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Full Length Research Paper

Levels of two oxidative stress indicators of human sickle erythrocytes incubated in aqueous extracts of *Anacardium occidentale, Psidium guajava* and *Terminalia catappa*

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The present *in vitro* study investigated the levels of oxidative stress indicators, namely, malondialdehyde (MDA) and methaemoglobin (mHb) when sickle erythrocytes were incubated in aqueous leaf extracts of *Anacardium occidentale, Psidium guajava* and *Terminalia catappa* for 12 h. At regular time intervals of 3 h, portions of the incubation mixture were withdrawn and spectrophotometric method was used to assay for erythrocyte MDA concentrations and percent (%) mHb. The control analysis showed that erythrocyte MDA concentration increased from 2.45±0.35 to 3.13±0.59 mmol/ml ($p > 0.05$; p value = 0.801176). Erythrocyte MDA concentrations in the presence of the three extracts were higher than the control samples at $t = 3$ h ($p > 0.05$; p value = 0.963253). However, compared with the control sample at the given time (t), aqueous extract of *T. catappa,* exhibited the highest capacity to cause reduction of erythrocyte MDA concentration [*T. catappa*] = 800 mg%; [MDA] = 2.89±0.33 mmol/ml; $t = 12$ h), representing 7.66% reduction of erythrocyte MDA concentration. Erythrocyte % mHb increased from 2.42±0.55 to 2.51±0.49% ($p > 0.05$; p value = 0.995171) in the control sample within the duration of 12 h. Incubation of sickle erythrocytes with aqueous extract of [*P. guajava*] = 800 mg% for 9 h caused reduction of Met.Hb% from 2.49±0.49% to 2.29±0.45%; $p > 0.05$; p value = 0.983519. Also, aqueous extract of *T. catappa* exhibited low capacity to cause reduction in erythrocyte %mHb. Aqueous extracts of *A. occidentale*, *P. guajava* and *T. catappa* exhibited variable capacities to hinder lipid peroxidation, but did not cause corresponding reduction in percent erythrocyte %mHb, as exemplified by negative correlation between the two oxidative stress indicators in the presence of *T. catappa* and higher concentrations of *A. occidentale*, *P. guajava.*

Key words: Malondialdehyde, methaemoglobin, erythrocyte, *Anacardium occidentale, Psidium guajava, Terminalia catappa.*

INTRODUCTION

Oxidative stress is caused by accumulation of reactive oxygen species (ROS) (Richards et al., 2007) produced as a normal by-product of cellular metabolism (Richards et al., 2007; Breusegem and Mittler, 2008), exposure to ionic/electromagnetic radiations and some environmental pollutants (ROS, 2008). These reactive species are capable of damaging diverse bio-molecules and cell structures (Saengkhae et al., 2007) in which lipids are probably the most susceptible (Clemens and Waller, 1987) when cellular levels are not controlled by appropriate antioxidant scavenging systems (Neupane et al., 2008). While erythrocytes of all genotypes are particularly sensitive to oxidative stress, when compared with normal erythrocytes; sickle erythrocytes spontaneously generate approximately twice as much superoxide ($\cdot O_2^-$), peroxide (H_2O_2) and hydroxyl ($\cdot OH$) radicals (Tamer et al., 2000). Increasing evidence suggests that lipid peroxidation may be an important factor in sickle cell anemia (Stone et al., 1990).

Specifically, sickle erythrocytes and their membrane structures are susceptible to endogenous free radical-mediated oxidative damage that correlates with the

proportion of irreversibly sickled erythrocytes (Rice-Evans et al., 1986). Furthermore, accumulation of hydrogen peroxide (H_2O_2) decreases the half life of erythrocytes by increasing oxidation of polyunsaturated fatty acids of membranes constituents (Nijs and De Meirleir, 2004) and can oxidize haemoglobin (Hb) to methaemoglobin (metHb) (Neupane et al., 2008; Chikezie, 2009). Methaemoglobin does not bind reversibly with oxygen. One of the toxic end products of lipid peroxidation is malondialdehyde (MDA) (Neupane et al., 2008). Sickle erythrocytes contain increased amount of MDA; and an evidence of abnormal amino group cross-linking by MDA has been demonstrated in lipid extract of sickle erythrocyte membrane preparations (Das and Nair, 1980).

