

# 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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## ABSTRACT

An *in vitro* study was carried out to investigate levels of oxidative stress indicators of sickle erythrocytes incubated in aqueous extracts of *Anacardium occidentale*, *Psidium guajava* and *Terminalia catappa* for 12 h. At regular time intervals of 3 h, portions of the incubation mixtures were withdrawn and spectrophotometric method was used to assay for levels of erythrocyte malondialdehyde (MDA) and methaemoglobin (Met.Hb%). The control analysis showed that within the experimental time, erythrocyte MDA increased from  $2.45 \pm 0.35$  to  $3.13 \pm 0.59$  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). Compared with the control samples at the given time ( $t$ ) intervals, extract of *T. catappa* exhibited the highest capacity to cause reduction of erythrocyte MDA [*T. catappa*] = 800 mg%; [MDA] =  $2.89 \pm 0.33$  mmol/ml;  $t = 12$  h). Erythrocyte Met.Hb% increased from  $2.42 \pm 0.55$  to  $2.51 \pm 0.49\%$  ( $p > 0.05$ ;  $p$  value = 0.995171) in the control samples within 12 h. Incubation of sickle erythrocytes with extract of [*P. guajava*] = 800 mg% for 9 h caused reduction of Met.Hb% from  $2.49 \pm 0.49\%$  to  $2.29 \pm 0.45\%$  ( $p > 0.05$ ;  $p$  value = 0.983519). Extracts of *A. occidentale*, *P. guajava* and *T. catappa* exhibited variable capacities to hinder lipid peroxidation, but did not cause corresponding reduction in erythrocyte Met.Hb%, as shown by negative correlation between the two oxidative stress indicators in the presence of *T. catappa* and higher concentrations of *A. occidentale* and *P. guajava*.

**Keywords:** Malondialdehyde, Methaemoglobin, Erythrocyte, *Anacardium occidentale*, *Psidium guajava*, *Terminalia catappa*.

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## INTRODUCTION

Oxidative stress is caused by accumulation of reactive oxygen species (ROS)<sup>[1, 2]</sup> produced as normal by-products of cellular metabolism<sup>[2, 3]</sup>, exposure to ionic/electromagnetic radiations and some environmental pollutants.<sup>[4, 5]</sup> These reactive species are capable of damaging diverse biomolecules and cell structures<sup>[6]</sup> in which lipids are probably the most susceptible,<sup>[7, 8]</sup> when cellular levels are not controlled by appropriate antioxidant scavenging systems.<sup>[9]</sup> Though erythrocytes of all human genotypes are particularly sensitive to oxidative stress, when compared with normal erythrocytes, sickle erythrocytes spontaneously generate approximately twice as much anion superoxide ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radicals ( $\text{OH}^\cdot$ )<sup>[10, 11]</sup> and increasing evidence suggest that lipid peroxidation may be an important factor in sickle cell anaemia.<sup>[12]</sup>

Specifically, sickle erythrocytes and their membranes are susceptible to endogenous free radical-mediated oxidative damage that correlates with the proportion of irreversibly sickled erythrocytes.<sup>[13]</sup> Furthermore, accumulation of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) decreases the half life of erythrocytes by increasing oxidation of polyunsaturated fatty acids of cell membrane<sup>[14]</sup> and can oxidize haemoglobin to methaemoglobin.<sup>[9, 15]</sup> Methaemoglobin does not bind reversibly with oxygen. One of the toxic end products of lipid peroxidation is malondialdehyde (MDA).<sup>[9]</sup> Sickle erythrocytes contain increased amount of MDA and evidence of amino group cross-linking by MDA, producing MDA-modified protein adducts has been demonstrated in lipid extract of sickle erythrocyte membrane preparations.<sup>[16]</sup>

