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Full Length Research Paper

Evaluation of the potency of certain substances as antioxidants in the assessment of red cell viability

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The effectiveness of certain antioxidant substances in the protection of red cells from oxidation and degradation with respect to their window times of survival have been analyzed using UV-Visible spectrophotometry. Antioxidant substances evaluated included; vitamin A, vitamin D, mixture of vitamins A and D, various species of Kolanut, *Garcinia kola, Prosopis africana* beans, beans shell extracts and eugenol, an oil extract from the clove plant. In the presence of an oxidizing agent; potassium ferricyanide, lysis of red cell membrane, oxidation of exposed hemoglobin and methemoglobin formation were observed within 12 h. When the red blood cells were exposed to antioxidants, red cells were found to survive oxidation above 12 h. The window times of survival of the red cells in the presence of the antioxidants were found to be as follows: eugenol ≥ 32 h, *G. kola* ≥ 32 h, *Cola acuminata* (white) ≥ 32 h, *C. acuminata* (red) ≤ 20 h, *C. acuminata* (pink) 24 h, *Cola nitida* (white) 20 h, *C. nitida* (red) 26 h, *C. nitida* (pink) ≥ 32 h, *Prosopis africana* beans (shell extract) 30 h, *P. africana* (beans extract) 24 h, vitamins A and D (mixture) 30 h, vitamin D 24 h and vitamin A ≥ 32 h. A synergistic relationship between vitamins A and D was established, eugenol was the most effective while *C. nitida* (white) showed the least potency and therefore effectiveness.

Key words: Antioxidant potency, evaluation, red cell, survival time.

INTRODUCTION

Red blood cell contained in blood has a distinct structure which gives it a special physiological property. Human adult red cells are manufactured in the bone marrow from nucleated stem cells which grow, divide and begin to synthesize hemoglobin. At this stage, they are called erythroblasts, containing hemoglobin, spectrin and other characteristic erythrocyte membrane proteins. The part of the red cell containing the nucleus and most of the intracellular membrane is pinched off and eventually degraded at this stage. The remaining non-nucleated cell; reticulocyte continues to synthesize hemoglobin and other erythrocyte proteins while the cell eventually loses its ribosomes and about a third of its plasma membrane, acquiring the biconcave disc-like structure of the mature erythrocyte which has no nucleus and intracellular membranes. It is very flexible, with a spongy cytoplasm enclosed in an elastic cell membrane. The life span of an adult red cell is about 120 days after which it is broken down by the liver.

Cell membranes are essential to the life of the cell. The plasma membrane encloses the cell, defining its boundaries and serves as the cell interface with its extracellular environment. Despite functional diversity, all biological membranes share a general structure. The membranes of human red blood cells are essentially composed of two parts; the lipid bilayer and the membrane skeleton (membrane protein). The red cell membrane consists of 50% protein, 40% lipid and 10% carbohydrate (Wolfe, 1993; Kopito and Lodish, 1985).

The lipid membrane is composed of 30% free unesterified cholesterol, 10% glycerides and free fatty acids and 60% phospholipids. Membrane lipids are amphipathic or amphiphillic; they have both hydrophilic and hydrophobic ends. The most abundant membrane lipids are the phospholipids; cylindrical in shape having a polar head group

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and two hydrophobic hydrocarbon tails. Due to their cylindrical shapes, they tend to spontaneously form bilayers in aqueous solutions, with a tendency to close on each other to form sealed compartments, thus eliminating free edges where the hydrophobic ends would be in contact with water. It is for this same reason that compartments formed by lipid bilayers tend to reseal when they are torn. Red cell membrane phospholipids are phosphatidyl choline, phosphatidyl ethanolamine, sphingomyelin phosphatidyl serine. All others are neutral with only phosphatidyl serine carrying a resultant negative charge at physiological pH of 7.4 (Wolfe, 1993). The phosphatidyl bilayer part of the membrane effectively determines the shape of a red cell.

The red cell membranes consist of 50% membrane protein and 10% carbohydrate, occurring only at the external surface of the red blood cell and are composed of glycoproteins and glycolipids (Hwang and Waugh, 1997). The two predominant integral membrane proteins; glycolphorin and band-3-proteins are glycoproteins which have exposed regions in the outer surface of the cell (Hunter, 1977). Other peripheral proteins are spectrin, ankyrin and actin which are confined to the cytoplasmic face of the membrane from the erythrocyte skeleton (Kopito and Lodish, 1985). Spectrin and actin are major protein components of the cytoskeleton whose removal causes two consequences; the formation of “ghost cell” in which a red blood cell has lost its cytoplasmic content being removed by cell lysis, so that only the outer cytoplasmic membrane remains and the ghost cell then loses its rigid shape and the membrane glycoprotein acquires lateral mobility. The cytoskeleton is the major determinant of the rigidity of the erythrocyte membrane and its activity is to restrict the lateral motion of membrane glycoproteins.