Erythrocytes like other cells are supplied with diverse protective antioxidant mechanisms in order to counteract the toxic effects of ROS (Forchetti et al., 2006; Chikezie, 2010a, b). Antioxidants function as modulators of cellular homeostasis including detoxification of oxy-radicals and metals as well as potent free radical scavengers. Reduced glutathione is one of the major non-enzymic endogenous antioxidants protecting tissues against ROS (Neupane et al., 2008). Other antioxidants are alpha-tocopherol (Horwitt, 1991), uric acid (Sautin and Johnson, 2008; Bowman et al., 2010), ascorbic acid (Sati et al., 2010), beta carotenoids (Paiva and Russell, 1999) and varieties of plant secondary metabolites such as flavonoids and related polyphenolic compounds (Rice-Evans, 2001). Notable erythrocyte enzymatic ROS scavenging systems include glutathione reductase (Forchetti et al., 2006), glutathione peroxidase (Manfredini et al., 2008), glucose–6-phosphate dehydrogenase (Ojo et al., 2006), superoxide dismutase (ROS, 2008), catalase (Pennings et al., 1999; Chandrasena et al., 2006), peroxiredoxins (Low et al., 2007) and NADH-methaemoglobin reductase (Mallory, 2003; Chikezie, 2010).

Antimicrobial properties activity of *A. occidentale* against *E. coli* (Kudi et al.., 1999; Akinpelu, 2001), *Pseudomonas* (Kudi et al.., 1999) and *Helicobacter pylori* considered to cause acute gastritis and stomach ulcers have been reported (Ofusori et al., 2008). Lectin chemicals in leaves of *P. guajava* have been shown to bind to *E. coli* (Coutino-Rodriguez et al., 2001), preventing its adhesion to the intestinal wall and thus preventing infection (Lozoya et al*.,* 1990). *P. guajava* fruit intake decreases blood pressure and serum high-density lipoprotein/cholesterol levels (Singh et al., 1993). Indian almond leaves contain several different flavonoids, including kaempferol and quercetin (Indian Almond and Folk Medicine, 2009). Flavonoids have been shown to possess a range of biological activities that are consistent with them contributing to the protection against degenerative diseases such as cancer, diabetes, cardiovascular diseases and cataract (Knekt et al., 2002).

Varieties of xenobiotics of plant origin such as fava beans extracts (*Fava fava*) have been reported as agents that can interfere with the redox status of human erythrocytes especially in those individuals with impaired glucose-6-phosphate dehydrogenase activity (Mayes, 1983; Ojo et al., 2006). In the same vein, scavenging activities for free radicals by natural products of plant origin have been widely reported (Lam et al., 2007; Buřičova and Reblova, 2008; Muanda et al., 2009; Veeru et al., 2009; Sati et al., 2010). This study therefore is aimed to ascertain the level of oxidative stress by estimating the concentrations of oxidative stress indicators, namely, MDA and methaemoglobin when sickle erythrocytes were incubated in aqueous extracts of three medicinal plants: *Anacardium occidentale, Psidium guajava* and *Terminalia catappa.*

MATERIALS AND METHODS

Collection of plant specimens

Fresh samples of *A. occidentale, P. guajava* and *T. catappa* leaves were harvested between July and August, 2010, from trees within the campus of Imo State University, Owerri, Nigeria. The plant specimens were identified and authenticated by Dr. F. N. Mbagwu of the Herbarium Unit, Department of Plant Science and Biotechnology. A voucher specimen was deposited at the Herbarium for reference purposes.

Preparation of aqueous extracts of plant specimens

The samples were washed under continous current of distilled water for 15 min and air dried at room temperature (24^{o}C) for 60 min. The separate leaves were dried for 5 h in an oven at 60^{o}C to become crispy, and ground with ceramic mortar and pestle. To each specimen, two grams (2 g) of the pulverized sample was suspended in 100 ml of distilled water and allowed to stand for 6 h at 37^{o}C. The aqueous extracts (2 g %) of *A. occidentale, P. guajava* and *T. catappa* leaves were obtained by simple filtration method with Whatman No. 2 filter paper. The filtrates were centrifuged at 1200 x *g* for 5 min to remove tissue debris. The supernatants were carefully harvested with Pasteur pipette into sterile test tubes and kept at 4^{o}C in a refrigerator for at least 24 h before subsequent tests. Serial dilutions of the aqueous extracts in the order of 200, 400, 600 and 800 mg% were used for analyses.

