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Oxidative Stress Indicators of Human Sickle Erythrocytes Incubated in Aqueous Extracts of Three Medicinal Plants

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ABSTRACT

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guajava* and *Terminalia catappa* for 12 h. At regular time intervals of 3 h, portions of the incubation mixtures were withdrawn and spectrophotometric method was used to assay for levels of erythrocyte Malondialdehyde (MDA) and methaemoglobin (Met.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 ± 0.35 to 3.13 ± 0.59 mmol mL⁻¹ ($p > 0.05$; 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 ± 0.33 mmol mL⁻¹; $t = 12$ h). Erythrocyte Met. Hb% increased from 2.42 ± 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 ± 0.49 to $2.29 \pm 0.45\%$; $p > 0.05$; $p = 0.983519$. 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%, 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*.

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

INTRODUCTION

Oxidative stress is caused by accumulation of Reactive Oxygen Species (ROS) (Shyur *et al.*, 2005; Richards *et al.*, 2007) produced as normal by-products of cellular metabolism (Richards *et al.*, 2007; Breusegem and Mittler, 2008), exposure to ionic/electromagnetic radiations and some environmental pollutants (Tiwari, 2001; Aqil *et al.*, 2006; Doss *et al.*, 2009). These reactive species are capable of damaging diverse biomolecules and cell structures in which lipids are probably the most susceptible (Saengkhae *et al.*, 2007; Okwu, 2007; Geetha *et al.*, 2007) when cellular levels are not controlled by appropriate antioxidant scavenging systems (Neupane *et al.*, 2008). Whereas erythrocyte of all genotypes are particularly sensitive to oxidative stress, when compared with normal erythrocytes, sickle erythrocytes spontaneously generate approximately twice as much superoxide (O_2^-), peroxide (H_2O_2) and hydroxyl (OH) radicals (Hebbel *et al.*, 1982) and increasing evidence suggest that lipid peroxidation may be an important factor in sickle cell anaemia (Tamer *et al.*, 2000).

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 (Aslan *et al.*, 2000). Furthermore, accumulation of hydrogen peroxide (H₂O₂) decreases the half life of erythrocytes by increasing oxidation of polyunsaturated fatty acids of membrane constituents (Nijs and De Meirleir, 2004) and can oxidize haemoglobin to methaemoglobin (Neupane *et al.*, 2008). Methaemoglobin does not bind reversibly with oxygen. One of the toxic end products of lipid peroxidation is Malondialdehyde (MDA) (Tamer *et al.*, 2000). Sickle erythrocytes contain increased amount of MDA and evidence of abnormal amino group cross-linking by MDA has been demonstrated in lipid extract of sickle erythrocyte membrane preparations (Dalle-Donne *et al.*, 2006).

Erythrocytes like other biological cells are supplied with diverse protective antioxidant mechanisms in order to counteract the toxic effects of ROS (Champe *et al.*, 2005; Forchetti *et al.*, 2006; Chikezie, 2011a, b). Antioxidants function as modulators of cellular homeostasis including detoxification of oxyradicals and metals as well as potent free radical scavenger. Erythrocytes reduced Glutathione (GSH) is one of the major non-enzymic endogenous antioxidants, protecting tissue against ROS (Dalle-Donne *et al.*, 2006). Other antioxidants are α -tocopherol (Muller, 2004; Zabri *et al.*, 2008), uric acid (Glantzounis *et al.*, 2005; Sautin and Johnson, 2008; Bowman *et al.*, 2010), ascorbic acid (Sati *et al.*, 2010), β -carotenoids (Lee *et al.*, 2000) and varieties of plant secondary metabolites such as flavonoid and related polyphenolic compounds (Middleton *et al.*, 2000; Rice-Evans, 2001; Srivastava *et al.*, 2011). Notable erythrocyte enzymatic ROS scavenging systems include glutathione reductase (Forchetti *et al.*, 2006), glutathione peroxidase (Manfredini *et al.*, 2008) and glucose-6-phosphate dehydrogenase (Champe *et al.*, 2005; Ojo *et al.*, 2006). Others are superoxide dismutase (Ekor *et al.*, 2006; Saengkhae *et al.*, 2007; Ojha *et al.*, 2010), catalase (Goth, 1991; Pennings *et al.*, 1999; Chandrasena *et al.*, 2006; Shanmugarajan and Devaki, 2008), peroxiredoxins (Neumann *et al.*, 2003; Low *et al.*, 2007) and NADH-methaemoglobin reductase (Mallory, 2003; Chikezie, 2011b).

