (Yamaguchi *et al*., 2003). The extent of this loss was dependent on the type of vegetable, boiling time and extraction solvent (Tables 2.1.1 and 2.1.2). Antioxidant property of jute mallow was least affected by boiling while that of pumpkin was the most affected. Yamaguchi *et al*. (2001) investigated the effect of cooking on vegetables and found both decreases and increases in flavonoid content after cooking. The present study found a significant increase in TPC, TFC, TAA and some flavonoids in extracts of pumpkin boiled for 60 min, which might have resulted from liberation of high amounts of antioxidant components due to thermal destruction of vegetable cell wall structures and sub-cellular compartments.

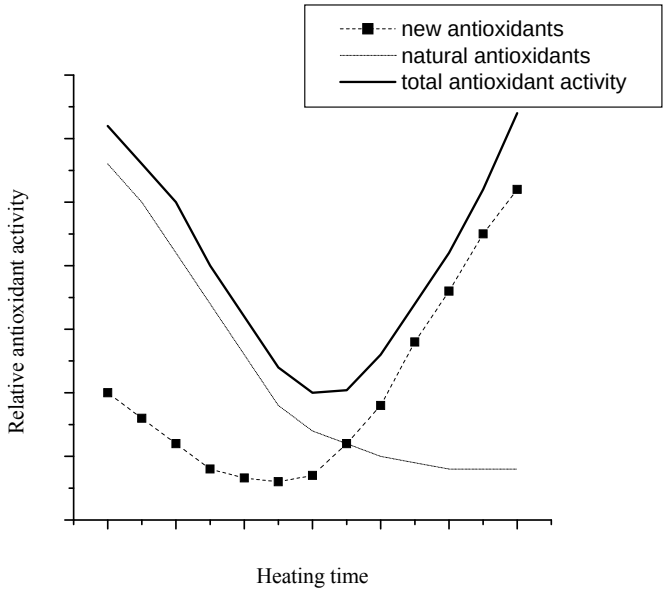

Figure 3.1 Changes in the overall antioxidant activity due to different and simultaneous events in a vegetable matrix subjected to heating (Nicoli *et al*., 1999).

The 75% acetone was more efficient in extracting phenolics from GLVs than water (Section 2.1). This was confirmed by significant strong correlations between TPC, TFC and antioxidant assays, especially of acetone extracts. This shows that phenolics and flavonoids content can be used as an indicator for antioxidant activity in GLVs. Extracts of 75% acetone also exhibited higher TPC yields than water and absolute acetone extracts (results not shown). These results were in agreement with those of Katsube *et al*. (2004), Sotillo *et al*. (1994) and Xu and Chang (2007) who found that organic solvents containing some percentage of water were more efficient in extracting polyphenols and antioxidants than water or pure organic solvents. In contrast, Zhao and Hall III (2008) found that water extracts of raisins contained significantly higher values of TPC than the extracts of organic solvents.

The results of the HPLC analysis revealed that water was able to extract most of the flavonoids although in less concentrations, while the 75% acetone extracts extracted mainly epicatechin and rutin and in higher concentrations especially for rutin (Section 2.2, Tables 2.2.1 and 2.2.2). These results also indicated that the concentrations of flavonoids detected in the GLV extracts were dependent on the type of vegetables. The effect of boiling was also dependent on the type of vegetable and the specific flavonoids present, which is in agreement with the results of antioxidant properties as discussed in Sections 2.1 and 2.3. In addition to the peaks of the identified flavonoids, the HPLC chromatograms showed the presence of peaks of other compounds that could not be identified in this study because of lack of standards, suggesting that the antioxidant properties of these GLV extracts cannot be attributed solely to the identified flavonoids. The results suggest that there were no distinct differences in concentrations and types of flavonoids detected in extracts of African GLVs in comparison to those in extracts of spinach. However, extracts of 75% acetone indicated higher amounts of total flavonoids in African GLVs in comparison to spinach.

The mechanisms of antioxidant activity of polyphenols in GLV extracts may be understood in terms of their structure-activity relationships, which are dependent on their structural features, especially the presence of a free 3-OH group. An increase in the number of phenolic OH groups has been found to correspond with an increase in antioxidant activity. Ammar *et al*. (2009) and Bors *et al*. (1987) proposed that three structural features are responsible for the radical scavenging effects of flavonoids (reviewed in Section 1.2, subsection 1.2.6). One of these features is the *ortho*-dihydroxy or catechol group in the B ring (3′,4′-OH), which confer a high stability to the radicals. Another is the conjugation of the B ring to the 4-oxo group via

the C2-C3 double bond, which ensures the electron delocalization from the B ring. The last is the 3- and 5-OH groups with the 4-oxo group, which allow electron delocalization from the 4-oxo group to both substituents. In addition, when the B ring possesses a third OH group (3′,4′,5′-OH), the central OH bond is the weakest, as the two other OH groups can form two hydrogen bonds with the flavonoid radical (Silva *et al*., 2002). Ishige *et al*. (2001) reported that in addition to the presence of the 3-OH group, the structural features of flavonoids crucial for antioxidant effects include an unsaturated C ring and hydrophobicity. The combination of all these structural features enables a higher electron delocalization, conferring a high stability to the present radicals.