Erythrocytes like other cells are supplied with diverse protective antioxidant mechanisms in order to counteract the toxic effects of ROS.<sup>[17-21]</sup> Antioxidants function as

modulators of the cell redox homeostasis detoxifying both radical and non-radical species as well as heavy metals. Erythrocytes-reduced glutathione is one of the major non-enzymatic endogenous antioxidants protecting tissue against ROS.<sup>[9, 22]</sup> Other antioxidants are alpha-tocopherol<sup>[23, 24]</sup>, uric acid<sup>[25-27]</sup>, ascorbic acid<sup>[28]</sup>, carotenoids<sup>[29, 30]</sup> and varieties of plant secondary metabolites such as flavonoid and related polyphenolic compounds.<sup>[31, 32]</sup> Notable erythrocyte enzymatic ROS scavenging systems include glutathione reductase,<sup>[17, 19]</sup> glutathione peroxidase,<sup>[33]</sup> glucose-6-phosphate dehydrogenase,<sup>[18, 34]</sup> superoxide dismutase,<sup>[5, 35]</sup> catalases,<sup>[36-38]</sup> peroxiredoxins,<sup>[39, 40]</sup> and NADH-methaemoglobin reductase.<sup>[21, 41-43]</sup>

Varieties of xenobiotics of plant origin such as fava beans extracts (*Vicia faba*) have been reported as agents that can modify the redox status of human erythrocytes especially in individuals with impaired glucose-6-phosphate dehydrogenase activity.<sup>[18, 34, 44]</sup> Similarly, scavenging activities for free radicals by natural products of plant origin have been widely reported.<sup>[1, 28, 45-49]</sup> This study has ascertained the level of oxidative stress by estimating the concentrations of two oxidative stress indicators, namely, MDA and methaemoglobin of sickle erythrocytes incubated in aqueous extracts of three medicinal plants: *Anacardium occidentale*, *Psidium guajava* and *Terminalia catappa*.

## MATERIALS AND METHODS

**Collection of Plant Specimens** Fresh leaf samples of *A. occidentale*, *P. guajava* and *T. catappa* were harvested between July and August, 2010, from trees within the environment of Imo State University, Owerri, Nigeria. The plant specimens were identified and authenticated by Dr. F. N. Mbagwu at the Herbarium, 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 continuous current of distilled water for 15 min and air dried at room temperature (24°C) for 60 min. The separate leaves were dried for 5 h in an oven at 60°C to become crispy, and ground with ceramic mortar and pestle. For 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°C. The aqueous extracts (2 g<sup>0</sup>%; w/v) 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°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<sup>0</sup>%; (w/v) 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 venipuncture 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 yrs 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 of the Declaration of Helsinki.

The erythrocytes were washed by centrifugation methods as described by Tsakiris *et al.*<sup>[50]</sup> Within 2 h of collection of blood samples, 2.0 ml aliquots 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 MgCl<sub>2</sub> 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 sediment 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.*<sup>[51]</sup> 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 Galbraith and Watts,<sup>[52]</sup> and Kamber *et al.*<sup>[53]</sup> The erythrocyte haemolysates were finally re-suspended in 1.0 ml of the buffer and stored at -70°C until analyses.<sup>[37]</sup>

**Experimental Design** A 0.2 ml aliquot of the 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 method described by Tjahjani *et al.*<sup>[54]</sup> 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 ( $\lambda_{\max}$ ) = 532 nm. The values of absorbance of the samples were converted to MDA concentrations using the MDA standard curve.<sup>[55]</sup>

Determination of methaemoglobin content of erythrocyte haemolysate was by modification of the method of Evelyn and Malloy,<sup>[56]</sup> as described by Akompong *et al.*<sup>[57]</sup> 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 0.7 ml aliquot of the supernatant fraction was used to measure the absorbance at  $\lambda_{\max}$  = 630 nm (the absorbance maximum for methaemoglobin), and

the measurement was recorded as first reading (S1). A volume of 0.05 ml of 10 g% KCN was added, and after 5 min at 24°C, a second reading (S2) 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 of the hemoglobin was converted to methaemoglobin, the absorbance of the sample at  $\lambda_{\max}$  = 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 percent of methaemoglobin (Met.Hb%) in the sample was calculated as  $[100(S1-S2)] / [10(T1-T2)]$ .