Energy utilization and possibly retention is very important in the anucleated adult red blood cells. This energy enables the red cells to survive and also carry out metabolic processes like the transportation of oxygen and carbon dioxide and also anion exchange processes. Mature red cells contain no sub-cellular particles and rely on anaerobic respiration, anaerobic conversion of glucose by the glycolytic pathway for the generation and storage of high-energy phosphates (Adenosine trinucleotide phosphate, ATP) and the production of reducing power as nicotinamide adenine dinucleotide hydrogenase, NADH. In addition, reducing power is transmitted as nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) produced via the pentose phosphate pathway. These sources of energy are sufficient to maintain the red cell membrane in a flexible and functional state, to maintain an environment which sustains hemoglobin in the reduced ferrous form which can combine reversibly with oxygen (Richard and Wouter, 2005).

Glycolysis involves the breakdown of glucose; the energy source of the red blood cell. The net gain of this process is 2 moles of ATP per mole of glucose. In addition, reductive power is generated in the form of NADH which can be used to reduce methemoglobin to hemoglobin. The membrane hemoglobin and proteins involved in the metabolic pathways of the red blood cells interact to modulate oxygen transportation to protect hemoglobin from oxidant-induced damage and maintain the osmotic environment of the cell. Because the red cells are not capable of generating energy via the oxidative Kreb’s cycle, they use the glucose by the glycolytic (Embden-Meyerhoff) pathway for the generation and storage of high-energy phosphate sugar and also the unique glycolytic by-pass of the Rapoport-Luebering shunt (Richard and Wouter, 2005). The reducing power transmitted as NADPH is produced via the pentose phosphate pathway (Gordon-Smith, 2006); an anabolic pathway which utilizes the six carbon atoms in glucose to generate five-carbon sugars and reducing equivalent. The pentose phosphate pathway (PPP) is an alternative pathway for the oxidation of glucose. NADPH is produced at the oxidative stage of this pathway. Glycolysis provides ATP for membrane ion pumps and NADH for reoxidation of oxidized hemoglobin. NADPH helps the red cells to maintain the produced glutathione. The inability to reduce glutathione in red cells leads to increased accumulation of peroxides, predominantly hydrogen peroxide (Michael, 2007).

Hemoglobin is a tetrameric protein found in the red blood cells (RBC). It is responsible for the binding of oxygen in the lungs and transporting of the oxygenated hemoglobin to various tissues. It also aids the return of deoxygenated hemoglobin which carries carbon dioxide back to the lungs. Hemoglobin in preference selects such molecules as oxygen and carbon dioxide which are capable of forming covalent bonds without destroying the electrical neutrality of the iron atom (Perutz, 1978). Methemoglobin occurs as a result of the oxidation of the iron centre changing the normal ferrous state to ferric state. An alteration of the molecule has occurred which has a resultant inhibition of its oxygen-binding property rendering it dysfunctional (Rachel, 2003). Under normal conditions, the body is subjected to continual production of methemoglobin. Common causes of methemoglobin formation include; reduced cellular defense mechanism in children younger than the age of four months exposed to various environmental agents which could induce methemoglobinemia; a disorder characterized by the presence of a higher than normal level of methemoglobin in the body, various pharmaceutical compounds such as nitroglycerin, antibacterial drugs, sulfonamides, phenazopyridine, industrial solvents, gun-cleaning products which contain nitrobenzene, laundry ink or dyes, aminophenol, environmental agents such as air pollutants, room deodourizers, propellants and also combustion processes (Deushaw-Burke-Mary, 2006).

Methemoglobin saturation in terms of total hemoglobin is expressed in percentage and the saturation summary with its physiological effects in the system is expressed as follows; 1-2% is a normal level, <10% shows no observable symptoms, 10 - 20% shows skin discolouration

only mostly notable on mucus membranes, 20 - 30% causes anxiety, headache, hypsnea, 30 - 50% causes fatique, confusion, dizziness, tachypinea, palpitation 50 - 70% causes coma, seizures, arrhythmiasis, acidosis and >70% may lead to death (Deushaw-Burke-Mary 2006).

