THE EFFECT OF CRUDE BETALAINS FROM BEET ROOT ON (*IN - VITRO*) HYPOCHLOROUS ACID INDUCED LOW DENSITY LIPOPROTEIN OXIDATION

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A Research Thesis Submitted to Graduate School in Partial Fulfillment of the Requirements for Masters of Science Degree in Food Science and Technology of Egerton University

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

I declare that this thesis is my original work and to the best of my knowledge it has not been submitted wholly or in part in this form or any form for a degree, diploma or certificate award at Egerton or any other university.

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Recommendation

This research thesis has been prepared under our supervision and has been submitted for examination as per Egerton University regulations with our recommendation and approval.

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ABSTRACT

Studies have shown a decreased risk of atherosclerosis linked to the intake of fruits and vegetables rich in bioactive phytonutrients. This study investigated the effect of crude beet root betalains on low density lipoprotein (LDL) oxidation induced by hypochlorous acid (HOCl). Crude betalains antioxidant potential was determined by use of 2, 2 – diphenyl - 1 – picrylhyrayl radical (DPPH assay) and was compared to lycopene and ascorbic acid. HOCl oxidizing ability and betalains antioxidant capabilities were determined by incubating heparin precipitated LDL from rat (*Rattus norvegicus)* blood serum with varying concentrations of HOCl or 10µM HOCl plus crude betalains at 37 ^{o}C in phosphate buffer saline (pH 7.4). The oxidation process was monitored by measuring conjugated dienes formed at 234 nm at 5 minute intervals over a 3 hour period using a Spectrum lab Gold S54T UV- Visible spectrophotometer. Lycopene was used as control. DPPH assay results indicated significant ($p < 0.05$) variation in antioxidant activity between crude betalains, lycopene and l - ascorbic acid and amongst varying concentrations of each sample. Generally, crude betalains had greater antioxidant ability than both lycopene and l – ascorbic acid. At 160µM concentration, crude betalains, lycopene and ascorbic acid had an antioxidant activity of 99%, 61.7% and 77.8% respectively. Tests on the oxidizing potential of HOCl revealed significant ($p < 0.05$) increase in LDL oxidation with time for HOCl concentrations 10µM, 30µM and 50µM but a significant ($p < 0.05$) drop in LDL oxidation activity below that of 10µM HOCl for HOCl concentrations 70µM and 100µM. At 50µM HOCl, LDL oxidation activity was 2.43% higher than that at 10µM HOCl and 1.46% higher than that at 30µM HOCl. However, at 70µM and 100µM HOCl, a drop in LDL oxidation activity of 1.64% and 1.85% respectively, was recorded compared to that of 10µM HOCl. At 100µM crude betalains showed significant ($p < 0.05$) inhibition of 10µM HOCl induced LDL oxidation. However, lycopene's ability to inhibit LDL oxidation surpassed that of crude betalains at all concentrations. Crude betalains of 100µM, 50µM and 10µM concentration registered a drop in LDL oxidation activity of 8.4%, 3.9% and 1.5% respectively against the control (10µM HOCl) while lycopene of similar concentrations had a drop of 39.9%, 27.6% and 19.5% respectively, against the control. Results confirm that crude betalains possess significant antioxidant ability and proposes that betalains may be useful in the reduction of *in - vivo* LDL oxidation.

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DEDICATION

I dedicate this thesis to Livingstone, Laura, Leila and Collins for their love and support.

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My sincere thanks go to God Almighty for giving me the strength and all the help I needed to bring me this far in my academic pursuit. I thank my supervisors Prof. Symon M. Mahungu and Dr. Mary Omwamba of Egerton University, department of Dairy and Food Science and Technology, who not only guided me through this research but in my desperate search for a research project helped me out. Their patience with me throughout this study is only God given. Thanks again. I am also grateful to all my classmates (Master of Science Food Science and technology 2010) for their advice and encouragement through the entire research and to my family for their financial support and encouragement during this study and always.

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LIST OF ABBREVIATIONS

ACAT	Acyl – CoA – cholesterol acyl transferase
BSA	Bovine serum albumin
CD	Conjugated dienes
DDW	Double distilled water
DPPH	2, 2 – diphenyl - 1 – picrylhyrayl
DPPH – H	2, 2 – diphenyl - 1- picrylhydrazine
FRS	Free radical scavengers / Antioxidants
HCl	Hydrochloric acid
HDL	High density lipoprotein
HMG - CoA reductase	β - hydroxy - β - methyl - glutaryl – CoA
HNE	4 - Hydroxynonenal
HOCl	Hypochlorous acid
IDL	Intermediate density lipoproteins
LDL	Low density lipoproteins
MDA	Malondialdehyde
MPO	Myeloperoxidase
RFM	Relative formular mass
TBARS	Thiobarbituric acid reactive substances

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CHAPTER ONE
INTRODUCTION

1.1 Background

Low density lipoproteins (LDL), the major cholesterol carrier in the human body are thought to become atherogenic after undergoing oxidative modifications. Atherosclerosis is initiated by an inflammatory process in the blood vessel walls in response to the damage caused by oxidized LDL after exposure to free radicals. Modifications of the apo - B protein by oxygen radicals makes the LDL particles less efficiently handled by the physiologic B-E receptor-mediated pathway (Ghaffari and Ghiasvand, 2011). They instead accumulate in macrophages through the scavenger receptor pathway (Kinlay *et al*., 2004). The mobility of these macrophages loaded with oxidized LDL out of the blood stream is inhibited. Macrophages grow, then rapture, depositing greater amounts of oxidized LDL (plaque) on the arterial wall. Plaque deposition causes thickening and hardening of arteries and eventual health complications like stroke and myocardial infarctions. Studies have shown a decreased risk of atherosclerosis associated with the intake of fruits and vegetables. Lycopene, a fat soluble antioxidant was shown to decrease the susceptibility of LDL to oxidation induced by copper (II) (Ghaffari and Ghiasvand, 2011). This study investigated the effect of crude beetroot betalains, on *ex-vivo* LDL oxidation induced by hypochlorous acid (HOCl). Lycopene was used as control. Currently such a report is unavailable.

Betalains are water soluble red and yellow indole - derived aromatic condensation products of a primary or secondary amine with betalamic acid. They are responsible for the color of flowers, fruits, leaves, stems and roots of plants like beet root (*Beta vulgaris ssp. crassa*), cactus fruits, colored swiss chard, bougainvillea and grains or leaves of amaranth. Betalains contained in red beet root are effective antioxidants (Wettasinghe *et al.,* 2002). They inhibit lipid peroxidation at very low concentrations (Kanner *et al*., 2001). Additionally, they are not degraded by the free radical mechanism (Attoe and Elbe, 1985). When consumed, betalain traces have been positively identified bound to LDL (Tesoriere *et al.,* 2003; 2004). These properties, plus betalains' strategic positioning at the source of LDL oxidation problem, make betalains a possible solution to the free radical LDL damage in the blood stream. However, kinetic investigations on betalains ability to inhibit LDL oxidation are few. Apart from their antioxidant abilities, betalains like betanin, isobetanin and vulgaxanthin lessen inflammation by inhibiting the activity of cyclo – oxygenase enzymes used by cells to produce messaging molecules that

trigger inflammation. Since atherosclerosis results due to chronic unwanted inflammation caused by oxidized lipids in the blood stream, betalains can possibly provide a two way solution to this problem.

Lycopene is a 40 carbon open chain carotenoid with 11 conjugated double bonds. This fat soluble pigment is found in tomatoes, red oranges, pink grape fruit, water melon, papaya, apricot and the pink guava. Lycopene is one of the most potent carotenoid antioxidants known to neutralize oxygen derived free radicals. Lycopenes' antioxidant activity is stronger than that of beta carotene while its singlet – oxygen quenching ability is twice as high as that of beta - carotene and ten times higher than that of alpha - tocopherol (Singh and Goyal, 2008).

Like betalains, lycopene from the gut is bound to LDL and transported to various tissue sites like the liver, adrenal, testis and prostrate. Since reports on kinetic investigations of LDL oxidation in the presence of crude betalains in comparison to lycopene are scarce, this study was intended to increase the understanding of the effect of water soluble and fat soluble antioxidants on lowering the risk of LDL oxidation especially in the aqueous phase. The study was also prompted by the current health trends of exploring natural and / or alternative health products, as opposed to modern drug therapy, in lowering the risk of disease occurrence. Empirical evidence obtained in this study will help establish betalains potential ability in inhibiting LDL oxidation induced by HOCl.

1.2 Statement of the Problem

Oxidation of LDL by free radicals in the blood stream results in destructive induction of the immune system and deposition of oxidized lipid materials (plaque) on blood vessel walls. Plaque deposition causes thickening and hardening of blood vessels. The sudden rapture of soft plaque results in the formation of a thrombus which rapidly slows or stops blood flow, leading to the death of tissues fed by the artery. Coronary thrombus causes myocardial infarction (a heart attack), while the same happening in an artery to the brain causes stroke. Insufficient blood supply to the legs causes claudication (pain, discomfort, tiredness) and is also known to cause ischemic gangrene of the extremities. At its worst, plaque related complications cause death of victims or their immobilization. This study explored the potential of crude betalain rich beet root extract in reducing significantly *ex-vivo* oxidation of LDL.

1.3 Objectives

1.3.1 General Objective

To assess the antioxidant ability of crude betalains from beet root and their effect on *ex-vivo* HOCl induced LDL oxidation in order to establish their capability to control LDL oxidation.

1.3.2 Specific Objectives

i) To determine the antioxidant activity of betalain rich beet root extract.

ii) To determine the effect of time and concentration of HOCl on LDL oxidation.

iii) To determine the effect of betalain rich beet root extract on HOCl induced LDL oxidation.

1.4 Hypothesis

i) H_o: Betalain rich beet root extract does not possess antioxidant activity.

ii) H_o: LDL oxidation is not dependent on time and concentration of HOCl.

iii) H_o: Betalain rich beet root extract does not inhibit HOCl induced LDL oxidation.

1.5 Justification

In the body, oxidative damage to proteins, lipids and deoxyribonucleic acids (DNA) is caused by high levels of free radicals (reactive oxygen and nitrogen species) resulting in cellular dysfunction and disease. Atherosclerosis starts early in life and progresses slowly and insidiously at times only becoming evident later in life due to complications like stroke and heart attacks. Treatment of atherosclerosis commonly involves last minute detection of disease, followed by use of expensive cholesterol – reducing drugs, prescribed diets and life threatening surgeries when the disease is far advanced.

Antioxidants stabilize free radicals by donating hydrogen radicals to them and inhibit oxidative damage of tissues by reacting faster with free radicals than lipids, proteins and / or deoxyribonucleic acids (DNA). This research will establish betalains role in inhibiting LDL oxidation. Oxidation of LDL in the presence of the betalain rich beet extract will provide valuable information on the role of beet root betalains in atherosclerosis risk reduction. Research information will be of use to nutritionist in the development of dietary recommendations for populations at risk of suffering from atherosclerosis.

CHAPTER TWO
LITERATURE REVIEW

2.1 Low Density Lipoproteins

LDL provides cholesterol to peripheral tissues. Each LDL particle circulating in the blood contains apo B -100, a protein that provides a recognition site for LDL receptors on the cell membranes of all cells of the body. Binding of LDL to an LDL receptor on the cells initiates endocytosis which brings both the LDL and its associated receptor into the cell within an endosome. The endosome fuses a lysosome which contains enzymes that hydrolyse cholesterol esters releasing cholesterol (incorporated into cell membranes) and fatty acids into the cytosol. Apo B -100 is degraded and the amino acids produced released to the cytosol. The LDL receptor escapes degradation and returns to the cell surface where it can again function in LDL uptake. High cellular cholesterol: inhibits intracellular synthesis of cholesterol by inhibiting HMG-CoA reductase an enzyme involved in cholesterol synthesis in the liver, it also triggers the esterification of excess cholesterol in the cytosol by ACAT for storage within cytosolic lipid droplets and also prevents the synthesis of new LDL receptors on cells reducing the absorption of additional LDL into the cell. A combination of all these processes helps the cell control its own internal concentration of cholesterol. The liver also takes up LDL for its own cholesterol requirements. This combined with the return of IDL and LDL to the liver maintains a high cholesterol concentration in the liver cells. Excess cholesterol in the liver inhibits the liver enzyme system synthesizing new cholesterol. The return of excess cholesterol to the liver therefore helps to diminish cholesterol synthesis in the body. LDLs are also taken up by a lower-affinity system in macrophages and some other cells. The LDL receptor on macrophages and related cells is called the scavenger receptor. The scavenger receptor is different from the LDL receptors on cells and has greater affinity for altered LDL. Macrophages preferentially take up LDL that has been modified by oxidation (Kinlay *et al*., 2004). Macrophages overloaded with oxidized LDL become 'foam cells' that are seen in early atherosclerotic lesions.

2.1.1 Isolation of Low Density Lipoprotein from Blood

2.1.1.1 Ultracentrifugation

The varying densities of lipoproteins can be used for their rapid isolation from a protein solution by density gradient centrifugation. When placed in a density gradient lipoproteins

sediment to the zone of identical density. However this gravitational sedimentation is counteracted by diffusion. To achieve separation grater force must be applied to a protein solution containing lipoproteins to cause separation hence the need for ultracentrifugation.

2.1.1.2 Precipitation Using Heparin

Heparin is a highly sulphated glycosaminoglycan carbohydrate that consists of sulphated repeating disaccharide units of D - glucosamine and uronic acid. Its many SO_3^- groups give it a strong negative electric charge. Heparin forms complexes with proteins in which its negative groups are bound to the positive groups of proteins. Under appropriate conditions the three classes of lipoproteins can be precipitated by heparin in the presence of one of the divalent cations like Ca^{+2}, Mg^{+2}, Mn^{+2} or Co^{+2}. However heparins' affinity for low density lipoproteins is considerably greater than for other proteins. At values near the isoelectric point of the lipoprotein heparin will precipitate low density lipoproteins completely without the addition of metal ions. At pH 5.05 – 5.25, heparin alone will precipitate only the low density lipoproteins. The precipitate formed is subsequently separated from the dissolved or soluble components by an appropriate solid-liquid separation techniques (Seidel *et al.*, 1990).

2.1.2 Determination of the Concentration of Proteins in Low Density Lipoproteins

The Lowry assay: 'protein concentration by Folin reaction' is commonly used to estimate low concentrations of proteins in solutions or in samples easily soluble in dilute alkali. The protein in a sample is first pre-treated with copper ions under alkaline conditions. This biuret reaction of protein with alkali cupric tartarate which produces color is followed by the copper - catalysed oxidation of aromatic acids (tyrosine, tryptophan) present in the treated sample and the subsequent reduction of phosphomolybdate-phosphotungistic acid reagent component in the Folin Ciocalteau reagent, to heteropolymolybdenum blue. The amount of proteins in the sample is then estimated by reading the absorbance at 750 nm of the end product of the Folin reaction against a standard curve of a selected protein solution (e.g. Bovine serum albumin) (Lowry *et al.*, 1951).

2.2 Oxidation of Low Density Lipoproteins

Oxidized LDL refers to LDL particles that have been exposed to an oxidant or may have lipid peroxides or products of lipid peroxidation associated with it.