**Statistical analysis** The results were expressed in terms of arithmetic mean ( $\bar{X}$ )  $\pm$  standard deviation (SD). The correlation coefficients between the results were determined with Microsoft Office Excel, 2007 version and data were analyzed by Student's t-test as described by Pearson and Hartley.<sup>[58]</sup> 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  $2.45 \pm 0.35$  to  $3.13 \pm 0.59$  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.

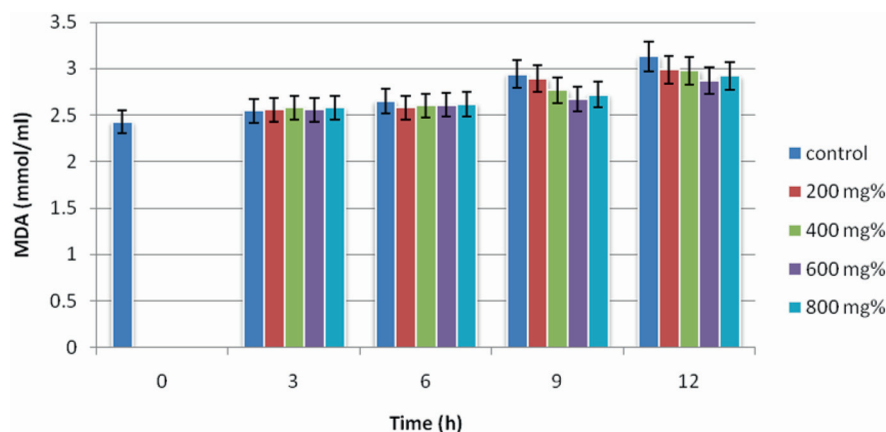


Figure 1. MDA concentrations of sickle erythrocytes incubated in aqueous extract of *A. occidentale*.

However, compared with the corresponding control samples at each 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 (Figs. 1-3). Specifically, when the erythrocytes were incubated for 12 h in aqueous extract of 800 mg% *A. occidentale*, MDA concentration was  $2.92 \pm 0.25$  mmol/ml (control =  $3.13 \pm 0.59$  mmol/ml;  $p > 0.05$ ;  $p$  value = 0.9776), representing a decrease of 6.71% of MDA concentration within the period of incubation (Fig. 1).

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] =  $2.89 \pm 0.33$  mmol/ml), representing 7.66% reduction in erythrocyte MDA concentration (Fig. 3). Whereas in the control sample, peak concentration of erythrocyte MDA concentration was  $3.13 \pm 0.59$  mmol/ml ( $t = 12$  h),

erythrocytes incubated in aqueous extracts of *P. guajava* and *T. catappa*, exhibited peak MDA concentration in the following specifications: ( $[P. guajava] = 800$  mg%, [MDA] =  $3.04 \pm 0.38$  mmol/ml;  $[T. catappa] = 200$  mg%, [MDA] =  $3.04 \pm 0.23$  mmol/ml) (Figs. 2, 3). Notably, Figure 3 showed that in a concentration dependent manner, aqueous extract of *T. catappa* caused decreased erythrocyte MDA concentrations between incubation time of 9 and 12 h. At 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 (Figs. 1, 2).