Both flavonol and flavones have identical OH configuration, however, the absence of the 3-OH group in the flavones explain the lower antioxidant activity of the latter. Burda and Oleszek (2001) reported that substitution of 3-OH by a methyl or glycosyl group decreases the activity of quercetin and kaempferol against ß-carotene oxidation in linolenic acid. In addition to a reduction in the number of OH groups, removal of the 3-OH group affects the conformation of the molecule. The torsion angle of the B ring with respect to the rest of the molecule also strongly influences free radical scavenging ability (Soobrattee *et al*., 2005). Flavonols and flavanols with a 3-OH are planar, while the flavones and flavanones, lacking this feature, are slightly twisted (Fig. 1.2.2). Planarity permits conjugation, electron dislocation, and a corresponding increase in flavonoid phenoxyl radical stability. Eliminating the hydrogen bond effect a minor twist of the B ring, abrogates coplanarity, conjugation and electron delocalization capacity, thereby compromising scavenging ability (Soobrattee *et al*., 2005). Due to this intramolecular hydrogen bonding, the influence of a 3-OH is potentiated by the presence of 3′,4′-catechol, explaining the potent antioxidant activity of flavanols and flavonols (Bors *et al*., 1990).

This study found that extracts of African GLVs especially cowpea, pumpkin and jute mallow exhibited higher antioxidant activity and protection against oxidative damage on plasmid DNA and cell cultures than those of spinach. The HPLC results showed that there were no distinct differences between the types of flavonoids detected in extracts of African GLVs and those in spinach. The major flavonoids detected in the water extracts of these GLVs were epicatechin, rutin and myricetin, while epicatechin and rutin were the main compounds detected in the 75% acetone extracts.

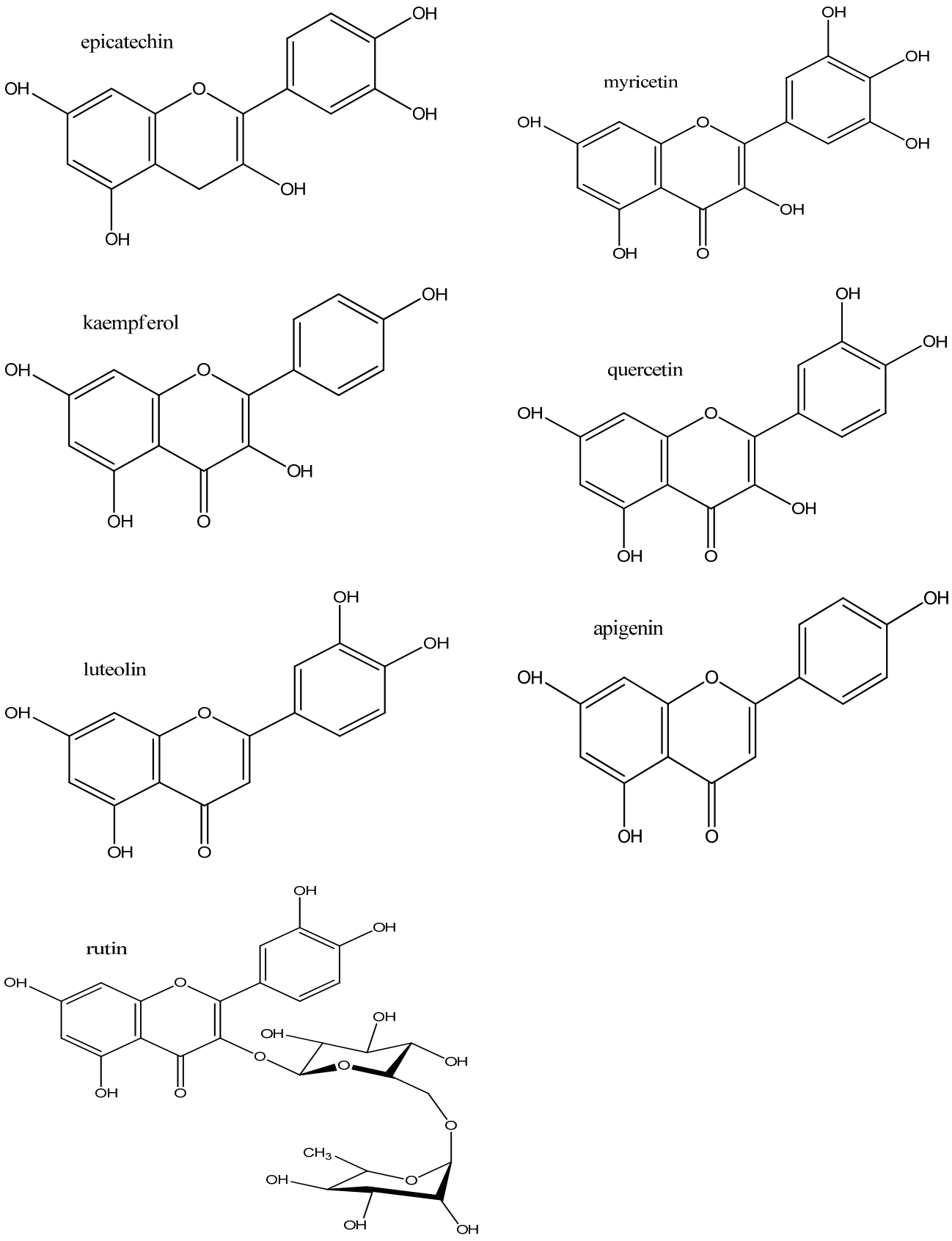

Figure 3.2 Chemical structures of the flavonoids detected in green leafy vegetable extracts (Heim *et al*., 2002).