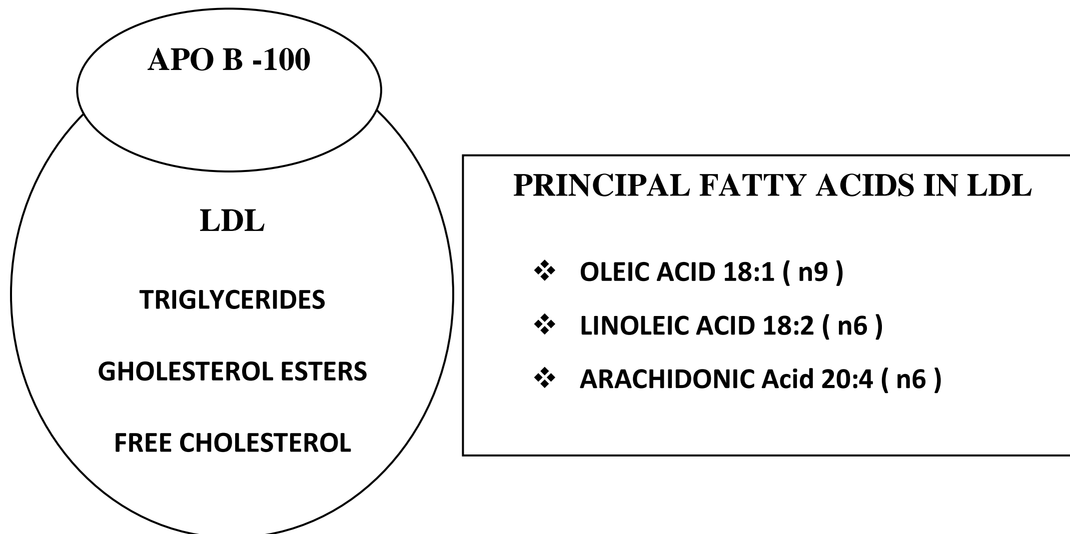
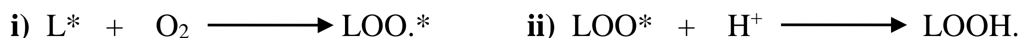


Figure 1: Components of Low Density Lipoproteins

Oxidation follows the following steps:-

i) Initiation: Unsaturated fatty acids oxidation is initiated when hydrogen is abstracted by free radicals from the fatty acids resulting in the formation of an alkyl or fatty acid radical (L*). This radical is stabilized by delocalization over the double bond(s) to form conjugated double bonds (conjugated diene). Polyunsaturated fatty acids contain methylene interrupted carbons hence have more sites for hydrogen abstraction.

ii) **Propagation**: The newly formed conjugated diene very easily reacts with oxygen. In the presence of oxygen the alkyl radical (**L***) binds covalently to oxygen to form a high energy peroxyl radical (**Loo***). The peroxyl radicals formed promotes the abstraction of hydrogen from another fatty acid molecule to form a new fatty acid radical while the addition of hydrogen to the



i) $L^* + O_2 \longrightarrow LOO.^*$ **ii)** $LOO^* + H^+ \longrightarrow LOOH.$

peroxyl radical leads to the formation of a fatty acid hydroperoxide. Since carbon – hydrogen covalent bonds on unsaturated fatty acids are weak they are susceptible to attack by peroxyl radicals. The newly formed alkyl radical propagates the repeat of the oxidation cycle. The reaction of fatty acid radicals with oxygen and hydrogen yields peroxides, hydroperoxides and cyclic endoperoxides all containg conjugated dienes that have an absorption maximum at 234 nm. Decomposition of lipid and hydroperoxides by transition metals (components of biological systems) leads to the production of a very high energy alkoxyl (**LO***) radical. This radical

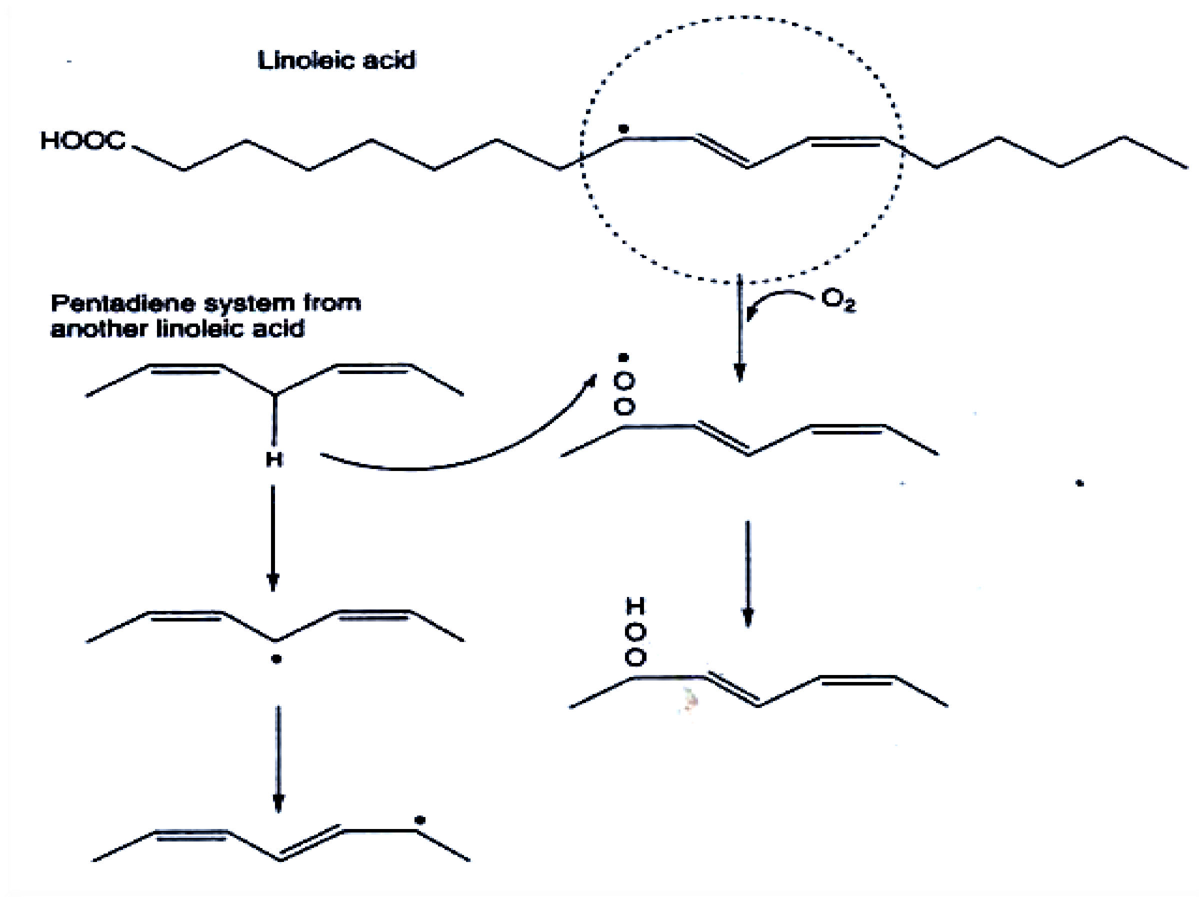


Figure 2: The Propagation Step of Lipid Oxidation for Linoleic Acid (McClements and Decker, 2007)

abstracts electrons from covalent bonds adjacent to it causing a cleavage of the aliphatic chain of fatty acids (B-scission reaction). This reaction breaks the aliphatic chain of the fatty acid to produce aldehydes (McClements and Decker, 2007). Aldehydes such as 2, 4 – decadienal (2, 4 - DDE) and malondialdehyde (MDA) produced during oxidation of LDL are known to be cytotoxic.

iii) Termination: Refers to the reaction of two radicals to form non – radical species. Under low oxygen concentrations the alkyl radicals combine to form fatty acid dimmers while in the presence of oxygen the peroxyl radical (predominant) combines with the alkoxyl radical.

Apo B - 100 comprises of 4536 amino acid residues and is the only protein associated with LDL. It extends over at least half of the lipoprotein circumference and functions by directing LDL to specific target tissue ensuring LDLs subsequent clearance from circulation. Oxidation of the apolipoprotein results in loss of specific amino acids. Additionally, aldehydes

produced in lipid oxidation reactions by alkoxyl radicals are known to interact with sulfhydryls and amines in proteins altering their functionality.

LDL bound cholesterol is in the form of cholesterol esters (80 – 90 %). Like unsaturated fatty acids the presence of a double bond in the cholesterol nucleus makes it prone to oxidation. Cholesterol oxidation may be due to direct abstraction of hydrogen by free radicals from the sterol nucleus or from the fatty acid attached to it. The association of triglycerides and phospholipids with cholesterol as is in LDL may accelerate the oxidation of the sterol nucleus and vice versa. Peroxides formed from polyunsaturated fatty acids during lipid oxidation may abstract hydrogen from the cholesterol double bond allowing the formation of a free radical into the cholesterol structure. This free radical may migrate to position 4 or 7 of the A and B rings allowing the reaction of molecular oxygen at positions 4, 5, 6 and 7. However the most preferred position is at carbon 7 where hydroperoxides may form either a hydroxyl derivative by

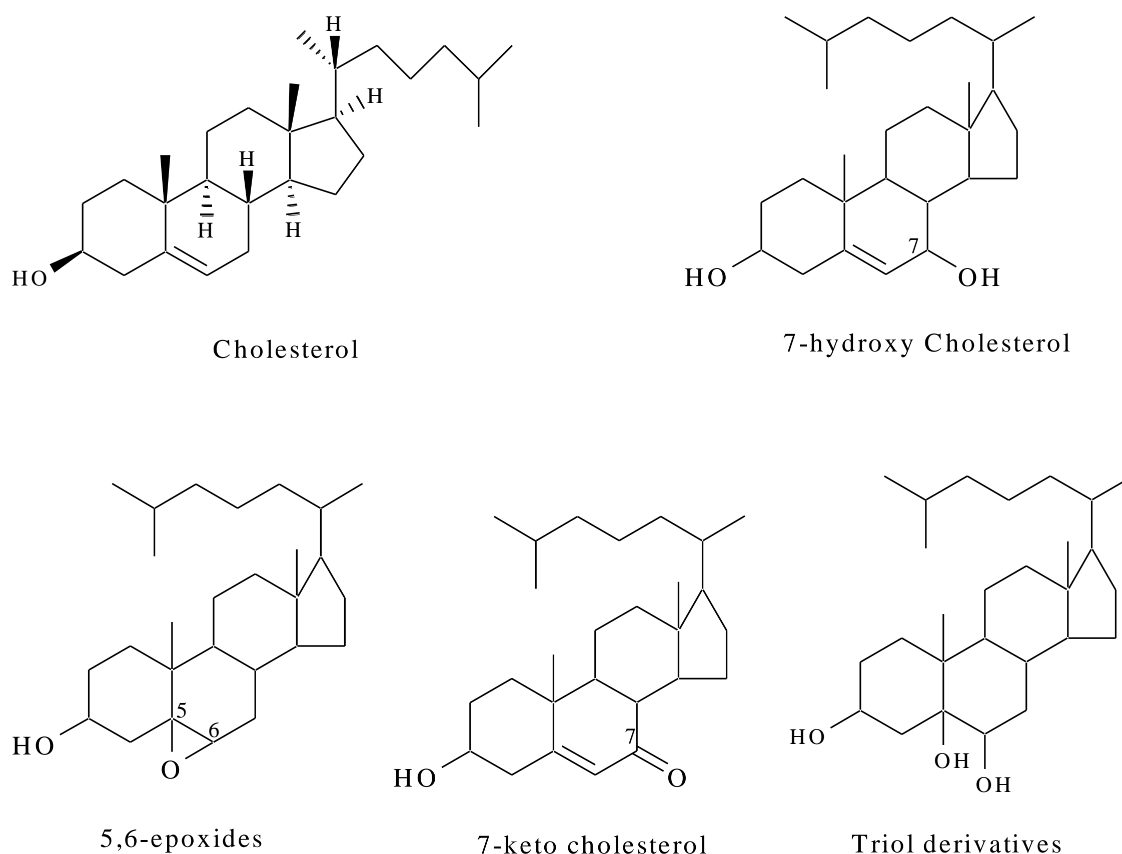


Figure 3: Cholesterol Oxidation Products (Valenzuela *et al.,* 2003)

decomposition (7-hydroxycholesterol) or a keto-derivative by dehydration (7-ketocholesterol). Other possibilities may involve cholesterol oxidation starting with the formation of a hydroperoxide at carbon 7 which undergoes decomposition into an alkoxyl radical that later undergoes rearrangement to 5, 6 epoxides, 7-hydrocholesterol and 7-ketocholesterol. Molecular oxygen free radicals may also react directly with the double bond forming a 5-6 epoxycholesterol by addition which by further dehydration can be transformed into a triol (cholestane - 3B, 5a, 6B - triol) (Figure 3). Oxidation at the lateral chain may produce either a 20-hydroxyl or a 25-hydrocholesterol derivative. Circulating oxysterols may be formed from lipoprotein oxidation at the blood stream while a large percentage comes from dietary sources. Dietary sources of oxysterol are heat processed cholesterol rich foods of animal origin like dairy, processed eggs and meats. Oxysterols may also be formed by intracellular enzymatic reactions (Bjorkhem, 2002). In human tissue endogenous production of oxysterols occurs during the conversion of cholesterol to bile acids and steroid hormones. These account for a significant percentage of oxysterols found in the serum and other tissues. Like cholesterol, oxysterols are transported by LDL to the tissues while HDLs are involved in oxysterol transport from the tissues to the liver. Oxidized LDL is potentially more atherogenic than native LDL because it is chemotactic (movement of monocytes in response to a chemical stimulus) for circulating monocytes, is an inhibitor of the motility of resident macrophages and is cytotoxic (agent / process that kills cells). Due to this LDL oxidation products are believed to initiate endothelial cell injury that leads to the accumulation of plaque and the eventual formation of blood clots**.**

2.2.1 Low Density Lipoprotein Oxidation by Hypochlorous Acid

Evidence shows that oxidants generated by the heme enzyme myeloperoxidase (MPO) play a key role in oxidation reaction of the artery wall. MPO produces hypochlorous acid by the reaction of hydrogen peroxide (H_2O_2) and chloride ions (Cl^-). This enzyme is abundantly present in neutrophils and monocytes (macrophages) and is released during inflammatory activation of immune cells. HOCl contributes to the antimicrobial activity of phagocytes. Unfortunately, chronic or prolonged production of HOCl initiates and propagates vascular diseases. HOCl is able to modify a great variety of biomolecules by chlorination and / or peroxidation. It reacts with lipids to produce chlorohydrins (major) and hydroperoxides. Lipid peroxidation by HOCl is proved by the presence of HNE, a chemotactic lipid peroxidation product (secondary oxidation product). HOCl can initiate lipid peroxidation in lipoproteins, producing conjugated dienes

(primary by - products), aldehydes and oxygen containing species. LDL oxidation by HOCl starts with a rapid modification of protein and is followed by an extended period of peroxidation during which further protein oxidation does not occur. Apo B -100, the only protein of LDL, is a hydrophobic protein with 4,536 amino acid residues (molecular mass 550 kDa). HOCl reacts readily with apo B lysine residues, resulting in the formation of *N*-chloramines, which are quantitatively the major products in HOCl - modified LDL. Other amino acid residues such as cysteine, methionine, tryptophan, and tyrosine, are also susceptible to oxidation by HOCl with lysine, cysteine and methionine exhibiting the highest reactivity. Chloramines from protein oxidation decay to give both nitrogen and carbon centered radicals (Panasenko, 1997) while the homolytic cleavage of preformed lipid hydroperoxides by HOCl generate high energy alkoxyl radicals which may induce oxidation of the LDL lipid component.