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

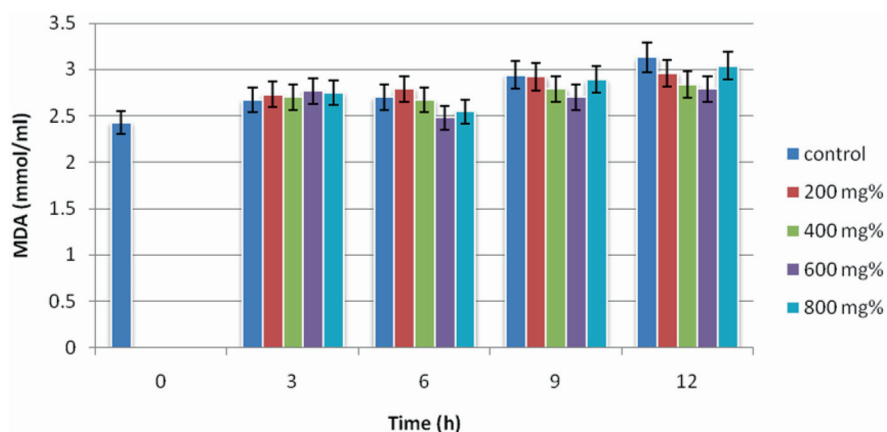


Figure 2. MDA concentrations of sickle erythrocytes incubated in aqueous extract of *P. guajava*.

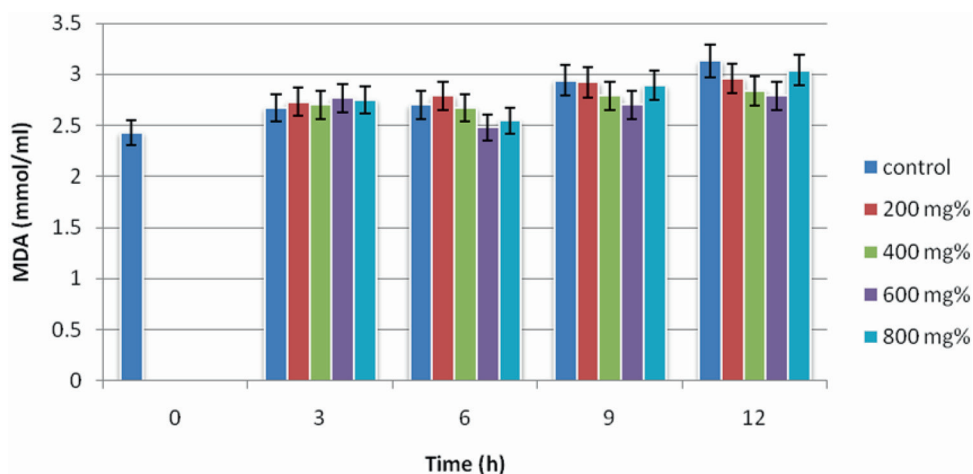


Figure 3. MDA concentrations of sickle erythrocytes incubated in aqueous extract of *T. catappa*.

Erythrocyte Met.Hb% increased from  $2.42 \pm 0.55$  to  $2.51 \pm 0.49\%$  in the control sample within the duration of 12 h (Figs 4-6). Although, at  $t = 3$  h, aqueous extract of *A. occidentale* (except 800 mg%) caused increasing of Met.Hb%, 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 (Fig. 4).

Although aqueous extract of [*A. occidentale*] = 200 mg% caused decreased erythrocyte Met.Hb% from  $2.49 \pm 0.49\%$  to  $2.34 \pm 0.65\%$  within 6 h of incubation, the value increased to  $2.55 \pm 0.43\%$  at  $t = 12$  hr (Fig. 4).

Incubation of sickle erythrocytes in aqueous extract of [*P. guajava*] = 800 mg% for 9 h caused the reduction of Met.Hb% from  $2.49 \pm 0.49\%$  to  $2.29 \pm 0.45\%$ . Incubation with [*P. guajava*] = 600 mg%, erythrocyte Met.Hb% gave value of  $2.55 \pm 0.43\%$  ( $t = 3$  h), which was not significantly ( $p > 0.05$ ;  $p$  value = 0.996121) higher than the control value (Fig. 5).