Varieties of xenobiotics of plant origin such as fava beans extract (*Fava fava*) have been reported as agents that can interfere with the redox status of human erythrocytes especially in individuals with impaired glucose-6-phosphate dehydrogenase activity (Champe *et al.*, 2005; Ojo *et al.*, 2006). In the same vein, scavenging activities for free radicals by natural products of plant origin have been widely reported Shyur *et al.* (2005), Aqil *et al.* (2006), Buricova and Reblova (2008), Muanda *et al.* (2009), Veeru *et al.* (2009) and Sati *et al.* (2010). This study ascertained erythrocyte level of oxidative stress by estimating the concentrations of 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 leaves samples of *A. occidentale*, *P. guajava* and *T. catappa* were harvested between July and August, 2010, from trees growing 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. 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°C. 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×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% 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 year 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 method as described by Tsakiris *et al.* (2005). To remove platelets and leucocytes, the sediment was re-suspended in 3.0 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 Galbraith and Watts (1980) and 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 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 MDA was by 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×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 (Schemuk *et al.*, 2002). Determination of methaemoglobin content of erythrocyte haemolysate was by modification of the method of Evelyn and Malloy (1938), as described by Akomopong *et al.* (2000).

Statistical analysis: The results were expressed in terms of arithmetic Mean±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 (1966). Values of $p < 0.05$ were considered statistically significant.

RESULTS

The control analysis showed that within the limit of experimental time 0-12 h, erythrocyte MDA concentration increased from 2.45 ± 0.35 to 3.13 ± 0.59 mmol mL^{-1} (Fig. 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

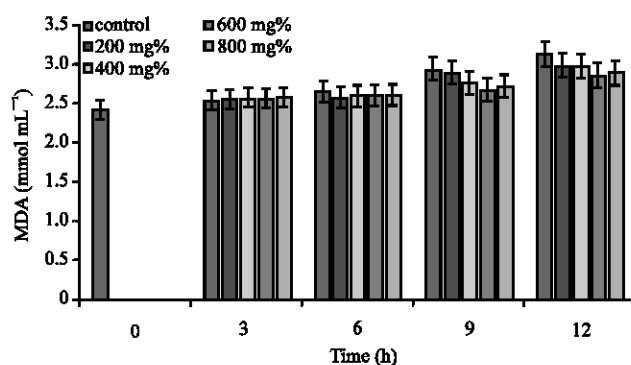


Fig. 1: MDA concentrations of sickle erythrocytes incubated in aqueous extract of *A. occidentale*

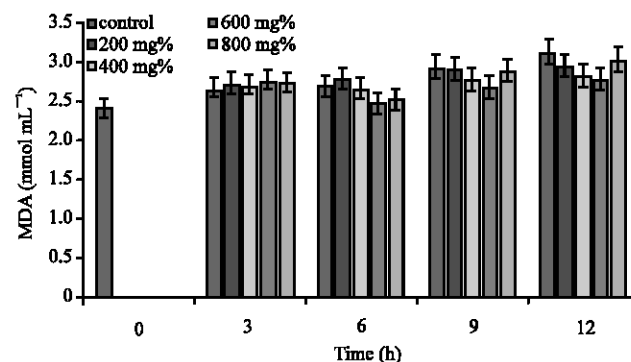


Fig. 2: MDA concentrations of sickle erythrocytes incubated in aqueous extract of *P. guajava*

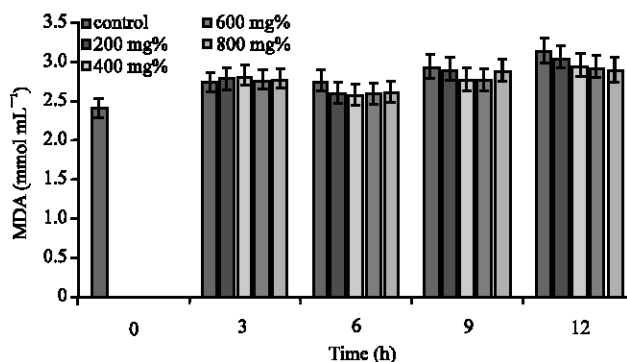


Fig. 3: MDA concentrations of sickle erythrocytes incubated in aqueous extract of *T. catappa*

2.92 ± 0.25 mmol mL⁻¹ (control = 3.13 ± 0.59 mmol mL⁻¹; $p > 0.05$; p value = 0.9776), representing a decrease of 6.71% of MDA concentration within the period of incubation (Fig. 1).

Whereas in the control sample, peak concentration of erythrocyte MDA concentration was 3.13 ± 0.59 mmol mL⁻¹ ($t = 12$ 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 ± 0.38 mmol mL⁻¹; [*T. catappa*] = 200 mg%, [MDA] = 3.04 ± 0.23 mmol mL⁻¹) (Fig. 2).

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⁻¹), representing 7.66% reduction in erythrocyte MDA concentration (Fig. 3). Notably, Fig. 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. 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 between *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; ($r = 0.271896$).

Erythrocyte Met. Hb% increased from 2.42 ± 0.55 to 2.51 ± 0.49 in the control sample within the duration of 12 h (Fig. 4-6). Although at $t = 3$ h, aqueous extract of *A. occidentale* (except 800 mg%) caused increased Met. Hb%, the values were not significantly different ($p > 0.05$) from the control samples. Further increases in incubation time ($t > 3$) engendered comparatively 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 ± 0.49 to 2.34 ± 0.65 within 6 h of incubation, the value increased to 2.55 ± 0.43 at $t = 12$ h (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 ± 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 (Fig. 5). Also, in the presence of aqueous extract of [*P. guajava*] = 200 mg%, value of erythrocyte Met. Hb%, 2.46 ± 0.43 %; at $t = 9$ h, was not significantly different ($p > 0.05$; $p = 0.999778$) from the control sample.