Among the water extracts, highest content of epicatechin was detected in amaranth, rutin in spinach, while myricetin, quercetin, kaempferol, luteolin and apigenin were detected in pumpkin. While, among the 75% acetone extracts, highest contents of epicatechin was also detected in amaranth and rutin in cowpea.

According to the structural features described above, myricetin is supposed to be the most efficient flavonoid antioxidant, followed by quercetin and epicatechin. The highest values of myricetin were detected in the water extracts of pumpkin. The water extracts of pumpkin also contained other flavonoids, whose combined effects and synergistic interactions probably contributed to its high radical scavenging and overall antioxidant property. However, it is not only the number and concentration of different flavonoids found in the extracts that contribute to overall antioxidant activity, but mainly the structure of flavonoids present especially with free 3-OH groups, as well as the interaction between these phenolic compounds (Rice-Evans *et al*., 1997). According to the HPLC results, water extracts of spinach contain higher amounts of total flavonoids than extracts of cowpea, however, the results of total antioxidant activity showed that the antioxidant activity of spinach was less than that of cowpea and other African GLVs. The possible reason of this discrepancy could be that the antioxidant activity of cowpea extracts was mainly contributed by the unidentified compounds with phenolic properties present in the extracts (see Section 2.2, Fig. 2.2.1). The results provided in this study are only for those flavonoids whose standards were available.

Although myricetin has all the above-mentioned structural characteristics for effective radical scavenging abilities (Fig. 3.2), it probably did not contribute most to the antioxidant properties of the extracts. It seems that the antioxidant properties of extracts are a result of a combination of reactions of all the phenolic compounds present and other components of the extracts. Soobrattee *et al*. (2005) found that quercetin exhibited more antioxidant capacity followed by myricetin and kaempferol. The presence of a third OH group in the B ring at C5 position (i.e. 5′-OH) did not enhance the effectiveness of myricetin compared to quercetin with simply an *o*-diphenolic structure (Fig. 3.2). Epicatechin was also detected in all the water extracts of GLVs. Epicatechin is a flavanol and lacks the 4-oxo group. Its contribution to total antioxidant activity and protection against oxidative damage in cell cultures is expected to be high because of its catechol group in the B ring.

Rutin, a glycoside of quercetin, is diglycosilated on the C-3 position of the C-ring, which is expected to have slightly lowered its antioxidant activity level (Kobus *et al*., 2009). Rutin was the main compound detected in the acetone extracts, probably because the presence of the two sugar parts increased the solubility of this compound in the 75% acetone extracts (Kim *et al*., 2005). Guo and Wei (2008) emphasized that rutin's polyphenol structure makes it very sensitive to changes in the surroundings, which may alter the planarity, hydrophobicity and electrostatic components and eventually lead to changes in its antioxidant properties.

Kaempferol was also detected in all the water extracts of raw samples. Kaempferol possesses one OH group in the B ring and exhibits the 3-OH and 5-OH groups with the 4-oxo group in the C ring in addition to the C2-C3 double bond (Fig. 3.2). Soobrattee *et al*. (2005) found that the presence of a single OH group in the B ring of kaempferol apparently made little contribution to antioxidant activity even in the presence of the conjugated double bond system and the 3-OH group. Regarding the flavones, higher contents of luteolin and apigenin were detected in water extracts of pumpkin than in other GLVs. Luteolin with the catechol group and 5,7-OH groups was found to be more potent than apigenin without the catechol group by Soobrattee *et al*. (2005). Based on the structural features discussed above, the contribution of these two flavones to total antioxidant activity of GLVs is supposedly minimal because of the absence of the 3-OH group. Overall, the resulting scavenging abilities of the extracts to scavenge the radicals are the sum of interactions and combinations of the reactions of all the components of the extracts.

Phenolics are also known to exhibit antioxidant actions through chelation of redox-active metals. Iron and copper are examples of metals having more than one redox state that can catalyze radical formation. Catechol moieties in flavonoid molecules possess a high affinity for metal ions. Metal chelation capacity of flavonoids is associated with (i) the presence of 3′- and 4′-OH groups in the B ring, (ii) the presence of 3- and 5-OH groups (Arora *et al*., 1998; Perron and Brumaghim, 2009), and (iii) the presence of the 4-oxo function (van Acker *et al*., 1996). However, efficient metal chelating action is not necessarily equivalent to a significant antioxidant action. For a flavonoid-metal complex to operate as an antioxidant, it has to be less efficient compared with the free metal as catalyst of free radical formation. It should be noted that a flavonoid-metal complex could also catalyze free radical formation acting in such cases as prooxidants (Perron and Brumaghim, 2009).