2.2.2 Measurement of Lipid Oxidation Products

2.2.2.1 Measurement of Conjugated Dienes (CD)

During lipid peroxidation the methylene - interrupted double bonds naturally present in unsaturated lipids are converted to conjugated double bonds (CD). All lipid oxidation products formed in the initiation and propagation stages like alkyl radicals, peroxyl radicals and hydroperoxides contain CD bonds. Conjugated double bonds or dienes absorb ultraviolet light strongly at 234 nm (Ghaffari and Ghiasvand, 2011). Continuous monitoring of the formation of CD bonds is used to measure the susceptibility of LDL to oxidation. LDL oxidation is induced *ex - vivo* by incubating native LDL with Cu^{+2} ions (or other oxidant) and is continuously measured by monitoring the change in the conjugate diene absorbance at 234 nm. (LDL oxidation initiated by metal ions is believed to be similar to the oxidative process occurring in vivo).

2.2.2.2 Measurement of Thiobarbituric Acid Reactive Substances (TBARS Assay)

The TBA assay is based on the reaction between TBA and carbonyls to form red-fluorescent adducts under acidic conditions. The oxidation product detected by TBA is malondialdehyde (MDA) that is usually produced by the degradation of polyunsaturated fatty acids. The TBA adduct absorbs strongly at 532 nm. TBA can also react with aldehydic lipid oxidation products other than MDA especially unsaturated aldehydes. The basic principle of the method is the reaction of one molecule of malonaldehyde and two molecules of TBA to form a red malonaldehyde - TBA complex that can be quantitated spectrophotometrically at 532 nm.

Other methods of measuring lipid oxidation products involve incorporating parinaric acid, a fluorescent and oxidative sensitive polyunsaturated fatty acid into LDL or monitoring fluorescence development during LDL oxidation caused by the reaction of protein amino groups with aldehydes generated during the decomposition of peroxidised fatty acids.

2.3 Antioxidants

The National academy of science defines a dietary antioxidant (free radical scavengers (FRS)) as a substance in foods that significantly decreases the adverse effect of reactive species such as reactive oxygen and nitrogen, on normal physiological functions in humans.

Antioxidants inhibit oxidation by reacting faster with free radicals than lipids, DNA and proteins. They stabilize free radicals before they harm the body by preferentially donating hydrogen (H^+) required by the free radicals for their stability and also helps promote the formation of fatty acid hydroperoxides ensuring no more alkyl radicals are formed, breaking the lipid oxidation chain reaction. Antioxidants efficiency is dependent on their ability to donate hydrogen to free radicals. The ability of an antioxidant to donate its hydrogen to a free radical is determined by its electron reduction potential. Any FRS that has a reduction potential lower than that of a free radical is capable of donating its hydrogen to that free radical unless the reaction is kinetically unfeasible. Chain breaking FRS, interact with peroxyl (LOO^*) and alkoxyl (LO^*) radicals as follows:-

$LOO^* + FRS \longrightarrow LOOH + FRS^*$ $\quad$ $LO^* + FRS \longrightarrow LOH + FRS^*$

FRS react mainly with lower energy radicals like the peroxyl radical (LOO^*) because they have a longer lifetime (less reactive) unlike the higher energy radicals like alkoxyl (LO^*) and hydroxyl (OH^*) radicals that react quickly with molecules closest to their site of production hence have a short lifetime, and are less likely to react with the lower energy hydrogen FRS.

Efficient FRS are determined by the energy of the resulting free radical scavenger radical (FRS^*). Effective FRS form low energy radicals owing to resonance delocalization. Lower energy FRS^* are less likely to catalyze oxidative reactions. FRS^* may participate in termination reactions with other FRS^* or lipid radicals to form non-radical species.

2.3.1 Betalains

The name betalain comes from the Latin name of common beet (*Beta vulgaris*) from which betalains were first extracted. Betalains are aromatic condensation products of a primary or secondary amine with betalamic acid (BA). They are a class of water soluble red and yellow

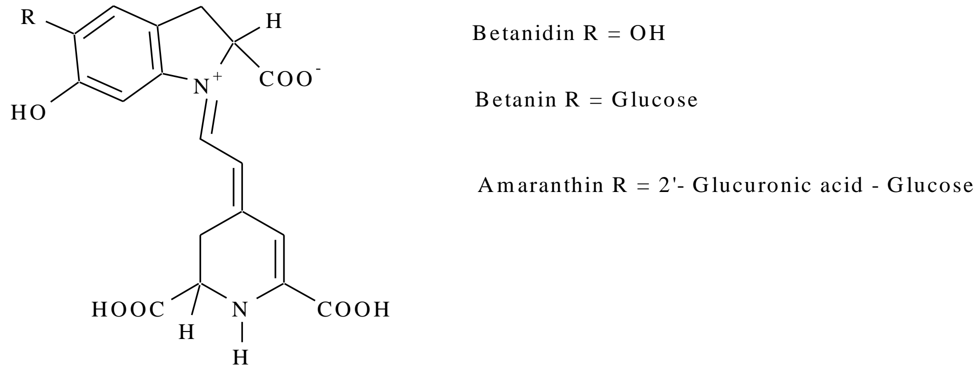


Figure 4: Structure of betacyanins (Schwarts *et al*., 2007).

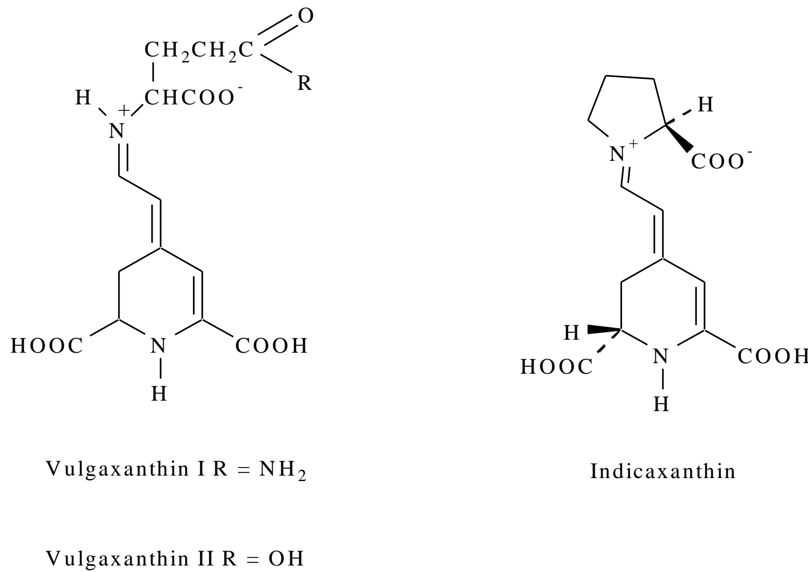


Figure 5: Structure of Betaxanthins (Schwarts *et al*., 2007).

indole - derived pigments found in plants of the caryophyllales which exist as internal salts (zwitterions) in vacuoles of plant cells. Betalains color petals of flowers, fruits, leaves, stems and roots of plants that contain them such as beet root, cactus fruits, bougainvillea, colored swiss chard and amaranth grains / leaves (Schwarts *et al.,* 2007). Betalains are classified into two

groups, the red-violet betacyanins and the yellow - orange betaxanthins. Red beet root varieties contain red-violet pigments like betanin, isobetanin, betanidin and isobetanidin while the yellow pigments are vulgaxanthin I and 11. Betacyanins have a maximum light absorption at 540 nm while the yellow - orange betaxanthins have a maximum light absorption at 480 nm. The major betacyanins in red beet are betanin and isobetanin while the two betaxanthins that have been isolated from beet are vulgaxanthin I and II. Gasztonyi *et al.* (2001) analysed five red beet varieties (Bonel, Nero, Favorit, Rubin and Detroit) and found that the red-violet pigments were betanin, isobetanin, betanidin and isobetanidin, while the yellow pigments were vulgaxanthin I and II. Red beets also contain several endogenous enzymes like B-glucosidase, polyphenoloxidase and peroxidases (Delgado *et al.,* 2000; Martinez and Munoz, 2001; Escribano *et al.,* 2002).

2.3.1.1 Properties of Betalains

Betalains are relatively stable over a broad pH range of 3 – 7. Their charge alters upon pH changes. Alkaline conditions cause aldimine bond hydrolysis (degrades to betalamic acid and cyclodopa-5-glucoside under mild alkaline conditions) while acidification induces recondesation of betalamic acid with the amine group. The optimum pH range for maximum betanin stability is 5 - 6 (Castellar *et al.,* 2003; Vaillant *et al.,* 2005).

Betalains are susceptible to oxidation. Their susceptibility to oxidation makes them effective antioxidants (Wettasinghe *et al.,* 2002). Molecular oxygen has been implicated as the active agent in the oxidative degradation of betanin. Betalains do not degrade by a free radical mechanism (Attoe and Elbe, 1985). Some antioxidants like ascorbic acid and ascorbate that scavenge molecular oxygen have been found to enhance betalain stability whereas those like phenolic antioxidants that act by terminating free radicals are ineffective, meaning that betalains do not degrade by a free radical mechanism. Oxidative degradation was promoted by light exposure while degradation was negligible under anaerobic conditions.

Betacyanins are more stable than betaxanthins at room temperature and upon heating (Herbach *et al.,* 2004). Upon heating betanin degrades by isomerization, decarboxylation and or cleavage.

The Optimum pH for enzymatic degradation of both betacyanins and betaxanthins is 3.4. The degradation products are similar to those of thermal, acid or alkaline degradations (Martinez and Munoz, 2001; Escribano *et al*., 2002; Stintzing and Carle, 2004).

Betacyanins are more susceptible to degradation by peroxidases than betaxanthins. Endogenous Β-glucosidase converts betanin glucosides to their respective aglycones. The aglycones formed are however more labile and prone to further oxidation leading to loss of colour and subsequent browning (Stintzing and Carle, 2004).

The stability of betacyanins was reported to increase after reducing the water activity (a_w) by concentration (Castellar *et al.*, 2006) and spray drying (Cai and Corke, 2000). This effect may be attributed to a reduced mobility of reactants or limited oxygen solubility (Delgado *et al.,* 2000). About 2% moisture content (dry weight basis) is recommended for the storage of pigments in beet root powder.

Metals like copper, iron, aluminum and tin accelerate betanin degradation.

LDL isolated 3 and 5 hours after ingestion of a betalain meal (500g pulp) incorporated betalains. A study by Tesoriere *et al.* (2004) showed that the hydrophilic betanin and indicaxanthin binds to LDL in vitro providing oxidative resistance. Further Tesoriere *et al.* (2005) showed that human red blood cells incorporated dietary betalains which may protect the cells and prevent oxidative hemolysis. Allegra *et al.* (2005) reported the effectiveness of betalains and indicaxanthin to scavenge hypochlorous acid (HOCl) the most powerful oxidant produced by human neutrophils. Betalains were able to act as reductants of the redox intermediates of myeloperoxidase which catalyses the production of HOCl. Kanner *et al.* (2001) reported the ability of both betanin and betanidin at very low concentrations to inhibit lipid peroxidation and heme decomposition in vitro while Gentile *et al.* (2004) observed the ability of betalains to protect an in-vitro model of endothelial cells from oxidation related to inflammation response. Lee *et al.* (2005) reported the ability of betalains to induce quinone reductase, a potent detoxification enzyme associated with cancer chemoprevention while Kapadia *et al*. (2003) showed significant inhibitory effect of beet root towards skin and lung cancer in mice and later demonstrated the suppression of skin and lung tumors in mice.

2.3.1.2 Extraction of Betalains from Beet Root

The major commercially exploited betalain crop is red beet root. It is abundant, inexpensive and produces up to 0.5g of betanin per kg of roots (Gastonyi *et al*., 2001). In the laboratory extraction of betalains can be done by diffusion – extraction, solid – liquid extraction, reverse osmosis and ultrafiltration. Betalains are concentrated mostly towards the outer parts of the roots decreasing in order from the peel, crown then flesh. Extraction of pigment from the

matrix is achieved by use of water or aqueous methanol or ethanol (20 - 50%). Betalains are the most soluble pigments in water hence easily leach from beet tissue (more hydrophilic than colourless phenolics) (Elbe, 2001). Use of aqueous methanol (60:40 or 80: 20) denatures the protein structure of enzymes present (polyphenoloxidase, peroxidase and B - glucosidase) inhibiting degradation reactions.

2.3.1.3 Determination of the Concentration of Betalains in Beet Root Extract

Betacyanin and betaxanthin concentrations of a freshly extracted betalain solution are determined by measuring their spectrophotometric light absorption at 538 nm and 476 nm respectively. The results are expressed as betacyanins (calculated as betanin) and betaxanthin (calculated as vulgaxanthin 1). These spectrophotometric measurements also include minor betacyanins and betaxanthins. Betanin and vulgaxanthin 1 make up 95% of the pigment concentration. Additionally all minor betalains have a maximum light absorption similar or very close to the light absorption of the two hence contribute to the light absorption at A538 and A476. Due to this the error involved when the betalain concentration is calculated in terms of the two major pigment components is negligible. Light absorption measured at 600 nm is used to correct small amounts of impurities.

The corrected light absorption of betanin and vulgaxanthin 1 will be given by:

$X = 1.095 \times (a-c)$ $\qquad$ $Y = b - z - x/3.1$ $\qquad$ $Z = a - x$

Where: -

a = light absorption of the sample at 538 nm

b = light absorption of the sample at 476 nm

c = light absorption of the sample at 600 nm

X= light absorption of betanin minus the colored impurities.

Y= light absorption of vulgaxanthin 1 corrected for the contribution of betanin and colored impurities.

Z = light absorption of the impurities.

The concentration of betanin and vulgaxanthin1 is calculated using each pigments adsorptivity value ($A^{1\%}$) e.g. Betanin = 1120 and Vulgaxanthin 1 = 750 and the appropriate dilution factor (Elbe, 2001).

2.3.2 Lycopene and Ascorbic Acid

Lycopene is a 40 carbon open chain polyisoprenoid carotenoid with 11 conjugated double bonds. About 80% of dietary lycopene is from tomatoes and is responsible for the characteristic deep - red color of tomatoes. Its name is derived from the tomato specie *Solanum lycopersicum.* Other sources of lycopene include; red fruits like the orange and pink grape fruit, water melon, apricot and the pink guava. Lycopene is one of the most potent carotenoid antioxidants known to neutralize oxygen derived free radicals. Although it has a structure similar to that of beta – carotene, its antioxidant activity is much stronger (Singh and Goyal, 2008). It has a singlet – oxygen quenching ability twice as high as that of beta-carotene and ten times higher than that of alpha - tocopherol. Lycopenes' superior antioxidant ability in comparison to other carotenoids is because of its unique structure of 11 conjugated double bonds and no cyclic groups. Lycopene absorbed from the gut is bound to LDL and transported to various tissue sites like the liver, adrenal, testes and prostrate.

