A COMPARATIVE STUDY ON CARBOHYDRATES AND ANTIOXIDANTS OF INDIGENOUS CROP BLACK JACK (*Bidens Pilosa L.*) AND SELECTED COMMERCIAL VEGETABLE CROPS

By

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Declaration

I, Nelani Simon Mbokazi, declare that the research reported in this thesis, except where otherwise indicated, is my original work. This thesis has not been submitted for any degree or examination at any other university.

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Nelani Simon Mbokazi
Final Date:

Certify that the above statement is correct.

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Dr. Samson Tesfay
Supervisor

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Abstract

The adequate consumption of African leafy vegetables has been closely associated with a strong reduction of chronically disease such as cancer, diabetes and cardiovascular diseases. The health benefits provided by African leafy vegetables are due to the presence of various primary and secondary metabolites. However, most of the epidemiological studies have indicated that very little is known about the antioxidant activity of African leafy vegetables, which is believed to be responsible for their therapeutic effect. In this present study the physiochemical and antioxidant properties were examined, in relation to other *Asteraceae*, commercial vegetables (lettuce and chicory). Firstly, the study investigated the physicochemical and antioxidant compounds during seed germination in black jack. Secondly examined the non-enzymatic and enzymatic antioxidants in black jack and compared them with lettuce and chicory.

In seed germination test, physicochemical seed quality properties for African leafy vegetable, black jack antioxidants accumulation during seed imbibition were reported. The results revealed that soaking of black jack seeds in water before sowing, induces germination. In this study black jack seeds that were soaked for 15 hours before germinating, showed a highest percentage of germination (72 %) in 5 days. The seeds also showed high considerable total antioxidants capacity of antioxidants (DPPH) and phenols (0.69±0.44 mg/g and 56.45±0.08 mg/g DW). The protein content was also high on the seeds (0.328±0.17 mg/g DW). However, there were high amounts of anti-nutritional factors noticed on the seeds, where the total tannins content was (416.36±1.14 mg/g DW).

The results further revealed that African leafy vegetable, black jack contained significant amount of non-enzymatic antioxidants at the early stages of growth than other leafy vegetables. The plant biomass per plant increased with the number of harvests. The plant DPPH antioxidant assay recorded black jack (0.73 ± 0.13mg/g DW - 0.29±0.083mg/g DW), lettuce (0.10±0.64mg/g DW - 0.29±0.03mg/g DW) and chicory (0.35±0.72 - 0.20±0.11mg/g DW). The plant phenolic content recorded black jack (155.46±0.07mg/g DW - 73.11±0.02mg/g DW) for lettuce it was found to be (13.24±0.05mg/g DW - 44.92±0.07mg/g DW) and for chicory (97.09±0.37mg/g DW - 17.88±0.22mg/g DW). However, as black jack all of the secondary metabolites were decreasing drastically when it was reaching maturity, while the phenols were increasing. For carbohydrates, black jack had the lowest concentration of the soluble sugars (glucose, sucrose and fructose). The enzymatic antioxidants of black jack were the lowest for most of enzymes, but SOD activity was higher. Although it decreased as the plant approaches maturity.

In conclusion, black jack accumulates different types of antioxidants and their concentration varied over plant developmental stages. The key findings of this study are; the African leafy vegetables have different antioxidant production trends compared to exotic vegetables. Depending on leaf positions and leaf stage, preferably young leaves of the ALVs, there might be sequential harvests, increases the food access for extended period for household consumption. The ALVs also experience higher SOD, CAT, POD activities during early growth stage. These plants have also displayed the highest antioxidant capacity during the early plant development, early stage high accumulation of the studied antioxidants most likely contribute to this antioxidant strength. Furthermore there adaptation to wild environment, exposed to various harsh

conditions, their tolerance to survive to this condition probably attributed to plants' antioxidant production characteristics.

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Literature review

1.1 Introduction

In most scientific works, indigenous crops are considered to be the highest group of underutilized crops as a result of their narrowly use. In general indigenous refers to the crop species or variety genuinely traditional or originated to the region (Weinberger, 2007). This definition also takes into account naturalized species or varieties that have evolved from materials introduced to the country from another geographical region over a long period of time (Hoeschle & Jaenicke, 2007). The crops that are domesticated or produced scientifically are not included in this definition. Humphry *et al.* (1993) argued that indigenous crops are referred to terms like neglected or underutilized, because they are believed to be poor man food, which is mostly consumed during times of hunger or droughts and /or during social unrest and wars.

The FAO (1996) reported that global food security depends largely on only few crop species. These crops almost provide about 95% of the dietary energy for most human beings, where maize, wheat and rice alone received more recognition worldwide more than any other crop. There are more than 7500 plant species that are cultivated around the world and they include mostly the vegetables and legumes and are also some estimated 70000 edible plants that can be found on earth of which most of these plant species are considered underutilized because they have never been planted on commercial or large scale farms (Hoeschle & Jaenicke, 2007).

The food base of many people especial those residing in rural areas of Africa have been shown to decline over years and that has resulted in many problems such as high levels of poverty. Malnutrition has also increased over time resulting in compromised well-being and high mortality rates among young children and women who are giving birth in rural Southern Africa as a result of insufficient food supply (WHO, 2003; Awino, 1989).

Most of the underutilized African leafy vegetables have been reported to have an excellent nutritional profiles compared to major commercial crops (Allemann, 2007). These leafy vegetables contain high protein, vitamins and mineral content that can contribute to the reduction of malnutrition among children as well as alleviation of food insecurity, especially in the low income communities (Engler & Altoos, 2001).

In most epidemiological studies, it has been indicated that, consumption of indigenous leafy vegetables can play a huge role in the reduction of risk of certain cancers, cardiovascular disease, cataract, mascular degeneration, and other age related diseases due to the high antioxidant activity of leafy vegetables (Viviane *et al.,* 2011). Most of the leafy vegetables have been shown to be the essential source of various dietary antioxidants, such as carotenoids (which are the main procuresors of vitamin A biosynthesis) of which the deficiency leads to blindness and other physiological disorders. Dietary antioxidants are also valuable for many other physiological functions for promoting good health such as boosting immune system, photoprotection and preventing aging of cells (Bhutkar & Bhise, 2011).

Engler & Altoveros (2001) argued that most of the indigenous crops mainly the vegetables ones are categorised as neglected or underutilized, because in most cases they are mainly planted in home gardens and used by very small groups of people in certain geographic areas. Engler & Altoveros (2001) further stated since the indigenous crops are underutilized or neglected, due to the lack of information among the people for their use, and more attention is given to high value commercial crops. Underutilized crops are

therefore facing danger of being replaced or lost and the indigenous knowledge associated with their cultivation, utilization and conservation is also endangered.

In South Africa, there are also many indigenous crops, currently termed underutilized as a result of their limited use.Vorster *et al.* (2002) states that in South Africa there are several lesser known plant species, which have remarkable potential to be used as commercial vegetables. The biggest advantage of these crops is that they do not require high intensive technology and input levels, they can be grown well on the diversity of climatic conditions of a country, at the same time reducing the issue of malnutrition among young and older people. Rensburg *et al.* (2007) stated that it is very important that promotion of underutilized crops in terms of poverty alleviation and nutritional improvement of the rural households to be given priority, due to their potential to bring economic empowerment in rural communities whilst it could also improve the well-being of urban communities. Subsequently, both rural and urban community poverty and food insecurity related challenges would be dealt with thereof.

1.2 General overview of antioxidants

According to Benzie & Straint (1996) biological antioxidants are any substance, when present at low concentrations compared to those of an oxidizable substrates significantly delay or prevent that substrate. On the other hand Chanda & Nagani (2010) defined antioxidants, as any compounds that inhibit the oxidation of other molecules by preventing the initiation or propagation of oxidizing chain. Antioxidants are generally involved in the prevention of reactive oxygen species (ROS) which are free radicals that are capable of causing huge damage in biological systems.

Potentially harmful reactive oxygen species (ROS) are produced as a result of normal metabolism and these free radicals are charged molecules which attack and damage cells, breaking cellular membranes by reacting with nucleic acids, proteins and the enzymes which are in the cell (Benzie and straint, 1996; Valko *et al.*, 2007 & Villamor *et al.*, 2004). Lushchak (2011; Ajaikumar *et al.* (2005) reported the oxidative damage caused by the action of free radicals, which is capable of initiating and promoting the progression of number of chronic diseases such as cancer, neurodegenerative disorders, cardiovascular diseases and ageing.

In nature there are two types of antioxidants, natural and synthetic ones. Natural antioxidant include various plants (fruits and vegetables) with significant scavenging properties as a result of being a source of secondary metabolites. Natural antioxidants have been reported to be safer than synthetic antioxidants, and they also possess anticancer, antiviral, anti-inflammatory, anti-tumour, anti-mutagenic and heptoprotective properties (Laxmi *et al.*, 2012). The major sources of natural antioxidant are all or any part of a plant such as fruit, vegetable leaves, seeds, stem, peels or roots.

Currently, the search for natural antioxidants in plant sources especially those with medicinal potential, has turned into a very interesting and attractive research field with the major focus of improving the quality of the human life. According to Jin & Russell (2010) polyphenols and phenolic acids, flavonoids, tannins and other significant plant metabolites are wildly distributed in plant foods and they all function as effective free radical scavengers. In healthy individuals the production of free radicals is balanced by the antioxidative defense system, but the oxidative stress is generated when equilibrium favors free radical generation as a

result of a depletion of antioxidants levels (Tesfay *et al.*,2010).

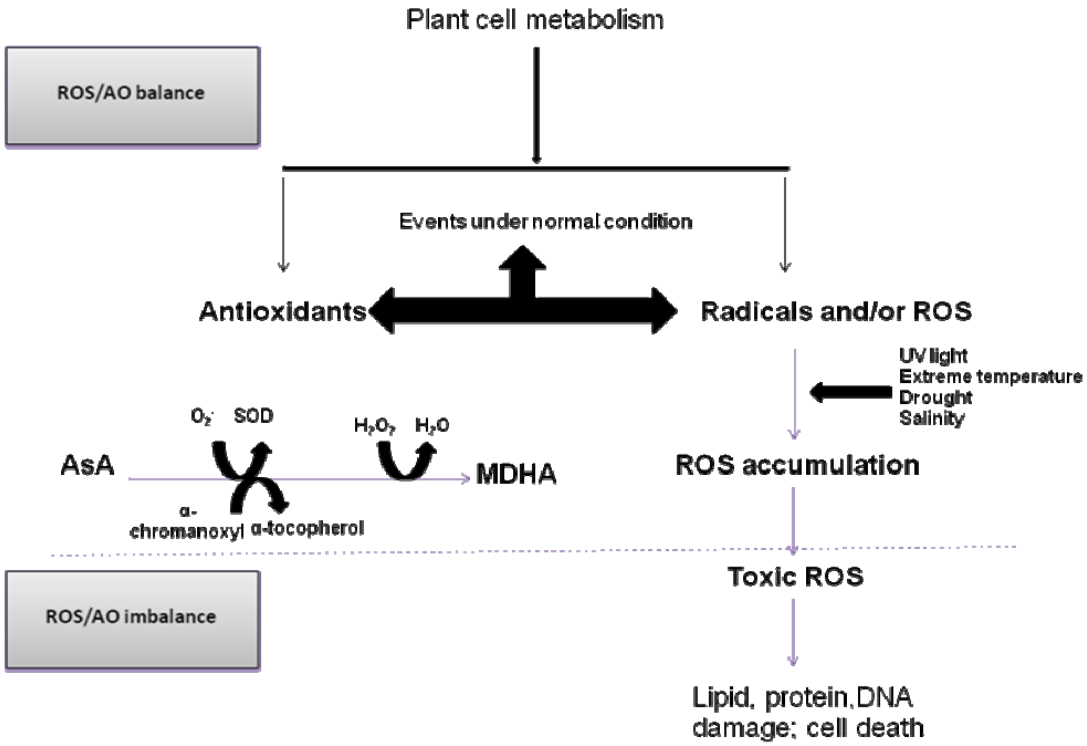


Figure 1: Schematic diagram of the antioxidant activity and free radical production under normal and stressful conditions (Tesfay *et al.*, 2010).

1.3 Classification and properties of major dietary antioxidants

Antioxidants are divided into enzymatic and non-enzymatic groups based on a system where they are found and functioning. The effectiveness of non- enzymatic antioxidants found in plants to act as free radical scavengers is related to three major groups which are vitamins, phenolics and carotenoids, where ascorbic acid and phenolics are termed water-soluble hydrophilic antioxidants, while carotenoids are termed water-insoluble lipophilic antioxidants (Marino *et al.*, 2001; Chong, 2010). On the other hand, the effectiveness of

enzymatic antioxidants to act as radical scavengers is also related to three major groups which are SOD, CAT and POD.

1.3.1 Ascorbate (Vitamin C)

Ascorbic acid (vitamin C) is a very important and essential vitamin in human diet, it is found in plant tissues where it is abundant with a chemical formula of ($C_6H_8O_6$). In plants ascorbic acid plays a very important role, functioning by protecting the plants from biological and environmental stresses.

Vitamin C is known as the most essential water soluble antioxidant in extracellular fluids, it functions on the neutralization of reactive oxygen species in the aqueous phase before lipid peroxidation is initiated (Patrica & Conklin, 2006). Ascorbic acid provides many other benefits except being a free radical scavenger, it is capable of regenerating other antioxidants such as tocopheroxyl and carotene radical cation from their core radical (Nangula *et al.*, 2010).

The properties of ascorbic acid to act as the effective antioxidant are attributed by its ability to reduce potentially damaging ROS and forming a relatively stable ascorbly free radical (Carr & Frei, 1999). Ascorbate has the greater ability to act as a reducing agent and, a single electron donated by ascorbate gives ascorbyl radicals which are known as monodehydroascorbate (MDHA) or semi dehydroascorbate (SDA), which further oxidized to dehydroascorbate (DHA) (Borut & John, 2009). DHA is a very unstable compound which further breakdown rapidly, resulting to diketo-L-gulonic acids which also disintegrate to

oxalic and L-threonic acid (Borut&John,2009).

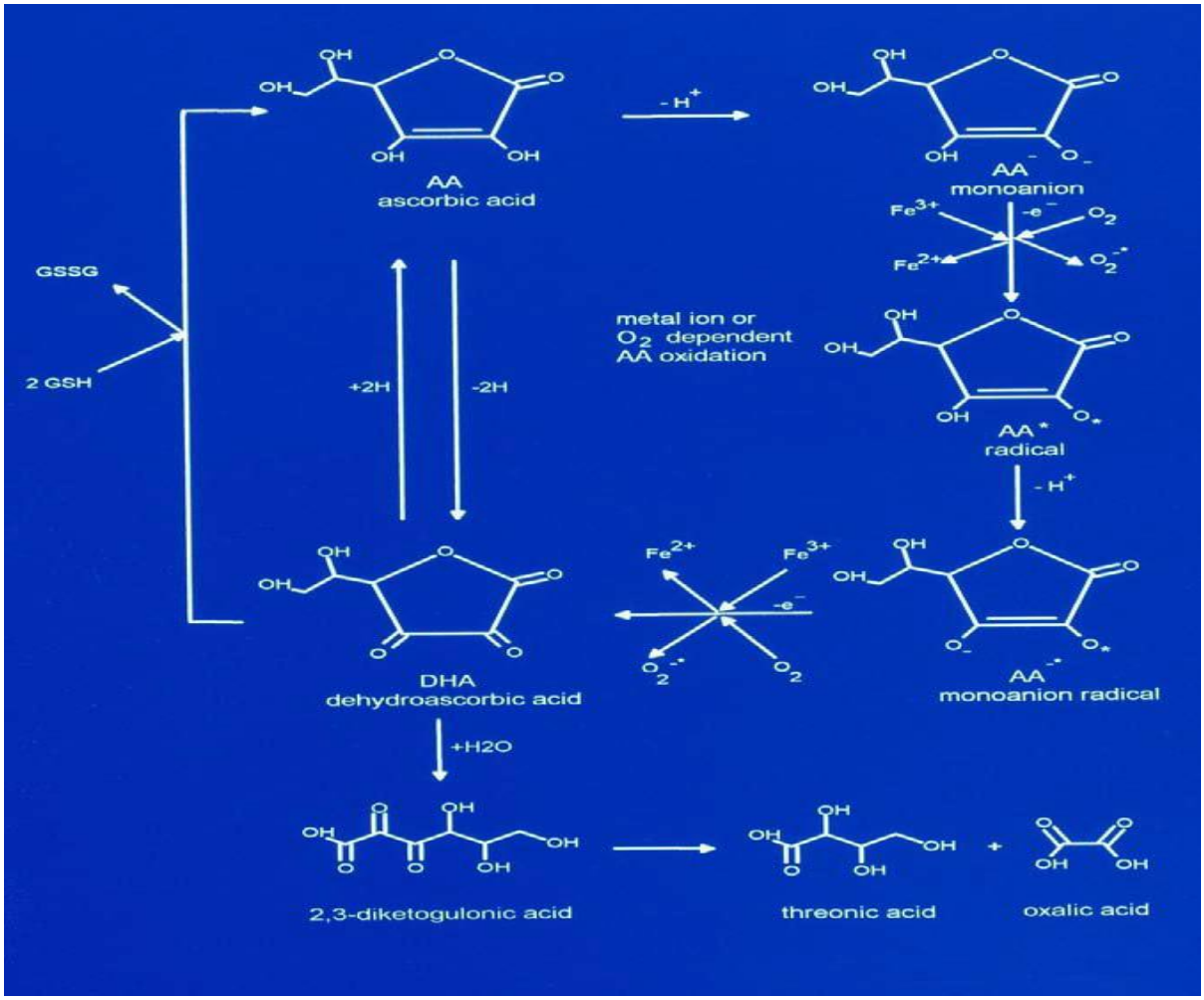


Figure 2: Ascorbate redox cycle and catabolic pathways (Borut & John, 2009).