In the absence of flavonoids (FOH) acting as antioxidant	In the presence of flavonoids (FOH) acting as antioxidant
Initiation LH → L•	***Initiation*** LH → L•
Propagation L• + O_2 → LOO•	***Propagation*** L• $\xrightarrow{O_2}$ LOO• LOO• $\xrightarrow{FOH}$ LH + FO•
LOO• + LH → LOOH + L•	LOO• $\xrightarrow{LH}$ LOOH + L• LOO• $\xrightarrow{FOH}$ LOOH + FO•
Termination L• + LOO• → non radical products L• + L• → non radical products LOO• + LOO• → non radical products	***Termination*** L• + LOO• → non radical products L• + L• → non radical products LOO• + LOO• → non radical products L• + FO• → non radical products LOO• + FO• → non radical products 2 FO• → non radical products

Figure 3.3 Sequence of reactions involved in the lipid oxidation chain process in the absence or presence of a flavonoid (FOH) acting as antioxidant. LH: lipid molecule, L•: lipid radical, LOO•: lipid peroxyl radical, LOOH: lipid hydroperoxyl, FOH: flavonoid: FO•: flavonoid derived free radical (Galleano *et al*., 2010).

Lipid oxidation, a major event in the oxidation of biological systems, occurs as a chain reaction involving lipids (LH) and molecular oxygen as substrates and metals as catalysts (Fig. 3.3). In cell models, the interactions of flavonoids with membrane lipids and proteins results in mediation of biological and antioxidant effects. In a review of antioxidant actions of flavonoids, Galleano *et al*. (2010) suggested that at cellular level, flavonoids can (i) interact with lipids at the surface of the bilayer, and/or (ii) insert into the bilayer and interact with hydrophobic chains of lipids. Direct interactions of flavonoids with cellular lipids could alter physical properties and modulate activity of membrane-associated enzymes, ligand-receptor interactions, ion and/or metabolite fluxes, and modulation of signal transduction. The uptake of flavonoids into lipid bilayers could bring these molecules in close proximity to lipid radicals, lipid peroxyl radicals and other lipid-soluble radicals. Flavonoids could then react with these radicals and prevent further propagation of free radical reactions that could potentially cause membrane damage and/or alter membrane functions (Fig. 3.3). As shown in Fig. 3.3, flavonoids (FOH) donates hydrogen atoms to lipid (L•) or lipid peroxyl (LOO•)

radicals to prevent lipid oxidation propagation. The products of these reactions are non radical species, i.e. the original lipid (LH), the corresponding lipid hydroperoxide (LOOH), and the radical derived from the flavonoid (FO•). Galleano *et al*. (2010) further emphasised that the effectiveness of FOH as a chain breaking antioxidant is dependent on (1) the occurrence of reaction between L• or LOO• and FOH that must be energetically favoured, and (2) the appropriate stability of the resultant radical (FO•), which should be relatively non-reactive.

The significant strong correlations found between assays of TPC and DNA plasmid or cell cultures (Section 2.3, Table 2.3.1) indicate that phenolic compounds possibly make a significant contribution to the biological and cellular antioxidant properties of GLV extracts. Although polyphenols probably play a significant role in chemical and cellular antioxidant effects of GLVs, these effects cannot be solely attributed to phenolics, but to all constituents of the extracts. It is assumed that unique constituents in each extract are responsible for their respective antioxidant properties, proliferation, cytotoxicity and/or cellular protective activities. GLVs contain other substances such as vitamin C and carotenoids that have been shown to have antioxidant functions and contribute to cellular protective effects.

The findings of the erythrocytes haemolysis assay show that all samples, except raw corchorus, offered some level of protection against haemolysis, but the percentage protection was dependent on the type of vegetables. There were no clear differences between treatments of African GLVs and spinach, as well as between raw and boiled samples. Protection against haemolysis represents prevention of oxidative damage to the cell membrane, which was mainly due to radical scavenging of peroxyl radicals. With regards to the plasmid DNA assay, African GLV extracts generally protected the plasmid DNA from oxidative damage better than extracts of spinach. The extracts varied in their relative abilities to protect the plasmid DNA from oxidative damage. The results of this assay correspond to those of TAA, possibly indicating that the protective effect observed in this assay was due to their respective antioxidant properties. These biological assays have different mechanisms and targets, which may explain the differences in results obtained in this study. Irrepairable DNA damage is involved in carcinogenesis, aging and other degenerative diseases (Cozzi *et al*., 1997). The results of this study therefore indicate that extracts of African GLVs could inhibit oxidative DNA damage and prevent cancer development.

When the GLV extracts were subjected to cell viability tests, the results of long-term cytotoxicity assays were completely different from the results of short-term cytotoxicity assay. Cytotoxic effects of extracts were not observed using the long-term cytotoxicity assays, however, the results of the short-term cytotoxicity assay showed that extracts of raw GLVs were significantly cytotoxic, more so in the extracts of spinach than in African GLVs. The findings of this study indicate that there was an initial cytotoxic effect as extracts of raw GLVs were added to the cells as observed with the DCF assay, however after about 72 h, the cells then recovered and started proliferating as usual, leading to this effect not being observed in the long-term cytotoxicity assays. Extracts of boiled GLVs offered better protection to the cells than extracts of raw samples, suggesting that boiling degraded the toxins or antinutritional factors present in extracts of raw GLVs. This observation is important because GLVs are consumed after boiling and therefore the effect of raw samples on cell cultures may not be observed *in vivo*. It is not known at this stage as to which specific antinutritional factors are present in these extracts as the determinations of antinutritional factors was not part of objectives of this study. The results of the cellular antioxidative protective assays indicated that extracts of African GLVs offered higher total protective abilities to cells in comparison to spinach, in both SC-1 fibroblast and Caco-2 cells (Table 3.1). The antioxidant activity and protective effects of plant extracts against oxidative stress in biological and cellular models may be attributed to the binding of flavonoids to the cell membrane (Blasa *et al*., 2007), H-atom abstraction from the phenolic groups (Deng *et al*., 2006), as well as iron chelation by polyphenols (Grinberg *et al*., 1997).