Under hypotonic ionic conditions, RBC may rupture and release their internal hemoglobin. This process is called hemolysis. Studies of the oxidative mechanisms of RBC have shown two major ways; lipid peroxidation and hemoglobin degradation (Atolaiye et al., 2006). Blood is continually exposed to reactive oxygen, nitrogen and other free radicals which gear up oxidative stress. RBC have been known to be subjected to oxidant stress by various means; oxidation of circulating red cells, dissociation of the superoxide anions from oxyhemoglobin, exposure to various oxidant drugs and chemicals such as potassium ferricyanide, hydrogen peroxide, quinones, drugs containing sulphanamides, anilines, insecticides, nitrates and nitrites (Atolaiye et al., 2006 and Rachel, 2003). The oxidants also include free radicals, peroxyl and alkoxyl radicals, and transition metal ions containing one or more unpaired electrons.

Lipid peroxidation is the oxidative degradation of lipids. It is the process whereby free radicals chelate electrons from lipids in the red cell membrane resulting in cell damage. Free radicals are postulated to be the major contributing factor to the degenerative processes responsible for decreasing the defense systems, causing damages to cell membrane and eventually leading to a cellular membrane breakdown or lysis of the cell (Dumaswala et al., 2000, 2001). It has been suggested that the human erythrocytes are very susceptible to oxidative/peroxidative damage. This is due to the fact that mature erythrocytes lack protein synthesis mechanism which makes them unable to replace damaged components and so they are geared towards reductive processes that combat the threat of oxidation (Dumaswala et al., 2001 and Wouter, 2005). If these reductive processes are inefficient, this could lead to oxidative damages to cellular constituents as a result of hemolysis (Gordon-Smith and White, 1974).

Recent studies have shown that RBC experience lipid peroxidation involving the cell membrane and also hemoglobin degradation. It has also been suggested that oxidative membrane damages appear to be the more important determinant factor which favor hemolysis in vitro than hemoglobin degradation (Robert et al., 1983). RBC was found to be prone to lipid peroxidation by virtue of their function as oxygen carriers and because of their lipid composition (Sawas and Pentyala, 2003; Tamir et al., 1983). Lipid peroxidation and hemoglobin degradation are processes of resultant oxidative stress on RBC. Hemoglobin can also undergo autooxidation to methemoglobin with concomitant production of superoxide radicals. The free radicals generated can further oxidize themselves with a resultant increase in accumulation of damaged non-functional proteins or attack membrane proteins and lipids (Dumaswala et al., 2001).

Work carried out on the effectiveness of antioxidants on red cell survival have predicted that certain substances known to have antioxidant properties can be used as antioxidants in red cells. Antioxidant effect of plasma quercetin metabolite has been identified and assessed. *In vitro* quercetin glucosides have been found to exhibit substantial protective effect on lipoprotein oxidation (Morand et al., 1998). Vitamin E has been found to show capability of protective effect on cell membranes via its ability to act as radical scavenger reducing cellular damages in the body while vitamin C exhibits antioxidant effects (Atolaiye et al., 2006). Antioxidants are also made within the body, for example, the enzyme superoxide dismutate.

Antioxidants are compounds which prevent or slow down oxidation reactions, they function as inhibitors, reacting with free radicals more efficiently to prevent oxidation (Dumaswala et al., 2001).

Kolanut is known for its economic importance. It is commonly consumed in West Africa and is a native plant of the tropical rain forest of Africa. Kolanut belongs to the family *Malvaceae*, subfamily; *stercutioideae* or *sterculiaceae*. The genus *cola* comprises of about forty species, but the most commonly used are; *Cola verticullata, Cola anomata*, *Cola nitida*, and *Cola acuminata*. The latter two are the most important species with greater economic and pharmacological importance (Lovejoy, 1980). *C. nitida and C. acuminata* are differentiated based on the number of cotyledons they possess; *C. nitida* is a dicotyledonous plant while *C. acuminata* has more than two cotyledons, possessing three to six (Mcllory, 1963). The key chemical components of kola nut are caffeine, theobromine, tannins and phenolics (Odebode, 1995). It also contains phlobaphens, anthrocyanins red pigment, betaine, protein and starch. The caffeine and theobromine provide stimulant effect, while the phenolics and anthrocyanins provide the antioxidative properties. The phenolic compounds have been discovered to be the main components which give this distinct antioxidative property (Dael-Rakotoarison et al., 2003).