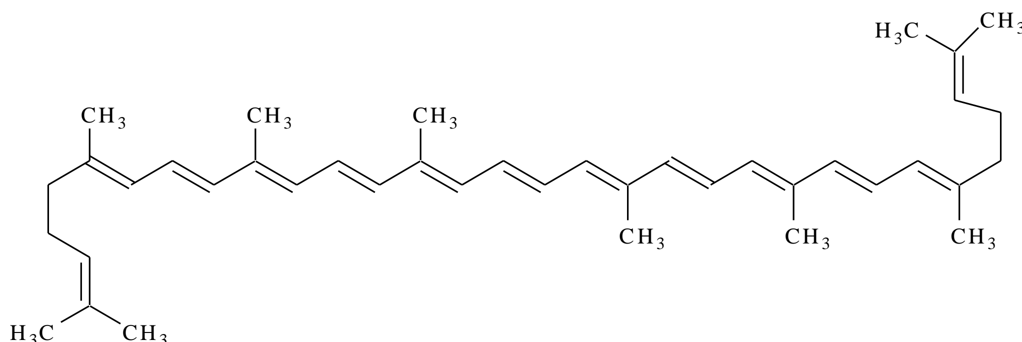


Figure 6: Structure of Lycopene (Gregory, 2007).

Vitamin C ($C_6H_8O_6$) is a potent water soluble antioxidant found in fruits like citrus and tomatoes. Its property of being an excellent electron donor accounts for all its known functions (Hacisevkđ, 2009). It blocks damage caused in the body by free radicals.

2.4 Determination of Antioxidant Activity of Antioxidants

Antioxidants or free radical scavengers (FRS) inhibit oxidation by reacting faster with free radicals than lipids, DNA and proteins. An antioxidants ability to scavenge free radicals in the body inhibits the oxidative mechanisms that lead to degenerative disease. The efficiency of antioxidants is therefore dependent on its ability to donate hydrogen to a free radical. This

efficiency or antioxidants scavenging ability can be measured by use of the DPPH radical (Brand *et al.,* 1995). Other tests that measure antioxidants efficiency to inhibit oxidation processes include metal chelating ability test and reducing capacity tests.

2.4.1 DPPH Radical Scavenging Activity Assay

This assay measures the relative antioxidant abilities of natural extracts to scavenge free radicals generated in the assay. DPPH (2, 2 – diphenyl - 1 – picrylhyrayl) radical is a good hydrogen abstractor. This radical has a violet color due to its unpaired nitrogen electron. Due to its odd electron DPPH gives a strong absorption maximum at 517 nm. After reaction with a hydrogen donor, DPPH yields its reduced counterpart DPPH - H (2, 2 – diphenyl - 1-picrylhydrazine) which is yellow. When DPPH color changes from violet to yellow its absorption strength is also decreased. The resulting decolorisation is stoichiometric with respect to the number of electrons captured. The color change is monitored spectrophotometrically at 517nm and in this way the antioxidant potential of a substance / plant extract can be determined (Brand *et al.,* 1995).

2.5 Spectroscopy

Spectroscopy deals with production, measurement and interpretation of spectra arising from the interaction of electromagnetic radiation with matter. The assay is based on the absorption or emission of radiation in the ultraviolet (UV), Visible (Vis), Infrared (IR) and Radio (NMR - nuclear magnetic resonance) frequencies. UV (200 – 350 nm) and Vis (350 – 750 nm) is the most encountered technique in food analysis. Absorbance of radiation is by pigments in the analyte or chemical reaction involving the analyte. Analytical determinations are based on the radiation being monitored e.g. absorbance or fluorescence. Quantitative UV - Vis absorption spectroscopy aims at determining the concentration of analyte in a given sample solution. Determination is based on the measurement of the amount of light absorbed from a reference beam as it passes through the sample solution. The analyte in the solution affects the amount of radiation transmitted through the solution hence the relative transmittance or absorbance of the solution may be used as an index of analyte concentration. The solution to be analysed is contained in an absorption cell placed in the path of radiation of a selected wavelength. The amount of radiation passing through the sample is then measured relative to a reference sample.

The relative amount of light passing through the sample is then used to estimate the analyte concentration.

Absorbance (A) = Log P_O / P where P_O - radiant power of incident beam

= - Log T P - radiant power of exciting beam

= 2 – Log % T. T - transmittance

Absorbance is directly proportional to the concentration of the absorbing species in the solution. The relationship between absorbance of a solution and the concentration of the absorbing species is known as Beers law.

A = abc where A = absorbance

b = path length through solution (cm)

a = absorptivity of a given species

c = concentration of absorbing species

A = εbc where ε = molar absorptivity and

c = molar concentration of analyte

Beers law is applicable to only dilute solutions up to 10mM for most analytes. Samples are homogenised to ensure a representative sample is used and clarified to prevent apparent absorption due to scattering of light by turbid solutions. The reference cell matches exactly the sample cell except it contains no analyte. Reference solutions are prepared by treating sample solvents in a manner identical to that of the sample. Sample holding cells or cuvettes should be composed of a material that does not absorb radiation in the spectral region being used (e.g. UV- Quarts or fused silica while Visible - silica glass or plastic). Absorption cell is $1cm^2$ and 4.5cm long with a 1cm path length. The minimum volume of solution for standard absorbance measurements is 1.5ml. The appropriate wavelength used is that which the analyte demonstrates maximum absorbance. The spectrophotometer is calibrated for 0% (distilled water) then 100% (reference cell). It is most convenient to make readings in absorbance since under optimum conditions absorbance is directly proportional to concentration (Penner, 2006).

CHAPTER THREE
MATERIALS AND METHODS

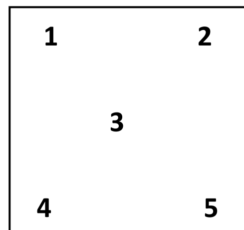
3.1 Sources of Reagents and Raw Materials

Lycopene, L - ascorbic acid, Heparin and Folin reagent [Sigma – Aldrich, St. Louis M.O, USA], Bovine Serum Albumine [Thermo Scientific, Mumbai, India], Celite [Celite Corporation, Lompoc Carlifonia, USA], Double distilled water [Nuclear Science department, Nairobi University, Nairobi, Kenya]. All other chemicals and reagents were of analytical grade unless otherwise stated.

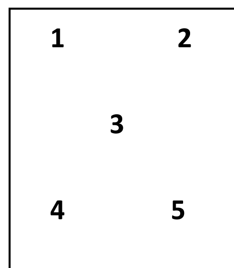
Rats [Technical University of Kenya biology department and National Public Health Laboratory Services (Kenyatta National Hospital)] and beet roots [Kangari, Gatiaini – Ngecha and Kanderendu areas of Muranga County].

3.2 Beet Root Sampling

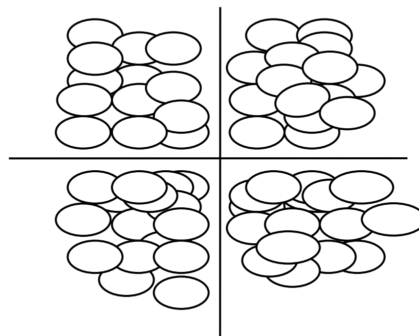
Beet roots were sampled from Muranga in central Kenya. Muranga offered an ideal climate for the beet roots in terms of rainfall, cooler temperatures and well drained soils compared to drier areas (Thika and Naivasha) were beets are also grown. Beets were ready for harvest 60 days after transplanting from the nursery (Average Detroit beet weight 90.8g).



Plot 1

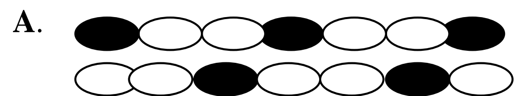


Plot 2

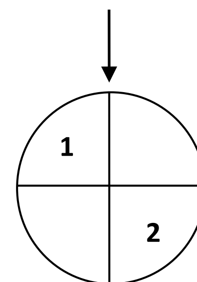


32 Beets / Variety

A.



B. Another 10 picked by ballot



Two opposite quarters used as half

Figure 7: Beet Root Sampling Procedures

Beet roots (*Beta vulgaris ssp. crassa*) were identified from seedling variety planted and were manually sampled from 4 plots using blends of simple random sampling procedures described by Proctor and Meullenet (2006). Samples were obtained from five locations in each plot along two diagonal lines. Harvested beets from each plot were mixed then subdivided into 4 lots and a choice of 8 beets randomly made per plot. The leaves and stems of chosen roots were cut off to prevent moisture loss and about 4cm of stems were left attached to prevent roots from bleeding. Beets were wrapped in brown paper, packed in an air tight plastic bag and transported to the laboratory. The 32 beet roots were each randomly assigned a number. Copies of these numbers were written on pieces of paper, the papers were mixed in a jar and ten randomly selected. Beet root units corresponding to these random numbers were then selected and used as a representative sample. Another batch of beets was randomly lined up and a beet root selected after skipping two beets in the line. Selected beets were also used as a representative sample.

3.3 Extraction of Crude Betalains from Beet Root Tissue

Extraction of Betalains from beet roots was carried out as described in a standard procedure by Elbe (2001) with slight modifications. Chosen beets were rinsed under cold running water and each cut into four quarters by use of a sharp knife. Two opposite quarters were selected (half), size reduced and extracted in a blender for 1 minute (1 minute extraction, limits the extraction of other solubles other than betalains from the beet tissue) with aqueous methanol (80:20 v/v). The puree was mixed with an equal amount of celite (Celite Corporation, Lompoc Carlifonia, USA) and the mixture transferred to a buchner funnel (Royal, Worcester, Netherlands) and filtered through a whatman no.1 filter paper using reduced pressure. The single strength extract was packaged in a dark colored air tight plastic container, flashed with nitrogen and stored in a freezer at -26 ^{o}C. Extraction was performed under nitrogen atmosphere to prevent degradation of the betalain pigments.

3.4 Determination of Betalain Concentration in Beet Root Extract

The concentration of betacyanin and betaxanthin in the crude extract was determined by measuring their spectrophotometric light absorption at 538 nm and 476 nm respectively (Elbe, 2001). Betacyanins were calculated as betanin while betaxanthins as vulgaxanthin 1. Absorption at 600 nm was used to correct small amounts of impurities. The spectrophotometer (Spectrumlab Gold S54T09014 UV- Vis, Shanghai Lengguang Technology Co. ltd, Shanghai, China) was

zeroed at 476, 538 and 600 nm using 0.05M phosphate buffer (pH 6.5) as the blank. The crude beet root extract was diluted using the 0.05M phosphate buffer (pH 6.5) such that the absorbance at 538 nm was between 0.4 and 0.5AU (the range is recommended to measure light absorption because in this range all solutions obeying beers law have the lowest relative error). The visible absorption spectrum of the extract was then obtained between 450 nm and 650 nm and the data used to plot a betalains content determination calibration curve from which corresponding values for 538 nm, 476 nm and 600 nm were determined.

The corrected light absorption of betanin and vulgaxanthin 1 were given by:

$X = 1.095\ (a-c)$ $\qquad$ $Y = b - z - x / 3.1$ $\qquad$ $Z = a - x$

Where: -

a = light absorption of the sample at 538 nm

b = light absorption of the sample at 476 nm

c = light absorption of the sample at 600 nm

X= light absorption of betanin minus the colored impurities.

Y= light absorption of vulgaxanthin 1 corrected for the contribution of betanin and colored impurities.

Z = light absorption of the impurities.

The concentration of betanin and vulgaxanthin1 was calculated using their pigment adsorptivity value ($A^{1\%}$) {Betanin = 1120 and Vulgaxanthin 1 = 750} and dilution factor using the formula below.

Absorbance = abc $\qquad$ {ab = ε} $\qquad$ therefore,

Absorbance = εc

c = (Absorbance / ε)

where:-

c = concentration of betanin or vulgaxanthin 1 and

ε = absorptivity value

The total betalain content in the crude extract was then obtained by adding the concentrations of betanin and vulgaxanthin 1.

3.5 Preparation of Antioxidant Working Solutions

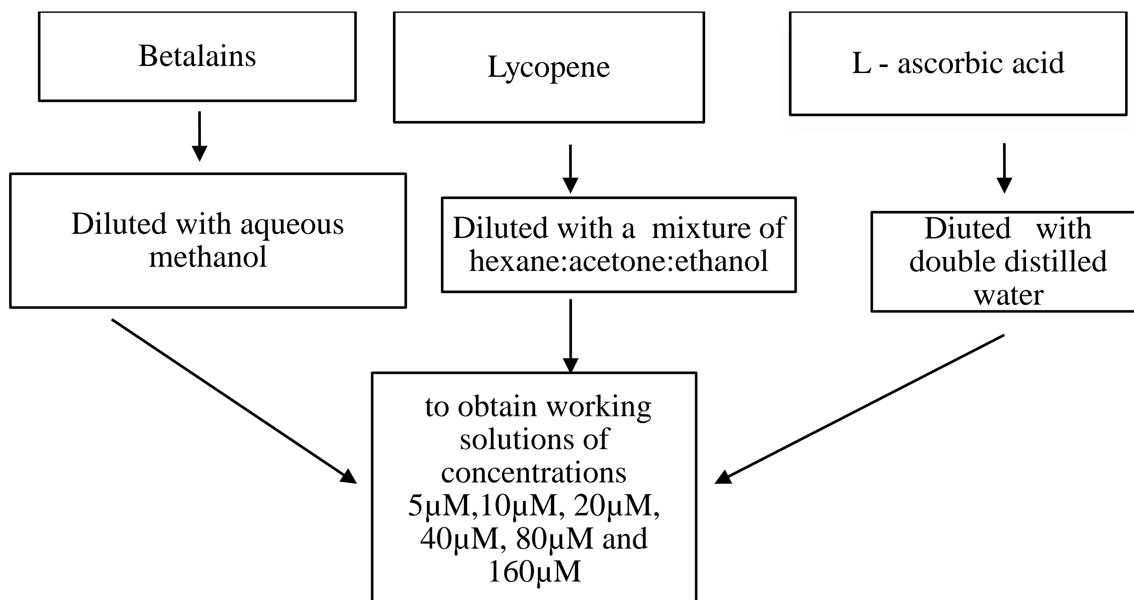


Figure 8: Crude Betalains, L - Ascorbic acid and Lycopene Antioxidant Working Solutions.

Crude betalains were diluted (stock concentration 542 µM) with aqueous methanol, a solvent previously used to extract betalains from beetroots and has the advantage over distilled water (in which betalains are very soluble) of inactivating beet root enzymes present in the extract, that would cause betalain deterioration. Lycopene (Mw.536.87) was diluted using a mixture of hexane, acetone and ethanol (at ratios which demonstrated the highest lycopene solubility) to avoid the option of using oil (lipid) as a diluting media since lycopene would later be used to inhibit the oxidation of another lipid (LDL). Ascorbic acid (Mw.176.12) was diluted in water (double distilled) in which it is readily soluble.