Overall, African GLV extracts exhibited higher phenolics, antioxidant activity and cellular protective effects against oxidative damage than extracts of spinach. However, there may be genetic variations between individual vegetable species that could result in altered gene expression, leading to various and different effects of these vegetables in the antioxidant defense systems (Arbos *et al*., 2008). Nonetheless, the findings of this study may be used to promote consumption of African GLVs especially in the poor urban areas where a significant increase in chronic diseases of lifestyle (CDL) has been observed. Consumption of these vegetables has a potential to reduce CDL in sub-Saharan Africa.

3.3 African GLVs may reduce chronic diseases of lifestyle

This section will discuss the merits and demerits of African GLVs as sources of natural antioxidants that can reduce CDL in sub-Saharan Africa.

Oxidative stress has been linked with development of several CDL (Govindarajan *et al*., 2005). In sub-Saharan Africa, diseases associated with a high sugar and fat and low fibre content in the diet such as diabetes, cardiovascular disease, hypertension and cancer are increasing due to significant changes in the diet in communities migrating from rural areas to cities (Addo *et al*., 2007; Parkin *et al*., 2008; Walker *et al*., 2002). However, the magnitude of CDL in these populations has been under-recognized and under-prioritized because of competing health priorities such as HIV/AIDS, tuberculosis and malaria (Parkin *et al*., 2008). Numerous studies suggest that phenolics such as those found in African GLVs provide bioactive mechanisms that can potentially reduce the risk of developing these diseases (Boyer and Liu, 2004; El Gharras, 2009; Hertog *et al*., 1992 and 1995). GLVs were strongly associated with an 11% reduction in risk of cardiovascular disease by Hung *et al*. (2004). Very little is known regarding the antioxidant status of the sub-Saharan African population. In a study of secondary data intake, Louwrens *et al*. (2009) reported that South Africans only consumed about half of their total antioxidant requirement. The findings of the present study indicate that African GLVs contain phenolics that have antioxidant properties (Sections 2.1 and 2.2). The burden of CDL could therefore possibly be reduced, in part, by promoting certain lifestyle factors such as consumption of African GLVs, which can potentially increase dietary polyphenols intake.

This study has shown that African GLVs contain higher phenolic content and antioxidant activity than spinach. Although this study found that boiling decreased the TPC, TFC and TAA, the levels remained appreciably high in boiled samples. African GLVs protected the biological molecules and cell cultures against oxidative damage better than spinach. Boiling has also been shown in Section 2.3 to have destroyed cytotoxins and decreased the potentially damaging effects of extracts to the cell cultures (Table 3.1). Antinutritional factors have been reported in African GLVs by Ejoh *et al*. (2007), Oboh *et al*. (2005) and Vanderjagt *et al*. (2000). Several antinutritional factors such as phytates may lead to mineral deficiencies, especially in individuals with largely plant-based diets. However, because these vegetables

are consumed after boiling, these antinutritional factors will be reduced and their effects may not be observed.

It remains unclear whether these beneficial antioxidant activities will deliver positive health effects after consumption of African GLVs *in vivo*. During absorption and distribution in the body, phenolics undergo high level of metabolism and biotransformation which greatly diminishes their H-donating and antioxidant capacity (Sies, 2010). Although little is known about the absorption and metabolism of phenolic GLVs components, improved *in vivo* antioxidant capacity has been reported in human subjects after consumption of some plant-based foods. Upon ingestion of the food, most polyphenols are metabolized by hepatic and intestinal enzymes and degraded and some part taken up into the bloodstream and other tissues (Galleano *et al*., 2010). Concentrations of TPC reaching plasma from a cup of green tea (1.2 g) estimated at approximately 1.6 - 2.0 µg/ml was reported by Grinberg *et al*. (1997). However, concentrations of antioxidants are reportedly transient (about 2 - 6 h) after consumption in humans and rodents (Holt *et al*., 2002; Schroeter *et al*., 2006).

Flavonoids can exert biological actions even when they are not taken up by cells (Erlejman *et al*., 2006 and 2008), i.e. within the gastrointestinal tract (Fig. 3.4). It has been estimated that flavonoids can reach concentrations higher than 300 µM in the gastrointestinal tract (Table 3.2) (Deprez *et al*., 2001). Figure 3.4 shows that even before absorption, polyphenols play an important role in protecting the gastrointestinal tract from oxidative damage, especially by direct prevention and quenching of oxygen radical species (ROS), which results in the delay of development of stomach, colon and rectal cancers (Halliwell, 2007; Halliwell *et al*., 2000). Some studies have shown that phenolics are extensively metabolized *in vivo*, mainly during transfer across the small intestine, by colonic microflora and in the liver, resulting in significant alteration in their redox potentials (Day and Williamson, 2001; Donovan and Waterhouse, 2003; Ginsburg *et al*., 2011). After undergoing phase I deglycosylation, the phenolic aglycones are believed to be converted to glucuronides, sulphates and O-methylated derivatives during phase II metabolism (Rice-Evans, 2001; Schroeter *et al*., 2003). Colonic bacteria play an important role in flavonoid metabolism and absorption and the resulting derivatives. The flavonoids and their metabolites are then responsible for the scavenging of reactive nitrogen, chlorine and oxidative species, generated by the gastrointestinal tract itself or by chemical reactions of dietary components such as iron, ascorbate, heme proteins, lipid peroxides and nitrite in the stomach (Amaral *et al*., 2009).

