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Research Article

Erythrocyte Membrane Stabilisation, Protease Activities And Antioxidant Properties of The Stem Bark Extract of *Alstonia boonei* (DC)

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Abstract
There is an increasing search for natural antioxidants because of the growing concern about the safety of the synthetic ones and their potency in stabilising biological membranes. Because of this, we investigated the membrane stabilizing effects, proteinase activities and antioxidant properties of extract and fractions of *Alstonia boonei*. The *in vitro* and *in vivo* antioxidant properties of *Alstonia boonei*, erythrocyte membrane stabilising and inhibitory effects of the extract and fractions of the plant on proteinase activity were assessed spectrophotometrically. Assessment of the antioxidant properties of extract and fractions of *A. boonei* showed that the plant exhibited significant antioxidant activities both *in vitro* and *in vivo*. In the *in vitro* antioxidant assessment of the extract and fractions of *Alstonia boonei*, the MF had the highest significant effects on nitric oxide inhibition (95.90±9.18), reducing power (1.311±0.054), and 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (90.00±0.010). The CF had the highest effect on percentage hydroxyl radical scavenging activity (79.72±6.84), and inhibition of lipid peroxidation (96.70±12.44). *In vivo*, extract and fractions of *Alstonia boonei* significantly altered the antioxidant status of the treated animals relative to control. The *n*-hexane fraction stabilized the red cell membrane in a concentration dependent manner and also significantly inhibited proteinase activity *in vitro*. These preventive effects may be as a result of the antioxidant properties of the extract and fractions of *Alstonia boonei* and the various fractions may find use either as natural antioxidants or as inhibitors of protease enzymes

Key Words: *Daniellia oliveri*, Cytochrome c, Mitochondria, Apoptosis.

INTRODUCTION

Herbal products encompass a variety of preparations of plant origin generally categorized as food, dietary supplements, cosmetics, and herbal medicinal products that are used in traditional medicine for the treatment and/or prevention of diseases (Kosalec, *et al*., 2009). *Alstonia boonei* is a common medicinal plant generally found in the sub-Saharan Africa and it is used for the treatment of ailments such as malaria and rheumatic pains. The folkloric use of the stem bark of *A. boonei* for analgesic, antipyretic and anti-inflammatory purposes is well documented (Abbiw, 1990). In recent times, the use of herbal products has increased in developing countries, due to the fact that orthodox medicines are not cost effective or not easily available.

The stability of red blood cells is the ability of erythrocytes to resist osmotic lysis against many harmful agents including chemotherapeutic drugs and lethal conditions including excessive temperature (Paraiso *et al.*, 2017). The structure of the membranes of red blood cells is important in order to maintain its normal function, stability and avoid cell death. It is evident that, like nucleated cells, anucleated erythrocytes undergo programmed cell death either to clear aged red blood cells, as a result of infection (such as malaria) or in the cause of therapy by drugs. Anemia in malaria as a result of excessive erythrocytic cell death is a major cause of maternal and child mortality in malarial-endemic regions in Africa (Totino *et al.*, 2016).

The modulatory effects of various antimalarials on hemolysis is debatable; while quinine, an antimalarial drug, precipitates acute hemolysis, its derivatives (quinaldine and quinoline) are inactive both *in vitro* and *in vivo* (Laser *et al.*, 1975). Although, sulfadoxine-pyrimethamine is a potent prophylactic antimalaria, its usage causes anemia which is detrimental to the mother and the foetus (Mockenhaupt *et al.*, 2003). Plant and plant products have been used in the treatment and prevention of diseases. In addition to this, plant extracts have been found to prevent hypotonic solution and heat-induced hemolysis (Hossain *et al.*, 2014; Anosike *et al.*, 2012).

It is believed that retaining the membrane integrity is a critical factor in the treatment and prevention of hemolysis in

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malarial treatment. Again, membrane stabilizing activity of plant extract may not be unconnected with their antioxidant activities. It is in this regard that we investigated the *in vitro* and *in vivo* antioxidant properties of various fractions of *Alstonia boonei* in addition to its membrane stabilizing activities.

MATERIALS AND METHODS

Collection of plant materials, preparation of extracts and fractions: The stem bark peels of *Alstonia boonei* were obtained at Ajibode Road, opposite University of Ibadan Second Gate. These peels were air-dried for two weeks, blended and soaked in sufficient absolute methanol for seventy two hours after which the concentrate were filtered and concentrated under reduced pressure using rotary evaporator at 40^{o}C to obtain dark brown concentrate. This was evaporated to dryness in a water bath at 40^{o}C to obtain the crude methanol extract. Part of this methanol extract (40 g) was partitioned successively between n-hexane, chloroform, ethylacetate and methanol to obtain their respective fractions. These were concentrated and kept in the refrigerator until used.