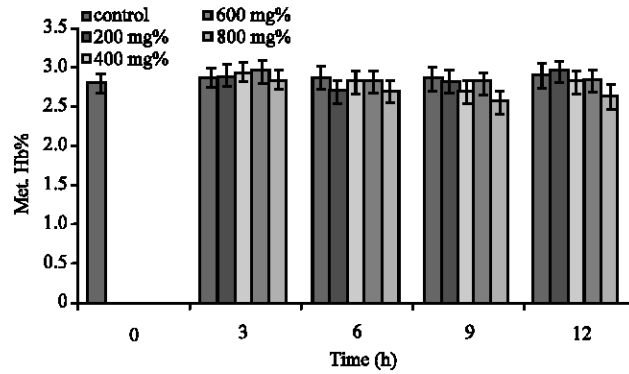


Fig. 4: Met. Hb% of sickle erythrocytes incubated in aqueous extract of *A. occidentale*

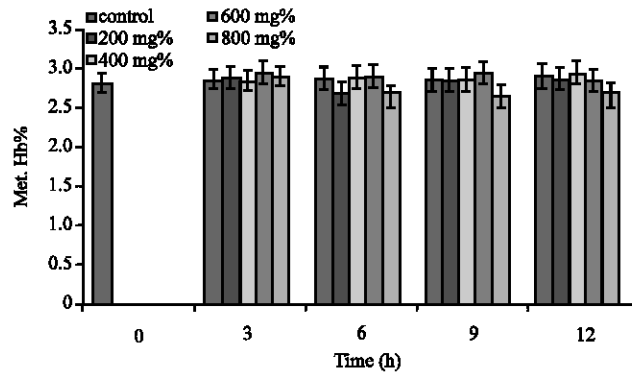


Fig. 5: Met. Hb% of sickle erythrocytes incubated in aqueous extract of *P. guajava*

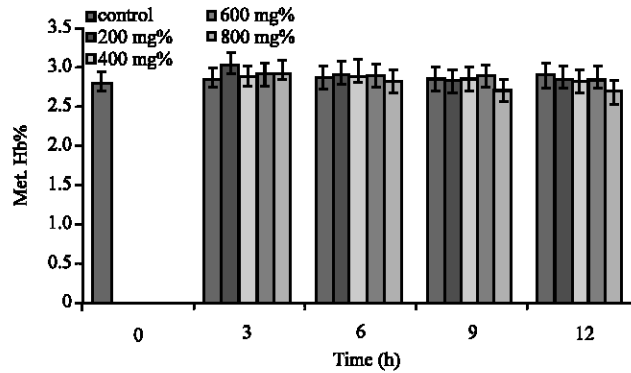


Fig. 6: Met. Hb% of sickle erythrocytes incubated in aqueous extract of *T. catappa*

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%. Statistical evaluation shows 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%.

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

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 extracts of *T. catappa* and concentrations of *A. occidentale* and *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. Tamer *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 the relatively high level of oxidative stressors in this erythrocyte genotype (Manfredini *et al.*, 2008; Repka and Hebbel, 1991; Dhalla *et al.*, 2000). From the results showed in Fig. 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. However, 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 γ -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* (L.) and *Rheum rhabarbarum* (L.) extracts to reduce lipid peroxidation to the presence of phenolic compounds in these plants (Aaviksaar *et al.*, 2003; Lam *et al.*, 2007). Also, membrane protective activities of diverse plant extracts have been attributed to their antioxidant content and capacity to impede membrane lipid peroxidation (Hu and Kitts, 2000; Okpuzor *et al.*, 2008; Buricova and Reblova, 2008; Mudgal *et al.*, 2010). However, since 800 mg% extract concentration of *A. occidentale* and *P. guajava* did not cause the anticipated level of hinderance to lipid peroxidation ($r = 0.845082$), 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 β -carotene) may exhibit adverse effects when present in high dose.

The present result showed that Met. Hb% of sickle erythrocyte was significantly ($p < 0.05$; $p = 0.0489323$) higher than normal physiologic concentration (Met. Hb% = 1.50) as reported by Tietz (1976) and Chikezie (2009) for HbAA erythrocyte genotype. Noteworthy, the level of erythrocyte methaemoglobin reported here is comparable to those presented elsewhere (Van Kuijk *et al.*, 1987; Chikezie, 2009; Chikezie, 2011b). 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 Kuijk *et al.*, 1987). The moderate reduction in Met. Hb% in erythrocyte incubated in aqueous extracts of *A. occidentale*, *P. guajava* and *T. catappa* is attributed to their antioxidant activity as earlier discussed. However, previous reports stated that 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 Met. Hb% (Mayes, 1983). These variant haemoglobin molecules are noted for tendency towards spontaneous oxidation *in vivo* and resistant to enzymic and non-enzymic reduction mechanisms.

CONCLUSION

Based on the levels of oxidative stress indicators 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 Met. Hb%, 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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