Garcinia kola, commonly called “bitter cola” is an *angiospermae* belonging to the family of *Guttriferal*. It is a plant found in the West African sub-region. It has a bitter, astringent and resinous taste (Atawodi et al., 1995). It is known for its medicinal value. The seeds are used in the treatment of liver disorder and diarrhoeae (Iwu et al., 1990 and Braide, 1991). It is used in the treatment of catarrh and colicky pain (Adefule et al., 2004). It also has antioxidant property (Olatunde et al., 2002). *G. kola* contains kolaviron, a biflavonoid complex containing *Garcinia* biflavone GB1, GB2 and kolaflavanone in an approximate ratio of 2:2:1 (Adaramoye et al., 2005). The evaluation of the protective effects of flavonoids from *G. kola* seeds on the oxidation of human low density lipoprotein (LDL) and their ability to scavenge reactive oxygen species in vitro showed a remarkable in vitro scavenging activity of these flavonoids. Kolaviron showed a remarkable reducing property on potassium ferricyanide

in vitro, implying that kolavirons are electron donors and could react with free radicals to convert them to stable products, thereby terminating terminal chain reaction (Yen and Chen, 1995).

Prosopis africana is a legume belonging to the family *Fabaceae* (Aremu et al., 2007). The bean seeds of this legume are commonly used as food condiments in Nigeria. They are also used as food and for medicinal purposes. They have been proposed to possess some physical and energizing properties (Akaaimo and Raji, 2006). Phytochemical screening of this plant shows the occurrence of saponin, tannins, phlobatannin, anthraquinone and cardic glucoside (Ojo et al., 2006). Experiment showed that the concentration of tannins was high in the leaf, stem and root of this plant. This could be related to the observed hepatoprotective potentials of the plant extract, showing antioxidative property (Ojo et al., 2006).

Vitamins are compounds necessary for the enhancement of life, growth and health of animals and humans. They cannot be produced by the body and hence must be supplied through dietary means. Vitamins are arbitrarily classified into two groups; fat-soluble and water-soluble vitamins. Examples of fat-soluble vitamins are vitamins A, D, E and K. Vitamins C and D are water soluble. The water-soluble vitamins have the ability to take part in reversible oxidation-reduction processes and thus form a part of various co-enzymes (Florkin and Stotz, 1963). Antioxidative property of vitamins A, B, C and E has been determined by various researchers. The physiological effect of vitamin E was found to be to help maintain cell wall integrity and preserve energy metabolism of the cell by inhibiting lipid peroxidation of cell membrane (Kalpana and Menon, 2004).

Atolaiye et al. (2006) suggested that the window time of survival of RBC exposed to vitamin E was found to be 30 and 22 h in the presence of vitamin C. Vitamin C is a water-soluble vitamin with antioxidant function taking place in aqueous body compartment. It helps to protect low density lipoprotein cholesterol (LDL-C) against free radical damage, while vitamin E is incorporated into the lipid portion of cell membrane and other molecules protecting these structures from oxidative damage and preventing the propagation of lipid peroxidation (Adam et al., 1999). Vitamin D, also known as calciferol is a fat-soluble vitamin which helps to maintain normal blood level of calcium and also in the absorption of calcium to form and maintain strong healthy bones. Antioxidative potency of vitamin D was determined by Wiseman (1993) who suggested that vitamin D was a membrane antioxidant with the ability to inhibit iron-dependent lipid peroxidation, therefore vitamin D serves as inhibitor of lipid peroxidation (Wiseman, 1993).

Eugenol is the principal constituent of clove oil with molecular formular $C_{10}H_{12}O_2$. It is an allyl chain substituted guaiacol; 2-methoxyl (-4(2-propenyl) phenol. It is a clear to pale yellow oily liquid extracted from essential oils especially from clove oil, cinnamon and nutmeg. It is slightly soluble in water and soluble in organic solvent. (Eugenol-Wikipedia the free encyclopedia, 2006). It is used to produce isoeugenol for manufacturing vanillin, analgesics, biocides, anti-inflammatory and antibacterial substances and manufacturing of stabilizers and antioxidants for plastics and rubber. Overdose of eugenol is possible and can cause a widw range of symptoms from blood in urine to convulsion, diarrhea, nausea, unconsciousness, dizziness or rapid heart beat. Eugenol is obtained from a plant called clove (*Szygium aromaticum*), it is aromatic and phenolic (Torel et al., 1986). The antioxidative potency of phenolic compounds depends on the chemical structure in particular and also electron delocalization on the aromatic nucleus (Kitagawa et al., 1992). Research carried out on the antioxidant effect of eugenol on CCl_4-induced erythrocyte damage in rats showed that eugenol inhibited the accumulation of lipid peroxide products in RBC and maintained the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glucose-6-phosphate hydrogenase atnormal levels. The CCl_4-induced increase in membrane fluidity was decreased by eugenol (Parasakthy et al., 1996). The antioxidant activity of eugenol was found to be comparable to that of natural antioxidant, α-tocopherol; vitamin E (Lee and Shibamoto, 2003).