Ascorbate in plants is used as coenzyme in the Fedioxygenase to function in the post-translational modification of cell wall proteins (Zhang, 2013). Fry (1986) argued that dehydroascorbate in plants, is capable of interacting with the side chain of arginine and lysine in the enzymes to prevent protein crosslinking. Ascorbate is shown to modulate plant growth, by controlling biological processes such as the biosynthesis of hydroxyproline rich proteins, which are required for the functioning of G1 and G2 phases of

the cell cycle and the plasmalemma redox reactions, which are functioning in the elongation mechanisms (Zhang, 2013). Ascorbate is also responsible for influencing senescence of plants by modulating the expression of SAGs (Olmos *et al.*, 2006). Barth *et al.* (2006) stated that in vegetables low levels of ascorbate induce the senescence, while higher levels of ascorbate prolongs senescence.

1.3.2 Phenols

Plant phenolics are the most broadly abundant secondary metabolites in plants with more than 8000 phenolic structures that are currently known ranging from simple molecules like phenolic acids to more highly specialized polymerized substances like tannins (Jin & Russell, 2010). Phenolics are compounds that are characterized by one or more aromatic rings with one or more hydroxyl groups (Jin & Russell, 2010). Phenols are synthesized from cinnamic acid which is created from phenylalanine through the action of L-phenylalanine ammonia-lyase (PAL), which is the branch point enzyme between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism (Michalak, 2006). Plant phenolics are mainly divided into three groups, which are notable as phenolic acids, flavonoids and tannins, but they are also less common groups such as stilbenes and lignans (Zbigniew, 2005). Phenolic acids can further be divided into two classes, which are the derivatives of benzoic acids such as gallic acid and the derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid (Zbigniew, 2005). The flavonols are the most common phenolics in the diets; they are characterized by 15 carbon atoms arranged in three rings (C_6-C_3-C_6). Flavonoids are also divided in several subgroups: flavones, flavanols, flavanones, isoflavones, and anthocyanins, based on the oxidation state of center C ring (Zbigniew, 2005). Tannins they belong to the major group of polyphenols which is also mostly abundant in the diet, they are divided into two main

groups: hydrolysable tannins, characterized by a central core of glucose or another polyol esterified with gallic acid and condensed tannins.

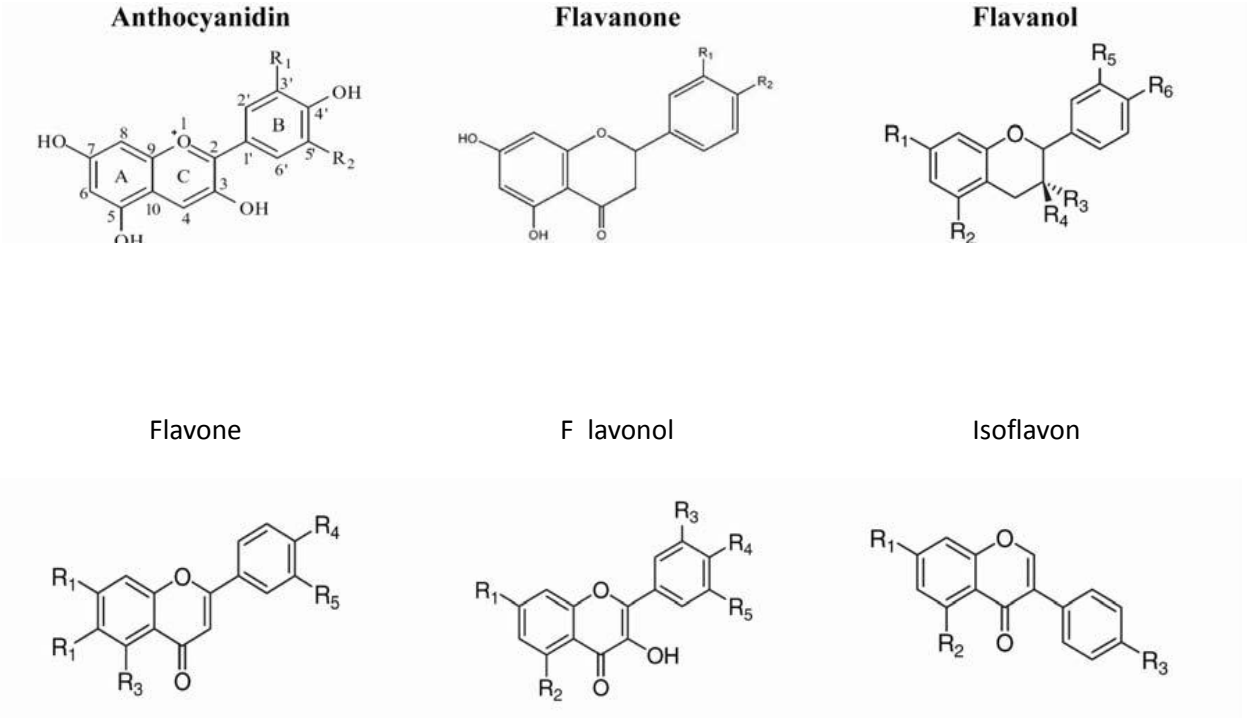


Figure 3: Different structures of phenolics, and flavonoids (Jin & Russell, 2010).

Phenolics in plants they serve many functions such as pigmentation, growth, reproduction, and the enhancement of phenylopropanoid metabolism and, the amount of phenolic compounds can be observed under different environmental factors and stress (Michalak, 2006). The isoflavones synthesis and other flavonoids are induced when the plants are infected or injured, or under low temperatures and low nutrient conditions (Michalak, 2006). Most of the plant phenols have antimicrobial activity and they also protect the plant from ultraviolet (UV) damage since the plants accumulate UV absorption flavonoids and other phenolic compounds mainly in vacuoles of epidermal cells and they also possess other physiological functions as they regulate cell elongation (Valentine *et al.*, 2003).

Phenols also have been reported to pose antioxidant activities (Zbigniew, 2005). The intensity and the ability of phenols to scavenge free radicals is dependent on many factors such as the number of hydroxyl groups bound to the aromatic ring and the number and places of double bonds in the molecule (Zbigniew, 2005). The ability of phenols to act as antioxidants is also contributed by the ability of phenols to chelate metals. Phenols they consist of hydroxyl and carboxyl groups which are capable of binding mainly iron and copper ions (Michalak, 2006).

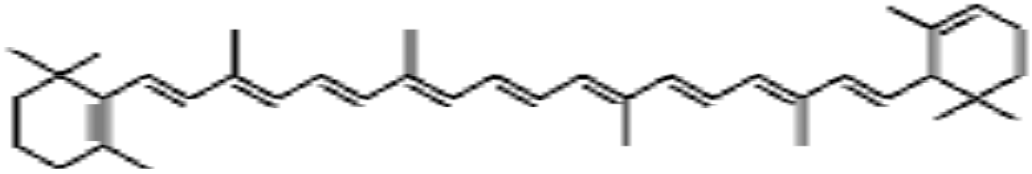
1.3.3 Carotenoids

Carotenoids are naturally occurring pigments that are found mainly on plants, and currently there are more than 750 carotenoids that have been identified in nature, mainly functioning in the coloration of many plants (i.e. yellow, orange and red colors) as well as several aromas in plants (Zhang *et al.*, 2011). In green plants, the colour of the carotenoids is masked by the more dominant pigment, chlorophyll and their concentration increases, becomes visible only plant growing results during the degradation of chlorophyll (Debjani *et al.*, 2005).

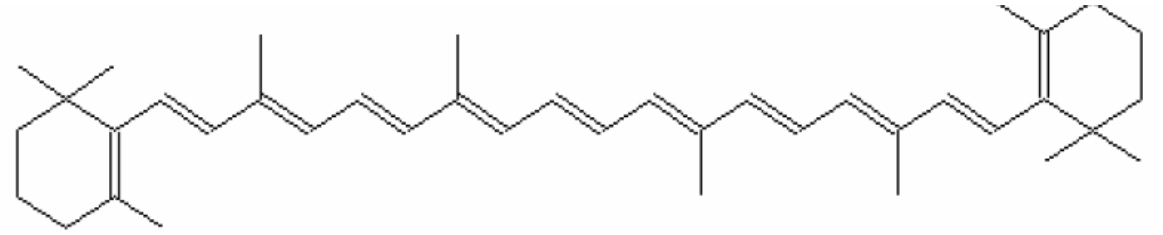
Carotenoids are lipophilic compounds, which are divided into two main groups, which are carotenes and /or hydrocarbon (Jafar & Qudah, 2009). Carotenoids only composed of carbon and hydrogen atoms and the second group are xanthophylls which are oxygenated hydrocarbon derivatives which contain at least one oxygen function such as hydroxyl-, keto-, epoxy, methoxy or carboxylic acid groups (Jafar & Qudah, 2009). Katri (1999) also defined carotenoids as the isopropanoid compounds that are biosynthesized by tail to tail linkages of two C_{20} geranylgeranyl diphosphate molecules. Which produces the parent C_{40} carbon skeleton where all the individuals variations are derived.

They widely known compounds on the first group of carotenoids (carotenes) are α-carotene, β- carotene and lycopene. B-carotene and α-carotene are the mostly known as provitamins. On the other hand, Di-mascio *et al.* (1989) stated that, Lycopene is not a provitamin but it is a more efficient antioxidant than β-Carotene. Luteine has been wildly reported to be also one of the major carotenoids found in leafy vegetables (Dragan *et al.,* 2011).

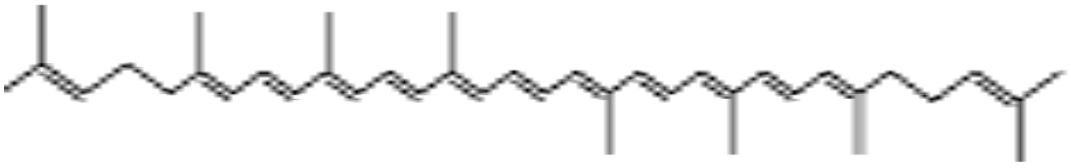
Jafar and Qudah (2009) further stated that lipophilic carotenoids display their structural characteristic by the conjugated double bond system, which influences their chemical and physical properties.



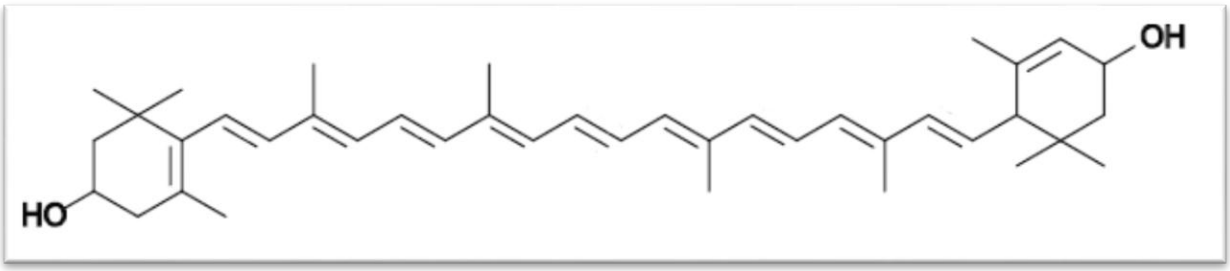
a Structure of α- carotene



b Structure of β- carotene



c Structure of lycopene



d Lutene

Figure 4: Chemical structures of various carotenoids of which (α-carotene) and b (β-carotene) are precursors of vitamin A (Jafar & Qudah, 2009).

The major ability of carotenoids to act as antioxidants is due to their ability to react and deactivate reactive oxygen species formed as results of free radicals, which may ended up producing harmful processes such as lipid peroxidation. The ability of β-carotenoids to act as an effective antioxidant is due to the electron rich conjugated double bond structure which permits β-carotenoid to physically prevent reactive oxygen species without degradation, during reacting with free radicals and for β-carotenoids to be instability towards oxidation (Briton, 1995).

Debjani *et al.* (2005) explained that there are four structural properties that contribute to antioxidant functions of carotenoids and they are:

- The availability of the multiplicity of tiny packed energy levels between the excited state and the ground state of the carotenoids, which permits the carotenoid to dissipate excited state of energy through small collisional exchange with solvents,
- The ability of the excited energy state of the carotenoid to be sensitized other molecules,
- The significance of the exciting state of the carotenoid to allow delocalization and stabilization of the excited state, and

- The multiple potential sites of the carotenoids to be attacked by the reactive oxygen species.

The ability of carotenoids to act as antioxidants is also based on the physical and chemical properties of carotenoids to prevent the formation of oxygen or by reacting with free radicals. The physical benefits of preventing the formation of oxygen species is that the carotenoids may act as the antioxidants without losing their own structure, whereas this reaction may only leads to energy dissipation as heat.

1.3.4 Chlorophyll

Chlorophyll is a photosynthetic green pigment found in most plants, chlorophyll is the most abundant of all natural pigments found in plants. It can exceed 1000 to 2000 ppm wet weight in some species (Mario *et al.*, 2007). Structural chlorophyll is a substituted tetrapyrrole with a central bound magnesium atom (Ferruzzi *et al.,* 2002). They are various forms of chlorophyll and all of them are characterized by the central bound magnesium atom, chlorophyll 'a' which is greenish to yellowish in solution, it is known as the primary photosynthetic pigment in most green plants for the transfer of light energy to chemical acceptors, which provides energy for photosynthesis.

One of these chemical acceptors is chlorophyll 'b' which is blue to green in solution, and it is prevalent in higher plants and green algae with chlorophyll 'a' (Sheer, 1991). In general, chlorophyll 'a' is more abundant over chlorophyll 'b' with a ratio of 3:1(Ferruzzi *et al.,* 2002).

Natural chlorophylls are catabolized into two related products: chlorophyllide and pheophorbide. The removal of the phytol tail from chlorophyll compound it forms chlorophyllide, and a removal of both the

phytol tail and chelated magnesium atom forms pheophorbide (Harttig & Bailey, 1998). The major structural differences between chlorophyll 'a' and chlorophyll 'b' is the methyl side chain in chlorophyll 'a', is replaced by a formyl group in chlorophyll 'b', so this result in converting chlorophylls into chlorophyllide 'a' (Chlide 'a') and chlorophyllide 'b' (Chlide 'b') or pheophorbide 'a' (Pho 'a') and pheophorbide 'b' (Pho 'b'), respectively (Ching – Yun *et al.*,2008)

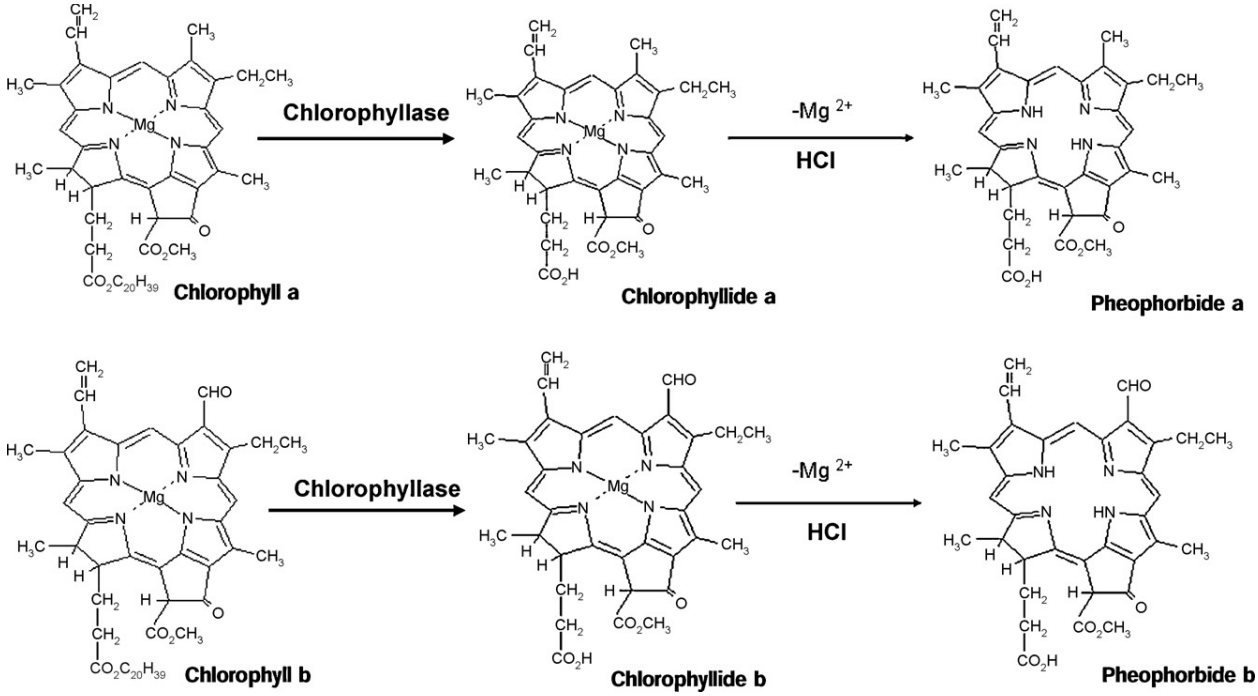


Figure 5: Chlorophyll a and chlorophyll b are converted into their respective derivative**.** Source (Ching – Yun *et al.*, 2008).