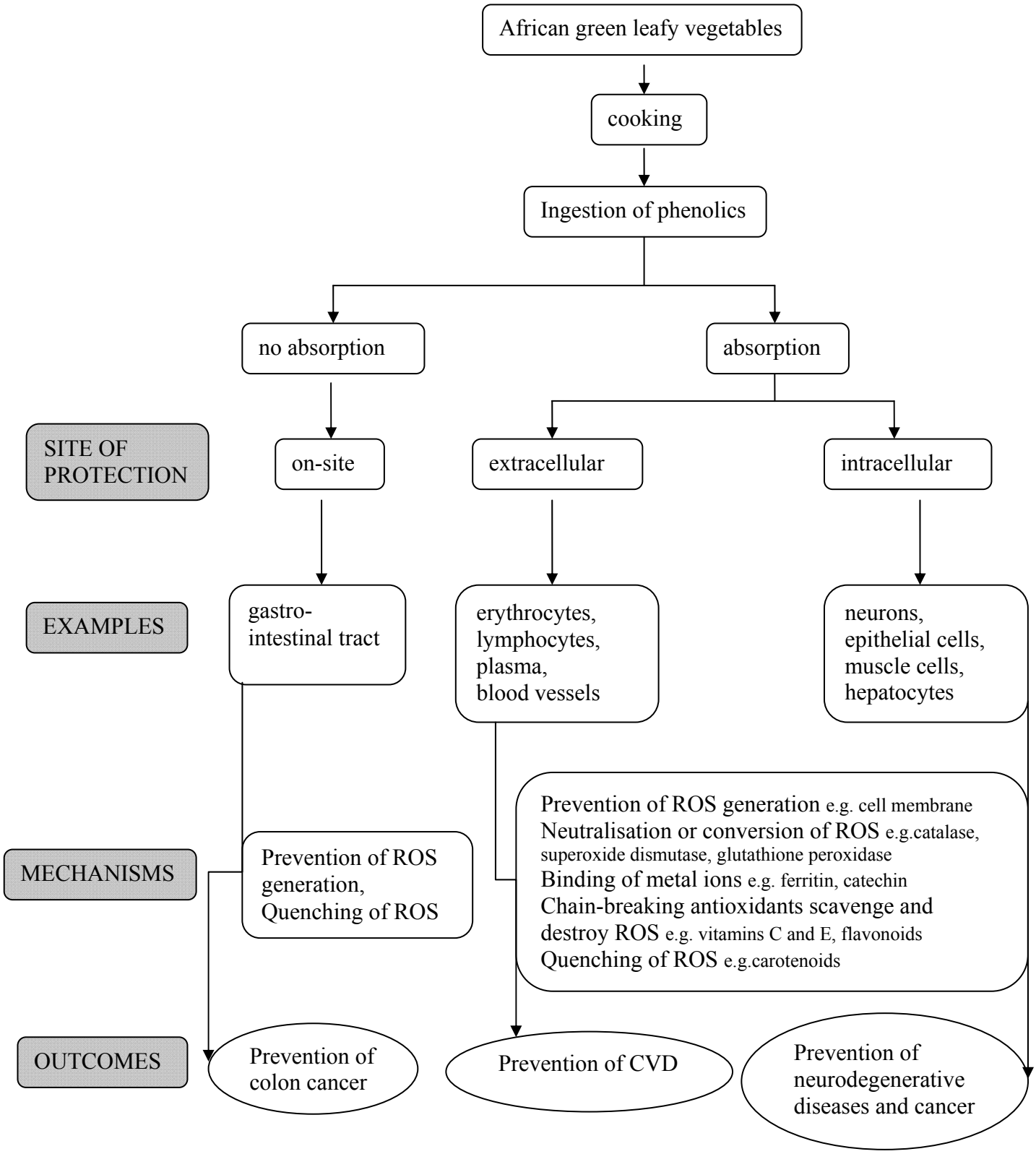

Figure 3.4 A schematic diagram illustrating the process involved in health-promoting effects of African green leafy vegetables.

The concentration of absorbed flavonoids depends on their absorption, distribution, retention, metabolism and safety in the cells (Noguchi *et al*., 2000). Figure 3.4 further illustrate that depending on the cellular compartment (i.e. extra- or intracellular), polyphenols are able to remove the ROS through enzymatic and non-enzymatic antioxidant reactions. ROS are formed within the body through various physiological processes (Benzie, 2003). Extracellularly, polyphenols bind to the cell membranes (Ginsburg *et al*., 2011), where they act as physical barriers preventing ROS generation or ROS access to the important biological biomolecules (Benzie, 2003). Superoxide, which is also a source of other ROS, is a key oxidant because it is produced constantly in the mitochondria from electron leakage during their passage along the respiratory chain. Superoxide reacts with nitric oxide, which is produced by endothelial cells, to give peroxynitrite that has a higher oxidizing potency. Superoxide can be converted to hydrogen peroxide by superoxide dismutase (SOD).