Collection of blood samples/preparation of erythrocyte haemolysate

Five milliliters (5.0 ml) of human venous blood samples of HbSS genotype were collected by venopuncture and stored in EDTA anticoagulant tubes. The blood samples were obtained between July and August, 2010, from nine (9) male volunteers (59-79 kg) between the age bracket of 21-34 years attending clinics at the Federal Medical Center (FMC), Imo State University Teaching Hospital (IMSUTH), Orlu, St. John Clinic / Medical Diagnostic Laboratories, Avigram Medical Diagnostic Laboratories, and Qualitech Medical Diagnostic Laboratories. These centers are located in Owerri, Imo State, Nigeria. The Institutional Review Board of the Department of Biochemistry, Imo State University, Owerri, Nigeria, granted approval for this study and all blood donors signed informed consent form. This study was in accordance with the ethical principles that have their origins in the Declaration of Helsinki.

The erythrocytes were washed by centrifugation methods as

described by Tsakiris et al. (2005). Within 2 h of collection of blood samples, portions of 2.0 ml of the samples were introduced into centrifuge test tubes containing 4.0 ml of buffer solution pH = 7.4: 250 mM tris (hydroxyl methyl) amino ethane–HCl(Tris-HCl)/ 140 mM, NaCl/ 1.0 mM and $MgCl_2$/ 10 mM glucose). The erythrocytes were separated from plasma by centrifugation at 1200 x *g* for 10 min and washed three times by the same centrifugation method with the buffer solution. To remove platelets and leucocytes, the pellet was re-suspended in 3 ml of phosphate-buffered saline (PBS) solution, pH 7.4, and passed through a column (3.5 cm in a 30 ml syringe) of cellulose-microcrystalline cellulose (ratio w/w 1:1) as described by Kalra et al. (1981). The eluted fraction was passed twice through a new column of cellulose-microcrystalline cellulose (ratio 1:1 w/w) to obtain erythrocyte suspension sufficiently devoid of leucocytes and platelets. The isolated erythrocytes were lysed by freezing/ thawing as described by Kamber et al. (1984). The erythrocyte haemolysates were finally re-suspended in 1.0 ml of the buffer and stored at -70°C until analyses (Pennings et al., 1999).

Experimental design

A portion of 0.2 ml aqueous extracts of *A. occidentale, P. guajava* and *T. catappa* of increasing concentrations in the order: 200, 400, 600 and 800 mg% w/v were added to corresponding test tubes containing 0.8 ml of erythrocyte haemolysate (ratio 1:4 v/v). The incubation mixture was allowed to stand at a regulated temperature of 37°C in a water bath. At regular time intervals of 3 h for 12 h; aliquots of 0.2 ml of the incubation mixture were withdrawn and used for the determinations of erythrocyte MDA and methaemoglobin concentrations.

Determinations of erythrocyte malondialdehyde and methaemoglobin concentrations

Determination of erythrocyte concentration of MDA was by the method described by Tjahjani et al. (2008) with minor modifications. A mixture of 20% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) in a ratio of 2:1 was added into a test tube. A volume of 0.2 ml of erythrocyte haemolysate was introduced in the mixture and boiled for 10 min in a water bath. After cooling to 24°C, the mixture was centrifuged at 3,000 x *g* for 10 min. The absorbance of supernatant was read with a spectrophotometer (SPECTRONIC 20, Labtech – Digital Blood Analyzer®) at maximum wavelength (λ_{max}) = 532 nm. The values of absorbance of the samples were converted to MDA concentrations using the MDA standard curve (Schmuck et al., 2002).

Determination of methaemoglobin of erythrocyte haemolysate was by modification of the method of Evelyn and Malloy (1938), as described by Akomopong et al. (2000). A total of 0.4 ml of 0.5 M phosphate buffer (pH = 6.5) was added to 0.6 ml of the cell lysate, and the mixture was centrifuged at 16,000 x *g* for 5 min to sediment debris. A total of 0.7 ml of the supernatant fraction was used to measure the absorbance at λ_{max} = 630 nm (the absorbance maximum for methaemoglobin), and the reading recorded as S_1. A volume of 0.05 ml of 10 g% KCN was added, and after 5 min at 24°C, a second reading (S_2) was recorded. KCN converts methaemoglobin to cyanomethaemoglobin, which does not absorb at 630 nm; hence, the difference between absorbance readings S1 and S2 represents the absorbance due to methaemoglobin. To measure total hemoglobin levels, all the hemoglobin was converted to methaemoglobin, the absorbance of the sample at λ_{max} = 630 nm was recorded, and then KCN was added to form cyanomethaemoglobin. Specifically, 0.07 ml of the supernatant fraction was diluted 10-fold into 0.6 ml of 0.1 M phosphate buffer (pH = 6.5). Next, 0.03 ml of freshly prepared 20 g% $K_3Fe(CN)_6$ was added and incubated for 5 min at 24°C and an initial reading (T1) was recorded. A total of 0.05 ml of 10% KCN was subsequently added, and a second reading (T2) was recorded. The % mHb in the sample was calculated as [100(S1-S2)] / [10(T1-T2)] (Akomopong et al., 2000).