The amount and distribution of chlorophyll content on vegetables and fruits is dependent on the variety of internal and external factors such as species, climatic conditions and pre-and postharvest treatment (Gross, 1991). Mario *et al*. (2007) stated that chlorophyll and its various derivatives are also known for their long established history of being successful used in traditional medicine for therapeutic purposes. In medicine chlorophyll is also known for the significant role on the reduction of inflammation, wound healing and oxidation (Tachino *et al.*, 1994). The medicinal properties of chlorophyll are believed to be due to the

presence of antioxidant properties on the derivatives of chlorophyll (Hoshina, 1998). Wanasundara (1998) also stated that other studies have indicated that chlorophyll and it derivatives were also responsible for the pro-oxidant effect on the oxidation of oils**.**

1.3.5 Sugars

Carbohydrates are primary metabolites; they are in the same category as amino acids, fatty acids and organic acids. Primary metabolites play a very important role in growth and development, photosynthesis, respiration, protein synthesis and hormone regulation (Hounsome *et al*., 2008).

In vegetables carbohydrates occur in a simple form of sugar monosaccharides (galactose, glucose and fructose), disaccharides (sucrose and maltose), oligosaccharites (raffinose and fructooligosaccharides), polysaccharides (starch, cellulose and hemicellulose) and sugar alcohols (mannitol and sorbitol) (Wang and Van Eys, 1981). Kuo *et al.*(1988, and Frias *et al.* 1999) stated that monosaccharides, polysaccharides and sucrose are present and found in all vegetables. Whereas, oligosaccharides such as raffinose are only present in legumes (beans and peas) (Hounsome *et al.*, 2008).

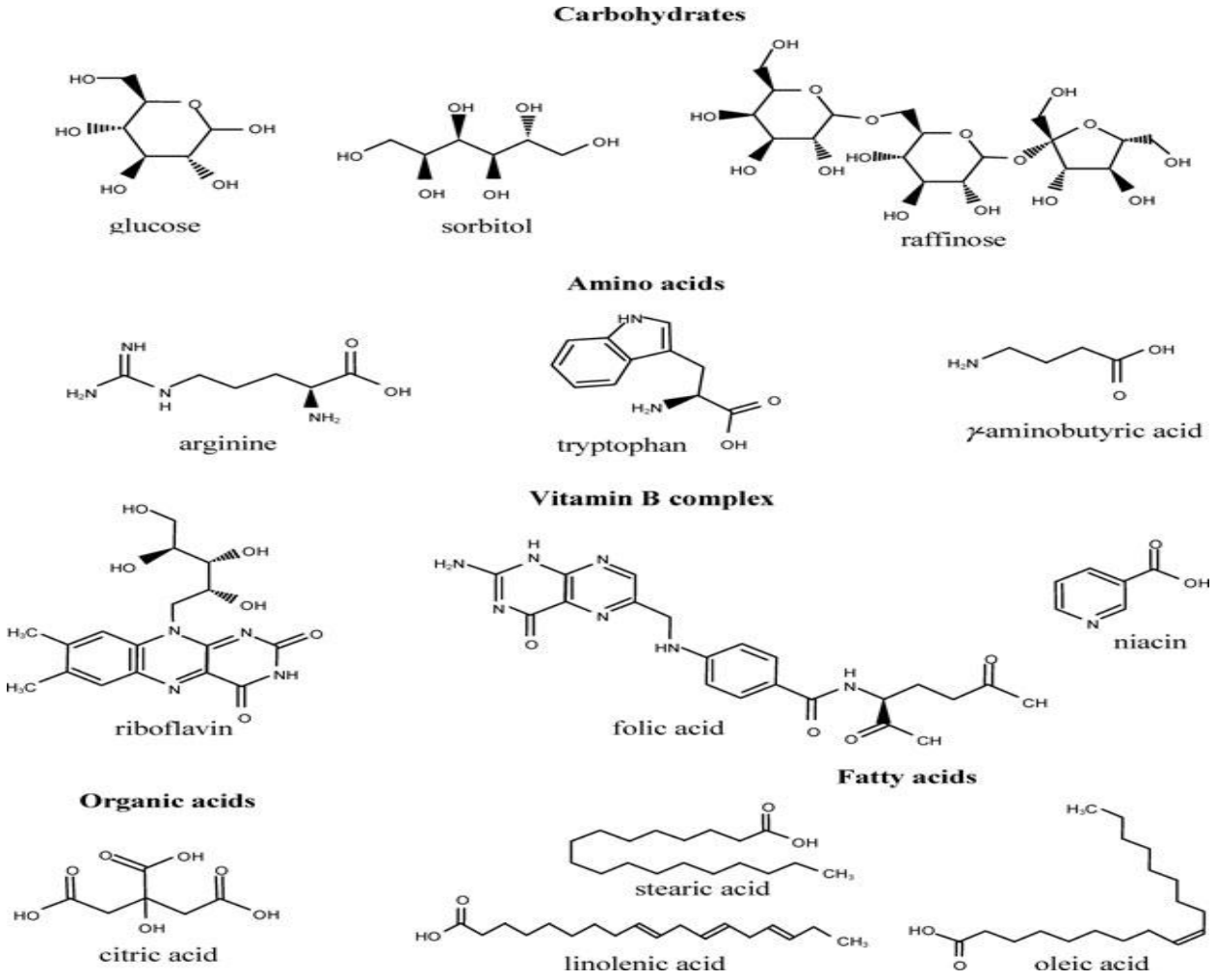


Figure 6: Chemical structure of primary metabolites (Carbohydrates) (Hounsome *et al.*,2008).

The most important soluble sugars in plant metabolism are sucrose and glucose; they are involved in the intermediary and respiratory processes of the plant. Sucrose and glucose also act as the primary substrate for synthesizing complex carbohydrates and as building blocks of amino acids (Filip *et al.*, 2002). Apart from providing energy for all complex metabolisms, sugars are also responsible for the control of gene expression related to plant stress resistance, growth and development (Ramond *et al.*,2008; Pego *et al.*, 2000). Sugar

status also modulates and coordinates internal regulators and environmental cues that govern growth and development (Sheen *et al.,* 1999; Smeekens, 2000).

During the internal processes of germination and seedling development, sugars play a very important role in the promotion and mobilization of nutrients shoot development, hypocotyl elongation and cotyledon greening and expansion (Dijkwel *et al.,* 1997; Perata *et al*., 1997 and Kurata and Yamamoto, 1998). However, Smirniff *et al.,* (2001) stated that during early seedling development high sugar accumulation may give rise to undesirable growth conditions.

Sugars also play a very important role in the synthesis of numerous antioxidant compounds, which protect the plants from the harmful reactive oxygen species (Asadi 1999; Tesfay *et al.,* 2010). During the normal process of photosynthesis plants produce both soluble sugars and ROS. However, Hounsome *et al.* (2008) stated that low levels of sugar accumulation can also leads to high production of ROS. Simple sugars such as, glucose are the primary initiator of certain antioxidant compounds, such as carotenoids and ascorbate synthesis (Tesfay *et al.,* 2010; Smirniff *et al.,* 2001; Smirniff 2000). According to Tesfay *et al.* (2010) glucose also yield, carbon skeletons of amino acids, such as cysteine, glycine and glutamate which are all the building blocks of antioxidant glutathione. And all of these systems are involved in defence mechanisms to prevent free radicals initiation through ascorbate- glutathione cycle (Tesfay *et al.,* 2010).

1.4 Enzymatic antioxidants

Plants also consist of a network of enzymatic antioxidants which also known as biological detoxifying systems that functions on the regulation of ROS. These antioxidant systems include enzymatic antioxidant compounds such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPX) (Apel & Hirt, 2004; Fridovich, 1983). Superoxide dismutase (SOD) functions by converting the highly active superoxide radicals (O_2^-) into hydrogen and peroxide (H_2O_2) and oxygen (O_2), and the accumulation of H_2O_2 is prevented in the cell by CAT and GPX; (Okezie & Aruoma, 2003; Gechev *et al.*, 2006).CAT and GPX are capable of converting H_2O_2 into water and oxygen (Purev *et al.*, 2010; (Foyer & Noctor, 2011).

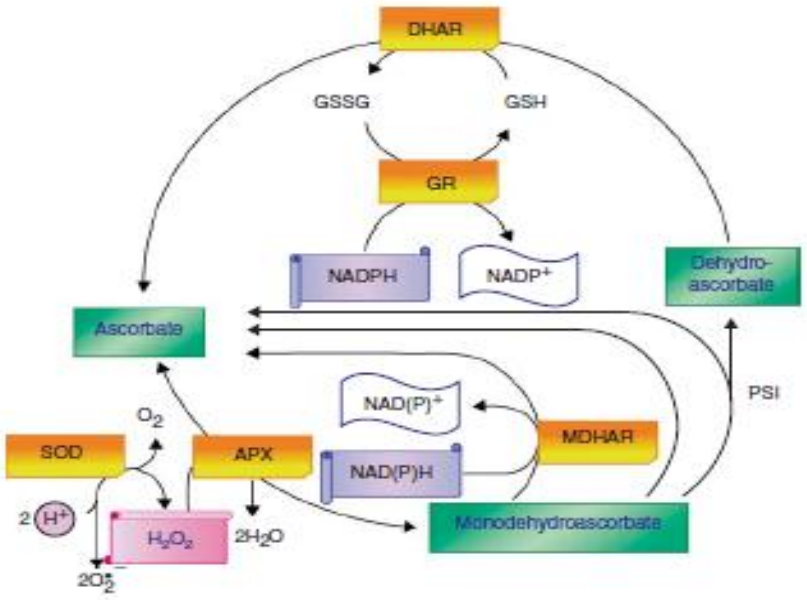


Figure 7: Ascorbate-glutathione cycle in terrestrial plants. Where enzymatic antioxidants scavenge H_2O_2 to H_2O and O_2 (Hossain *et al.*, 2011).

APX is also one of the most important enzymatic antioxidant present in plants and functions on the AsA–GSH cycle and in plant defense against oxidative damaged caused by the ROS (Hossain & Fujita, 2011).

For APX to function effectively reduced ascorbate must be present and the effectiveness of GPX is determined by the phenolic compounds such as guaiacol in the plant tissue (Ho-Min & Saltveit, 2001). Glutathione reductase (GR) requires the regeneration of reduced ascorbate after it has been converted to monodehydroascorbate by APX in order to function effectively (Ho-Min & Saltveit, 2001).

1.5 The role of the seed in plant development and food security

The seed plays a very important role in the plant life, such as permitting the propagation of new plants, allowing the success of new plants and it also allows the new generation of plants to adapt or inhibit new environments which are distance from the parent plant (Deirdre *et al.,* 2014). Hartmann *et al.* (1997) also argued that in depth information about the chemical reactions of the seed is very important since the seed allows for propagation of root stock that give rise to hybrid plants that are useful in breeding studies. Many scholars indicated that most of our knowledge about the chemical composition of seeds is for those current cultivated species since they make a large share of our food sources and they also contribute the raw materials for industries. However, information on the seed of indigenous species is relatively scarce.

Seed plays a very important role in meeting the human demand for food and nutrition security. Humans heavily rely on seeds for nutrition value and survival as food in the form of cereal grains and legumes (Prakash *et al.,* 2014). However, this heavy dependency of humans in seeds in a form of cereal based, such as maize, sorghum and millet, have been strongly criticized by many researchers, who indicated that this imposes a danger of malnutrition, especial in rural areas. Most epidemiological studies have shown that, the cereal based diets are deficiency in various micro nutrients such as vitamins and minerals (Gibson *et al.*, 2006). Nevertheless, the recent advancements in food technologies, such as food fortification and

micronutrients supplementation, decrease the risk of malnutrition in cereal based foods (Ruel, 2008; Kiess *et al.,* 2001; Olsen & Wendel, 2013; Beddingtonm, 2010).

Many of the indigenous seeds have been shown to be good sources of secondary metabolites that can contribute to the removal of reactive oxygen species that are responsible for many diseases. Seeds of indigenous plants that were previously ignored and neglected, such as those of amaranth, have been shown to contain more proteins and phenols than other well-known grains such as maize, sorghum and wheat they are also good sources of antioxidants, minerals and vitamins A and E (Okoth *et al.,* 2011; Pawel *et al.,*2009; Yilmaz & Toledo, 2004).

The seed can allow for the proper conservation and storage of African leafy vegetables from time to time, allowing for the crops to be available when they are needed (Meissa *et al.,* 2007). Therefore, proper information on the seed biology and technology is very vital when it comes to preservation of indigenous African leafy vegetables.

1.6 Plant morphological and nutritional properties

1.6.1 Black Jack

Bidens Pilosa L. (*Asteraceae*) is an annual erect, branched herb capable of reaching a maximum height of about 1.5 m, the plant has yellow flowers with the diameter of around 5-15mm (Hutchinson & Dalziel, 1963). The plant is commonly known as black jack in most scientific works, however in rural KZN (South Africa) it is known as ‘Uqadolo’. Black jack is native in South America, but today it is widely distributed in

the tropical and subtropical regions in Africa (DAFF, 2011). The plants strive very well on fertile soils on the wild and in disturbed land, planting fields and home gardens.

In South Africa the plant is found mainly in waste places, abandoned crop fields, river banks and along the road sides. It is commonly known as the troublesome weed. In most cases black jack is not cultivated; it grows on its own. Faber (2003) stated that in rural South Africa black jack is naturally available, especially during winter when most of the other natural indigenous leafy vegetables are not available. Black jack is also considered nutritious by many rural households.

Faber (2003) further stated that the leaves of black jack cannot be stored for more than one day. However, if the refrigerators are available they can be stored for longer periods, although this has the quality implication. Most of the rural households preferred or consumed black jack by the time it is young on the first or second week after emergence, because when it start flowering it turn to be very bitter.

Black jack is also known for its wide use in traditional medicines. Geissberger & Sequin (1991) indicated that black jack was widely used in traditional medicines to treat malaria and it was found to have anti-inflammatory activity. Processed leaves (extract) of black jack were also used by ‘Zulu’ people for the treatment of diarrhea, flue, high blood pressure, dysentery and colic (Rabe & Staden, 1997; Faber, 2003). The medicinal properties of black jack are believed to be due to the present of high antioxidant and phenolic compounds in black jack tissues. However, very little that has been published about the antioxidant and nutritional content of black jack, and their role in improving the human health.

1.6.2 Lettuce

Lactuca sativa L (*Asteraceae*) is considered as one the most important vegetable in a group of leafy vegetables, it is a cool season crop originated in the Mediterranean region (Kristkova *et al.,* 2008). Lettuce is also one of the most common vegetables that are known for growing well in hydroponics systems (Santos *et al.,* 2012). Hydroponics is the production methods of growing plants in water without the soil. Lettuce in most cases is exclusively used as the fresh salad vegetable that is consumed raw, however other varieties of lettuce can also be cooked.

Lettuce is also one of the world most commercially produced vegetables in many countries. It is known for its superior taste on it raw form. Lettuce is also known for its high nutritional value and also regarded as the good source of phytonutrients (Khoo *et al.,* 2011). However, Tomas *et al.* (1997) stated that the leafy tissues of lettuce contain small amounts of phenolic compounds when compared with other leafy vegetables.

Nevertheless, Koudelal & Petrikova (2008) indicated that the nutritional content of lettuce depend on many factors such as the growing conditions, namely temperature, nutrition and means of fertilization, irrigation, cultivation methods and the cultivar. However, lettuce is a good source of fiber, folate, manganase, chromium and potassium. Lettuce is also regarded as an excellent source of beta carotene, vitamin A, K and C, but lettuce is low in calories because it is made by the high fraction of water. Federico *et al.* (1997) further state that, the antioxidant activity of lettuce also provides several health benefits such as reduction in the risk of heart disease, cataracts and stroke, so it is very important that lettuce must be included in each daily diet.

1.6.3 Chicory

Cichorium intybus L. (*Asteraceae*) is a small aromatic biennial or perennial herb it is commonly known as chicory (Nandagopal & Kumari, 2007). Chicory is widely distributed in Asia and Europe, and it is used as a salad by many people (Bais and Ravishankar, 2001). Chicory as the whole plant possess a number of great medicinally important compounds such as alkaloids, flavonoids, tannins, inulin, esculin, unsaturated sterols, chlorophyll pigments, and vitamins (Nandagopal & Kumari, 2007; Molan *et al*., 2003).

Other parts of chicory are also known for the great commercial values the tuberous root of chicory also contains a number of medicinal important compounds such as inulin, bitter sesquiterpene lactones and coumarins (Varotto *et al.,* 2000). Nandagopal & Kumari (2007) further stated that the root of chicory are also used as anti-hepatotonic, anti-inflammatory, appetizer, stomachic, liver tonic, cardiotonic and diuretic. Many researchers also suggested that the roots of chicory are rich in alkaloids which form very important ingredients for coffee. Afzal *et al.* (2009) also indicated that the medicinal properties of chicory are noticeable in different parts of the world where chicory is used. Chicory has been traditionally used in many countries where it is available to treat illnesses such as different fevers, diarrhoea, jaundice and gallstones (Molan *et al*., 2003).