The aim of this work is to determine and compare the effectiveness of certain easily accessible and relatively cheap substances such as plants (kola nuts, *G. kola* called bitter kola), food plants (*P. africana*, locally called locust beans), vitamins (A and D) and some essential oils (eugenol, from clove oil) as antioxidants in the assessment of red cell survival in relation to the window time of survival of RBC when they exposed to them.

MATERIALS AND METHOD

Kola nut seeds were obtained commercially from Keffi in Nasarawa state, Nigeria. The kolanut seeds were identified by Mr. Onovo J.C of the Plant Science and Biotechnology Unit of the Biological Sciences Department of the Nasarawa State University, Keffi-Nigeria. The kola species were identified as *Cola nitida* and *Cola acuminata*. They were each further divided into three based on their colour pigments; pink, red and white. The three different samples of each kola nut species were separately lacerated into tiny pieces and air-dried at room temperature; 27°C for 48 h. Extraction was carried out using 70% methanol as solvent at a boiling temperature of 50 – 60°C using a soxhlet extractor over a period of 8 h. The extract collected was further concentrated using a water bath. This process was used in each case and the extracts were kept in the refrigerator at 10 - 18°C.

G. kola seeds were obtained commercially also from Keffi, Nasarawa state of Nigeria. The seeds were peeled, lacerated and air-dried at room temperature of 27°C for 48 h. Extraction was carried out using the procedure of Iwu et al. (1990) as adapted and modified by Adaramoye et al. (2005). The extraction was carried out on the powdered seeds using light petroleum ether at a boiling point of 40 - 60°C in a soxhlet extractor for 24 h. The defatted, dried samples were repacked and extraction continued using 70% methanol. The methanolic extract was concentrated and diluted to twice its volume with distilled water and further extraction was carried out using ethyl acetate (6 x 250 mL). The ethyl acetate fraction gave a yellowish solution of kolaviron.

P. africana bean seeds with their shells, obtained commercially

from a locality called Angwan Mala in the Kokona Local Government Area of Nasarawa state, Nigeria were pretreated and prepared according to the method of Aremu et al. (2006). Both *P. africana* bean seeds and shells were milled into flour. Extraction was carried out using 70% methanol in a soxhlet extractor in each case.

Vitamins A and D were both obtained commercially in Keffi, Nasarawa state, Nigeria. Vitamin A capsules; USP produced by Olive Health Care Company in India (containing vitamin A as palmitate), 50,000 I.U., equivalent to retinol 15mg in water-soluble form in each sample were purchased. Vitamin D purchased was produced by Manson Company. Vitamins A and D needed no further treatment. Apart from the separate exposure of RBC to vitamins A and D, vitamins A and D were mixed in order to establish the possibility of a synergistic relationship.

Reagents used in this study, sodium chloride (for normal isotonic saline), potassium ferricyanide (oxidizing agent), trisodium citrate dihydrate, citric acid monohydrate and anhydrous dextrose (the latter three in anticoagulant solution) were all prepared by BDH company, United Kingdom.

Normal isotonic saline was prepared by dissolving 9.50 g of sodium chloride in 1 dm^3 of distilled water.

Potassium ferricyanide solution was prepared by dissolving 0.25 g of the salt in 250 mL of the normal isotonic saline solution.

The anticoagulant, ACD (Acid Citrate Dextrose) was prepared by dissolving the mixture of 5.10 g of trisodium citate dihydrate, 1.60 g of citric acid monohydrate and 2.40 g of anhydrous dextrose in distilled water and then making it up to 200 mL. The ACD was prepared a few hours before use and kept refrigerated until required for use. ACD is preferred freshly used and cold. Blood samples were collected into ACD to prevent coagulation/agglutination of blood and probable lysing.