3.6 Drawing Blood Samples from Rats

The rats (*Rattus norvegicus*) were housed in a standard cage at room temperature and were provided with standard rat food or cubes (Unga Feeds Ltd Kenya), water and wood chippings as beddings. Rats were chosen because they offered more blood than **mice**, were easy available, affordable and are known to be good responders to various research variables. Rats of both sexes were used because atherosclerosis is not gender based.

Blood was drawn from 20 overnight fasted, 4 – 5 months old rats. They were stunned by use of

chloroform dubbed onto cotton wool and enclosed in a desiccator. Unconscious rat was laid on its back on a board and its limbs secured onto the board by use of tuck pins. The skin was torn around the sternum by use of a sharp blade, the sternum was exposed and the diaphragm severed to impede breathing. The heart was exposed, punctured using a syringe and all the blood in the heart (5 - 8ml) gently drawn out using the syringe. The blood was emptied into a centrifuge tube and let to clot (Hoff, 2000).

3.7 Precipitation of Low Density Lipoproteins from Rat Blood Serum Using Heparin

LDL was extracted from rat blood following the method described by Seidel *et al.*, (1990) with some modifications. Coagulated blood was centrifuged (Omega 12, Ferrum Inc. Centrifuge Technology, Houston, Texas) at 2800g for 15 minutes to recover serum**.** Serum and phosphate buffer pH 5.13 (containing 1000 units / ml heparin) were mixed in test tubes at ratios of 5:1 respectively**,** gently nitrogen flashed (to displace oxygen from the tubes), stoppered and let to stand in a closed cabinet until a precipitate was formed. The tubes were centrifuged at 3000g for 20 minutes at room temperature to separate the precipitate from the supernatant fluid. The separated fluid was siphoned out using a dropper and a few drops (4ml) of Phosphate buffer (pH 7.4) added to the precipitate to disperse lumps and re - dissolve the lipoprotein precipitate. The LDL solution was then transferred to a glass tube which was flashed with nitrogen and stoppered firmly. The tube was covered with aluminum foil and kept refrigerated at 4 ^{o}C. Bulk samples were thoroughly mixed before use.

3.8 Determination of the Concentration of Proteins in Isolated Low Density Lipoproteins

The Lowry assay, Lowry *et al*. (1951) was used to determine the protein concentration in the LDL solution. Lowry solution was prepared using mixtures of solutions A, B and C (v/v) at the ratio of 100:1:1 respectively. Where:

A - mixture of 0.0715M NaOH and 0.135M Na_2CO_3 solutions at ratios of 1:5 respectively,

B - 0.057M solution of $CuSO_4.5(H_2O)$ and

C - 0.124M solution of $Na_2C_4H_4O_6.2(H_2O)$

Bovine serum albumin (BSA) was diluted using double distilled water (DDW) to make a BSA stock solution of a final concentration of 100mg BSA / L. This stock solution was diluted with double distilled water to make triplet BSA dilutions of varying protein concentration in 15ml

glass tubes as shown in Table 1 below. A triplet assay for the LDL samples was also set up as for the BSA dilutions. LDL and BSA sample dilutions (0.5ml) were transferred to 10ml glass tubes and 0.7ml Lowry solution added to each of the tubes. The tubes were incubated at room temperature for 20 minutes in the dark. At the end of incubation 0.1ml Folin reagent (5ml of 2M Folin and Ciocalteu's phenol reagent mixed with 6ml of double distilled) was added to each tube and incubated for 30 minutes at room temperature in the dark. At the end of incubation 1.3ml from each tube was transferred to a semi - micro disposable cuvutte and the absorbance determined at 750 nm. A standard curve was plotted using the absorbance readings of the BSA dilutions verses the mg of protein contained in each tube. The protein content in the LDL sample

Table 1: Bovine Serum Albumine and Low Density Lipoprotein dilutions

	DDW (ml)	BSA (ml)	Protein concentration (mg/l)
BSA dilutions	10	0	0
	8	2	20
	6	4	40
	4	6	60
	2	8	80
	0	10	100
LDL dilutions	9	1	unknown
	8	2	unknown

solutions was calculated in mg BSA / L from this curve. This LDL stock solution (2.1g protein / litre LDL) was later diluted using double distilled water to make an LDL solution of 50µM protein / ml concentration.

3.9 Preparation and Determination of the Concentration of Hypochlorous Acid

Hypochlorous Acid was prepared using procedures described in a Corning Limited Laboratory division manual. Manganese oxide (0.1g) was mixed with excess concentrated hydrochloric acid to produce chlorine (Cl_2) gas. The gas was dissolved in 200ml double distilled water containing mercury oxide (orange yellow). Chlorine in water forms hypochlorous acid which disassociates to form hydrochloric acid. Mercury oxide (HgO) reacts with HCl to form a black colored precipitate of mercury chloride (HgCl). Hypochlorous acid was used immediately after preparation.

Chemical Reactions Involved in the Production of HOCl.

$MnO_2 + 4HCl \longrightarrow MnCl_2 + Cl_2 + 2H_2O$ MnO_2 (RFM 86)

1mole 1 mole

$Cl_2 + H_2O \longleftrightarrow HCl + HOCl$ HOCl (RFM 52.5)

1mole 1mole

$HCl + HgO \longrightarrow HgCl_2 + H_2O$

(s) **(s)**

Using mole ratios of MnO_2 : Cl_2 : HOCl (1:1:1) involved in the reaction, hypochlorous acid concentration was determined. The 0.0058M HOCl stock solution was diluted with double distilled water to make 10µM, 30µM, 50µM, 70µM and 100µM HOCl solutions.

3.10 Determination of the Antioxidant Activity of Crude Betalains

The antioxidant activity of crude betalains of concentrations 5µM, 10µM, 20µM, 40µM, 80µM and 160µM was determined by use of the 2, 2 – diphenyl - 1 – picrylhyrayl (DPPH) radical scavenging activity method (Brand *et al.,* 1995) with slight modifications. Crude betalains (0.2ml) was mixed with 0.8ml DPPH (100µM in ethanol) in a cuvette and let to stand for 30 minutes at room temperature in the dark. After incubation the absorbance of each cuvette sample was measured at 522 nm. The solvent was used as blank, while DPPH in ethanol minus extract served as a positive control. Lycopene and l – ascorbic acid of the same concentrations were used as negative control.

3.11 Determination of the Susceptibility of Low Density Lipoproteins to Hypochlorous Acid Induced Oxidation

LDL oxidation was induced *ex - vivo* by incubating LDL with HOCl. The conjugated dienes formed during the oxidation process were continuously measured at 234 nm using a UV - spectrophotometer as described by Ghaffari and Ghiasvand (2011) with slight modifications. The sample cuvette contained LDL (50µg protein / ml), phosphate buffer (pH 7.4) and HOCl (10µM). The blank cuvette did not contain LDL. The cuvettes were incubated at 37 ^{o}C for 3 hours and the absorbance at 234 nm measured at 5 minute intervals using a UV - spectrophotometer (Spectrumlab Gold S54T09014 UV- Vis, Shanghai Lengguang Technology Co. ltd, Shanghai, China). The same was repeated for HOCl concentrations 30µM, 50µM, 70µM and 100µM.

3.12 Determination of the Effect of Crude Betalains on Hypochlorous Acid Induced Low Density Lipoprotein Oxidation

LDL was oxidized using 10µM HOCl in the presence of crude betalains of concentrations 10µM, 50µM, and 100µM as described by Ghaffari and Ghiasvand (2011) with slight modifications. The sample cuvette contained phosphate buffer (pH 7.4), LDL (50µg protein / ml), HOCl (10µM) and 10µM crude betalains. The blank did not contain LDL. Both sample and blank cuvettes were incubated at 37 ^{o}C for 3 hours in an incubator with intermittent absorbance readings done at 234 nm at 5 minute intervals using a UV – spectrophotometer (Spectrumlab Gold S54T09014 UV- Vis, Shanghai Lengguang Technology Co. ltd, Shanghai, China). The same was repeated for crude betalain concentrations 50µM and 100µM. Lycopene of concentrations 10µM, 50µM, and 100µM was used as control.

3.13 Data Analysis

The data was entered in Microsoft excel (version 2007) sheets. It was analyzed using Prism Graph pad software Inc., (San Diego CA) version 5.01 and a P value of <0.05 considered statistically significant. Significance of difference between means for antioxidants activity, HOCl induced LDL oxidation efficiency and antioxidant LDL oxidation inhibition was determined by one way ANOVA and Tukey post hoc test was performed to evaluate differences among group means. Graphs were plotted to show the trend of various response variables. The data are expressed as means ± standard error of means of triple determinations.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Betalain Concentration in Crude Beet Root Extracts

The concentration of crude betalains extracted from Detroit (dark red flesh / Detroit dark red cultivar) and Crimson Globe (ox - blood flesh / Detroit cultivar) beet root tissue are shown in Table 2. The beet varieties were chosen because they are industrially processed varieties (due to their regular shapes), are widely consumed, have high betalain content (obviously indicated by their deep color) and are available locally. They are also a favorite for commercial growers since they are high yielding, tolerant to diseases and do well in many soil types.

Table 2: Concentration of Betalains in Detroit™ and Crimson Globe™ Beet Root Extracts

Type of Betalains	Detroit Beet	Crimson Globe
Betacyanins	21.0	16.0
Betaxanthins	8.80	7.12
Total betalain content (mg / 100 ml)	29.80	23.12

The red - violet betacyanins were determined as betanin at 538 nm while the yellow - orange betaxanthins were determined as vulgaxanthin 1 at 476 nm. The crude betalain content in Detroit beet root extract was 29.8 mg /100 ml while that of Crimson globe variety was 23.12 mg /100 ml. In both varieties the concentration of betacyanins was more than double that of betaxanthins. Studies by Lee *et al.* (2014) showed that beet root cultivars greatly vary in betalain content while Gasztonyi *et al*. (2001) found that betacyanins were higher in content in red beet root varieties than betaxanthins. The betacyanins content ranged from 0.44 g – 0.60 g / kg while the betaxanthin content (vulgaxanthin 1) ranged from 0.32 g – 0.42 g / kg. In this research, extraction of beet tissue with aqueous methanol was done for only one minute. This brief extraction process did not remove all color or betalains from the beet tissue. This was done to limit the extraction of other solubles (other than betalains) from the beet tissue that would affect the activity of betalains. Additionally, betalains are susceptible to oxidation, promoted by light exposure and room temperature conditions, hence the limit in extraction time. Due to this, the content of betalains in the extract as determined does not equal to the total (%) betalain content in the beet variety used as determined by the other researchers (since this would be erroneous).

However, the betalain concentration in the extract was adequate for the purpose intended and permitted further dilutions to be made for use in the research.

4.2 Antioxidant Activity of Crude Betalain Rich Beet Root Extract

The antioxidant activity of Detroit crude betalains was determined by measuring the free radical scavenging ability of the crude extract using the DPPH (2, 2 – diphenyl - 1 – picrylhyrayl) radical. The antioxidant activity of varying concentrations of crude betalains is presented as the percentage reduction of DPPH *(Q),* also referred to as "inhibition" or "quenching" (Figure 9). Lycopene and l - ascorbic acid were used for comparative evaluation.

The use of very low antioxidant concentrations and in micro molar quantities was ideal in mimicking low doses of antioxidant available to consumers from food sources and was also informed by the fact that excess intake of antioxidant could have some side or toxic effects.

There was an increase in inhibition with increase in sample concentration in all the three samples. However, crude betalains had a significantly higher rate of inhibition compared to lycopene and l - ascorbic acid. These results reveal that crude betalains, lycopene and l – ascorbic acid have antioxidant properties even at very low concentrations of 5µM. At 5µM concentration the antioxidant activity of all samples was over 40% meaning that even lower antioxidant concentrations would possibly still exhibit outstanding antioxidant capabilities. At 5µM concentration, the antioxidant activity of ascorbic acid, crude betalains and lycopene was 49%, 46.2% and 40.7% respectively. The highest antioxidant activity at 160µM concentrations was that of crude betalains at 99%, followed by ascorbic acid at 77.8 % and the least was lycopene at 61.7 % at the same concentration. Overall, the crude betalains extract had the highest antioxidant activity. However, betalains superior antioxidant abilities were true from 10µM concentrations upwards and not below. Lycopene had the lowest antioxidant activity at all concentrations except at 20µM were it registered 56% inhibition against ascorbic acid's 55.4%. At lower concentrations of 5µM and 10µM, crude betalains antioxidant activity was very significant (p<0.05) against lycopene but not significant against ascorbic acid. At 20µM there were no significant differences between the means of all the three antioxidants. However, at concentrations 40µM, 80µM and 160µM crude betalains antioxidant activity was significant (p<0.05) against lycopene and l - ascorbic acid while the antioxidant activity of Lycopene against l - ascorbic acid was not significant except at160µM. Generally, crude betalains of

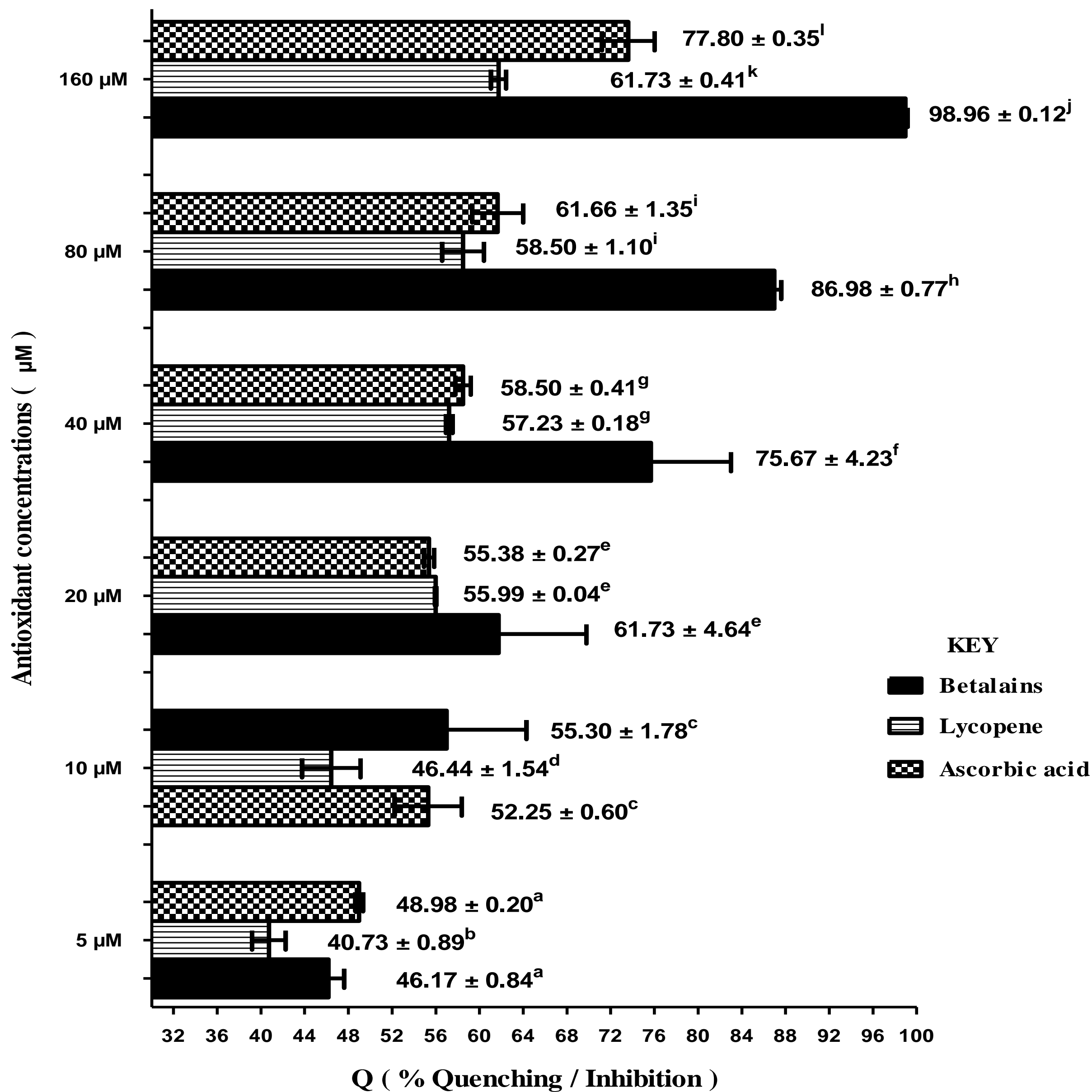


Figure 9: Detroit Crude Betalains, Lycopene and L - Ascorbic Acid Inhibition Effect of Equal Sample Concentrations.