ROS such as alkoxyl and peroxyl radicals are generated through the decomposition of hydroperoxides (hydrogen peroxides) by transition metals such as iron and copper. Enzymatic antioxidants such as glutathione peroxidase and catalase reduce the hydrogen peroxide, thereby preventing the formation of ROS. Certain proteins such as ferritin and flavonoids such as catechin are also known to prevent the formation of ROS by sequestering transition metal ions (Arora *et al*., 1998). Non-enzymatic antioxidants such as carotenoids, α-tocopherol and vitamin C directly scavenge ROS and act as quenchers of singlet oxygen which oxidizes unsaturated lipids to give hydroperoxides. All these antioxidant actions take place extra- and intracellularly, resulting in the prevention of cardiovascular diseases (CVD) (Schroeter *et al*., 2010), as well as neurodegenerative diseases and cancer (Boyer and Liu, 2004), respectively (Fig. 3.4).

The potential health effects of flavonoids have recently been challenged, because of low concentrations of flavonoids in the systemic circulation and in tissues, as compared to other endogenous and exogenous antioxidant compounds and enzymes (Justino *et al*., 2004). Although, studies on the assessment of tissue and cellular concentrations of flavonoids are limited, studies on plasma bioavailability and cell cultures have shown relatively low tissue and cellular concentrations of flavonoids (Table 3.2).

Table 3.2 Estimated flavonoid presence in different body compartments (modified from Galleano *et al.*, 2010).

	Gastrointestinal tract	Extracellular/vascular compartments	Intracellular compartments
Total flavonoid content[a]	300 µM[b]	≤ 3 µM[c]	< 3 µM
Flavonoid oligomers or polymers	High concentration	Low concentration	Very low concentration or absence
Glycosylated/methylated flavonoids	High amount	unknown	unknown
Metabolites from breakdown of flavonoids	Principally from colonic microflora	Principally from enterocytes and extracellular metabolism	Principally from intracellular metabolism

[a] Considered as the sum of the different forms (original compound and metabolites) in which each individual flavonoid can be measured in human tissues and fluids, [b] Deprez *et al.*, 2001, [c] Benzie, 2003.

As indicated in Table 3.2, it is estimated that after consumption of flavonoid-rich foods, concentrations of flavonoids and their metabolites in the gastrointestinal tract remain high, leading to higher free radical scavenging capacity of flavonoids in the gastrointestinal tract. In blood, concentrations of flavonoids and their metabolites are estimated to be low because of poor bioavailability (Rahman *et al*., 2006), strict regulatory mechanisms preventing excess toxic polyphenols to the circulation, as well as competition from other relevant antioxidants such as catalase, SOD and haemoglobin (Ginsburg *et al*., 2011). In plasma and cells, the concentrations of flavonoids are even lower because of further competition with endogeneous antioxidants such as α-tocopherol, ascorbate and glutathione (Galleano *et al*., 2010). It is estimated that cells have around 10^7 - 10^{10} molecules of glutathione, and less than 10^3 flavonoid molecules, making the scavenging of radicals by flavonoids in the cells very limited (Galleano *et al*., 2010). However, regardless of body tissue or cellular compartment, flavonoid levels in humans are transient, and therefore require frequent consumption of flavonoid-rich foods to sustain a steady state concentration.

Polyphenols at concentrations of 2 - 20 µM were found to have effective protection effects against cytotoxicity in a number of studies (Blasa *et al*., 2007; Deng *et al*., 2006; Grinberg *et al*., 1994 and 1997). These low concentrations possibly indicate polyphenols binding to low molecular iron species rather than direct $HO^{\bullet}$ scavenging activity that requires high concentrations of antioxidants. Some biological effects of flavonoids that only require small concentrations have been linked to modulatory actions in the cell, by influencing the cellular processes of signal transduction mediated by oxidants (Rahman *et al*., 2006).

Even at low concentrations, consumption of African GLVs could strengthen and protect biological molecules from free radical-induced oxidative damage by increasing plasma and cellular levels of antioxidants. Increased plasma total antioxidant capacity after consumption of vegetables may partly be attributed to the antioxidant activity of the phenolics, predominantly because of the free OH groups (Sies, 2010). After reviewing a number of studies on dietary antioxidants and their beneficial effect on oxidatively damaged DNA, Moller and Loft (2006) suggested that protective effect of antioxidants may be seen only in the presence of an oxidative stressor. Frei (2004) also confirmed that the antioxidant intake does not reduce oxidative stress biomarkers appreciably in healthy individuals, but that the intake would lower such biomarkers in subjects with malnutrition, increased oxidative stress and diseases related to oxidative stress. It is therefore possible that an increased consumption

of African GLVs would only enhance antioxidant functioning of cell biomolecules in individuals with deficient antioxidant systems and those with underlying chronic diseases.

Overall, the physiological significance of African GLV phenolics depends on their mechanisms and the total sum of absorption, distribution, metabolism and excretion (ADME). If absorbed, these compounds could yield health-promoting benefits by increasing the concentration of endogenous and exogeneous antioxidant compounds and enzymes in the systemic circulation and in tissues. If not absorbed, these GLVs could still provide good health effects in the gastrointestinal tract by directly scavenging the free radicals, leading to prevention of cancers such as colon cancer. It is therefore important that these GLVs are consumed in higher quantities in order to effect these health-promoting properties, especially in individuals with low antioxidant status, those with underlying chronic diseases, as well as vulnerable groups such as the old-aged and children.