Statistical analysis

The results were expressed in terms of arithmetic mean (X) ± standard deviation (SD). The correlation coefficients between the results were determined with Excel software (Microsoft, 2007 version) and data were analyzed by Student's t-test as described by Pearson and Hartley (1966). Values of $p < 0.05$ were considered statistically significant.

RESULTS

The control analysis showed that within the experimental time 0-12 h, erythrocyte MDA concentration increased from 2.45±0.35 to 3.13±0.59 mmol/ml (Figures 1-3). Also, incubation of sickle erythrocytes in aqueous extracts of *A. occidentale, P. guajava* and *T. catappa,* showed increasing erythrocyte MDA concentrations with progression of incubation time.

However, compared with the corresponding control samples at every given time (*t*) interval, when $t > 3$ h, erythrocytes suspended in the three aqueous extracts exhibited decreased MDA concentrations ($p > 0.05$; *p* value = 0.963253). Erythrocyte MDA concentrations in the presence of the three extracts were generally higher than the control samples at $t = 3$ h. Specifically, when the erythrocytes were incubated for 12 h in aqueous extract of *A. occidentale*, MDA concentration was 2.92±0.25 mmol/ml (control = 3.13±0.59 mmol/ml; $p > 0.05$; *p* value = 0.9776), representing a decrease of 6.71% of MDA concentration within the period of incubation.

At $t = 12$ h, aqueous extract of *T. catappa,* exhibited the highest capacity to cause the reduction of erythrocyte MDA concentration ([*T. catappa*] = 800 mg%; [MDA] = 2.89±0.33 mmol/ml), representing 7.66% reduction in erythrocyte MDA concentration whereas in the control sample, peak concentration of erythrocyte MDA concentration was 3.13±0.59 mmol/ml ($t = 12$ h), erythrocytes incubated in aqueous extracts of *P. guajava* and *T. catappa,* exhibited peak MDA concentration in the following: ([*P. guajava*] = 800 mg%, [MDA] = 3.04±0.38 mmol/ml; [*T. catappa*] = 200 mg%, [MDA] = 3.04±0.23 mmol/ml). Notably, Figure 3 showed that, in a concentration dependent manner, aqueous extract of *T. catappa* caused decreased erythrocyte MDA concentrations between the incubation time of 9 and 12 h. The levels of erythrocyte MDA in the presence of 800 mg% concentration of *A. occidentale* and *P. guajava* were elevated compared to lower concentrations of the same extracts.

Erythrocyte MDA concentrations in the presence of 800 mg% of *A. occidentale* and *P. guajava* ($r = 0.845082$) showed a higher positive correlation than *A. occidentale* and *T. catappa* ($r = 0.746903$). The MDA contents of

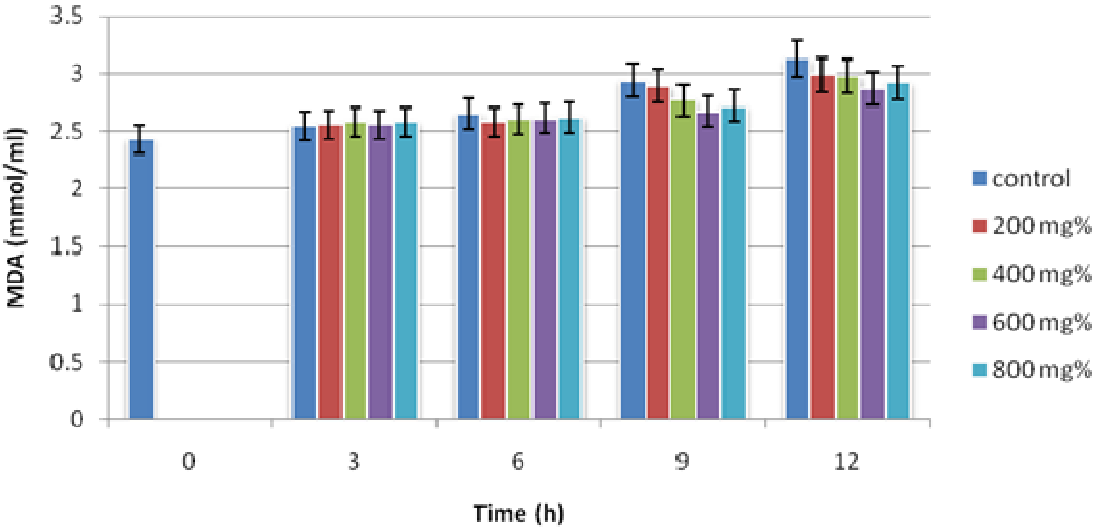

Figure 1. *MDA* concentrations of sickle erythrocytes incubated in aqueous extract of *A. occidentale.*