Data presented as means ± S.E.M of triple determinations. Means not sharing a common superscript letter are statistically different (P<0.05).

concentration 10µM to 160µM had exemplary antioxidant ability compared to both lycopene and l - ascorbic acid at the same concentrations. These results are in agreement with those of Cai *et al*. (2003) who using a modified DPPH method, found that amaranth betalains exhibited a stronger antioxidant activity than ascorbic acid. Georgiev *et al*. (2010) reported that extracts of Detroit dark red hairy root cultures tended to have a higher antioxidant activity associated with increased concentrations of phenols contained in the extract, which may have synergistic effects with betalains. In this study however, the concentration of phenols and other solubles in the beet extract was minimized by extracting the beet tissue with methanol for only one minute.

4.3 Hypochlorous Acid Induced Low Density Lipoprotein Oxidation

4.3.1. Effect of Hypochlorous Acid Concentration on Low Density Lipoprotein (50µg protein\ml) Oxidation

LDL oxidation increased from HOCl concentrations 10µM, 30µM to 50µM. Oxidation was highest at 50µM HOCl. Despite the increase in HOCl concentration, a drop in oxidation activity occurred for HOCl concentrations 70µM and 100µM which registered LDL oxidation activity below that of 10µM HOCl (Figure 10). At 50µM HOCl, the mean value of induced LDL oxidation was 2.43% and 1.46% higher than that of 10µM and 30µM HOCl respectively while at 30µM HOCl the mean value increased by 0.96% against that of 10µM HOCl. However, at 70µM and 100µM HOCl concentrations, the mean values dropped by 1.64% and 1.85% respectively against that of 10µM HOCl. The highest drop of mean values of induced LDL oxidation of 4.17% and 3.97% occurred at 100µM and 70µM HOCl respectively compared to that of 50µM HOCl. LDL oxidation activity of HOCl concentrations 10µM, 30µM and 50µM was significant ($p<0.05$) against each other and that of 70µM and 100µM concentrations. No significant differences existed between the LDL oxidation activity of 70µM and 100µM HOCl. These findings agree with those of Panasenko *et al.* (1994a) who using HOCl concentration ranges of 0.1- 1.0mmol / L had a linear increase of TBARS (secondary oxidation products) formation observed while at concentrations above 1mmol / l the yield of TBARS decreased. Both these findings indicate reduced HOCl induced LDL oxidation activity at higher HOCl concentrations when either primary (CD) or secondary (TBARS) oxidation products are quantitated. Findings on the effect of HOCl concentration on LDL oxidation imply that uncontrolled or excessive build up of HOCl leads to increased LDL oxidative damage at least up

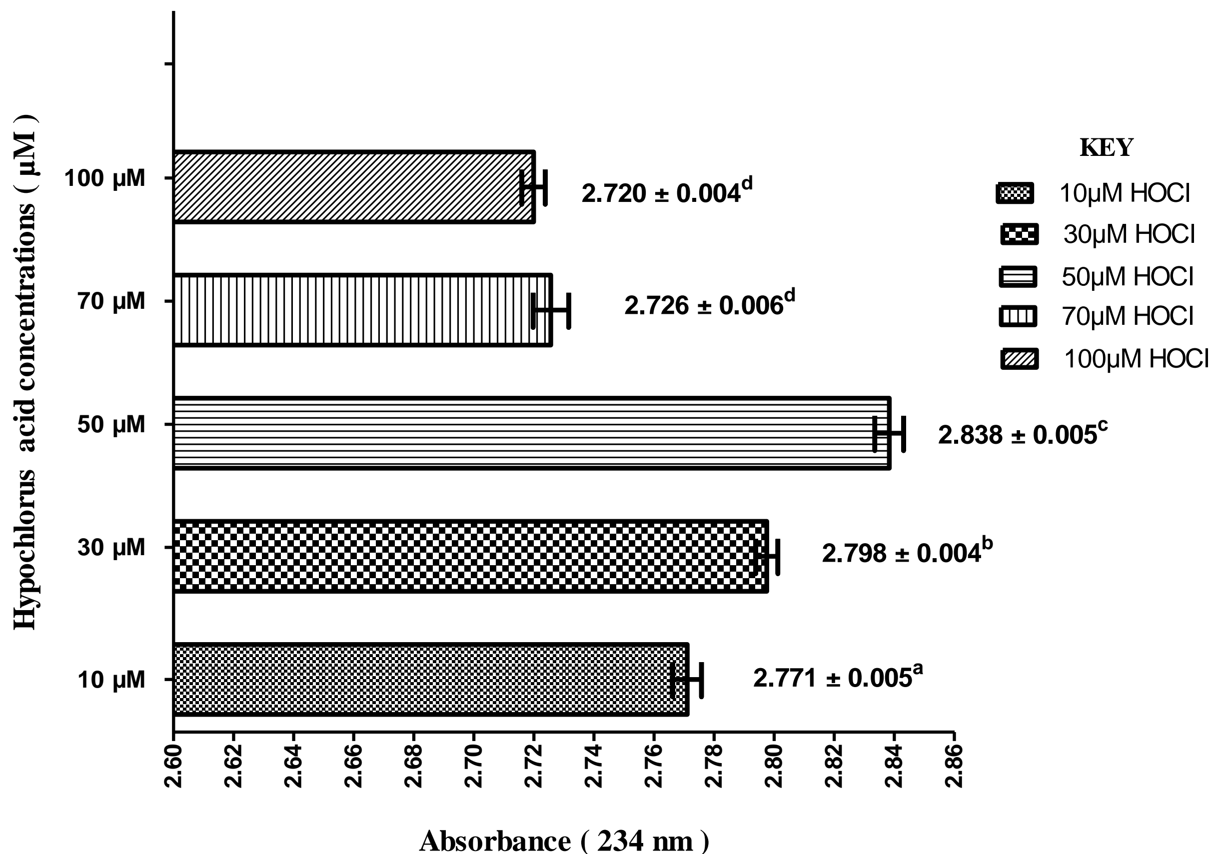


Figure 10: Oxidation of Low Density Lipoprotein (50µg protein \ ml) Using Varying Concentrations of Hypochlorous Acid.

Data presented as means±S.E.M of triple determinations. Means not sharing a common superscript letter are statistically different ($P<0.05$).

to 50µM HOCl. Products of HOCl induced LDL oxidation have been shown to include majorly chlorohydrins (Spickett *et al*., 2000), chloroamines and a great variety of oxysterols known to be cytotoxic (Schaur *et al*., 1994). Unfortunately, unlike other factors that cause an up - surge of free radicals in the body like exposure to herbicides, radiation and smoking, macrophage activity, being part of the vital immune system cannot be controlled while the ability of the body's natural antioxidant systems to quench or inhibit excessive destructive oxidative activity is limited and decreases with age.

Interestingly LDL oxidation was characterized by the rapid increase and decrease of conjugated dienes measured for all HOCl concentrations. This behavior was probably due to the conversion

of some of the conjugated diene products formed, to chlorohydrins since HOCl is known to modify biomolecules by chlorination (major) and / or peroxidation (Panasenko, 1997). Due to this the temporal change in absorbance at 234 nm lacked the distinct characteristic lipid oxidation phases i.e. the lag, propagation and decomposition phases that would result due to accumulation of oxidation products formed.

4.3.2 Effect of Time on Hypochlorous Acid Induced Low Density Lipoprotein Oxidation

4.3.2.1 Half Hourly Non – Cumulative Hypochlorous Acid Induced Low Density Lipoprotein Oxidation

Half hourly non - cumulative LDL oxidation results show increased LDL oxidation with time for HOCl concentrations 10µM, 30µM and 50µM. This is shown by increase of mean values between 30 minutes to 150 minutes for all the three HOCl concentrations with a slight slow - down shown by 10µM HOCl concentration between 30 minutes and 60 minutes (Figure 11). Higher HOCl concentrations 70µM and 100µM showed decreased LDL oxidation activity below that of 10µM HOCl throughout the three hour period. Optimum LDL oxidation activity was attained at the 150th minute and a fall noted at the end of the three hours, except for concentrations 70 and 100µM. The initial fall in oxidation for 10µM HOCl is probably because at low HOCl concentrations, the prioritized activity is the oxidation of the protein moiety of LDL and not the unsaturated lipids that contain carbon carbon double bonds, whose oxidation would have lead to the production of conjugated dienes measured in this study**.** This finding is explained by Vissers *et al*. (1998) who reported that at lower doses of HOCl (5µM / L) modification of membrane proteins (lysine residues) occurs than chlorohydrin formation of phospholipids and cholesterol while Jerlich *et al*. (1998) observed a rapid degradation of apo B - 100 tryptophan residues and a low rate of TBARS formation under the same conditions. Apo B - 100 the only protein associated with LDL comprises of 4536 amino acid residues and extends over at least half of the lipoprotein circumference. This drop in LDL oxidation likely implies that the modification of apo - B proteins also takes precedence over lipid peroxidation at 10µM HOCl. This finding is somewhat confirmed by Jerlich *et al*. (1998) although there is a difference in the concentration of HOCl used (5µM / l) and the end products measured (TBARS). If this reaction is replicated in the human body, then at these low HOCl concentrations, HOCl or

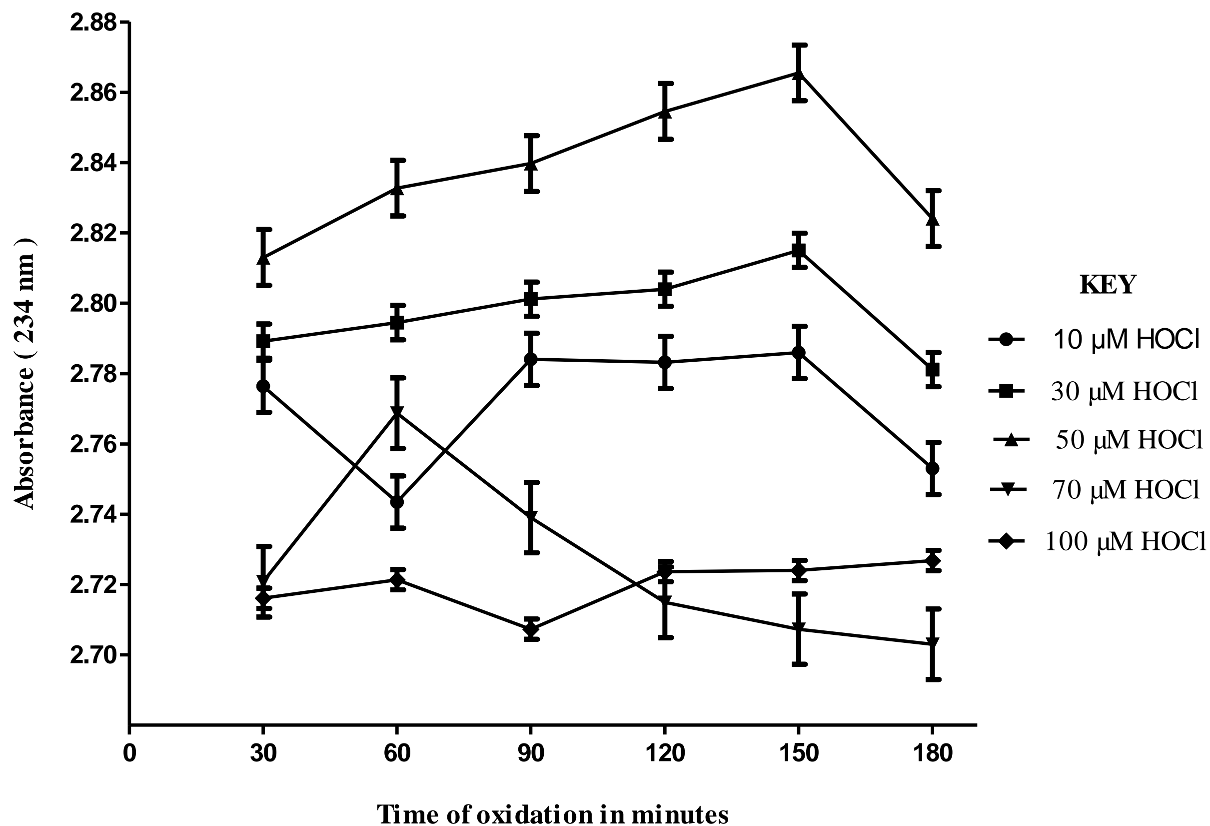


Figure 11: Half Hourly Non - Cumulative Hypochlorous Acid Induced Low Density Lipoprotein Oxidation.

macrophage LDL damage shown to occur within 30 to 60 minutes or exposure will most likely ensure that LDL particles remain stranded in the blood stream. Apo – B 100 functions by directing LDL to specific target tissue ensuring LDLs subsequent clearance from circulation. Any apo – B 100 protein damage, will hinder this clearance and increase LDLs chances for further oxidation by radicals in the blood stream. Increased LDL oxidation activity at the end of the assumed degradation of apo B – 100 most likely was due to the presence of excess HOCl which continued the peroxidation process. Additionally chloroamines formed during the oxidation of apo – B 100 and chlorinated hydroperoxides could decompose yielding free radicals that could initiate further, the oxidation of LDL.