Fresh blood sample was collected into prepared anticoagulant. The blood was spinned in a cold or temperature controlled centrifuge at maximum speed, not less than 10,000 revolutions per minute (r.p.m.) for 20 min at 5°C and supernatant containing plasma was discarded and residue washed thrice with normal isotonic saline solution with spinning after each wash at a speed of 9,000 r.p.m for 15 min each turn. Normal isotonic saline solution was added to RBC volume per volume. The supernatant was discarded after each wash. Residue collected contained whole red blood cells only.

Method of analysis

0.5 - 1 ml of RBC was transferred into labeled sample tubes according to antioxidant to be analyzed. To each labeled tube, an equal volume of antioxidant was added and left to stand for 10 min, after which the oxidizing agent [K_3 Fe $(CN)_6$] was added, volume per volume. The time it takes for RBC to survive oxidation by potassium ferricyanide after exposure to each antioxidant (window time of survival) was monitored. The process was terminated after 32 h which was greater than the 30 h suggested for vitamin E by Atolaiye et al., 2006. After each length of monitoring hour (for example 12, 20, 22, 24, 26, 30 and 32 h), each sample was spinned and supernatant decanted and optical density/absorbance analyzed using a Cecil BioQuest UV-Visible Spectrophotometer

A reference sample was prepared in which RBC were exposed to oxidizing agent in the absence of antioxidant. This sample was exposed to the same conditions as other samples above except that no antioxidant was added. Oxidation and methemoglobin formation was identified by the change in colour of red cells from bright red to dirty brown at the monitoring wavelength of absorbance of 630 nm. The optical density or absorbance recorded was used as the reference for monitoring all other samples. The formation of methemoglobin indicated lysis of cell membrane and oxidation of Fe^{2+} in hemoglobin to Fe^{3+}. All analyses were monitored at a wavelength of 630 nm; the wavelength of aquomethemoglobin and the absorbance values of all supernatants collected from RBC exposed to antioxidants before oxidizing agent were compared with absorbance of methemoglobin at 630 nm.

RESULTS AND DISCUSSION

From Table 1, it is shown that in an isotonic environment the probability of lysis and oxidation with methemoglobin formation is very minimal, it has been reported that cells which are washed three times in isotonic saline do not lyse easily. RBC exposed ordinarily to oxidants, even those that are very mild are oxidized instantly. RBC in isotonic saline exposed to oxidizing agent was only oxidized after 12 h. Methemoglobin formation was observed by the change in colour from bright red to dirty brown of methemoglobin at 630 nm. The absorbance of methemoglobin at this wavelength was 0.187. All absorbance values equal to and greater than this value were assumed to show lysis of cell membrane, oxidation of hemoglobin and methemoglobin formation. The choice of monitoring from 20 h was to determine the effectiveness or potency of antioxidants in increasing the window time of survival of the red cells since oxidation occurred at 12 h.

Recent research has been carried out to determine the antioxidative potency of various plant extracts and vitamins. Atolaiye et al. (2006) reported that red blood cells exposed to vitamin E as antioxidant had a window time of survival of 30 hours while red cells exposed to vitamin C as antioxidant had a window time of survival of 22 h.

With respect to the vitamins; A, D and mixture of A and D, it was observed that the order of potency was as follows; A>D>A+D. At 32 h red cells exposed to A were still viable as no methemoglobin formation had been observed, those exposed to A+D were still viable at 30 h while at 25 h, cells exposed to D were still viable, showing that A was the most potent of the three and was even more potent than vitamin E which showed a survival time of 30 h (Atolaiye et al., 2006).

Considering the *P. africana* beans and shell extracts; it was observed that the shell extract was more potent or effecttive than the beans extract as shown by the corresponding window time of survival of the red cells exposed to them (PABS; 30 h and PAB; 26 h). Order of potency was therefore; PABS>PAB. This is not far-fetched as the presence of saponnins in *P. africana* as reported by Ojo et al. (2006) is most likely to be significantly present in the shell than in the beans as it was not significantly present in the leaves.

In the *Cola nitida* species, it was observed that the order of potency in terms of increasing effectiveness was as follows; CNP > CNR > CNW, with 32, 26 and 20 h as window times of survival exhibited by red cells expos-ed to them respectively.

In the *Cola acuminata* species, the order of antioxidative potency was as follows; CAW > CAP > CAR.