Drug for membrane stabilisation assay: Antimalarial drugs (Artesunate and Chloroquine) were obtained from a registered Chemist at Ibadan, Oyo State and acetylsalicylic acid was obtained from SCP, Needham Market, Suffolk, England. These were dissolved in appropriate solvent system and graded concentrations were used.

Preparation of erythrocyte suspension: Fresh bovine blood (25 mL) collected in EDTA bottle was centrifuged at 3,000 rpm for 15 min. This was washed repeatedly with isosaline until the supernatant was clear. Thereafter, a 2% erythrocyte suspension was prepared by adding 49 mL of isosaline to 1 mL of packed red blood cells. The reconstituted red blood cells were used for membrane stabilization assay.

Procedure for in vitro assay

Nitric oxide radical scavenging activity: This was carried out according to the method of Garrat, 1964. Sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore which was formed in the reaction was measured at 546 nm.

Lipid peroxidation: Post mitochondrial fraction of rat liver homogenate (0.5 mL) was added to 0.1 mL of the extract (10 µg/mL). The volume was then made up to 1.0 mL with distilled water. Thereafter, 0.05 mL of 60 µM $FeSO_4$ was added and the mixture was incubated at 37^{o}C for 30 min. Then, 1.5 mL of acetic acid was added, followed by 1.5 mL of TBA in SDS. The resulting mixture was vortex mixed and heated at 95^{o}C for 1 hour. After cooling, 5 mL of butan-1-ol was added and the mixture was centrifuged at 3000 rpm for 10 min (Ruberto *et al.,* 2000). The absorbance of the organic upper layer was measured at 532 nm and the percentage inhibition was calculated with the formula:

$$Percentage\ inhibition\ of\ lipid\ peroxidation = Ao - A1/Ao\ x\ 100$$

Where: Ao is the absorbance of the control and A1 is the absorbance of the sample

Hydroxy radical scavenging activity: All solutions were freshly prepared before the assay. One (1) mL of the reaction mixture contained 100 µL of 2.8 mM 2-deoxyribose (dissolved in phosphate buffer (10 mM), pH 7.4), 500 µL solution of various concentrations of the extract (10 to 40 µg/mL), 200 µL of 200 µM $FeCl_3$ and 1.04 µM EDTA (1:1 v/v), 100 µL of H_2O_2 (1.0 mM) and 100 µL of ascorbic acid (1.0 mM). After incubation period of 1 hour at 37^{o}C, the extent of deoxyribose degradation was measured by TBA reaction (Halliwell *et al.,* 1987). The % inhibition of hydroxyl radical was calculated as

$$Percentage\ inhibition\ of\ hydroxy\ radical = Ao - A1/Ao\ x\ 100$$

Where Ao= Absorbance of control, A1= Absorbance of sample

Total flavonoid content: The total flavonoid content was measured according to the method of Marinova *et al.,* 2005. An aliquot (1 mL) of extracts or a standard solution of quercetin (20, 40, 60, 80 and 100 mg/L) was added to a 10 mL volumetric flask, containing 4 mL of distilled deionized water (dd H_2O). To the flask was added 0.3 ml 5 % $NaNO_2$. After 5 min, 0.3 ml 10 % $AlCl_3$ was added. At the sixth minute, 2 mL 1 M NaOH was added and the total volume was made up to 10 ml with dd H_2O. The solution was mixed well and the absorbance was measured against a prepared reagent blank at 510 nm. The data of the total flavonoid contents were expressed as milligrams of quercetin equivalents.

The reducing power: This was measured as follows: Various concentrations of the plant extracts in 1.0 mL of deionized water were mixed with phosphate buffer (2.5 mL) and potassium ferricyanide (2.5 mL) and incubated at 50^{o}C for 20 min. Aliquots of trichloroacetic acid (2.5 mL) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared ferric chloride solution (0.5 mL). The absorbance was measured at 700 nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power (Oyaizu, 1986).