4.3.2.2 Cumulative Half Hourly Hypochlorous Acid Induced Low Density Lipoprotein Oxidation

Cumulative Half Hourly HOCl induced oxidation of LDL showed increased LDL

oxidation with time between 0.5 hours to 2.0 hours for HOCl concentrations 10µM, 30µM and 50µM and decreased LDL oxidation activity below that of 10µM HOCl at higher HOCl concentrations 70µM and 100µM throughout the three hour period (Figure 12). The 10µM HOCl concentration still showed the brief slow - down in oxidation activity in the first hour consistent with the explanations given above. However, the oxidative activity of 70µM HOCl remained below that of 10µM and well above that of 100µM unlike in the non - cumulative results which showed its activity above that of 10µM in the initial stages and a fall below that of 100µM in the later stages (Figure 11). Increase in LDL oxidation for concentrations 10µM, 30µM and 50µM HOCl was significant (*p<0.05*) against each other from 1.5 hours to the end of the three hours.

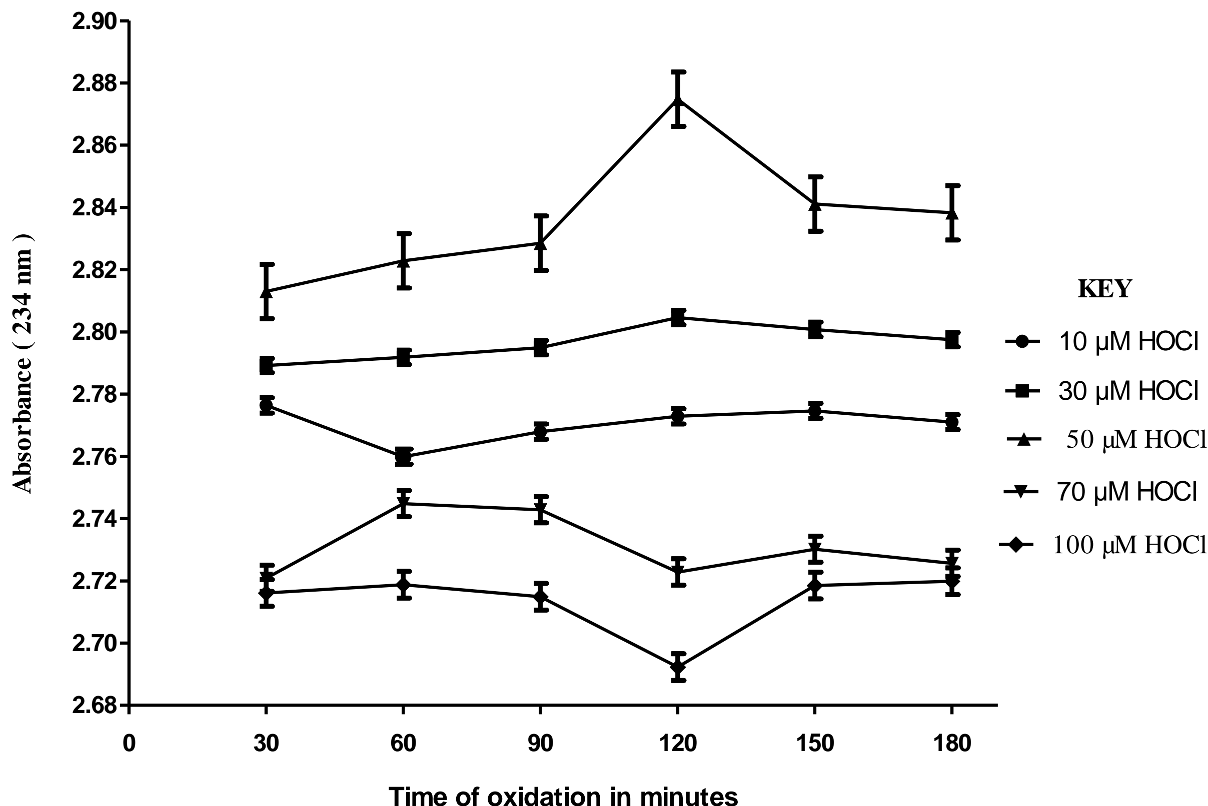


Figure 12: Cumulative Half Hour Hypochlorous Acid Induced Low Density Lipoprotein Oxidation

LDL must be oxidized to promote vascular disease. Results obtained from the non cumulative and cumulative time effects of HOCl induced LDL oxidation may possibly imply that prolonged HOCl induced LDL oxidative damage could enhance plaque formation and worsen the

atherosclerosis condition. This deduction is augmented by earlier research in a rat model that showed activated neutrophils form the chemotactic lipid product 4 – hydroxynonenol (HNE) (Schaur *et al*., 1994) proving that neutrophils and monocytes oxidize LDL making it cytotoxic. This situation is further aggravated by the fact that more high energy radicals also emerge from the by - products of this oxidation process. Chloroamines subsequently break down into chlorine and nitrogen centered radicals (Hawkins and Davies, 1998) and chlorinated hydroperoxide intermediates decompose to yield high energy alkoxyl radicals (Panasenko and Arnhold, 1999). This means that the onset of HOCl or macrophage (and / or free radical) induced oxidation of LDL gets worse with time. However, *in – vivo* studies may not follow the partten of *in – vitro* results.

4.4 Effect of Crude Betalains on 10µM Hypochlorous Acid Induced Low Density Lipoprotein Oxidation

LDL oxidation induced by 10µM HOCl was lowered following the inclusion of either crude betalains or lycopene (Figure 13). Both crude betalains and lycopenes LDL oxidation inhibitory effects were concentration dependent. Higher concentrations attained higher LDL oxidation inhibition and vice versa. The mean values of induced LDL oxidation of 100µM, 50µM and 10µM crude betalain groups dropped by 8.4%, 3.9%, and 1.5% respectively against that of the control (10µM HOCl) . Amongst the different concentrations of crude betalains, the mean values of induced LDL oxidation of 100µM crude betalains decreased by 7% and 4.7% against those of 10µM and 50µM crude betalains respectively while 50µM crude betalains had a mean value drop of 2.4% against that of 10µM crude betalains. These findings agree with those of Wettasinghe *et al.* (2002) who confirmed that betalains are effective antioxidants. Kanner *et al.* (2001) also reported on the ability of both betanin and betanidin at very low concentrations to inhibit lipid peroxidation and heme decomposition while a study by Tesoriere *et al.* (2004) showed that the hydrophilic betanin and indicaxanthin binds to LDL in vitro providing oxidative resistance. Though all betalain groups lowered LDL oxidation, only 100µM crude batalain's LDL oxidation inhibitory effects were significant ($p<0.05$) against the control. The 100µM lycopene group registered the highest percentage drop in mean values of induced LDL oxidation of 39.9% against the control (10µM HOCl) while 50µM and 10µM lycopene group had a 27.6%

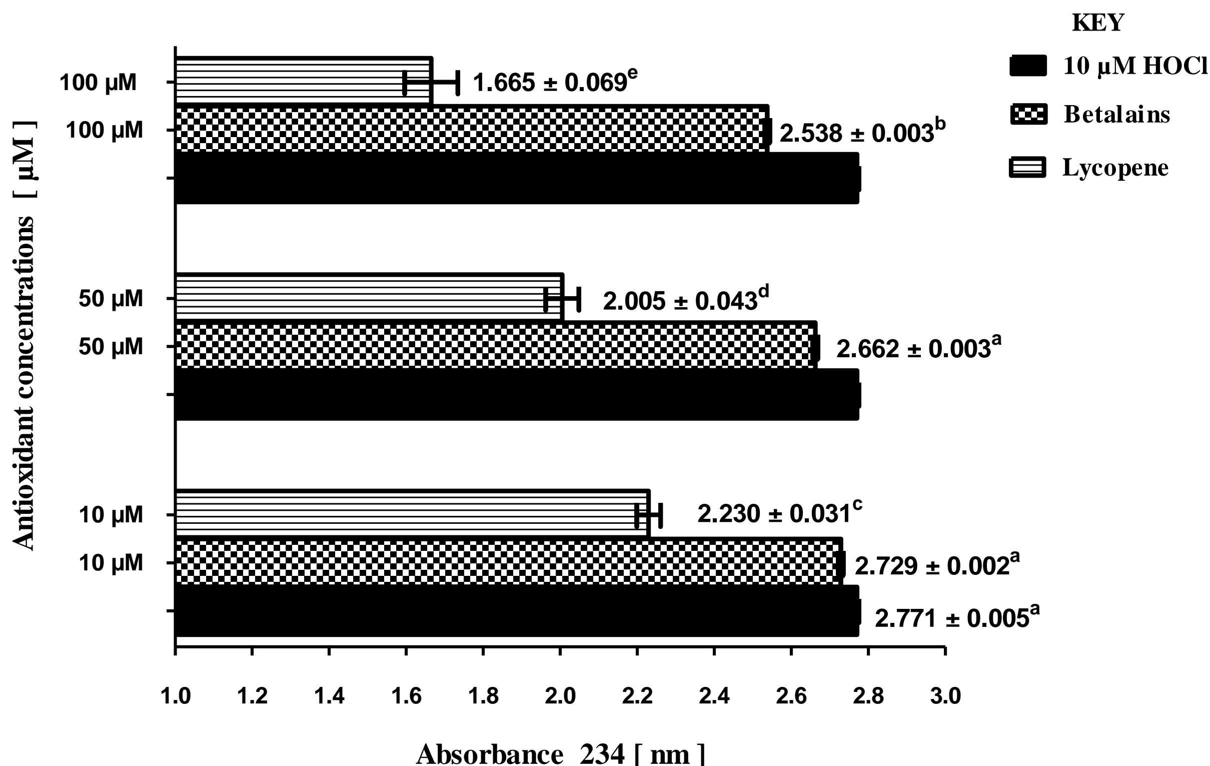


Figure 13: Effect of Varying Concentrations of Detroit Crude Betalains and Lycopene on 10µM Hypochlorous Acid Induced Oxidation of Low Density Lipoproteins.

Data presented as means±S.E.M of triple determinations. Means not sharing a common superscript letter are statistically different (P<0.05).

and 19.5% mean value drop respectively, against the control. These results imply that lycopene is a potent antioxidant. Lycopene lowered LDL oxidation by 18.3%, 24.7%, and 34.4% at 10µM, 50µM and 100µM respectively against crude betalains of equal concentrations. Lycopenes LDL oxidation inhibitory effects, were statistically significant (*p<0.05*) against crude betalain groups and the control. Crude betalains and lycopenes ability to inhibit LDL oxidation demonstrated in this study is consistent with findings by Panasenko *et al.,* (1994b) who reported that in the presence of α – tocopherol and butylated hydroxy toluene (BHT) a concomitant decrease of double bonds was noted and an inhibited accumulation of TBARS. Panasenko and Arnold (1999) also reported that accumulation of TBARS was inhibited by the presence of BHT.

At the beginning of the oxidation process all lycopene concentrations had a massive decrease of conjugated dienes compared to the control. This effect reduces gradually with time. This gradual decrease of lycopenes antioxidant activity with time indicates that lycopene is probably degraded

during the oxidation process. Conversely, all crude betalain concentrations lowered the LDL oxidation activity by a small margin from the start of the oxidation process to the end of the three hours. Each betalain concentration had almost equal antioxidant activity from the beginning to the end of the three hours. This indicates that the concentration of betalains probably remained somewhat the same throughout the process. This finding is confirmed by Attoe and Elbe (1985), who stated that betalains do not degrade by a free radical mechanism.

Antioxidants stabilize free radicals by donating electrons to them and inhibit oxidative damage to tissues in the body by reacting faster with free radicals than lipids, proteins and / or DNA. This research has established that crude betalains have the ability to inhibit *(ex - vivo)* HOCl induced LDL oxidation significantly ($p<0.05$) at higher concentrations of 100µM. Betalains therefore, would probably help reduce plaque deposition (*in – vivo*) an undertaking that is of paramount importance in reducing the severity of atherosclerosis.

Although higher antioxidant concentrations attained higher LDL oxidation inhibition giving the impression that more antioxidant intake may be more beneficial to health, this could possibly be a false impression since some antioxidants or excesses have been known to have side effects or to be counter - productive (Beta carotene* radicals that form during oxidation are known to act as prooxidants while Vitamin A in excess doses causes blindness). Some betalains (betanin, isobetanin, vulgaxanthin) have been shown to contain anti - inflammatory properties. Although this is beneficial to some extent and especially regarding LDL oxidation and atherosclerosis, betalain's anti - inflammatory properties could impair sensitivity of the immune system to desired inflammation that helps protect the body against infection.

Both betalains and lycopene effectively inhibit HOCl induced LDL oxidation (even at low concentrations). This could in effect mean that the intake of food sources rich in these phytonutients could be useful in the prevention of LDL oxidation and could possibly reduce or replace the use of drugs and surgeries in the fight against atherosclerosis.

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The following conclusions are drawn from this study:

i) Detroit crude betalain rich beet root extract possess superior antioxidant ability against the DPPH radical than lycopene and ascorbic acid antioxidants. This possibly implies that crude betalains could be useful in the control of free radicals in the blood stream.

ii) HOCl induced LDL oxidation increases with the concentration of the acid and time up to 50µM HOCl, but falls from 70µM HOCl. However, it has been suggested that adequate damage to the apo – B 100 moiety could occur at very low HOCl concentrations and short time exposures which would likely cause the LDL particles to remain stranded in the blood stream, increasing LDLs chances of further oxidation by free radicals. This indicates that any HOCl and / or free radical induced LDL oxidation carried out at levels beyond that which the body's natural antioxidant systems can control, could result in oxidative stress and is potentially detrimental to health.

iii) LDL oxidation induced by HOCl can potentially be lowered by crude betalains. However, crude betalains have a lower oxidation inhibitory ability against HOCl induced LDL oxidation than lycopene. Betalains lower performance against lycopene in the inhibition of HOCl induced LDL oxidation (done at 37 ºC and at a pH of 7.4) is contrary to earlier findings when the two antioxidants were tested against the DPPH radical (done at room temperature 24 - 25 ºC). Betalains lower performance could have been caused by the temperature increase (they degrade at room temperature), PH conditions (they degrade under mild alkaline conditions) and their probable tendency to partition into the extended aqueous phase in the lipoprotein (LDL) phosphate buffer mixture (betalains are polar), where they were less able to protect the lipids. However, the increase in temperature could have initiated a thermally induced conversion of trans - lycopenes to cis - lycopene isomers known to have a greater bioactivity, while lycopenes nonpolar nature could have caused it to be retained in the lipoprotein phase or accumulated at the oil – water interface, giving better protection to the lipids.

iv) Since betalains are not degraded by free radicals and are known to reside in the blood stream for up to 12 hours, they probably would offer prolonged oxidation inhibition without the need of frequent replenishment unlike lycopene. Additionally, some betalains (betanin, isobetanin,

vulgaxanthin) have been shown to contain anti - inflammatory properties. Since atherosclerosis results due to chronic unwanted inflammation caused by oxidized lipids in the blood stream, betalains would possibly provide more than one way of combating atherosclerosis, compared to lycopene.