1.7 Indigenous crops and nutritional profile of African people

Adequate consumption of leafy vegetables has been closely associated with a high reduction of chronically illnesses such as cancer, diabetes and cardiovascular disease (Steinmetz & Potter, 1996). These health

benefits which are provided by indigenous crops are due to the high nutritional and antioxidant activity of many indigenous leafy vegetables (Lila, 2006). Most epidemiological studies proved that Africa is the leading continent in the world for being rich in the biodiversity of indigenous crops especial the leafy ones. However, of all the benefit that are provided by biodiversity, African people especial those who are residing in rural areas are still living below the acceptable standards that are recommended by the world health organization (FAO, 2009).

A limiting factor for dearth of information is lack of research output that contributes for crop improvement and its consequence towards consumer's perception of their significance as these vegetables provide health promoting antioxidants.

The world health organization (WHO) recommends that a healthy daily diet of an individual must contain a variety of nutrients such as protein, carbohydrates, lipids, vitamins, and minerals. WHO (2003) also indicated that at least the diet of an individual must contain 400g of vegetables and fruits to protect against the chronic diseases. However, most of the people especially those who are residing in rural areas they cannot afford the type of diet that is recommended by WHO. Rose *et al.* (2002) argued that, the current recommended diet by the WHO is like doubling the amount of food currently consumed by the average South African.

Smith & Eyzaguirre (2007) suggested that due to nutritional properties of indigenous crops especially the leafy vegetables, could play a very crucial role in the global initiative of WHO for increasing the consumption of vegetables and fruits.

Currently, most of the research that has been done on the area of indigenous crops focus on the general health benefits of indigenous crops and on the conservation and protection of indigenous knowledge associated with their cultivation. Very little is known about the physiochemical and antioxidant activity of

indigenous crops. The objective of this study was to investigate the nutritional and antioxidant properties of black jack, in comparison with two known commercial vegetables lettuce and chicory. The second objective was to examine the germination potential of black jack seeds and their nutritional quality.

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Chapter 2

The physiochemical and antioxidant mobilization of black jack (*Bidens pilosa L*) seeds during seed germination

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2.1 Abstract

Black jack (*Biden Pilosa L*) is the herbaceous leafy vegetable that is consumed in many African countries. It is also considered as the most valuable medicinal plant that is used for the treatment of various disorders. The black jack plant is propagated through seeds; however poor germination of black jack seeds has been reported in some studies. The present study was conducted with the purpose of examining the physicochemical properties of black jack seeds, after soaking the seeds in water for several hours.

The black jack seeds were experiencing dormancy, they needed to be soaked or steeped in the water before sowing or germinating. The result showed seeds that were soaked for 15 hours before germinating had a highest percentage of germination (72 %) in 5 days, than the rest of other seeds. The dry matter was also increasing as the time and number of germination hours progressing and steeping time increases.

Black jack seeds exhibited a high percentage of antioxidant capacity (DPPH) and phenols (0.69±0.44 mg/g DW and 56.45±0.08 mg/g DW respectively). The protein content on the seeds was (0.328±0.17 mg/g DW). Anti-nutritional, factors were also determined, total tannins content was (416.36±1.14 mg/g DW). These seeds responded to above soaking treatments by instant accumulation various antioxidants. Sugars had significant effect in the plant developmental phases. In conclusion, plant antioxidants have vital role in seed germination and the synergy of these compounds can be regulated by seed imbibition, mainly water.

2.2 Introduction

The seed plays a very important role in the plant life such as ensuring the survival of the plant species over time. Success for the new seedling plant is largely determined by the physiological and biochemical features of the seed (Bewley, 1994). Currently, there is very little information about the internal processes by which the embryo develops and emerges from the seed to complete germination (AOSA, 1993; Bewley, 1997).

Seed development is one of the most vital processes in the plant life. The process of seed development is divided into the number of distinct stages which are marked by morphological, genetical and physiological parameters (Bewley, 1994). The first stage is embryogenesis which is a morphogenesis stage, characterized by the formation of a single cell zygote and ends when all embryo structures have been established (Mayer *et al.,* 1991). The next stage is the period of seed maturation, this is the stage where cell division in the embryo arrest, and during where food reserves accumulation characterized by major changes in embryo size and in fresh and dry weight (Raz *et al.,* 2001). During the process of normal seed development the embryo typically acquires the characteristics that are necessary for survival and growth such as desiccation tolerance and in many species dormancy mechanisms (Baskin *et al.,* 2000).

When assessing seed quality, there are two most important seed attributes that need to be considered, firstly it is the germination capacity and vigor potency, both are determined during seed development and maturity phases (Harada, 1997). Secondly, primary and secondary metabolites particular antioxidants are also very good indicators of seed quality and vigor (Carla *et al.*, 2005; Aalen, 1998). However, there is a huge scarcity of information to the role of antioxidants, during the phases of seed development up to the attainment of seed desiccation tolerance (Christophe *et al.*, 2001). There is also very little known about the biological activities of black jack seeds. So the aim of this study was designed to investigate physicochemical and antioxidant compounds during seed germination.

2.3 Materials and methods

2.3.1 Plant material

The black jack seeds that were used during the study were collected from the University of KwaZulu-Natal Pietermaritzburg campus.

2.3.2 Germination test

The black jack seeds were counted and placed in petri dishes. There were 100 seeds per petri dish and the total of 15 petri dishes. The seeds in petri dishes were then soaked for 5, 10, 15, 20 and 24 hours respectively (3 petri dishes per soaking period treatment) and then placed in germination chamber set at 30 ^{o}C. Seeds were soaked by completely submerging them in water, and water was disposed in petri dishes, moist filter papers were placed in petri dishes to prepare the seeds for germination. Seed soaking was

applied due to black jack's dormancy characteristics, to facilitate seed germination. The seeds were then evaluated from the germination chamber every 24, 48, 72, 96 and 120 hours respectively (for 5 days), and the germination percentage was calculated. Mean time to germination (MGT) was also calculated accordingly using the formulae by (Ellis and Robert, 1981; Panwar & Bhardwaj, 2005).

$MGT = \sum^{\wedge}Dn / \sum n$ Equation 1

Where:

MGT = mean germination time,

n = the number of seed which were germinated on day D, and

D = number of days counted from the beginning of germination

2.3.3 Dry Matter

The dry matter was determine based on the method that was described by AOAC (1995) the seeds inside the paper bags after being soaked and germinated for (0, 24, 48, 72, 96, and 120) respective hours. The seeds were placed in an oven drier set at the temperature of $80^{o}C$ for 48 hrs. The difference between dry matter of original seeds (unsoaked and ungerminated seeds) and that of soaked and germinated seeds was used to calculate the dry matter.

2.3.4 Growing and identifying sprouts

The seeds were soaked in distilled water for 24 hours. Then they were placed in petri dishes with moist filter paper inside. The petri dishes were then transferred to a germination chamber set at 30 ^{o}C for five days

where they were watered each day using distilled water. On the fifth day, all of those seeds which were sprouted (approximate 1mm radical length) were cut and immediately snap shocked with liquid N_2 and crushed into powder using mortar and pestle. Samples were then transferred to plastic containers and stored at -70 ^{0}C for further analysis.

2.3.5 DPPH radical scavenging activity

The method by Priyanka *et al.* (2011) was used to measure the scavenging activity of black jack using a DPPH antioxidant assay with slight modification. A 0.1mM solution of DPPH (1,1-diphenyl- 2-picrylhydrazyl) in methanol (0.044g in 1L) was freshly prepared. One ml plant extracts was added to 3mL of DPPH (0.1 mM in methanol) a control consisting of 3ml DPPH was also prepared at the same time. The reaction mixture was mixed well and was then allowed to stay in the dark at room temperature for 20 min and a decrease in the purple colouration of a reaction mixture was measured at 517nm using a UV- 1800 (Shimadzu UV Spectrometer). The assay was done in triplicate. The following formula was used to calculating the radical scavenging activity of the plants: % Radicle Scavenging Power = Absorbance [Control – (Sample – Blank)] / Absorbance of Control × 100.

2.3.6 The ferric reducing power (FRAP) assay

Total antioxidant capacity (TAOC) was determined according to Benzie and Strain (1996) with slight modifications. These authors developed the FRAP assay which is based on the reduction of the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe (II)-TPTZ) complex by a reductant, therefore determining the combined antioxidant capacity of antioxidant molecules present in the

tissue under investigation. Aliquots of 0.1g freeze-dried plant material were extracted with 1N perchloric acid, vortexed and centrifuged at 12,400g for 10 min at 4 ^{0}C. A fresh FRAP reagent solution (300 mM sodium acetate buffer pH 3.6,10 mM Fe (II)-TPTZ prepared in 40 mM HCL, 20 mM $FeCl_3 \times 6H_2O$ (10:1:1) was prepared prior to measurement. Subsequently an aliquot of the samples (30 µL) was mixed with 900 µL FRAP reagent solution and the absorbance was measured at 593nm after 10 min. The total antioxidant capacity was expressed as µmol $FeSO_4 \times 7H_2O \times g^{-1}$ DW equivalent.

2.3.7 Total Phenol content

The total phenolic content of all leafy vegetables was estimated spectrophotometrically by the method that was described by (Eghdami & Sadeghi 2009;Gulcin *et al.*, 2007) with some slight modifications. Proper diluted methanolic extracts (200µl) was subsequently mixed with 900 µl Folin-Ciocalteau reagent. The reaction mixture was incubated at the room temperature for 10 min to permit the reaction mixture to react completely with the oxidizable substrate. Following the incubation 2 ml of 7.5% sodium carbonate was added to the mixture, and the mixture was final diluted to 7ml with deionized water. The reaction mixture was allowed to stand on the dark at room temperature for 2hrs. The absorbance was measured at 765nm (UV- 1800 (Shimadzu UV Spectrometer) using methanol as a blank. The results were expressed as mg gallic acid equivalent (GAE)/100 g DW.

2.3.8 Total flavonoid content

The total flavonoid content was also determined according to the method that was described by Eghdami & Sadeghi (2009*)* with slight modifications. The appropriately diluted methanolic extract, of the plant samples (0.1 ml) was added to distilled water. Subsequently followed by adding 5% $NaNO_2$ (0.03ml). And the reaction mixture was allowed to stand on a room temperature for 5min. 0.03ml of 10% $ALCL_3$ was added to the samples and the reaction mixture was incubated for 6min, after that 0.2ml of 1 mM NaOH was added to the samples. And the reaction mixture was finally diluted to 1 ml by distilled water. The absorbance of the reaction mixture was read at 510nm, against methanol used as blank. The results were finally expressed as mg quercetin (QE)/g DW.

2.3.9 Protein determination

The proteins were determined on the method that was described by (Marinova *et al.,* 2002) with slightly modifications. A 0.5g of the leafy tissue was extracted in 5ml of 50 mM Tris-HCL buffer. The extraction was carried on ice and the mixture was centrifuged at 18000 rpm for 10 min. The supernatant were transferred on the small test tubes which were placed on ice. For enzyme determination, Bradford Microassay was used, 1ml of the Bradford reagent was mixed with 30 µL of the plant extracts in the cuvettes. And the reaction mixture was allow to stand for 5 min, and the protein concentration was determine Spectrophotometrically by reading at 595nm. The protein standard curves were prepared separately by using bovine serum albumin (BSA).

2.3.10 Tannin determination

Tannin content was determined according to Lai & Liew-Kang (2000). Eighty micro-litre of sample extracts (0.02g/20 ml) were packed into a beaker and volume was topped up to 1 ml using distilled water. Then, 0.5 ml of Folin-Ciocalteaureagent together with 2.5 ml of $NaCO_3$ (20%) were added into the solutions. The mixture was allowed for 40min at room temperature before measured at 725 nm using UV-spectrophotometer.

2.3.11 Statistical Analysis

The experimental results were analyzed using GenStat (Version 17.0; VSN International, Hemel Hempstead,UK). They were expressed as mean ±standard deviations in triplicate measurements. The differences were considered significant when $p < 0.05$.

2.4 Results and discussion

2.4.1 Dry matter

There was an increase in the dry matter as the number of hours of germination increase and the steeping time (Fig1.). A positive correlation between the steeping time and the amount of dry matter was further observed. Seeds that were steeped for few hours showed a low percentage of dry matter gain i.e. (Control, 5hrs and 10 hrs) compared to the rest. The highest dry matter gain was observed during the 72 hour to 120 hours of germination. Similar results were also reported by Zivile *et al.* (2009) who noted that there was a slow increase in dry matter as the hours of germination were reduced in broccoli seeds. Zielinski *et al*. (2006) argued that the dramatic dry matter increase during germination is caused by the intensive imbibition

of water by the seed which triggers other physiological processes in the seed, resulting in emergence which further degrades the storage reserves of the seed.

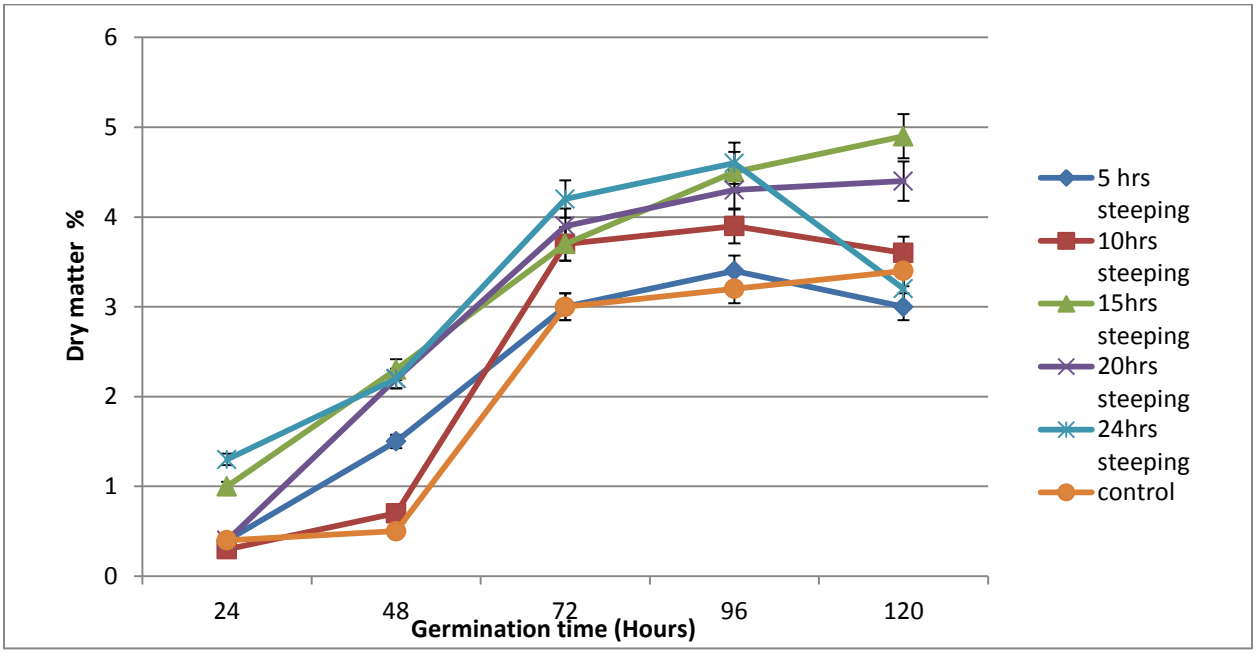

Figure 1: The effect of soaking and germination period on the dry matter of black jack. Vertical bars represent s.e of the mean value (n = 5).

2.4.2 Germination

In this experiment germination percentage significantly ($P<0.01$) increased with length of soaking hours (Fig.2). There was a direct proportional relationship between the germination percent and the soaking time. The seeds that were soaked for longer hours showed higher percentage of germination, than those which were soaked for fewer hours. The highest germination percentage was observed on the seeds that were soaked for 15 hours, and the lowest was observed on the control and seeds that were soaked for 5 hours. In

contrast, seeds that were soaked for 24 hours also showed less germination percentage than those seeds which were soaked for 10 and 20 hours. It is therefore speculated that dormancy in black jack seeds can be broken by soaking the seeds for approximately 10 and 15 hours before sowing. In the experiment that was conducted by Forsyth & Brown (1982) the results showed that 100 percent germination was achieved on black jack seeds that were germinated for 4 days on dark light at 25 ^{o}C.

Seed germination commences with water uptake by the dry seed (imbibition) and followed by the start of the expansive growth of the embryonic axis, usually radicle (Leubner, 2003). The rupture of the seed coat and the emergence of the radicle are considered as the end of germination. These findings concur with Bewley (1994), who stated that during the process of germination there are other numerous events that are taking place, protein hydration, respiration and subcellular structural changes. Furthermore, Luna *et al*. (2009) argued that most of the seeds of indigenous species are difficult to germinate, because they are dormant. Plants used dormancy as the survival mechanism, to protect themselves from external factors. Dormancy in many plant species is controlled by environmental factors such as light temperature and time of dry storage.