Red cells exposed to eugenol were found to have survived oxidation at 32 h. Based on this; it can be assumed that eugenol is more effective as antioxidant than vitamin

Table1. Method of analysis.

S/N	SAMPLE IDENTITY
1	RBC+NIS
2	RBC+K
3	RBC+NIS+K
4	RBC+A+K
5	RBC+D+K
6	RBC+A+D+K
7	RBC+PAB+K
8	RBC+PABS+K
9	RBC+CNP+K
10	RBC+CNR+K
11	RBC+CNW+K
12	RBC+CAP+K
13	RBC+CAR+K
14	RBC+CAW+K
15	RBC+E+K
16	RBC+G+K

RBC; Red blood cells, NIS; Normal isotonic saline, K; K_3 Fe $(CN)_6$, A; Vitamin A, D; Vitamin D, A+D; Mixture of Vitamins A and D, PAB; *Prosopia africana* beans extract, PABS; *Prosopis africana* beans shell extract, CNP; *Cola nitida* (Pink), CNR; *Cola nitida* (Red), CNW; *Cola nitida* (White), CAP; *Cola acuminata* (Pink), CAR; *Cola acuminata* (Red), CAW; *Cola acuminata* (White) E; Eugenol and G; *Garcinia kola*

E which had a window time of survival of 30 h (Atolaiye et al., 2006).At 32 h, RBC exposed to *G. kola* was found to be still viable, implying that *G. kola* has very potent antioxidative effect as reported by Farombi et al. (2004).

Farombi et al. (2004) reported that *Garcinia kola* exhibits protective effect against oxidative damage due to molecular target via scavenging of free radicals and iron binding. Adaramoye et al., (2005) also proposed that *G. kola* has protective effect on the oxidation of human low density lipoprotein (PDL) and has the ability to scavenge reactive oxygen species *in vitro.*

With respect to values of absorbance, Eugenol, *G. kola*, Vitamin A, *C. nitida* (pink) and *C. acuminata* (white) were all effective even at 32 hours with the following values of absorbance: 0.072, 0.138, 0.152, 0.048 and 0.148 respectively. They may therefore be arranged in the following pattern of increasing potency: CNP> Eugenol> *G. Cola*>Vitamin A>CAW.

With respect to the window times of survival; at 32 h red cells exposed to Vitamin A, CNP, CAW, E and G were still viable, at 30 h; vitamin A, A+D, PABS, CNP, CAW,E and G were still viable, at 26 h; Vitamins A, A+D, PABS, CNP, CNR, CAW, E and G had not experienced lysing and oxidation, at 24 h; Vitamin A, D, A+D, PAB, PABS, CNP, CNR, CAP, CAW, E and G were viable, at 22 h; A, D, PAB, PABS, CNP, CNR, CAP, CAR, CAW, E and G were also still viable and at 20 h; A, D, PAB, PABS, CNP, CNR, CNW, CAP, CAW, E and G were viable.

At 32 h the pattern of potency of the antioxidants according to their window times of survival were as follows; CNP > G >A > CAW > A. All other antioxidants were ineffective.

At 30 h; E > PABS > A+D > A > G > CAW > CNP, all others were not effective.

At 26 h; G > E > A > CAW > PABS > CNP > A+D > CNR, all others were not potent.

At 24 h; E > G > CNP > A > PAB > D > CAW > CAP > A+D > PABS, all others had no potency.

At 22 h; E > A > G > PAB > A+D > PABS > CNP > CAW > D > CNR, all others were not potent.

At 20 h; E > G > CAW > CNR > A > PAB > CNP > A > A+D > D > CAP > PABS > CNW, at 20 h CAR had lost its potency.

A synergistic relationship between A and D was indicated when red blood cells exposed to vitamins A and D survived for 30 h without lysis, oxidation and formation of methemoglobin. The antioxidant potency of vitamin D was improved in the presence of vitamin A.

The effectiveness of *G. kola* extract as antioxidant has been shown to be as a result of the synergistic relationship of the combination of *Garcinia* biflavonone GB1 and GB2 and kolaflavonone in the *G. kola* extract (kolaviron) which are in the ratio 2:2:1 (Adaramoye et al., 2005, Iwu; 1985 and 1987). This also shows why *G. kola* extract was effective as antioxidant. The kolaviron has a combined effect because of the reducing power it exhibits as electron donor which reacts with free radicals. RBC exposed to *G. kola* has a window time of survival greater than 32 h.