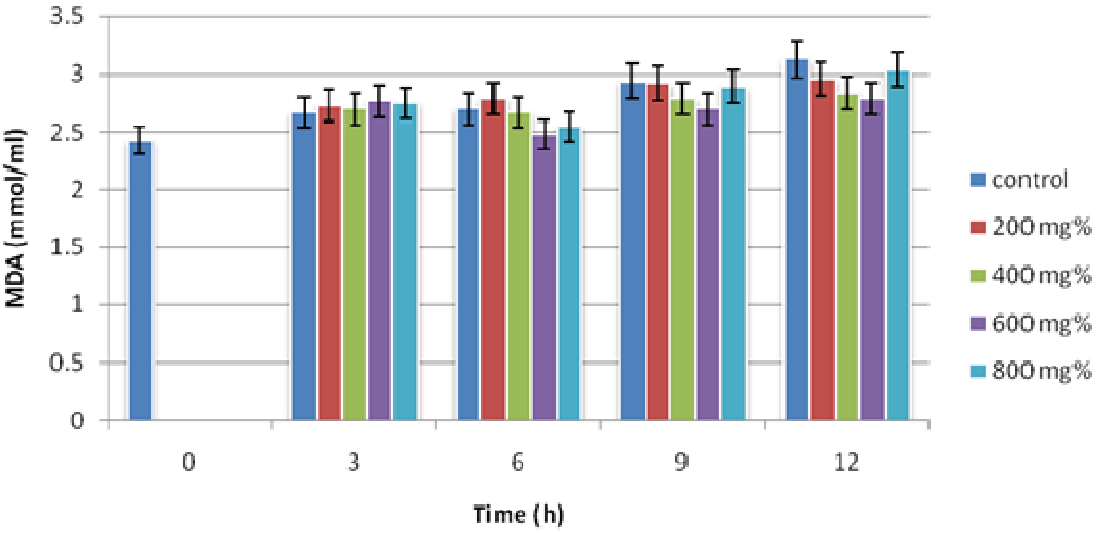

Figure 2. *MDA* concentrations of sickle erythrocytes incubated in aqueous extract of *P. guajava.*

sickle erythrocytes incubated in the four experimental concentrations of *A. occidentale* and *P. guajava* exhibited low positive correlation at $t = 3$ h; ($r = 0.271896$).

The percent erythrocyte %mHb increased from 2.42±0.55 to 2.51±0.49% in the control sample within the duration of 12 h (Figures 4-6). Although at $t = 3$ h, aqueous extract of *A. occidentale* (except 800 mg%) caused increased % mHb, the values were not significantly different ($p > 0.05$) from the control samples. Further increases in incubation time ($t > 3$) engendered declining levels of erythrocyte Met.Hb% except at $t = 12$ h.

Although aqueous extract of [*A. occidentale*] = 200 mg% caused decreased erythrocyte %mHb from 2.49±0.49% to 2.34±0.65% within 6 h of incubation, the value increased to 2.55±0.43% at t = 12 h.

Incubation of sickle erythrocytes in aqueous extract of [*P. guajava*] = 800 mg% for 9 h caused the reduction of Met.Hb% from 2.49±0.49% to 2.29±0.45%. At [*P. guajava*] = 600 mg%, erythrocyte Met.Hb% gave value of 2.55±0.43% ($t = 3$ h), which was not significantly ($p > 0.05$; p value = 0.996121) higher than the control value.