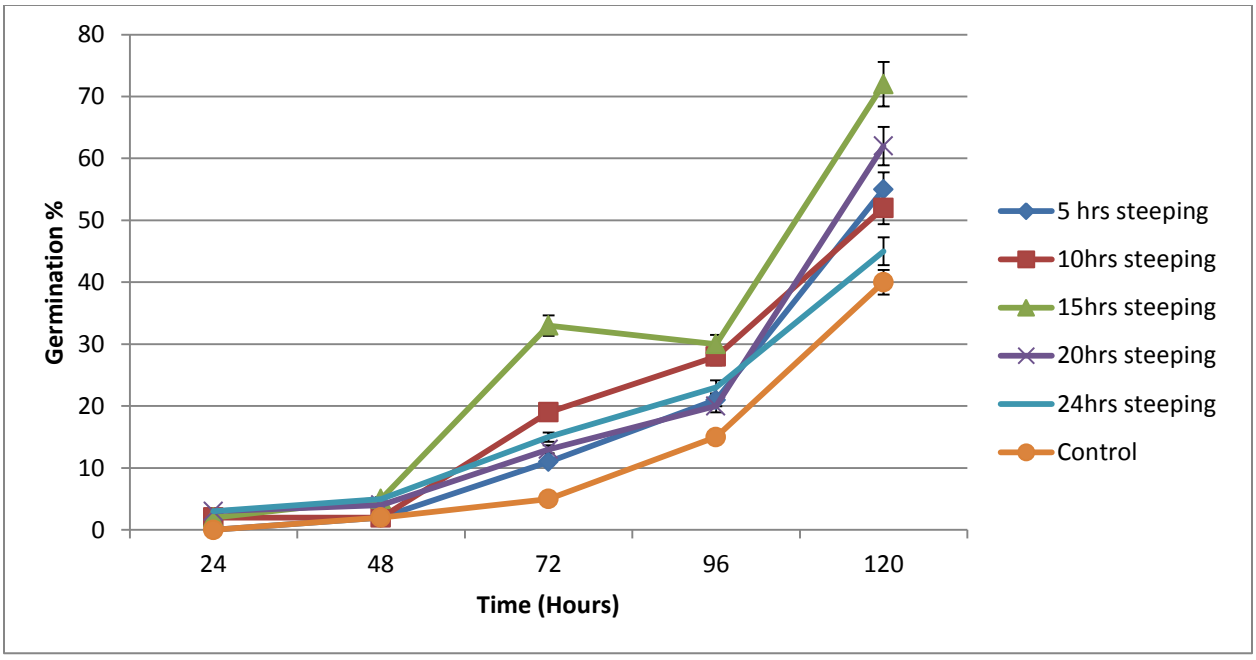

Figure 2: The germination percentage of black jack, against the steeping time. Vertical bars represent s.e of the mean value (n = 5).

2.4.3 Chemical compounds

Table 1 below shows the variation in the compositions of antioxidants and tannins in black jack seeds and sprouts. The total antioxidant content (FRAP) on black jack seeds and radicle was found statistically significant at $P<0.05$ and the content was (0.45±0.97 and 0.30±0.07 µmol $FeSO_4 \times 7H_2O \times g^{-1}$ DW). The total antioxidant on black jack seeds and radicle from another assay (DPPH) $P<0.01$, was (0.69±0.44 mg/g and 0.76±0.77 mg/g DW). The total phenolic content in black jack seeds and radicles was (56.45±0.08 GAE/g and 36.24±0.29 GAE/g DW respectively). The total flavonoid content for black jack seeds and radicles was (352.74±0.39 mg/g and 295.02±0.30 catechin mg/g DW respectively). Protein content as determined by Bradford assay was 0.328±0.17 mg/g DW in seeds, and 0.226±0.12 mg/g DW on radicles. The tannin content, which is the major of anti-nutritional factors in black jack seeds and radicles was (416.36±1.14 GAE/g and 69.05±0.05 GAE/g DW respectively). Plants use the tannins to defend themselves

from harmful environmental conditions and from the parasite. However, the high accumulation of tannins in plant tissues can negatively affect plant palatability.

Table1: Antioxidant activity, polyphenols and protein content investigated in black jack seeds and sprouts.

	FRAP	**DPPH**	**Phenol**	**Flavonoids**	**Protein**	**Tannin**
Seeds	0.45±0.97	0.69±0.44	56.45±0.08	352.74±0.39	0.328±0.17	416.36±1.14
Sprout	0.30±0.07	0.76±0.77	36.24±0.29	295.02±0.32	0.226±0.12	69.05±0.05
±se (n=5)	FRAP in µmol $FeSO_4$ 7H2O g -1 DW	DPPH in mg/g DW	Phenols mg (GAE)/100 g DW	Flavonoids mg (QE)/g DW	Protein in mg/g DW	Tannin in mg GAE/100g DW

2.5 Conclusion

The results have revealed that soaking of black jack seeds with water improves seed germination. The results also proved that black jack seeds and radicles contain relatively high levels of antioxidants, phenols and flavonoids. In conclusion, plant antioxidants have vital role in seed germination and the synergy of these compounds can be regulated by seed imbibition, mainly water.

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Chapter 3

Evaluation of phytochemicals and antioxidant properties of leafy vegetables, black jack (*Bidens Pilosa L*) and some Asteraceae species: A comparative study.

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3.1 Abstract

Natural Antioxidants are very essential substances in nature; they function on the protection of biological systems against the damaging effect caused by free radicals. Only few information is found on antioxidant potential of black jack. The main aim of this study was to examine the antioxidant activity of black jack in comparison with other *Asteraceae* commercial vegetables (chicory & lettuce) over the growing period. The plant metabolites that were used for comparisons include TAO as determined by both DPPH and FRAP assays, TP, flavonoids, chlorophyll pigments and carotenoids. All of the tested parameters were determined using Spectrophotometer. Total soluble sugars were also examined using HPLC-RID. The results revealed that black jack recorded significant amounts of non-enzymatic antioxidants. The maximum amount of TAO of black jack, using DPPH assay was (0.73 ± 0.13mg/g DW) in week one, this was similar again on the other assay FRAP which showed the maximum amount of the TAO (0.47±0.22 µmol $FeSO_4 7H_2O$ g^{-1} DW) in week one. The TP content of black jack was also the highest, compared to other leafy vegetables that were used for comparison, it reached the maximum value of (155.46±0.07 mg GAE/g DW) in week one. The chlorophyll content and carotenoids were also among the highest in black jack, although they were

decreasing as black jack was reaching full maturity. The only exception was on sugars, black jack was found to be not a good source of soluble sugars, for all the examined sugars black jack was the lowest. The study reports nutritional value of African leafy vegetables is higher for immature leaves.

3.2 Introduction

The consumption of vegetables and fruit provides the most vital nutrients for human quality life and health. The African leafy vegetables have a potential role to play in this regard on the African continent; especially when alleviating malnutrition for rural households. Globally the African continent is rated on the top for having the highest malnutrition in the world, particularly among the pregnant, lactating women and young children below the ages of twelve (Uusiku *et al*., 2010).

Most researchers agreed that, the food insecurity issues in the African continent can be reduced or easily be solved by encouraging the communities to increase the consumption of ALVs (IPGRI, 2005). The low intake of vegetables and fruits is associated with the high mortality rate in the African continent. The ALVs constitute very essential functional food components that contribute minerals, vitamins and other active biologically ingredients which are associated with dietary activities. Most epidemiological studies also indicated that, increase intake of ALVs in the diet is associated with the decreased risk of a number of chronic health conditions such as certain cancers, cardiovascular and neurodegenerative, mascular degeneration, counteract and other age related diseases (Moyo *et al.,* 2013). The beneficial effects of ALVs are due to the wide range of compounds they comprise such as antioxidants, phenols, carotenoids, vitamins, saponins and flavonoids (Dinda *et al.,* 2007; Gerber *et al.,* 2002).

In particular, dietary antioxidants are the most notable important indicates of the nutritional quality of food, because of the role they play in protecting the cells against the damaging effect of free radicals (Hesam *et al.,* 2012; Deivamaru *et al.,* 2013). Free radicals are charged molecules and they have a significant role in

causing several diseases, by reacting with nucleic acids, enzymes and proteins present in the cell (Uttara *et al.*,2009). This damaging effect by free radicals is known as oxidative stress. Indigenous plants especially the ALVs are believed to be the rich source of antioxidant compounds that can contribute to the protection of the body from the damaging effect of free radicals (Harnafi & Amrani, 2008).

The *Asteraceae* family plants are well known for their antioxidant effect when it comes to free radical scavenging (Shad *et al.,* 2013). Black Jack (*Bidens pilosa L*) is also a member of this family, black jack is indigenous to SA where it freely grows in cultivated areas, disturbed soils and along the road sides. Black Jack in SA is known to be consumed by indigenous people in rural areas, especial in winter where most of the cash or commercial vegetables are scarce (Faber, 2010).

Black Jack is also known to be used in African traditional medicines to cure many diseases, such as diabetics, cholera and it is thought to lower high blood pressure (Faber, 2010; Geissberger & Sequin, 1991). Other parts of black jack are used in the anti-diarrhea and anti-parasite formulations. The aim of the study was to evaluate the antioxidant activity of black jack extracts using the leaves and compare them with other common commercial grown vegetables from the *Asteraceae* family (Lettuce and Chicory).

3.3 Materials and methods

3.3.1 Plant culture

The experiment was conducted under greenhouse conditions, at the University of KwaZulu-Natal (Experimental field) Pietermaritzburg, South Africa (29^0 35'S, 30^0 25'E). Three leafy vegetables were used during the study black Jack (*Bidens Pilosa L*), chicory (*C.intybus L cv*) and lettuce (*Lactuca sativa*).

All of the seeds were first planted on the seedling trays, after emergence they were transplanted to plastic seedling pots. The plastic seedling pots were consisting of a diameter of 24cm and 20cm in heights.

The entire seedling pots were filled with a peat moss based potting without the use of any agrochemicals. All the seedling pots were placed on the greenhouse rolling benches. There were a total of 150 seedling pots per each cultivar (there were 50 seedling pots per replication). The experiment was conducted using randomized complete block design.

Leafy samples were collected fortnightly for seven consecutive weeks. The samples were snap shocked with liquid N_2 and freeze dried for further laboratory analysis (non-enzymatic and enzymatic antiocidants).

3.3.2 DPPH radical scavenging activity

The method by Priyanka *et al.* (2011) was used to measure the scavenging activity of black jack using a DPPH antioxidant assay with slight modification. A 0.1mM solution of DPPH (1,1-diphenyl- 2-picrylhydrazyl) in methanol (0.044g in 1L) was freshly prepared. One ml plant extracts was added to 3mL of DPPH (0.1 mM in methanol) a control consisting of 3ml DPPH was also prepared at the same time. The reaction mixture was mixed well and was then allowed to stay in the dark at room temperature for 20 min and a decrease in the purple colouration of a reaction mixture was measured at 517nm using a UV- 1800 (Shimadzu UV Spectrometer). The assay was done in triplicate. The following formula was used to calculating the radical scavenging activity of the plants: % Radicle Scavenging Power = Absorbance [Control – (Sample – Blank)] / Absorbance of Control × 100.

3.3.3 The ferric reducing power (FRAP) assay

The FRAP was determined by using the method according to Weng *et al* (2011), with slight modifications. Briefly the stock solution was made by preparing three reagents: 300mM acetate buffer (dissolve 20g Sodium acetate in 20ml acetic acid, dilute to 500ml H_2O, PH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) in 40 mM HCl and 20 mM FeCl3.6H2O solution were prepared. The FRAP reagent was then prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5ml $FeCl_3$ solution at the ratio of 10:1:1 (v/v/v), respectively, and they were incubated at 37^{o}C before use. One N perchloric acid extracts (30µl) were allowed to react with 900µl of the FRAP solution for 10min in the dark conditions and the readings of the reaction were taken at 593nm, against FRAP reagent without extract used as blank. The results were expressed as µmol $FeSO_47H_2O$ g^{-1} DW.

3.3.4 Total phenol content

The total phenolic content of all leafy vegetables was estimated spectrophotometrically by the method described by (Eghdami & Sadeghi, 2009) with some slightly modifications. A proper diluted methanolic extracts (200µl) was subsequently mixed with 900 µl Folin-Ciocalteau reagent. The reaction mixture was incubated at the room temperature for 10 min to permit the reaction mixture to react completely with the oxidizable substrate. Following the incubation 2 ml of 7.5% sodium carbonate was added to the mixture and the mixture was final diluted to 7ml with deionized water. The reaction mixture was allowed to stand on the dark at room temperature for 2hrs. The absorbance was measured at 765nm (UV- 1800 (Shimadzu UV

Spectrometer) using methanol as a blank. The results were expressed as mg gallic acid equivalent GAE)/100 g DW.

3.3.5 Total flavonoid content

The total flavonoid content was also determined according to the method that was described by (Eghdami & Sadeghi, 2009) with slightly modifications. The appropriately diluted methanolic extract, of the plant samples (0.1 ml) was added to distilled water. Subsequently followed by adding 5% $NaNO_2$ (0.03ml). And the reaction mixture was allowed to stand on a room temperature for 5min. A 0.03ml of 10% $AlCl_3$ was added to the samples and the reaction mixture was incubated for 6min after that 0.2ml of 1 mM NaOH was added to the samples. And the reaction mixture was finally diluted to 1 ml by distilled water. The absorbance of the reaction mixture was read at 510nm, against methanol used as blank. The results were finally expressed as mg quercetin (QE)/g DW.

3.3.6 Total carotenoid and chlorophylls

Total chlorophylls and carotenoids were determined according to the method that was described by Manuela *et al.* (2012) with slight modifications. Two grams of frozen powder leafy tissues were homogenized in 100% methanol using an Ultra–Turrax (2 minute burst twist). The homogenate were then centrifuged at 3500 rpm for 10 min at 4 ^{o}C in Beckman centrifuge model. Subsequently followed, by filtering all of the samples in a 0.45µm filter paper. All of the extraction procedures were performed under dim light.

The absorbance of supernatant was performed with UV- 1800spectrophotometer (Shimazu) wavelengths: 665.2 and 652.4nm for chlorophyll a and b and 470 nm for Carotenoids. All of the measurements were performed in triplicate and the results were expressed as (mg/ml of extract solution using the following equations described by (Lichtenthaler, 1987):

Concentration of chl a: $Ca = (16.72 \times A665.2) - (9.16 \times A652.4)$

Concentration of chl b: $Cb = (34.09 \times A652.4) - (15.28 \times A665.2)$

Concentration of Ca+b: $= (1.44 \times A665.2) + (24.93 \times A652.4)$

Concentration of carotenoids: $Cx+c = (1000 \times A470) – (1.63 \times Ca) – (104.96 \times Cb) / 221$

3.3.7 Soluble sugars

Soluble sugars were determined according to Tesfay *et al*. (2010) with slightly modifications. A 0.2 g of a freeze- dried, ground material was mixed with 10 ml of 80% ethanol (v/v) and vortexed for 60s. The reaction mixture was then incubated in an 80^0C water bath for 60 min and then kept at 4 ^{0}C overnight. The mixture was centrifuged at 12,000 x g for 20 min at 4 ^{0}C was then filtered through glass wool and moved to dryness to a savant Vacuum Concentrator (SpeedVac, Savant, NY,USA). After few hours, dried samples were resuspended in 2ml of distilled water and then filtered through 0.45 µm nylon filters. The sugars were analysed using HPLC-RID (LC–20AT, Shimazu Corporation, Kyoto, Japan) equipped with a refractive index detector. The authentic sugar standards were used to determine the concentration of individual sugars

3.3.8 Statistical analysis

The experimental results were analyzed using GenStat (Version 17.0;VSN International, Hemel Hempstead,UK). They were expressed as mean ±Standard deviations in triplicate measurements. The differences were considered significant at $p < 0.05$.

3.4 Result and discussion

3.4.1 Antioxidants

Plants antioxidants are gaining more attention from the consumers, as they are considered to be safer with multifunctional uses than synthetic antioxidants when it comes to preventing oxidation from biological systems (Elmastas *et al.,* 2005). Natural antioxidants are present in plants that possess polyphenolic compounds (Stoilova *et al.,* 2007). Therefore, medicinal and indigenous plants are believed to be potentially rich in natural antioxidants. In this study the total antioxidant activity of the studied plants were significantly ($P \leq 0.05$) different.

Black jack was found to have a highest antioxidant capacity as compared to other leafy vegetables on the first week after transplanting. However, it started to decrease gradually on the second week as shown in figure 1, and then it started to pick up again at a constant increasing rate. They were no large significant differences on the total antioxidant activity of chicory and lettuce. For both chicory and lettuce the trend for the total antioxidant capacity was increasing on the first weeks of the experiment, as plants approached to maturity it then decreased. However, for chicory the total antioxidant activity started to pick up again on the fifth week, showing a similar trend to that of black jack. The results that were observed during this study were similar to that which were shown in other studies (Tesfay *et al.,* 2014).