The DPPH radical scavenging activity: The Diphenyl picryl hydrazyl radical scavenging activity of the extracts and fractions of the stem bark of *Alstonia boonei* was assessed using stable 2,2-diphenyl-1-picrylhydrazyl(DPPH) radical (Yokozawa, *et al*., 1998).Test samples were dissolved in MeOH and mixed with ethanol solutions of DPPH (0.15%) in test tubes, following incubation at 37^{o}C for 30 min. DPPH reduction was estimated at 517nm. Final concentrations of test materials were typically in a range from 250 to 2000 µg/ml. Percentage inhibition by the sample treatment was determined by comparison with a MeOH-treated control group. All experiments were carried out in triplicates.

The free radical scavenging activity was analyzed using linear regression analysis followed by the percentage inhibition

calculated by using the formula, inhibition (%)=[1−(A−B)/C]×100,
Where: A is the absorbance of 1.5 mL of the crude extract/control solution mixed with 2 mL of the DPPH solution; B the absorbance of 2 mL of the crude extract solution mixed with 1.5 mL of methanol and C is the absorbance of a blank prepared by mixing 2 ml of the DPPH with 1.5 mL of methanol.

Total phenol content: One mL each of the test solution was added to 0.2 ml each of Folin-Ciocalteu reagent and 2 ml of distilled water. One mL of 15% Na_2CO_3 was mixed with the solution. The solutions were incubated at 40^0C for 30 min and the absorbance was read at 760 nm. Total phenol content was expressed as µg/mg of gallic acid equivalent (GAE) (Slinkard and Singleton, 1977).

Procedure for *in vivo* assay

Animal treatment and sample collection:

The rats were treated and handled according to the ethics of animal handling for experimental purpose in accordance with the institution's guideline and criteria for animal care. Seventy-five male albino rats were used for this assay. The animals were obtained from the Preclinical Animal House in Physiology Department, University of Ibadan. They were housed in the Department of Biochemistry, University of Ibadan Animal House and were acclimatised for two weeks. After acclimatisation, the animals were grouped into five groups of fifteen animals each. Each group were subdivided into three groups of five animals each and were treated with 50, 100 and 200 mg/kg body weight of the methanol extract (ME), n-hexane (HF), chloroform (CF), ethylacetate (EF) and methanol fractions (MF) of *Alstonia boonei*. Five animals were treated with the vehicle only. All the animals were treated for twenty-eight days after which they were sacrificed. Blood was collected from the heart and was put in plain sample bottles. These were spanned in a table top centrifuge and the serum was separated into Eppendorf tubes using Pasteur pipette. The serum was then kept in the refrigerator until used.

Determination of protein:

Protein was determined according to the method of Lowry *et al.*, 1951. Specific volume (3 mL) of alkaline copper solution (a mixture of 2% Na_2CO_3 in 0.1M NaOH, 2% Na-K-Tartrate, and 1% $CuSO_4.5H_2O$ in ratio 100:1:1 respectively) was added to 10 µL of serum sample, mixed and allowed to stand at room temperature for 10 minutes. A 1:1 ratio dilution of Folin reagent (0.3 mL) was then added to the mixture and then shaken to ensure a thorough mixing. The mixture was left for another 30 minutes for colour development after which the absorbance was read at 750nm using a spectrophotometer. A reagent blank was used to standardise the spectrophotometer.

Determination of catalase activity:

Catalase activity was determined according to the method of Claiborne (1985). Hydrogen peroxide (2.95 ml of 19 mM solution) was pipetted into a 1 cm quartz cuvette and 50 µl of sample added. The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm every minute for 5 min.

Catalase activity

$= \Delta A_{240}/\text{min} \times \text{reaction volume} \times \text{dilution f actor}$

$0.0436 \times \text{sample volume} \times \text{mg protein/ml}$

$= \mu\text{mole } H_2O_2/\text{min/mg protein}$

Assessment of lipid peroxidation:

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the test sample according to the method of Varshney and Kale (1990). An aliquot of 0.4 mL of the test sample was mixed with 1.6 mL of Tris-KCl buffer to which 0.5 mL of 30% TCA was added. Then 0.5 ml of 0.75% TBA was added and placed in a water bath for 45 minutes at 80^0C. This was then cooled in ice to room temperature and centrifuged at 3000 rpm for 10 min. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. The MDA level was calculated using an extinction coefficient of 0.156 $\mu M^{-1}cm^{-1}$ (Adam-Vizi and Seregi, 1982).