Also, in the presence of aqueous extract of [*P. guajava*] = 200 mg%, erythrocyte % mHb value of 2.46±0.43%; at t = 9 h, was not significanly different ($p > 0.05$; p value = 0.999778) from the control sample. In addition, 200 mg% of *T. catappa* caused increased erythrocyte %mHb within the experimental period of 6 h (Figure 6). Generally, aqueous extract of *T. catappa* exhibited low capacity to cause reduction in erythrocyte %mHb. Statistical

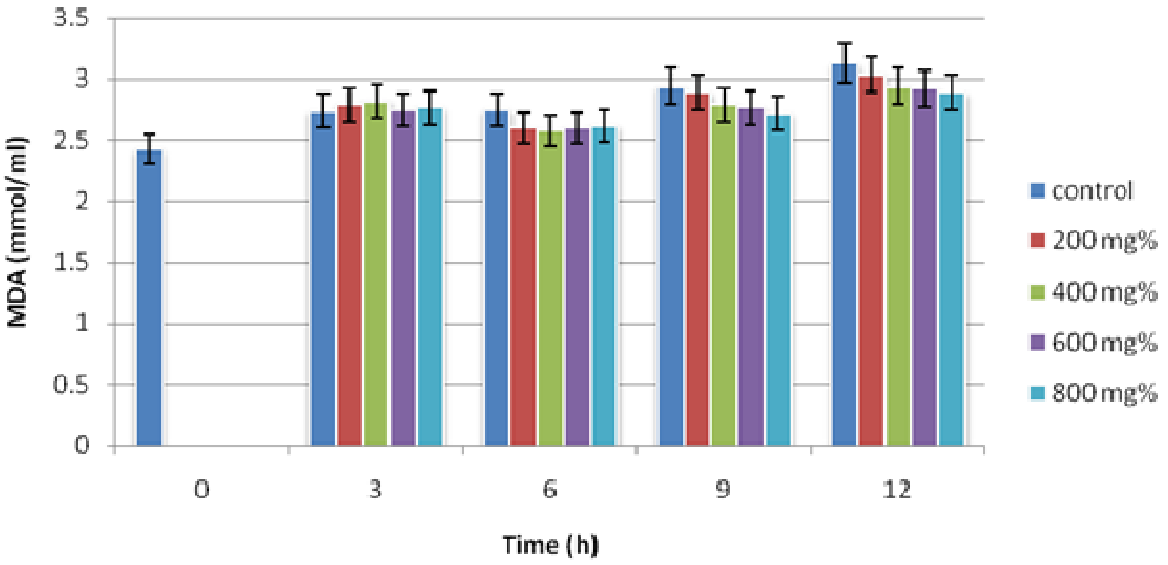

Figure 3. *MDA* concentrations of sickle erythrocytes incubated in aqueous extract of *T. catappa*.

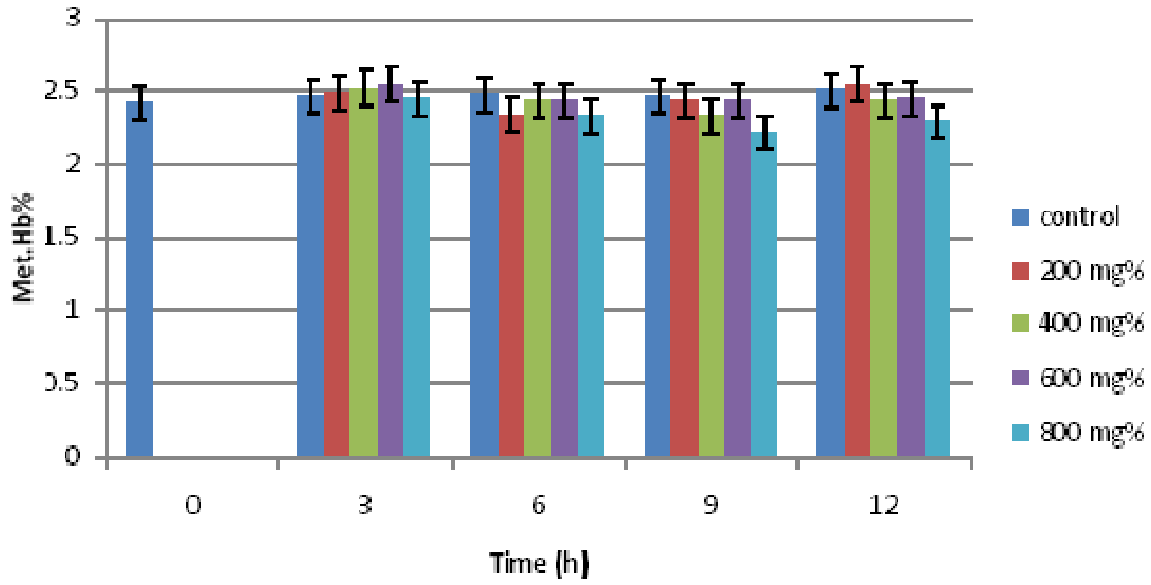

Figure 4. Percent mHb of sickle erythrocytes incubated in aqueous extract of *A. occidentale*.