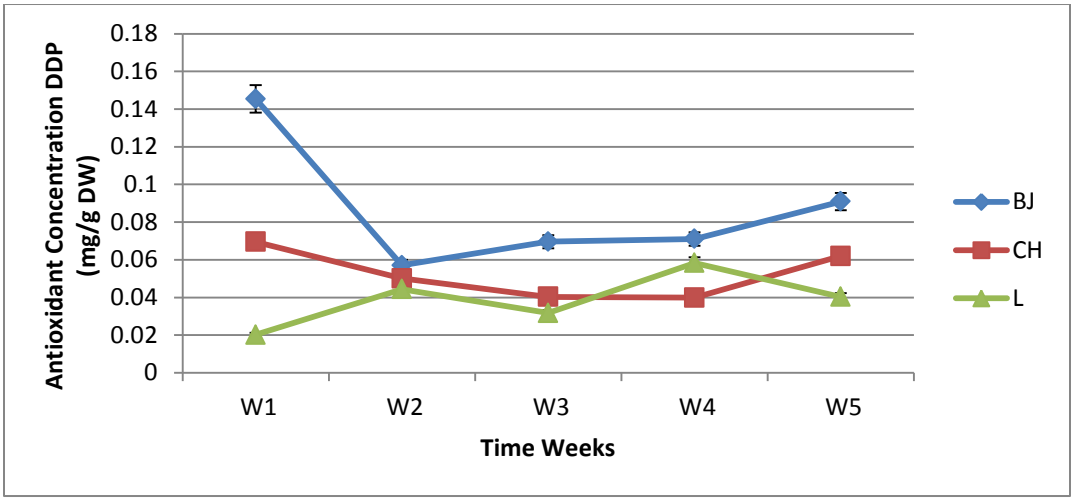

Figure 1: Total antioxidant capacity (DPPH) black jack, chicory and lettuce over growing period. Vertical bars represent ±SE. (n=5).
Where BJ=Black Jack, CH=Chicory and L=Lettuce

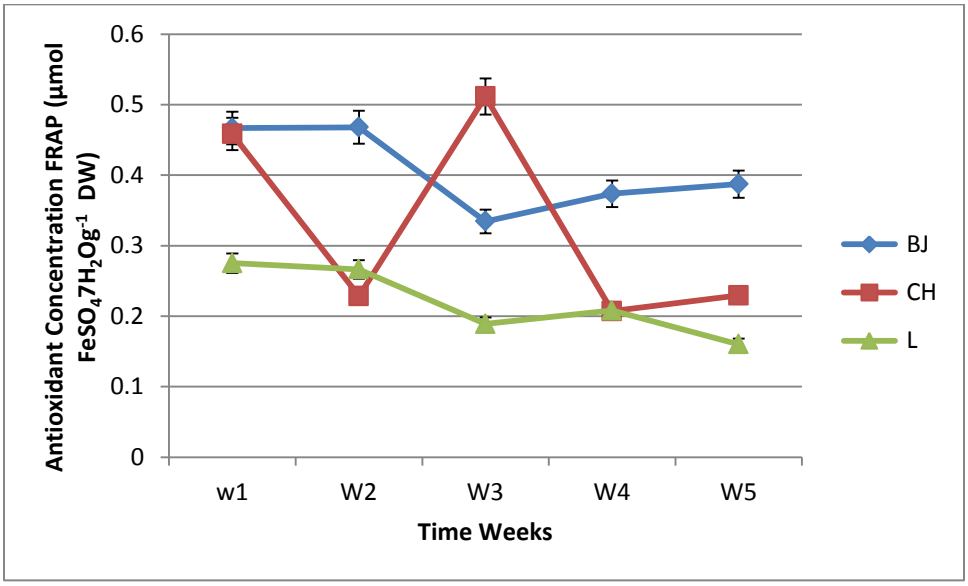

Figure 2: Total antioxidant capacity (FRAP) black jack, chicory and lettuce over a growing period. Vertical bars represent ±SE. (n=5).

3.4.2 Phenols

For both phenols and flavonoids the results were significant for all plant extracts ($P \leq 0.01$). In this present study the phenolic content of black jack was fluctuating greatly. It was the highest on the first week (155.46±0.07 mg/g DW). For chicory, the maximum phenolic content was on the first week after transplanting, then it decreased gradually until week three, from there it started to pick up until the end of the experiment figure 3. Lettuce had the lowest phenolic content than all the leafy vegetables that were examined during the study. Total phenolic content of black jack was found to reach to the maximum value of 538.10 ± 0.96 mg/g dry weight (Wen – Chin *et al.,* 2013). Phenolic and flavonoid content of chicory were reported in other studies. Shad *et al.* (2013) reported the phenolic content of matured leaves of chicory, recorded from 0.47±0.07 to 2.52±0.26g/100g dry weight to 0.05± 0.03g/100g dry weight. Srivastava *et al.* (2013) found the phenolic content of fully matured lettuce leaves to be 118.20 mg/GAE/100g. In overall the total phenolic content of all the leafy vegetables in this study showed a high level of variation in some leafy vegetables. Black jack and chicory, the phenolic content was increasing when the plants were reaching maturation, and this high phenolic content in black jack may be a good reason to explain why full matured black jack leaves are very bitter when they are consumed at their reproductive stage. Grubben & Denton (2004) also this argument by indicating that *Biden pilosa* full matured leaves are not suitable for consumption because of a strong bitter stringent taste.

Plant phenols are known for their antioxidant, depending on its dominance for its type; govern plant taste which will impact consumers' perception. Rahman *et al.* (2008) reported phenolic compounds influence seed bitterness and astringency as well as the overall quality of plant. Although African leafy vegetables produce high concentration of phenols, in rural communities people still prefer using them as vegetables for

household consumption. Then it was thought plant bitterness as well as astringency could be improved, as this plant produces high concentration of carbohydrates, mainly fructose. However, these results were in contrast with Tesfay *et al.* (2014) who reported that a phenolic content of many leafy vegetables, including black jack decreases as the plants are reaching maturity.

The flavonoid content of all the leafy vegetables was found showing a similar trend that was displayed in figures 3 & 4. This was in accordance with what was stated by Maisuthisakul *et al.* (2007) who indicated that there was a positive correlation between the phenolic content and the flavonoid content. Maisuthisakul *et al.* (2007) also observed positive correlation between phenolic and flavonoid content.

Srivastava *et al.* (2013) found the flavonoid content on lettuce leaves around 76.80 mg CE/100g DW. In this present study black jack was found superior as well in the flavonoid content more than lettuce and chicory. A significant amount of secondary compounds could be due to environmental or geographical location which may be caused by changes in seasonal patterns, temperature, abiotic or biotic factors (Saurabh *et al.*, 2011).

Polyphenolic compounds are known for their antioxidant activity when it comes to defending the biological systems from the harmful effect of free radicals, this is contributed by the presence of the hydroxyl group in their structures (Milala *et al*., 2009). Phenols and flavonoids are the important group of polyphenolic compounds; they have attained great attraction because of their anti-inflammatory, antiviral, antibiotic and antioxidant activity (Wang *et al.,* 2003).

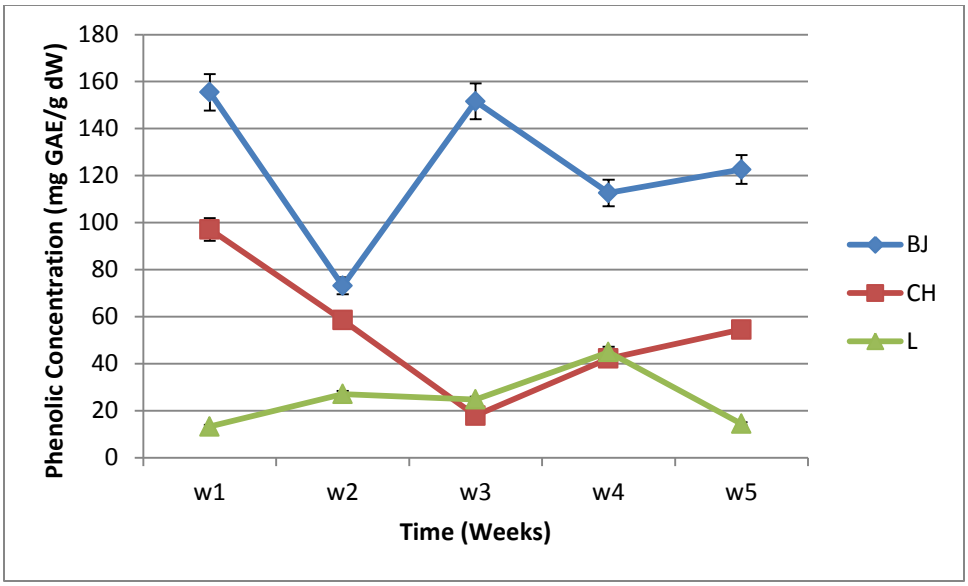

Figure 3: Total phenol content in black jack, chicory and lettuce over the growing period. Vertical bars represent ±SE. (n=5).

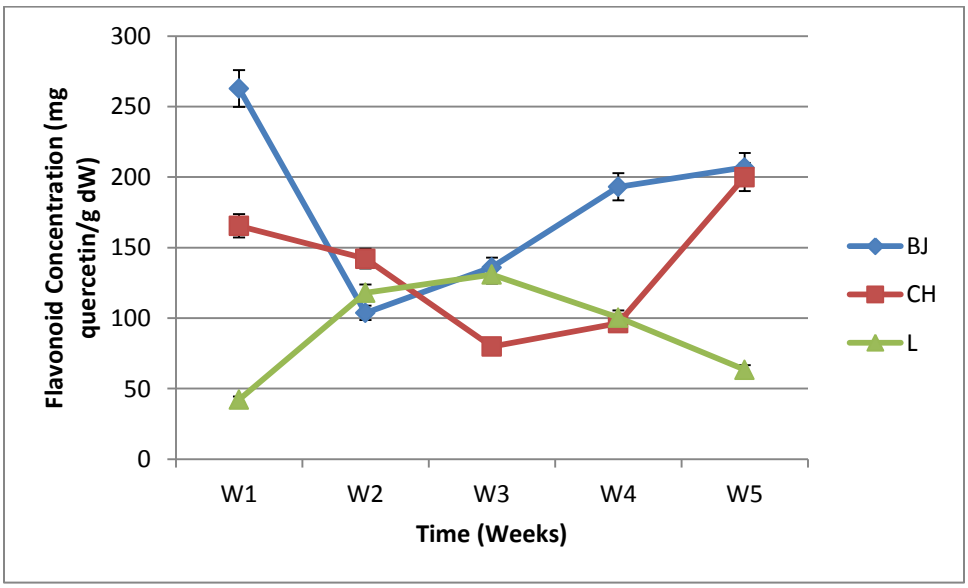

Figure 4: Total Flavonoids content in black jack, chicory and lettuce over the growing period. Vertical bars represent ±SE. (n=5).

3.4.3 Carotenoids and chlorophylls

In this present study, chicory had the highest chlorophyll ‘a’ content of all the vegetables (figure 5).Then followed by black jack and lettuce respectively (Figure 7). Similarly, chicory had also the highest chlorophyll ‘b’ content of all the vegetables (figure 6). And it was also noted that as this chlorophyll was decreasing as growing towards maturity (Figure 7). Results were found in agreement with Tesfay *et al.* (2014) they reported leafy pigments concentration for African leafy vegetables decreasing towards maturity, and might have an effect to formation of other types of pigments, such as carotenoids.

Chlorophyll is the most abundant pigment in nature, its functions to absorb and transfer of light energy in autotrophs, and it is also involved in the transfer of electrons which are all the vital processes in photosynthesis (Manuela *et al.,* 2012). Many epidemiological studies indicated that chlorophyll provides green leafy vegetables with their antioxidant and nutritional benefits. The antioxidant benefits of chlorophyll are thought to lower the risk of cancer, prevent carcinogenesis and strengthening the whole immune system (Chairat *et al.,* 2013; Sangeetha & Baskaran, 2010). Leafy vegetables’ chlorophyll and other chlorophyll related compounds are among the best chemicals that are responsible for the general plant protection during their development stages (Ching-Yun *et al.,* 2013).

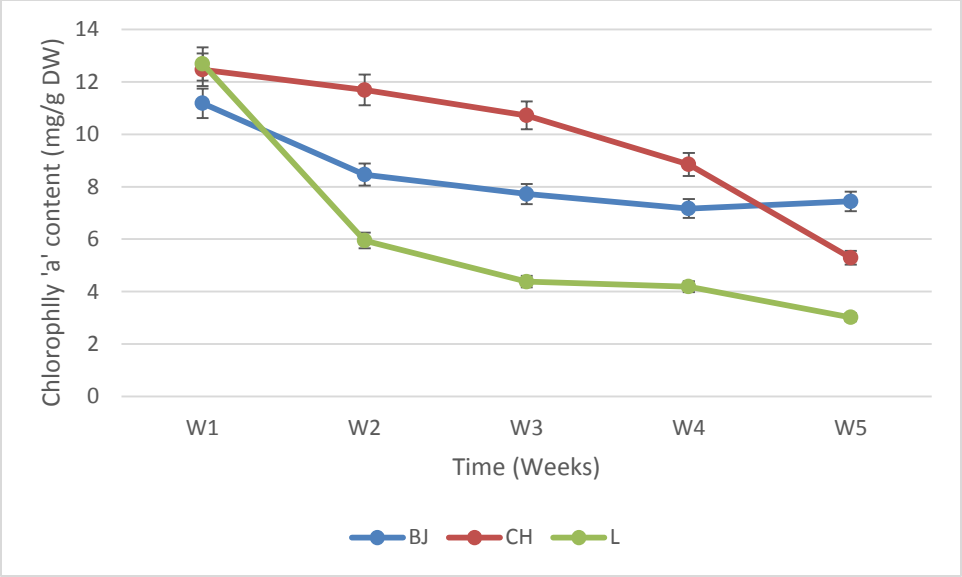

Figure 5: Chlorophyll ‘a’ content for all leafy vegetables over the growing period. Vertical bars represent ±SE. (n=5).

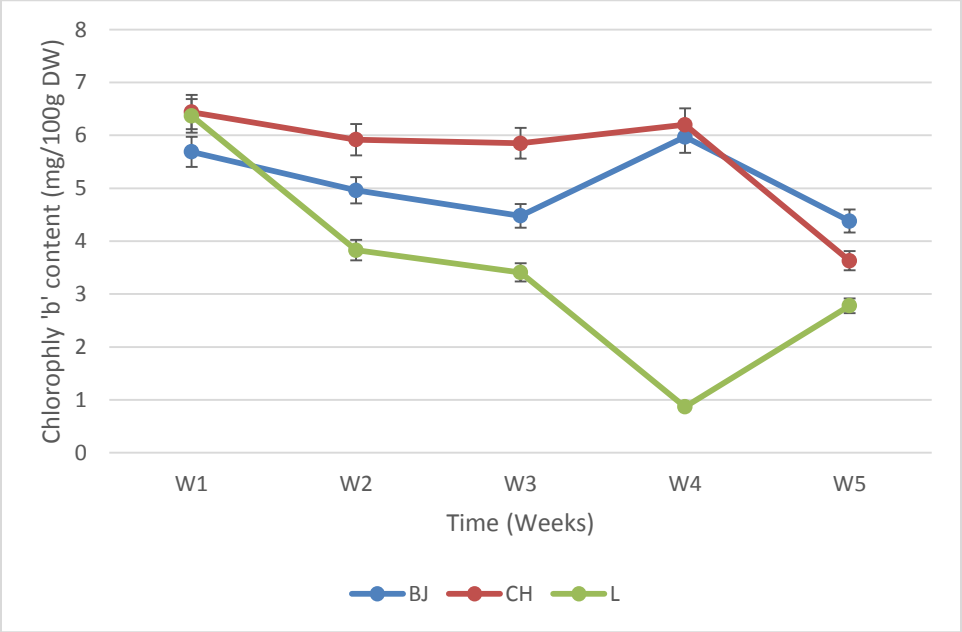

Figure 6: Chlorophyll ‘b’ content for all leafy vegetables over the growing period. Vertical bars represent ±SE. (n=5).

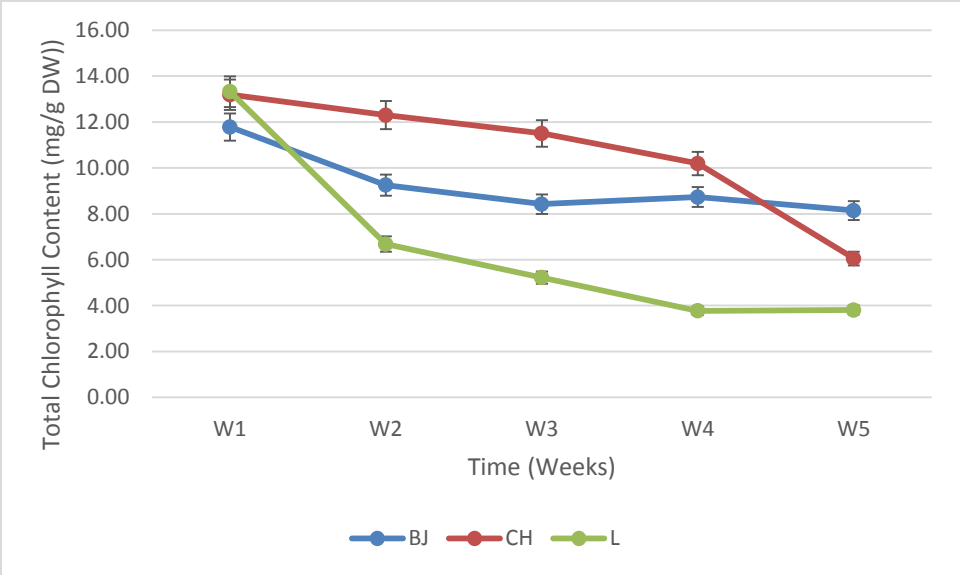

Figure 7: Total Chlorophyll content for all leafy vegetables over the growing period, vertical bars represent ±SE. (n=5)

3.4.3 Carotenoids

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The carotenoid content of all the leafy vegetables was significantly different. For black jack the carotenoid trend was fluctuating greatly, it reached the maximum peak on the first week after transplanting and it started to decrease gradually towards maturity. The results were found in agreement with Znidarcic *et al.* (2011) they reported carotenoid content of chicory to be around (3.94±0.65 mg/100g) in fully matured leaves.