Lipid peroxidation (nmole MDA/mg protein)

= Absorbance × volume of mixture

$E_{532nm} \times$ volume of sample × mg protein/ml

Estimation of reduced glutathione (GSH) level:

The method of Beutler *et al.*, (1963) was followed in estimating the level of reduced glutathione (GSH). 0.4 mL of sample was added to 0.4 mL of precipitating solution which was vortexed and centrifuged at 4000 rpm for 5 minutes. Thereafter, 0.5 mL of the supernatant was added to 1.5 mL of Ellman's reagent. The absorbance of the reaction mixture was read at 412 nm against a reagent blank.

Determination of superoxide dismutase (SOD) activity:

The activity of SOD was determined by the method of Misra and Fridovich (1972). A 50 µL of sample was added to 2.5 mL of 0.05M carbonate buffer (pH 10.2) and 0.3 mL of epinephrine in a cuvette, mixed by inversion and change in absorbance monitored every 30 sec for 2.5 min at 480 nm. The reference cuvette was the same as for the samples with water replacing the samples.

Percentage inhibition =

$$100 - \frac{(100 \times \text{Increase in absorbance per min for sample})}{\text{Increase in absorbance per min for blank}}$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the autooxidation of epinephrine.

Estimation of glutathione S-transferase activity:

Glutathione S-transferase activity was determined according to Habig *et al.*, (1974). Sample test tubes contained 150 µL of 20 mM 1-Chloro-2,4-dinitrobenzene (CDNB), 30 µL of 0.1 M reduced glutathione (GSH), 2.79 ml of 0.1 M phosphate buffer (pH 6.5) and 30 µL of serum sample. The blank test tube contained no sample, 2.89 µL of phosphate buffer and equal volume of CDNB and GSH. The reaction was allowed to run for 3 min with readings taken every 60 seconds against the blank at 340 nm.

GSH S-transferase activity

$$= \frac{\Delta A_{340}/\text{min} \times \text{reaction volume} \times \text{dilution factor}}{9.6 \times \text{sample volume} \times \text{mg protein/L}}$$

= µmole/min/mg protein

Assay for glutathione peroxidase activity:

Glutathione peroxidase (GPX) activity was measured according to the procedure of Rotruck *et al*., (1973) with some modifications. To 0.5 mL of phosphate buffer in a test tube was added 0.1 mL of NaN_3, 0.2 mL of GSH, 0.1 mL of H_2O_2

and 0.5 mL of sample (added last). The reaction mixture was incubated for 3 min at 37˚C after which 0.5 ml of TCA was added and the final mixture centrifuged at 3000 rpm for 5 min. To 1 mL of the supernatants, 2 mL of K_2HPO_4 and 1 mL of DTNB were added and the absorbance read against a reagent blank of 1 mL distilled water, 2 mL of K_2HPO_4 and 1 mL of DTNB at 412 nm. GSH consumed = initial GSH amount (129.39 µg) – GSH remaining (µg/mL × 4 mL)
GPX activity = GSH consumed/mg protein
= µg GSH/mg protein

Assessment of xanthine oxidase activity:
The activity of xanthine oxidase was assessed by the method of Bergmeyer *et al.,* (1974). Into cuvettes, 1.9 mL of phosphate buffer, 1.0 mL of xanthine solution and 0.1 mL of sample were pipetted. The mixture was quickly inverted to mix and absorbance monitored every minute for 3 minutes at 290 nm. A blank was made by replacing 0.1 mL of sample with distilled water. Xanthine oxidase activity was calculated as follows:
Xanthine oxidase activity = ΔA_{290}/min × reaction volume × dilution factor 12.1 × sample volume × mg protein/mL
= µmole/min/mg protein
The extinction coefficient of uric acid at 290 nm = 12.1 $mM^{-1}cm^{-1}$

Membrane Stabilization Assay:
The effects of extract and fractions of *Alstonia boonei* on red blood cell hemolysis induced by heat and hypotonic solution was evaluated using the method of Shinde *et al.,* (1989).

Heat induced hemolysis:
Graded concentrations of the extract (0.5-2 mg/mL) were dissolved in 20 percent DMSO. Suspended red blood cells (0.1mL) were added into test tubes containing the extract and fractions of *Alstonia boonei* and the volumes were made up in duplicates accordingly. These were incubated at 56ºC for 30 min in a water bath. The tubes were thereafter cooled to room temperature and then centrifuged at 3, 000 rpm for 5 min. The absorbance of the supernatant was read at 560 nm in a spectrophotometer. The vehicle (20% v/v DMSO) and acetylsalicylic were similarly treated and used as negative and positive controls respectively.
Percentage membrane stability was expressed as
Percentage membrane stability
$$= \frac{\{(\text{Ab. of drug test value} - \text{Ab. of drug control value}) \times 100\}}{\text{Ab. of Control value}}$$

Where Ab. is the absorbance read at 560 nm in each case.