evalution shows that erythrocyte %mHb in the presence of 600 mg% of *P. guajava* and *T. catappa* displayed high positive correlation (r = 0.965535). Also, 800 mg% of *A. occidentale* and *P. guajava* exhibited postive correlation (r = 0.878868) with respect to erythrocyte Met.Hb%. From the data presented in Table 1, erythrocyte Met.Hb% and MDA concentration showed significant positive correlation in the presence of aqueous extracts of 200 mg% *A. occidentale* and 400 mg% *P. guajava*. Notably, erythrocytes incubated in aqueous extract of *T. catappa* and concentrations of *A. occidentale, P. guajava* > 400 mg% showed negative correlation.

DISCUSSION

The increasing erythrocyte MDA concentrations of the control samples with time were obvious reflection of production and accumulation of ROS, engendered by normal metabolic processes in these cells. Tarmer et al. (2000) had earlier reported that sickle erythrocytes generate ROS spontaneously and contain high level of MDA, a by-product of lipid peroxidation. It is worthwhile to note that several abnormalities associated with sickle erythrocyte are inextricably connected with high level of oxidative stressors in this erythrocyte genotype

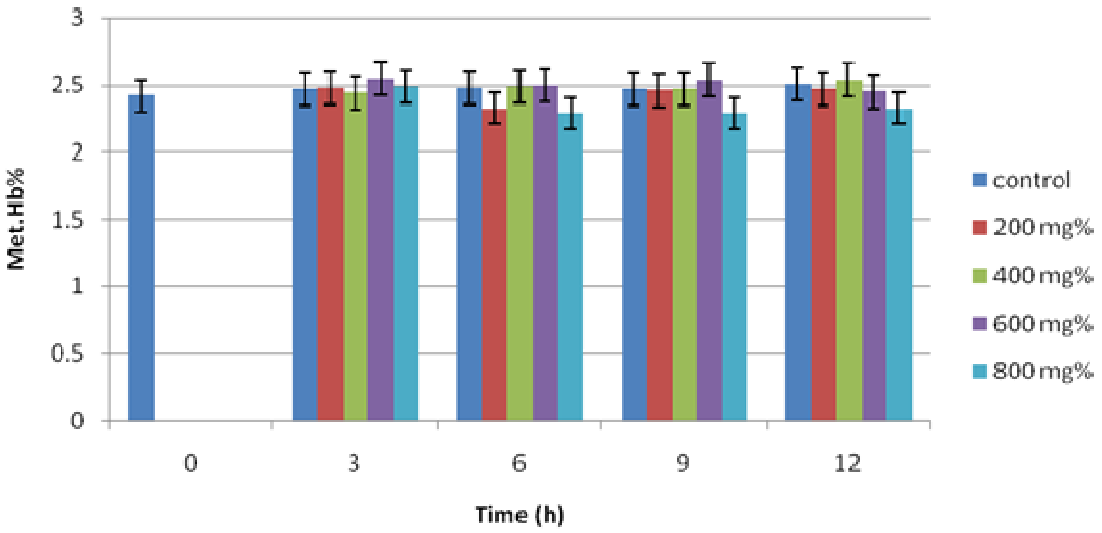

Figure 5. Met.Hb% of sickle erythrocytes incubated in aqueous extract of *P. guajava.*