Also, in the presence of aqueous extract of [*P. guajava*] = 200 mg%, erythrocyte Met.Hb% value of  $2.46 \pm 0.43\%$ , at  $t = 9$  h, was not significantly different ( $p > 0.05$ ;  $p$  value = 0.999778) from the control sample (Fig. 5). In addition, 200 mg% of *T. catappa* caused increased erythrocyte Met.Hb% within the experimental period of 6 h (Fig. 6). Generally, aqueous extract of *T. catappa* exhibited low capacity to cause reduction in erythrocyte Met.Hb % (Fig. 6). Statistical analysis showed that erythrocyte Met.Hb% 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 positive 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.

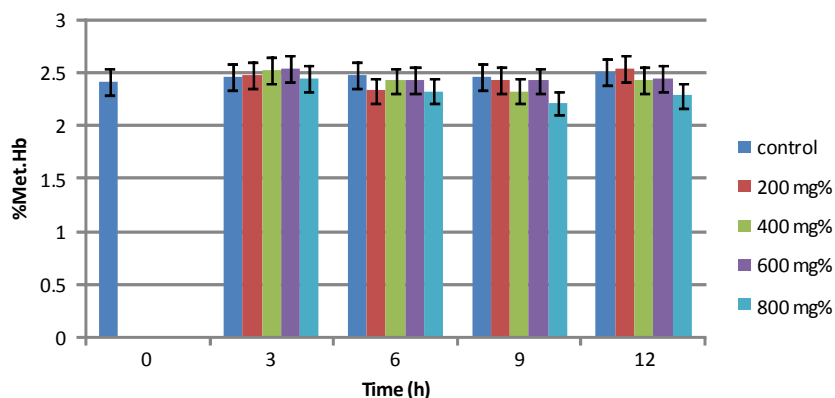


Figure 4. Met.Hb% of sickle erythrocytes incubated in aqueous extract of *A. occidentale*.

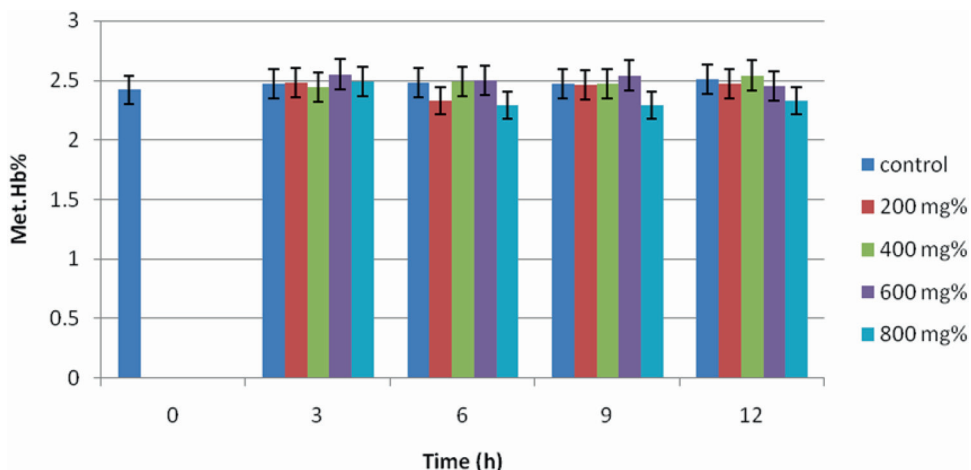


Figure 5. Met.Hb% of sickle erythrocytes incubated in aqueous extract of *P. guajava*.