5.2 Recommendations

The following recommendations are drawn from this study:

i) Owing to their ability to suppress HOCl induced oxidation of LDL (*ex-vivo*), betalains and lycopene antioxidants, plus foods ingredients that contain them should be pursued as dietary supplements to modulate conditions associated with oxidative stress caused by free radicals in the blood stream.

ii) Betalain and lycopene pigments can also be targeted in the production of natural food colorants that can be used to enhance the health benefits of ready to drink beverages like soft drinks, yoghurt, sour milk, bottled drinking water and sweets. The production and consumption of carbonated beet root juices should also be promoted.

iii) Having confirmed that crude betalains can suppress HOCl induced LDL oxidation *ex-vivo* using LDL extracted from rats, further studies should be conducted to concluded on their effect in humans (*in – vivo*) and their antioxidant effect when combined, to establish whether they have a synergistic or antagonistic effect.

iv) Crude betalains showed superior antioxidant abilities to those of lycopene with the DPPH assay but the vice versa was realized when their antioxidant inhibitory abilities were tested on 10μM HOCl induced oxidation of LDL**.** Further studies therefore, should be conducted to establish why this is so and whether betalains superiority as an antioxidant would be revealed if the LDL oxidation process was extended beyond three hours.

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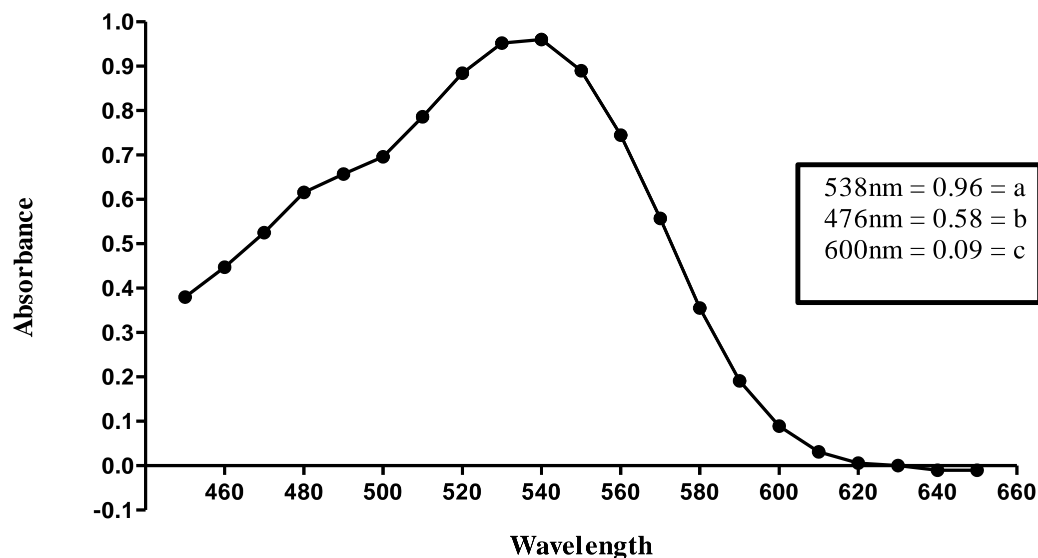
APPENDICES

APPENDIX I: Crude Betalains Concentration Determination Calibration Curve



APPENDIX II: Calculated Betalain Concentration in Beet Root Extract

Betacyanin and betaxanthin concentrations of the freshly extracted crude betalain solution were determined by measuring their spectrophotometric light absorption at 538nm, 476nm and 600 nm respectively. Betalain concentration was expressed as betacyanins (calculated as betanin) and betaxanthin (calculated as vulgaxanthin 1). Light absorption measured at 600 nm was used to correct small amounts of impurities (Elbe, 2001).

The corrected light absorption of betanin and vulgaxanthin 1 was given by:

$X = 1.095\ (a-c)$ $\qquad$ $Y = b - z - x / 3.1$ $\qquad$ $Z = a - x$

Where: -

a = light absorption of the sample at 538nm

b = light absorption of the sample at 476nm

c = light absorption of the sample at 600nm

X= light absorption of betanin minus the colored impurities.

Y= light absorption of vulgaxanthin 1 corrected for the contribution of betanin and colored impurities.

Z = light absorption of the impurities.

Absorbance readings determined for betanin (a), vulgaxanthin 1(b), and impurities (c) is as given below.

a = 0.96 at λ 538 nm

b = 0.58 at λ 476 nm

c = 0.09 at λ 600 nm

The corrected light absorption of betanin, vulgaxanthin 1 and impurities was calculated as follows:

X (betanin) = 1.095 (a - c)

= 1.095 (0.96 – 0.09)

= 1.095 (0.87)

= 0.953

Z (impurities) **=** (a - x)

= (0.96 – 0.953) = 0.007

Y (vulgaxanthin 1) = b – Z – (X) \ 3.1

= 0.58 – 0.007 - (0.953) \ 3.1

= 0.58 – 0.007 – 0.307

= 0.58 – 0.314

= 0.266

The concentration of betanin and vulgaxanthin1 was calculated using each pigments adsorptivity value ($A^{1\%}$) {Betanin = 1120 and Vulgaxanthin 1 = 750} and the appropriate dilusion factor.

Betanin concentration

Abs = abc

ab = ε

Abs = εc

c = (Abs \ ε)

where:

Abs = absorbance of betanin = 0.953

c = concentration of betanin

ε = absorptivity value of betanin =1120

Betanin concentration (c) = (Abs / ε) D.F where D.F = dilution factor

= (0.953 / 1120) 24.75

= 0.021059

= 0.021g / 100ml

= 21mg / 100ml

Vulgaxanthin 1 concentration

Abs = (0.266) ε = 750 D.F = 24.75

Therefore c = (0.266 / 750) 24.75

= 0.0088g / 100ml

= 8.8 mg / 100ml

Total betalain content = betanin + vulgaxanthin 1 content

= 21mg + 8.8mg

= 29.8mg / 100ml

APPENDIX III: Q Values of Varying Percentages of Crude Betalains

Crude Betalain Extract

				Q					**Q**
Extract Conc.	**(A_c)**	**0.865 - A_c**	**/ 0.865**	**x 100**	**Extract Conc.**	**(A_c)**	**0.865 - A_c**	**/ 0.865**	**x 100**
5µM					**40µM**				
Tr. 1	0.453	*0.412*	0.47630	47.630		0.154	0.711	0.82196	82.196
Tr. 2	*0.466*	0.399	0.46127	46.127		0.279	0.586	0.67746	67.746
Tr. 3	0.478	0.387	0.44740	44.740		0.198	0.667	0.77110	77.110
10µM					**80µM**				
Tr. 1	0.416	0.449	0.51907	51.907		0.113	0.752	0.86937	86.937
Tr. 2	0.380	0.485	0.56069	56.069		0.107	0.758	0.87630	87.630
Tr. 3	0.364	0.501	0.57919	57.919		0.118	0.747	0.86358	86.358
20µM					**160µM**				
Tr. 1	0.257	0.608	0.70289	70.289		0.008	0.857	0.99075	99.075
Tr. 2	0.341	0.524	0.60578	60.578		0.008	0.857	0.99075	99.075
Tr. 3	0.395	0.470	0.54335	54.335		0.011	0.854	0.98728	98.728

The data represents triple trials (Tr.1 - 3) of absorbance readings at 522 nm.

APPENDIX IV: Formula for the Determination of Antioxidant Activity {**Q**} of Betalains

$$Q = 100 \{ A_0 - A_c \} / A_0$$

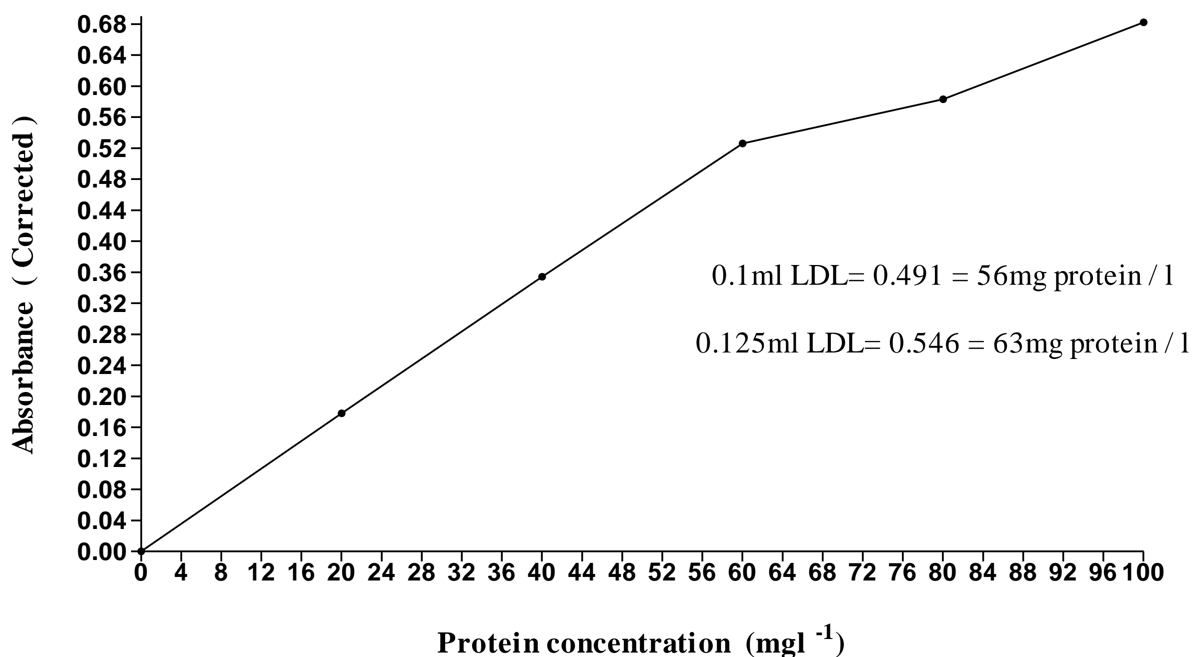
Where: Q - is the resulting antioxidant activity determined in terms of the percentage reduction of DPPH, also referred to as "inhibition" or "quenching"

A_0 - is the absorbance of the control

A_c - is the absorbance of sample

Note: All absorbance readings were measured by use of a spectrophotometer at 522 nm following the reaction of antioxidant test extracts with 100µM DPPH (www.medallionlabs.com).

APPENDIX V: Bovine Serum Albumin Standard Calibration Curve



i) From graph readings 0.1ml LDL sample had an absorbance reading of 0.491 = 56mg protein / litre. The whole LDL extract sample = 3.0ml. Therefore, the concentration of protein in the whole sample = $(3.0 \times 56mg / l^{-1}) / 0.1$

=1680mg protein / litre

=1.68g protein / litre.

ii) From graph readings 0.125ml LDL sample had an absorbance reading of (0.546) = 63mg protein / litre. The whole LDL extract sample = 3.0ml. Therefore, the concentration of protein in the whole sample = $(3.0 \times 63mg / l^{-1}) /0.125$

=1512mg protein / litre

=1.512g protein / litre

iii) From graph readings 0.02ml LDL sample had an absorbance reading of (0.541) = 12mg protein / litre (calculated from a different BSA standard graph). The whole LDL extract sample = 3.5ml. Therefore, the concentration of protein in the whole sample

= (3.5 x 12mg / l^{-1}) /0.02

= 2100mg protein / litre

= 2.1g protein / litre.

APPENDIX VI: Composition of Phosphate Buffered Saline (Ph 7.4)

Component	Quantity
0.14M NaCl	8.00g / L
2.70mM KCl	0.20g / L
1.50mM KH_2PO_4	0.20g / L
8.10mM Na_2HPO_4	1.15g / L

All components were dissolved in 1 litre double distilled water and the pH adjusted by use of dilute hydrochloric acid. Storage was at room temperature.

APPENDIX VII: Preparation of 1000 units Heparin / ml Phosphate Buffer (pH 5.13)

Heparin (182 Units mg^{-1})

50000 Units = 50000 x 1mg / 182

= 274.725mg (confirmed mass = 0.293g)

1mg heparin = 182 Units (dissolved in 10mls phosphate buffer)

= 18.2 Units / ml

182 Units: 18.2 Units / ml = X Units: 1000 Units / ml

182/ 18.2 = X / 1000

X = 182 (1000) / 18.2

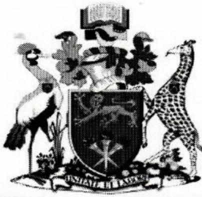
= 10000Units heparin (dissolved in 10ml phosphate buffer)

182 Units = 1mg heparin

10000 Units = 10000 (1mg) / 182

= 54.95mg (dissolved in 10ml phosphate buffer)

APPENDIX VIII: Animal Studies Methodology Review by Kenyatta National Hospital/ University of Nairobi Ethical Review Commission



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KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/ 01/MISC/43

7th September 2015

Saru Charles-Mwaighacho
KM16/2794/10
Technical University of Kenya
Food Science Department
P O BOX 52428-00200
NAIROBI

Dear Saru

Re: Request for ethical clearance to use rats in research – the effect of crude betalains from beet root on hypochlorous acid induced low density lipoprotein oxidation"

Your letter of request dated 14th July 2015 refers.

It is noted that:

i. You are registered as Masters student at the Egerton University Food Science Department. Egerton University has an Ethics Committee that should have reviewed your proposal. In the event that they did not have the expertise to review animal based studies, the institutional ERC should have formally written to the KNH- UON ERC requesting for external review. This is allowed and it regularly happens between the accredited Ethics Committees.

ii. Your proposal was submitted for review after the work was already done. Ethically it is not right to give retrospective approvals. Your able supervisors should have guided on this.

iii. Considering the prevailing situation you are in as a student, the KNH/UON ERC reviewed the methodology followed during the conduct of the animal studies and notes that there was no major/gross violation of ethical principles regarding animal studies.

Conclusion:
Your work may therefore be examined in line with the institutional procedures.

Yours sincerely

PROF. A.N GUANTAI
CHAIPERSON, KNH/UON-ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS
Supervisors: Prof. Symon M. Mahungu, Dr. Mary Omwambia

Protect to discover

APPENDIX IX: Letter from Research Practical Work Station

TECHNICAL UNIVERSITY OF KENYA

OFFICE OF THE DIRECTOR, SCHOOL OF PHYSICAL SCIENCES AND TECHNOLOGY

Selassie Avenue, P. O. Box 52428, 00200 NAIROBI Tel: +254-20-343672 Fax: +254-20-2219689

Date: October 15, 2015

TO WHOM IT MAY CONCERN

Dear Sir/Madam,

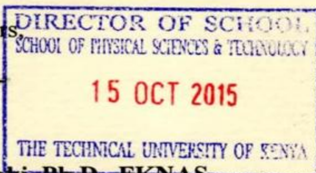
RE: SARU CHARLES MWAIGHACHO –MSC LABORATORY WORK

The above named is a member of staff of the Technical University of Kenya. She is a Masters Degree student in the Department of Dairy and Food science and Technology of Egerton University (Registration number: KM16/2794/10). In the 2013-2014 period, she carried out her research in laboratories at the Technical University of Kenya in the following Departments:

- **Biology and Biochemistry labs** (Animal House) - Extraction of rat blood samples and precipitation of LDL from rat blood and LDL oxidation as well as antioxidant oxidation inhibition.
- **Biomedical Laboratory-** Centrifugation of blood samples
- **Chemical Science and Technology labs-** Practical preparation, extraction of betalains, LDL protein analysis and DPPH Assay.

This note serves to confirm that the laboratory work mentioned above was carried out with the help of our Technicians within the School.

Sincerely yours,



Prof. Paul Baki, Ph.D., FKNAS

Director & Professor of Physics