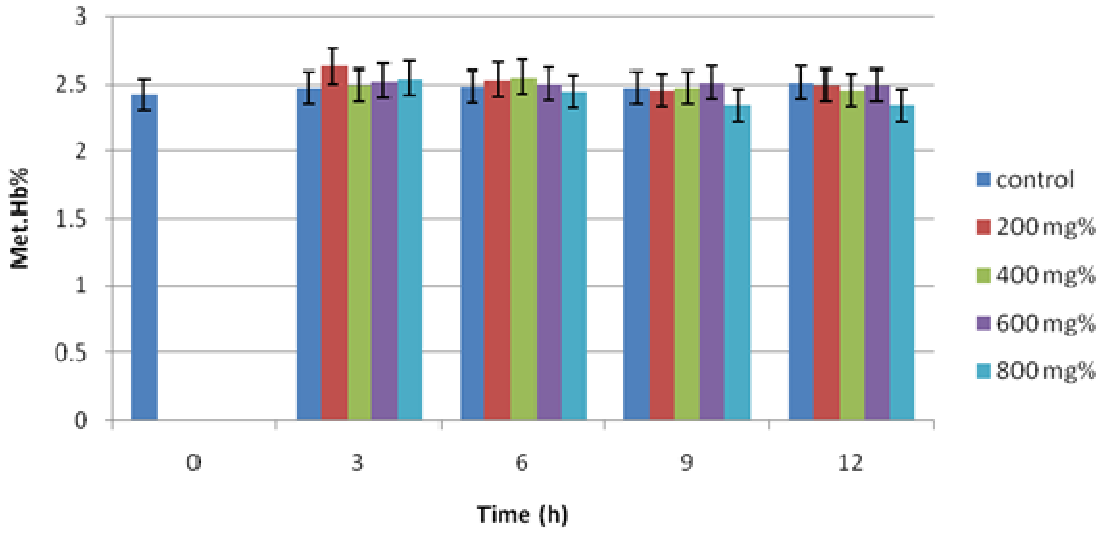

Figure 6. Percent %mHb of sickle erythrocytes incubated in aqueous extract of *T. catappa.*

Table 1. Correlation between changes in MDA *concentration* and %mHb of sickle erythrocyte incubated in aqueous extracts of *A. occidentale, P. guajava* and *T. catappa for 12 h.*

	Correlation coefficient (r)		
[Extract] mg%	***A. occidentale***	***P. guajava***	***T. catappa***
200	0.578986	0.284634	-0.40214
400	-0.42167	0.624449	-0.95828
600	-0.48163	-0.02065	-0.33534
800	-0.5715	-0.03693	-0.27915

Manfredini et al., 2008). From the results in Figures 1-3, the increasing erythrocyte MDA concentrations of the control and test samples was an indication of time dependent progression of lipid peroxidation in both

samples. Furthermore, relatively lower MDA concentrations of the test samples compared to the control samples at the given time intervals revealed that lipid peroxidation was retarded by the three plant extracts in connection with their corresponding concentrations. The present findings are in concord with the report of Lam et al. (2007).

They noted that gamma irradiation induced oxidative stress indicators were significantly reduced in rats after oral administeration of *Aloe vera* extract. Pre-vious findings have attributed the capacities of *Zingiber officinale, Aloe vera* (L) and *Rheum rhabarbarum* (L) extracts to reduce lipid peroxidation to the presence of phenolic compounds in these plants (Lam et al., 2007). Also, membrane protective activities of diverse plant extracts have been attributed to their antioxidant content and capacity to prevent membrane lipid peroxidation (Okpuzor et al., 2008; Buřičova and Reblova, 2008). However, since 800 mg% extract concentration of *A. occidentale* and *P. guajava* did not cause the anticipated level of hindrance to lipid peroxidation (r = 0.845082). Therefore, it is envisaged that higher concentrations of the two plant extracts may promote it. It is worthwhile to mention that Paiva and Russell (1999), had previously reported that antioxidant activity of carotenoids (including ß-carotene) may exhibit adverse effects when present in high dose.

The present result showed that mHb% of sickle erythrocyte was significantly ($p < 0.05$; p value = 0.489323) higher than normal physiologic concentration (%mHb = 1.50) as reported by Chikezie (2009) for HbAA erythrocyte genotype. Noteworthy, the level of erythrocyte methaemoglobin reported here is comparable to those presented elsewhere (Van Kiujk et al., 1987; Chikezie, 2009; 2010). The primary reason for the relatively high concentration of oxidized haemoglobin is also connected with excessive production and accumulation of ROS compared with other human erythrocyte genotypes (Van Kiujk et al., 1987). The moderate reduction in %mHb in erythrocyte incubated in aqueous extracts of *A. occidentale*, *P. guajava* and *T. catappa* is attributed to their antioxidant activity as earlier discussed. However the association of certain methaemoglobinopathies such as HbM_{Boston}, HbM_{Iwate}, $HbM_{Hydepark}$ and $HbM_{Hammersmith}$ with sickle erythrocytes contributed to insignificant reduction in erythrocyte %mHb. These variant haemoglobin molecules are noted for their tendency towards spontaneous oxidation *in vivo* and resistant to enzymic and non-enzymic reduction mechanisms (Mayes, 1983). Based on the levels of oxidative stress indictors in the present study, aqueous extracts of *A. occidentale*, *P. guajava* and *T. catappa* exhibited variable capacities to hinder lipid peroxidation, but did not cause corresponding reduction in erythrocyte %mHb, as exemplified by negative correlation between the two oxidative stress indicators in the presence of *T. catappa* and higher concentrations of *A. occidentale* and *P. guajava.*

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