African GLVs are seasonal and, unlike their exotic counterparts, are not readily available in the urban areas. It is therefore difficult to obtain these vegetables in the urban areas where the prevalences of CDL are increasing as found by Cronjé *et al*. (2009). Furthermore, these vegetables are often considered to be inferior in their taste and nutritional value compared to exotic vegetables such as spinach and cabbage (Weinberger and Msuya, 2004). Many people in sub-Saharan Africa cannot afford expensive chemotherapy medicine necessary to reduce or manage these CDL. Owing to the possibilities of diverse biological activities of the phenolics in African GLVs discussed above and the need for natural dietary chemopreventive compounds that are able to provide health-promoting effects, there is a need to promote these vegetables because of their ability to protect against cellular oxidative damage. African GLVs are cost effective, natural and have antioxidant properties that can assist to decrease the risk, development and progression of these diseases.

Oxidative stress leads to development of CDL. African GLVs have been shown to reduce oxidative damage to biomolecules and cell cultures. The benefits of consuming African GLVs rather than spinach are therefore promising. These vegetables grow in the wild and are well adapted to harsh environmental conditions prevalent in sub-Saharan Africa. They also grow well with minimal water and poor soil fertility. It is therefore convincing that African GLVs should be promoted, cultivated, consumed and possibly commercialized for the benefits of the population at risk of developing CDL in sub-Saharan Africa.

CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

This study indicates that African GLVs, especially jute mallow and cowpea, contain higher total phenolics and antioxidant activity than spinach. Phenolics make a large contribution to the antioxidant activity and biological protective effects of these GLVs. Boiling decreases the phenolic content and antioxidant activity of these vegetables. In comparison to water, the 75% acetone is more effective in extracting antioxidants from the GLVs. In practice, the results of water extracts are the better representatives of actual antioxidant properties obtained from GLVs after consumption. Because African GLVs have higher antioxidant properties, they have a potential to reduce CDL associated with oxidative stress, due to their increased radical scavenging abilities.

The type and content of flavonoids present in GLVs seem to be dependent on the type of vegetable and extraction solvent. The effect of boiling on these phenolics is also dependent on the type of flavonoid and GLVs. GLVs that exhibit higher antioxidant activity and cellular protective abilities against oxidative damage have appreciable levels of total flavonoids, especially their 75% acetone extracts. The types of flavonoids found in African GLVs are similar to those found in spinach, although with varying levels depending on the extraction solvent.

Extracts of African GLVs exhibit significant superior protection against oxidative damage in plasmid DNA and cell cultures than spinach. Phenolics in GLVs seem to contribute to these protective effects because the GLVs containing high levels of phenolics protect against oxidative damage in biological molecules and cell cultures better than those with low levels of phenolics. Water extracts of raw jute mallow have low levels of phenolics and cause damage to the erythrocytes, while raw spinach which also has low levels of phenolics exhibits more cytotoxicity to the SC-1 fibroblast and Caco-2 cells. Caco-2 cells are more sensitive to the toxicity in extracts of raw samples than the SC-1 fibroblast cells. Boiling reduces the cytotoxins found in raw extracts, because extracts of boiled GLVs show less toxicity and offer considerably superior protection of the cell cultures, possibly partly due to the antioxidant activity of the phenolics.

The study indicates that African GLVs are valuable plant foods. Their nutritional content and antioxidant properties are in most cases higher than for the exotic vegetables such as spinach. Although some of the extracts of raw African GLVs possibly have cytotoxic compounds and antinutritional factors that may cause cytotoxicity, boiling has been shown to reduce these factors. These vegetables have appreciable levels of antioxidant properties including protection against oxidative damage to plasmid DNA and cell cultures as identified in this study, which can be largely attributed to their phenolic compositions.

African GLVs are drought-tolerant and well adapted to the harsh environment of sub-Saharan Africa. They grow in the wild and not readily available in urban areas because they are not grown for commercial reasons. Based on the findings of this study, the phenolic compositions of these GLVs are higher than those of spinach, which is an exotic vegetable and commonly consumed in the region. The *in vitro* chemical, biological and cellular protective effects and radical scavenging abilities observed in extracts of African GLVs suggest that these vegetables have the potential to reduce CDL associated with oxidative stress.

Further studies should be conducted to identify and characterize the specific phenolics present in the extracts of African GLVs, using the HPLC/DAD/MS or GC/MS. This will allow for a more specific determination of other phenolic compounds apart from the ones identified using the HPLC method in this study. The results will be used to relate the phenolic structure with antioxidant activity and cellular protective effects. Elucidation of the mechanisms of action of African GLV extracts in several physiological processes including cellular signal transduction, cell differentiation, apoptosis and inflammation would also yield important insights into their total prophylactic uses.

The findings from this study should form the basis to promote consumption of these vegetables, especially in rural areas where these vegetables grow in the wild as well as among urban dwellers. A campaign is needed to demystify the perception that exotic vegetables have more nutritional and health benefits than indigenous ones. This is important especially with the increasing prevalence of CDL in communities that traditionally consume these vegetables, and because consumers are now advocating for natural products with health-promoting properties. Studies on bioavailability of phenolics from African GLVs as well as

epidemiological studies should also be done to determine if the protective effects observed in this study will in fact result in reduction of CDL after consumption of these vegetables.