In the study of the pattern of potency used in this work, certain properties were contributing factors to their effecttiveness. Such properties included solubility (fat or water soluble) and types of phenolic compounds present. Vitamins A and D are fat-soluble vitamins and so have the ability to penetrate through the lipid membrane of the RBC, therefore increasing cellular membrane fluidity. Similarly, eugenol which is fat-soluble is also incorporated into the membranes thereby preventing free radical attack. This maintains the activity of the antioxidant enzymes at normal level (Parasakthyl et al., 1996). The combined effect of the phenolic nature and fat-soluble properties of eugenol makes it more effective than vitamins A, D, E and the mixture of vitamin A and D.

G. kola is known to possess a biflavonoid complex which is more effective than *C. acuminata* and *C. nitida* species. This is because flavonoids are the most potent of all phenolic antioxidants. *G. kola* (kolaviron) is water-soluble but has the capacity of incorporating into the red cell membrane, which makes it more effective than the corresponding water-soluble vitamins such as vitamin C.

Conclusion

From the results shown in Table 2, it is observed that Eugenol, *G. kola*, Vitamin A, Vitamins A+D, Vitamin D, *C. acuminata* (white), *C. nitida* (pink) and *C. nitida* (red) are

Table 2. Survival time of red cells (h) and absorbance.

S/N	Sample identity	Absorbance at window time of survival (h)						
		12	20	22	24	26	30	32
1	RBC+NIS	NO METHEMOGLOBIN FORMATION						
2	RBC+K	INSTANTENOUS METHEMOGLOBIN FORMATION						
3	RBC+NIS+K	0.187 (REFERENCE)						
4	RBC+A+K	--	0.043	0.042	0.061	0.089	0.115	0.152
5	RBC+D+K	--	0.071	0.104	0.123	0.755	0.525	0.276
6	RBC+A+D+K	--	0.070	0.085	0.140	0.127	0.103	0.196
7	RBC+PAB+K	--	0.062	0.080	0.089	0.224	0.206	0.225
8	RBC+PABS+K	--	0.100	0.086	0.163	0.117	0.016	0.256
9	RBC+CNP+K	--	0.065	0.088	0.060	0.123	0.147	0.048
10	RBC+CNR+K	--	0.035	0.120	0.087	0.158	0.219	0.291
11	RBC+CNW+K	--	0.141	0.232	0.265	0.270	0.242	0.274
12	RBC+CAP+K	--	0.096	0.108	0.138	0.195	0.202	0.269
13	RBC+CAR+K	--	0.301	0.656	0.290	0.253	0.362	0.478
14	RBC+CAW+K	--	0.033	0.091	0.137	0.108	0.137	0.148
15	RBC+E+K	--	0.002	0.002	0.009	0.063	0.005	0.072
16	RBC+G+K	--	0.027	0.046	0.038	0.061	0.136	0.138

RBC; Red blood cells, NIS; Normal isotonic saline, K; K_3 Fe $(CN)_6$, A; Vitamin A, D; Vitamin D, A+D; Mixture of Vitamins A and D, PAB; *Prosopia africana* beans extract, PABS; *Prosopis africana* beans shell extract, CNP; *Cola nitida* (Pink), CNR; *Cola nitida* (Red), CNW; *Cola nitida* (White), CAP; *Cola acuminata* (Pink), CAR= *Cola acuminata* (Red), CAW; *Cola acuminata* (White) and E; Eugenol.

effective as antioxidants in red cell survival and viability. Their potency or effectiveness is shown as follows; Eugenol > *G. kola* > Vitamin A > Vitamin D > *C. acuminata* (white) > *C. nitida* (pink) > *C. nitida* (red).

Recommendation

Research work into the world of natural antioxidants has paved a way for discoveries of their potencies and effecttiveness in biological systems. Because of their very high effectiveness and minimal side effects since most of them are naturally occurring plants and plant parts, they may be substituted for synthetic antioxidants. They may also be converted into food supplements.

This study can therefore be used as a basis for other findings such as the conversion of such plant extracts and vitamins into more effective forms that can be used as drugs. The eugenol extract may be extracted, refined and used in safe doses.

All the effective antioxidants may be used in the assessment of diseased cells and treatment in diseased conditions of cells such as sickle cell anemia, cancer, diabetes, asthma, viral infections and most common diseases that reduce the life span of cells because of their scavenging and antioxidative abilities. They scavenge agents of oxidation like peroxides, oxidant drugs, printer's dyes, insecticides, anilines and other zenobiotics.

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