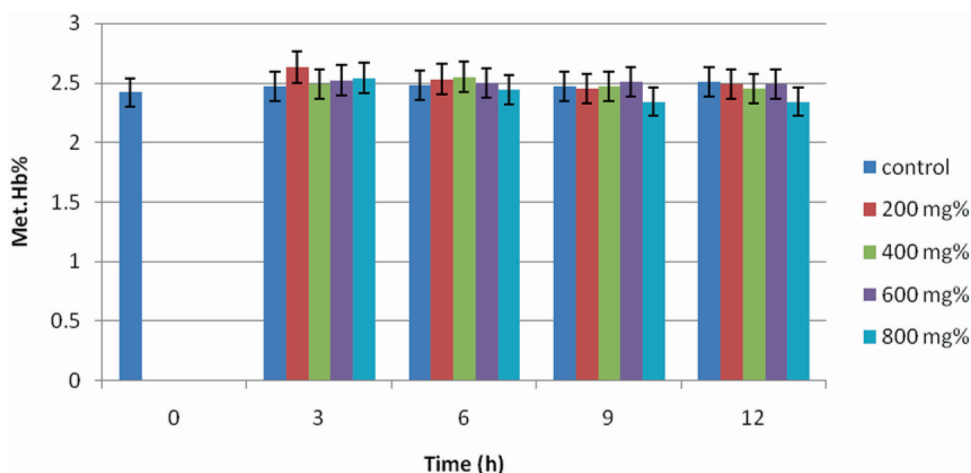


Figure 6. Met.Hb% of sickle erythrocytes incubated in aqueous extract of *T. catappa*.

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

[Extract] mg%	Correlation coefficient (r)		
	<i>A. occidentale</i>	<i>P. guajava</i>	<i>T. catappa</i>
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

## 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. Tarner *et al.* [59] had previously 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 [33, 60, 61]. From the results showed in Figures 1-3, the increasing erythrocyte MDA concentrations of the control and test samples are indication of the time-dependent progression of lipid peroxidation in both samples. Furthermore, relatively lower MDA concentrations of the test samples, compared to the control ones, at the given time intervals, revealed that lipid peroxidation was retarded by the three plant extracts in relation to their corresponding concentrations. The present findings are in concord with the report of Lam *et al.* [46]. They noted that gamma

irradiation-induced oxidative stress indicators were significantly reduced in rats after orally administered with *Aloe vera*. Previous findings have attributed the capacities of *Zingiber officinale*, *Aloe vera* and *Rheum rhabarbarum* extracts to reduce lipid peroxidation to the presence of phenolic compounds in these plants [46, 62]. Also, membrane protective activities of diverse plant extracts have been attributed to their antioxidant content and capacity to impede membrane lipid peroxidation [47, 63, 64]. However, when compared to lower experimental concentrations of *A. occidentale* and *P. guajava*, 800 mg% concentration of the two plant extracts did not cause an anticipated reduction of lipid peroxidation ( $r = 0.845082$ ) (Figs. 1, 2). Therefore, it is envisaged that higher concentrations of the two plant extracts may promote peroxidation. It is worthwhile to mention that Paiva and Russell [30] previously reported that antioxidant activity of carotenoids (including  $\beta$ -carotene) may exhibit adverse effects when present in high dose.

The present results have shown that Met.Hb% of sickle erythrocytes was significantly ( $p < 0.05$ ;  $p$  value = 0.489323) higher than normal physiologic concentration (Met.Hb% = 1.50) reported for HbAA erythrocyte genotype [15, 65]. Noteworthy, the level of erythrocyte methaemoglobin reported here is comparable to those presented by other authors [15, 21, 66]. 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 [66]. The moderate reduction in Met.Hb% in sickle erythrocytes 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<sub>Boston</sub>, HbM<sub>Iwate</sub>, HbM<sub>Hydepark</sub> and HbM<sub>Hammersmith</sub> with

sickle erythrocytes must have contributed to the poor and insignificant capacities of the three plant extracts to reduce the levels of erythrocyte Met.Hb%. These variant haemoglobin molecules are noted for tendency towards spontaneous oxidation *in vivo* and resistance to enzymatic and non-enzymic reduction mechanisms [44].

## CONCLUSION

Based on the levels of oxidative stress indicators considered in the present study, aqueous extracts of *A. occidentale*, *P. guajava* and *T. catappa* exhibited variable capacities to hinder lipid peroxidation, but they did not cause corresponding reduction in erythrocyte Met.Hb%, as shown 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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