Carotenoids act as antioxidants which function in the protection of cell membranes against the damaging effects of free radicals which results in the retardation of ageing processes (Merzlyak & Solovchenko, 2002). In plants that are exposed under the excess of light, carotenoids have been shown to provide the protection from the photo-oxidative damage this is achieved by preventing the excitation of energy from the singlet or triplet state chlorophyll by removing the highly reactive oxygen species (Muller *et al.*, 2001). According to Van den Berg *et al.(*2000) in plants the carotene content will depend on various factors such as cultivar, environmental conditions, production practices, stage of maturity, climatic and environmental conditions such as temperature, irradiance and soil characteristics

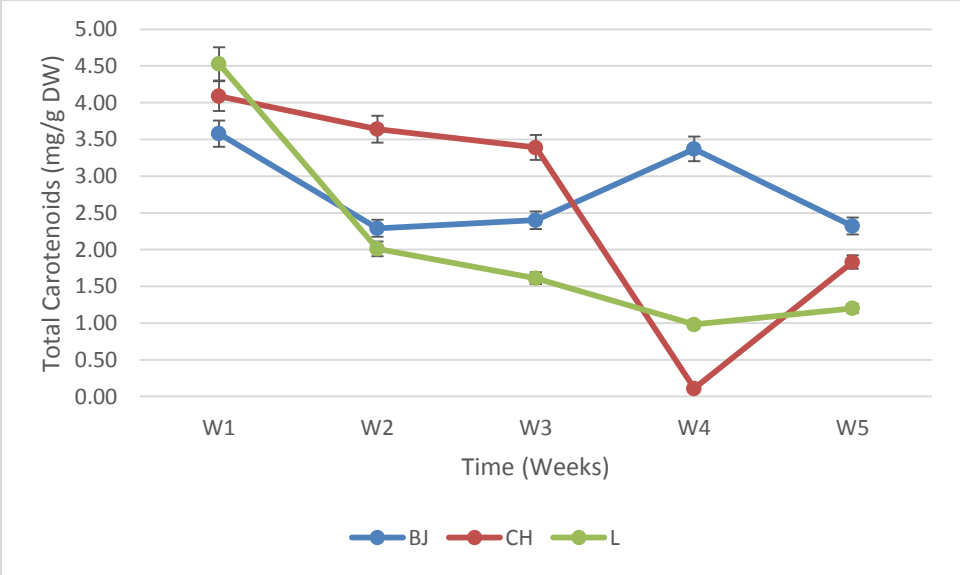

Figure 8: Total Carotenoids content for all leafy vegetables over the growing period. Vertical bars represent ±SE. (n=5).

3.4.4 Soluble sugars

Leafy vegetables carbohydrates were significantly ($P < 0.01$) different. Black jack records the lowest carbohydrates of all the vegetables. Chicory had a highest carbohydrates concentration on the second week after transplanting, lettuce was on the first for both; it started to decrease gradually until last week (figure 9). Our result was also found in agreement with Angela *et al.* (2010) sugar levels were found very low in leafy vegetables.

Carbohydrates play a very important role as sources of energy for all living organisms. In biological systems carbohydrates are converted into glucose, sucrose and other simple polysaccharides, to supply energy that is vital for all metabolism (Kavya *et al.,* 2012). Sugars are very essential cellular nutrients that serve as metabolite regulatory modulators of gene expression in plants, animals and yeast, they acts as nutrients and signal molecules in plants (Reza-Bolouri *et al.,* 2010). Glucose and sucrose are also responsible for the control of gene expression related to plant stress resistance, growth and development (Ramon *et al.,* 2008; Pego *et al*., 2000).

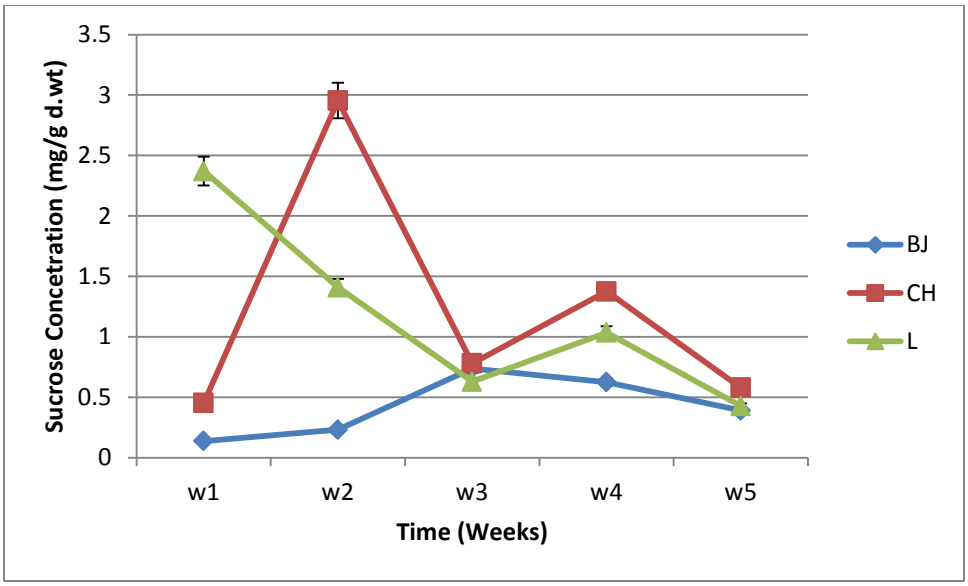

Figure 9: Sucrose content of black jack, chicory and lettuce over the growing period. Vertical bars represent ±SE. (n=5).

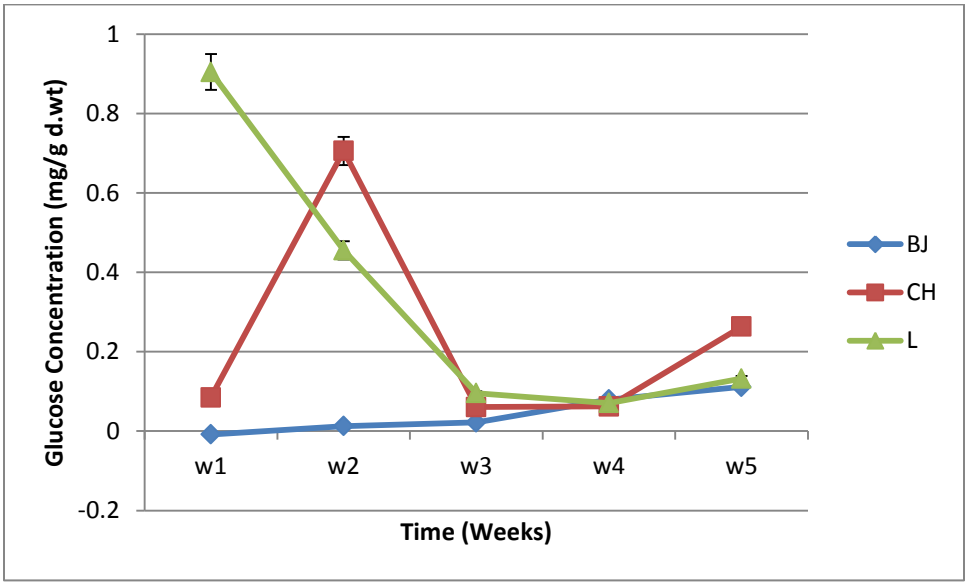

Figure 10: Glucose content of black jack, chicory and lettuce over the growing period. Vertical bars represent ±SE. (n=5).

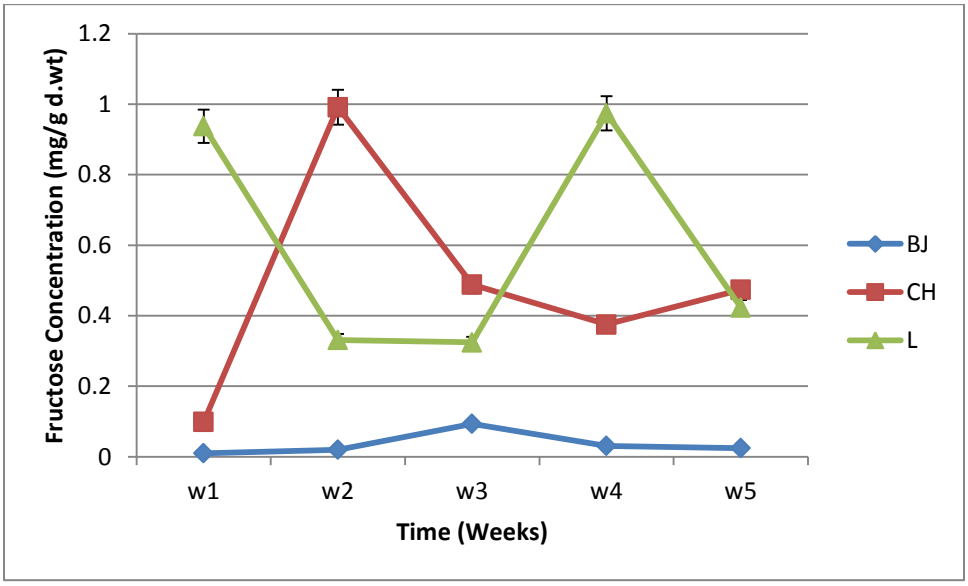

Figure 11: Fructose content of black jack, chicory and lettuce over the growing period. Vertical bars represent ±SE. (n=5).

3.5 Conclusion

In conclusion black jack (*Bidens pilosa L*) leaves especial at early vegetative stage (baby leaves), contain high considerable amounts of secondary metabolites phytochemicals than other *asteracea* commercial, leafy vegetables (lettuce and chicory). The accumulation of different antioxidants compounds varies greatly overplant developmental stages in African leafy vegetables. Therefore, proper harvest time of indigenous leafy vegetables is vital.

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Chapter 4

Study on enzymatic antioxidant: Emphasis on Black Jack (*Bidens Pilosa L*) in relation to other *asteraceae* leafy vegetables.

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4.1 Abstract

Black jack (*Bidens Pilosa L)* is an indigenous leafy vegetable in many sub-Saharan countries including South Africa. The medicinal properties of black jack are believed to be due to high antioxidant activity in black jack tissues. A study was conducted to investigate the enzymatic antioxidant activity of black jack and exotic vegetables. The results revealed that black jack accumulated these enzymatic antioxidants, their production is regulated by growth conditions. The antioxidant enzymes, its accumulation was observed in early plant developmental stages, and declined as approaching towards maturity. In conclusion, plant enzymatic antioxidants function in relation to other non-enzymatic ones, result in higher activity in early stage.

4.2 Introduction

The oxidative damage caused by the action of reactive oxygen species (ROS) is responsible for the progressive of the number of chronic disease such cancer, diabetes, aging, neurodegenerative diseases, atherosclerosis, cardiovascular diseases and the peroxidation of polyunsaturated lipids in cellular membranes (Smirnoff, 1995; Finkel and Holbrook, 2000; Esposito *et al.,* 2002; Valko*et al*., 2006). ROS are generated as the results of essential aerobic metabolisms, such as photosynthesis and respiration which are considered as the essential requirements for acquiring energy for catabolism (Masoko and Eloff, 2007). ROS are formed in the body as a result of oxygen production and they include free radicals such as singlet oxygen species ($_1O_2$), hydroxyl radical (˙OH), superoxide anion and molecules such peroxide (·OOH) and peroxyl radicals (RCOO·**)** (Zarban & Asghar, 2008).

It is well documented that plant species especially the African leafy vegetables are the good sources of natural antioxidants, both enzymatic and non-enzymatic (Elmastas *et al.,* 2007). There is a vast amount of safe natural compounds present in African leafy vegetables, which has been reported to scavenge free radicals. This is attributed by the availability of the hydroxyl groups in the chemical structure of ALVs (Valko *et al*., 2006). The natural antioxidant defense system found in plants include enzymes such as catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione peroxidase (GP_X), superoxide dismutase (SOD) and many none enzymatic compounds such as polyphenolocs compounds (Huang *et al.,* 2005). The aim of the study was to evaluate the enzymatic antioxidant activity of black jack compared with other *Asteraceae* commercial leafy vegetables(lettuce and chicory).

4.3 Materials and Methods

4.3.1 Antioxidants

4.3.1.1 Peroxidase

Peroxidase activity was estimated by the method that was described by (Nakano & Asada, 1981) with slight modifications. Briefly, 0.5 g of the plant tissue was homogenized in 3M of 0.1M phosphate buffer at the PH of 7. The homogenates were centrifuged at 18000 rpm at 5 ^{o}C for 20min. A reaction mixture of 3ml buffer solution, 0.05ml guaiacol solution, 0.1ml enzyme extract and 0.03ml hydrogen peroxide solution was pipetted on the cuvette. The reaction mixture was allowed to stand for about 10 min before reading. The rate of formation of guaiacol dehydrogenation was followed spectrophotometrically at 436nm. Enzyme activity was reported in Units/mg protein using protein standards.

4.3.1.2 Ascorbic acid oxidase

Ascorbic acid oxidase was estimated by the method that was described by (Nakano & Asada, 1981) with slight modifications. Briefly, 0.5 g of the plant extract was homogenized in 3M of 0.1M phosphate buffer at the PH of 7. The homogenates were centrifuged at 18000 rpm at 5 ^{o}C for 20min. The reaction mixture involving 3ml of the substrate solution (8.8mg ascorbic acid in 300ml phosphate buffer (pH 5.6) and 0.1 ml of the enzyme extract was pipetted on the cuvettes, and the reaction was allowed to stand on the dark for about 10min before reading. The decrease on the absorption caused by the oxidation by ascorbic acid was

read at 265nm using the spectrophotometer. Enzyme activity was reported as Units/mg protein using protein standards.

4.3.1.3 Ascorbate peroxidase (APx)

Ascorbate peroxidase (APx) activity was also determined by the method that was described by (Nakano & Asada, 1981). Briefly, 0.5 g of the plant extract was homogenized in 3M of 0.1M phosphate buffer at the pH of 7. The homogenates were centrifuged at 18000 rpm at 5 ^{o}C for 20min. The reaction mixture containing 0.1mM EDTA and 0.1ml of enzyme extract was allowed to stand for 5 min at room temperature before reading. The decrease in the absorbance of ascorbate was measured at 290nm spectrophotometrically. Enzyme activity was reported as Units/mg protein using protein standards.

4.3.1.4 Catalase

The catalase activity was described by the method that was stated by (Sadasivam & Manickam, 1996), with slightly modifications. Briefly, 0.5 g of the plant tissue was homogenized in 0.067 M (pH 7) phosphate extraction buffer on cold conditions. The homogenates were centrifuged a 10,000 rpm for 15min at 4 ^{o}C. The activity was determined by observing the disappearance of hydrogen peroxide. The reaction mixture containing 3ml hydrogen peroxide - phosphate buffer (Dilute 0.16ml of hydrogen peroxide to 100ml with phosphate buffer) and 0.02ml of plant extract, was allowed to stand for 10min and the absorbance was read at 240nm using the spectrophotometer. Enzyme activity was reported as Units/mg protein using protein standards

4.3.6 Superoxide dismutase (SOD)

SOD activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), as described by Giannopolitis & Ries, (1977). The reaction solution (3mL) contained 1.5 mM NBT, 0.12 mM riboflavin, 13mM methione, 0.1 M EDTA, 67 mM phosphate buffer (pH 7.8), and contained 10 to 100 µL enzyme extract. Riboflavin was added last and the tubes were shaken and placed under fluorescent lighting with an intensity 8.42 µmol m-2 s-1. Enzyme extract dilutions and controls were determined by withholding illumination and without addition of enzyme, respectively. The absorbances of the illuminated and non-illuminated solutions were determined spectrophotometrically at 560 nm. One unit of SOD activity was defined as the amount of enzyme inhibiting 50 % NBT photoreduction. The results were expressed as Units/mg protein standards.

4.3.7 Statistical analysis

The experimental results were analyze using GenStat (Version 17.0;VSN International, Hemel Hempstead,UK). They were expressed as mean ±Standard deviations in triplicate measurements. The differences were considered significant when $p < 0.05$.

4.4 Result and discussion

Reactive oxygen species accumulation can be induced by unfavorable environmental conditions, which result to the damage of plant cells and their organelles (Caverzan *et al.*, 2012; Sang *et al.*, 2005). Plants and other biological systems are able to counteract this effect through the use of ROS scavengers such as

enzymatic and non-enzymatic antioxidant (Scandalios, 2005; Ho-Min *et al.*, 2001). Plant enzymatic antioxidants were significantly different ($P \leq 0.01$) among different vegetables.

APx of black jack was found ranging from (0.20±0.04 to 0.06±0.002 U/mg of protein) and the highest value was observed in week one (0.20±0.04 U/mg of protein). For chicory the total antioxidant activity (APX) was found ranging from (0.19±0.01 to 0.06±0.007 U/mg of protein) and again the highest values were observed in week one and week two. For lettuce the total enzymatic antioxidant content (APX) was found ranging from (0.01±0.001 to 0.16±0.003 U/mg of protein).

Black jack leaf catalase was found ranging from (0.02±0.001 to 0.03±0.001 U/mg of protein) and the highest value was only observed in week four after transplanting (figure 3).For chicory the total enzymatic antioxidant activity (CAT) was found ranging from (0.02±0.003 to 0.08±0.001 U/mg of protein) and the highest values were observed in week one after transplanting. For lettuce the total enzymatic antioxidant activity (CAT) was found ranging from (0.001±0.001 to 0.04±0.002 U/mg of protein).

The plant enzymatic antioxidants had significant differences over maturity. Plant SOD was dominant for black jack as compared to other commercial vegetables (Figure 4). Their activity started by increasing on the first week after transplanting reaching the maximum value of (1.6 U/mg of protein) in black jack, and declined as approaching maturity. Chicory followed, started by increasing at the constant rate until week 4, and then it decreased gradual, until the end of the experiment.

Peroxidases are formed by the variety of enzymes which includes NAD peroxidase, NADP-Peroxidase and fatty acid peroxidases and other structural enzymes known as POD. They break down or biodegrade a number of organic molecules which includes phenolics and aromatic amines (Sadasivam & Manickam, 1996). Peroxidase enzymes (POD) function on the oxidation of some phenolic compounds into chinone,

they also break down hydrogen peroxide by oxidation into molecules of water and oxygen (Weisany *et al.*, 2012).

The peroxidase activity on many plants has been reported to increase during physiological maturation and leaf senescence (Farkas *et al*., 1964; Maraite, 1973; Parish, 1968). The total peroxidase on black jack was found ranging from (0.0018 ± 0.01 to 0.021 ± 0.008 U/mg of protein) and the highest value was on week five after transplanting (figure 5). And for chicory the total peroxidase activity was found ranging from (0.0032±0.021 to 0.040±0.003 U/mg of protein) and the highest value was on week five. In lettuce the total peroxidase activity was found to range from (0.0080 ± 0.005 to 0.026±0.006 U/mg of protein). And the highest value was on week five again. The peroxidase results were online with what most of the writers have claimed about the peroxidase activity of leafy vegetables. Parish (1968) also made a claim that the increase of the peroxidase activity is the reliable indicator of plant maturity and senescence.

The plant enzymatic antioxidants had significant differences over maturity. Plant SOD was dominant for black jack compared to the commercial vegetables. Their activity increased the first few weeks after transplanting and declined as approaching towards final phase. CAT and POD also responded in a similar way where the two ALVs appeared dominating. The increase in enzyme activity during the first weeks of plant development could be associated with plant high metabolic rate, result in accumulation of oxidants and the plant has also responded by involving these antioxidants, and then creates redox balance within the cell. The SOD may have dismutase singlet oxygen radicals into hydrogen peroxide, and the CAT activity can then possibly increase as regulated by accumulation of H_2O_2. H_2O_2 accumulation in early to middle developmental stages possibly coincided with increased SOD, CAT and POD activities, which may infer that H_2O_2 acts as a signalling molecule. Thereafter, the gradually decreasing SOD, CAT and POD activities presumably led to an increase in $^{\cdot}OH$ levels in later developmental stages. We suspect that, in the later

stages, more H_2O_2 is likely to transform to $^{\cdot}OH$ so as to breakdown polysaccharide and mediated the lignification of old fully matured leaf.

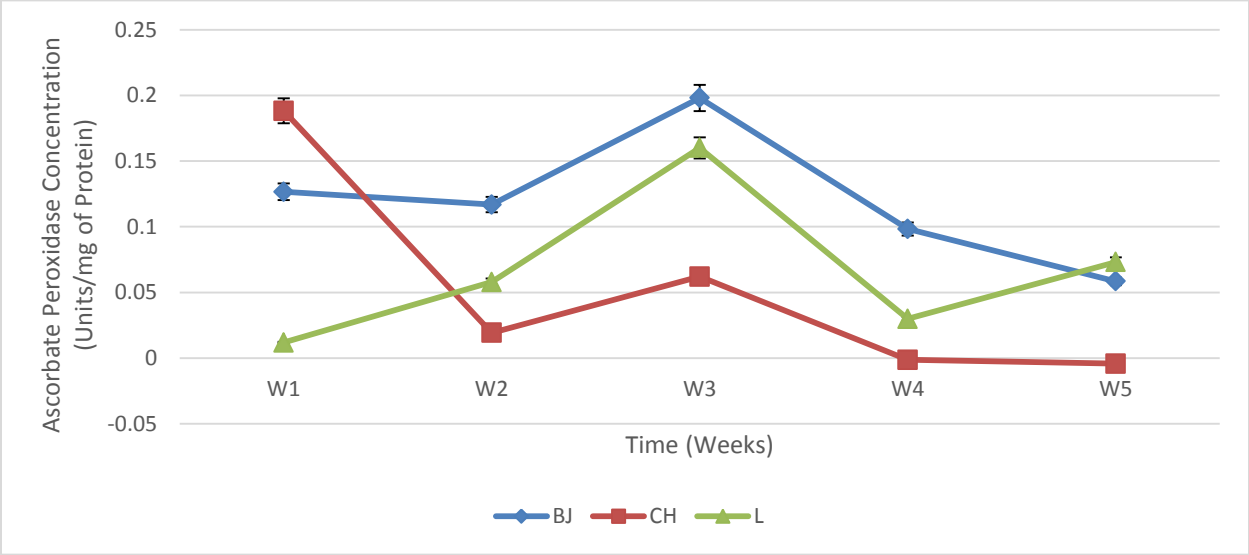

Figure 1: Ascorbate Peroxidase activity for all leaf vegetables over the growing period, vertical bars represent ±SE. (n=5).
Where BJ = Black Jack, CH=Chicory and L=Lettuce

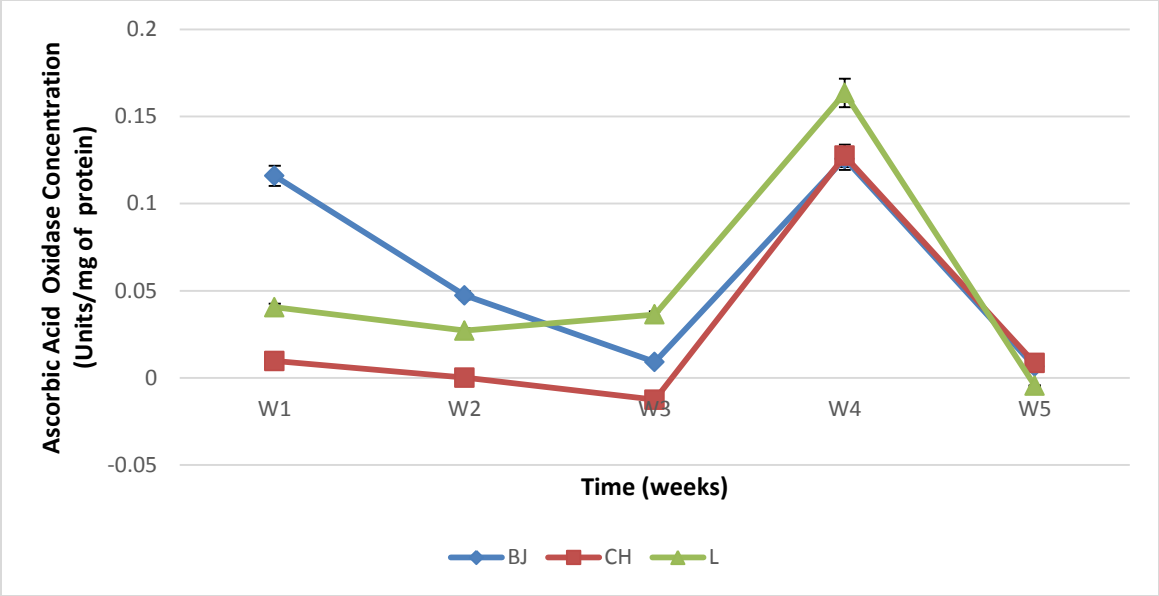

Figure 2: Ascorbic Acid Peroxidase activity quantity over the growing period, vertical bars represent ±SE. (n=5).

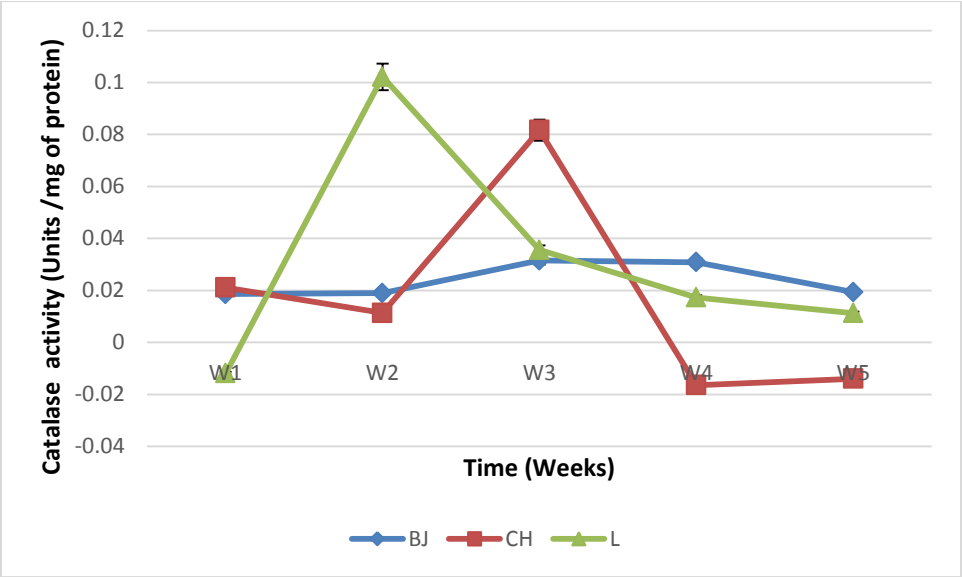

Figure 3: Catalase activity of all leafy vegetables over the growing period, vertical bars represent ±SE. (n=5).

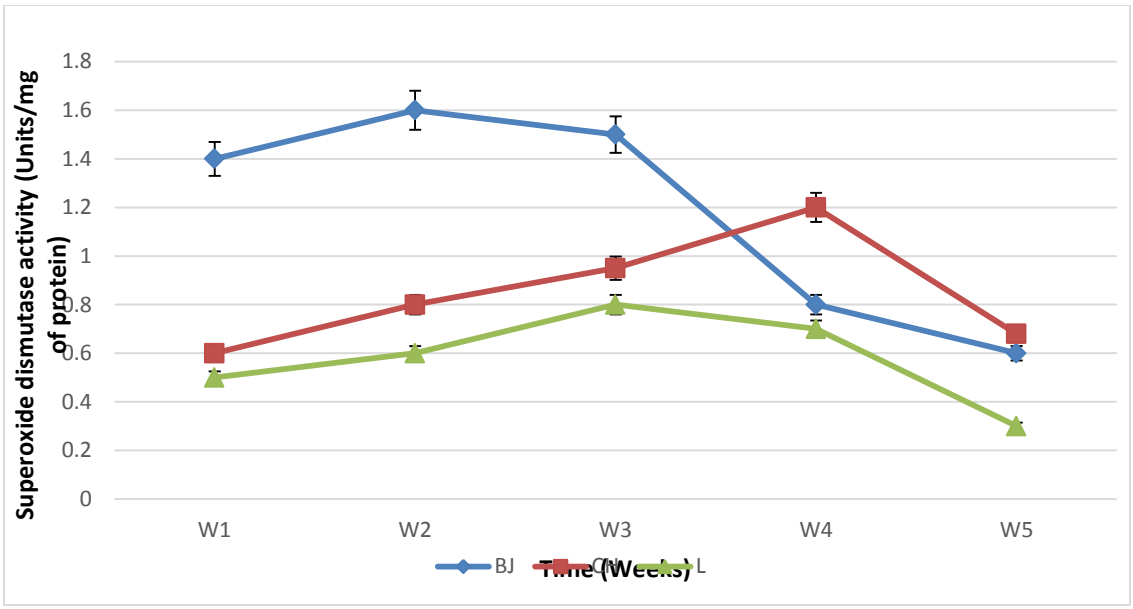

Figure 4: Superoxide dismutase activity of all leafy vegetables over the growing period, vertical bars represent ±SE. (n=5).

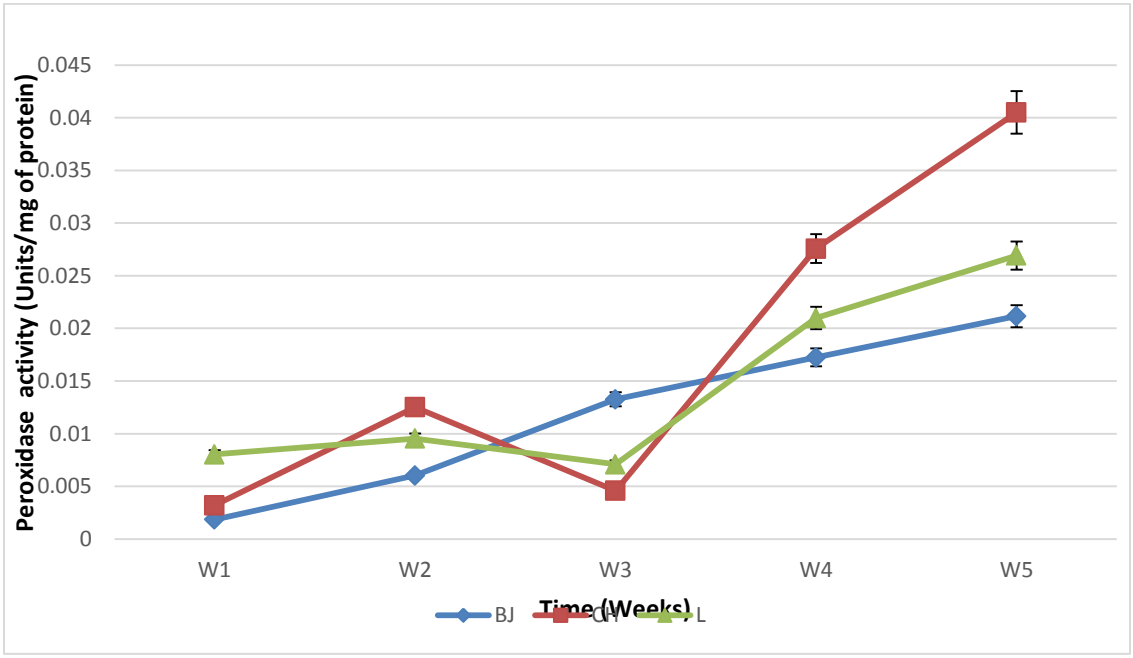

Figure 5: Peroxidase activity of all leafy vegetables over the growing period, vertical bars represent ±SE. (n=5).

4.5 Conclusion

It can be concluded that accumulation of enzymatic antioxidants varies over different growing stages, in both indigenous and exotic vegetables. The growth conditions also have a major effect on the accumulation of enzymatic antioxidants. There is a high concentration of enzymatic antioxidants in younger leaves than older leaves. Therefore, for household food security sequential harvest of younger leaves is recommended.

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Chapter 5

5. General Discussion and Conclusion

Reactive oxygen species are produced as a result of oxidation reactions, in some instances they are produced during normal metabolism. They comprise of very reactive short lived chemicals such as hydroxyl radicals, superoxide anion, and peroxides (Uttara *et al*., 2009). The reactive oxygen species, are highly reactive and they cause huge damage in many biological cells, by initiating lipid peroxidation which results in the deactivation of enzymes (Bagchi *et al.,* 2000) .The deleterious effect of reactive oxygen species, is believed to be responsible for many dieses such as cancers, cardiovascular disease, cataracts, mascular degeneration, and other age related diseases (Gulcin *et al.* 2009; Oktay *et al.*, 2003).

Shui & Leong (2004) stated that most of the indigenous leafy vegetables are equipped with a strong antioxidant system, to cope with the oxidative stress. Primary and secondary metabolites in vegetables, such as simple sugars, phenols, flavonoids, vitamins and carotenoids are believed to be responsible for this antioxidant system. The antioxidant system of many indigenous leafy vegetables plays a very important role in protecting the biological systems from the damaging effect of free radicals. Antioxidant compounds that are found in indigenous plants are valuable for many physiological functions such as promoting good health, by assisting immune system, photoprotection and preventing aging of cells (Christopher, 2011).

Indigenous plants are recommended by many authors and researchers because they are readily available, and they do not require high intensive technology and input levels; they can be grown well on the diversity of climatic conditions of a country, at the same time contributing to good nutrition. In this present study, a germination test and the bio-chemical, phytochemical and antioxidant composition of an indigenous plant

black jack (*Biden Pilosa L*) were examined and compared with other *Asteraceae* commercial vegetables (chicory & lettuce) over the growing period. The black jack seeds were found to possess comparatively high levels of total phenolics, flavonoids, tannins and total antioxidants. The protein content was also high on the seeds. Similar results were also observed on the radicals, the only noticeable differences were on the anti-nutritional compounds, tannins which were lower on the radicals as compared to the seeds. This implies that black jack radicals are nutritious; they can contribute to the general health of human beings.

Black jack leaves were also found to possess, high levels of secondary metabolites, as compared to other crops that were used during the study. However, all of the beneficial metabolites in black jack were found during the early stages of growth, from first to the third week after transplanting. They were considerable high amounts of total phenols, flavonoids, carotenoids and various chlorophyll pigments as well as enzymatic and non-enzymatic antioxidants. The study concluded by recommending young leaves of black jack to be consumed by all households. Black jack seeds and leaves can also be used by pharmaceuticals, to develop more drugs that have a human value.

5.1 Recommendations

- More future studies on the postharvest handlings of black jack leaves to minimize loss of essential nutrients could add value, in future food security strategies.
- More field studies are needed to assess the antioxidant activity of black jack, in different soils and climatic conditions.

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