Hypotonic solution-induced hemolysis:
Graded concentrations (0.5-2 mg/mL) of the extract and fractions of *Alstonia boonei* used in this assay were treated as stated previously. The entire assay volume (5 mL) contained the extract and fractions of *Alstonia boonei* and 0.1 mL of suspended red blood cells. The reaction mixture was incubated at 37ºC for 1 hr and afterwards centrifuged at 3, 000 rpm for 15 min and the absorbance of the supernatant was read at 560 nm in a spectrophotometer against negative control (20% v/v DMSO) and positive control (acetylsalicylic acid). In each case, (heat-induced and hypotonic solution-induced hemolysis), parallel test tubes, in duplicates were similarly treated for the drug control values. Here, the test tubes contained all the reagents except the red blood cells suspension. Hemolysis produced in the presence of distilled water was taken as 100% destabilisation (i.e., zero stabilisation of red blood cells). The percentage membrane stabilisation was calculated as follows:
Percentage membrane stability
$$= \frac{\{(\text{Ab. Of drug test value} - \text{Ab. of drug control value}) \times 100\}}{\text{Ab. of Control value}}$$
Where Ab. Is the absorbance read at 560 nm in each case.

Inhibitory effects of extract and fractions of *Alstonia boonei* on proteinase activity:
The proteinase inhibition assay was carried out according to the methods of Govindappa *et al.,* (2011) and Sakat *et al.*,(2010) with little modifications. Briefly, each assay test tube contained 0.06mg trypsin, 20 mM Tris HCl (pH 7.4) and graded concentrations of extract and fractions of *Alstonia boonei* in an assay volume of 2mL. Thereafter, the reaction mixture was incubated at 37°C for 5 min after which 1mL of 0.8% casein was added. The reaction was inhibited for additional 20 min after which 2mL perchloric acid (70%) was added to stop the reaction. The final volume was centrifuged at 5, 000 rpm for 15 min and the absorbance of the supernatant was read at 210 nm using a spectrophotometer. The Tris buffer was used as blank. The extent of protease inhibition was calculated as follows:

Percentage Protection
$$= 100 - \frac{(\text{Absorbance of treated sample})}{\text{Absorbance of control}) \times 100}$$

Statistical analysis
Data were mean ± standard deviation of triplicate determinations and were analysed using two way ANOVA and Duncan's multiple range tests. Values along the columns with different superscripts are significantly different at 95 percent degree of freedom.

RESULTS

***In vitro* antioxidant activity:**
Table 1a showed the inhibitory effects of the extract and fractions of *Alstonia boonei* on the nitric oxide generation. The result obtained show that MF had the highest inhibitory effects at the highest concentration compared with other fractions and extract while *n*-hexane fraction had the least effect. Interestingly, the level of inhibitory effect increases with increasing polarity of the fractions and the effect of the methanol fraction of *Alstonia boonei* is significantly higher than the crude methanol extract. Similar result was obtained for the reducing power and DPPH radical scavenging activities (Tables 1b and 1c).

The percentage hydroxyl radical scavenging activities, *in vitro* lipid peroxidation, total flavonoid and phenolic contents of the extract and fractions of the stem bark of *Alstonia boonei* were represented in tables 1d through to 1g. The results showed that chloroform fraction had the highest activities in all except that ethylacetate fraction had the highest flavonoid content while *n*- hexane fraction still had the least activities in all the parameters assessed. The activities of both chloroform and ethylacetate fractions were significantly higher than the activities of the methanol fraction showing that fractionation

enhanced the activities of the two fractions. It was also observed that the antioxidant activities of these fractions are concentration dependent.

Table 1a:
Nitric oxide inhibition assay for various solvent fractions of the stem bark of *A. boonei*

Conc (µg/ml)	ME	HF	CF	EF	MF
100	63.70 ±4.62	21.30 ±1.69	52.40 ±3.07	61.14 ±3.8	84.10 ±6.31
200	73.20 ±4.74	29.10 ±1.80	65.20 ±4.16	73.20 ±4.00	86.23 ±7.12
300	74.16 ±4.83	34.40 ±1.92	65.90 ±4.43	74.41 ±4.26	92.20 ±7.88
400	75.12 ±4.92	44.30 ±2.16	67.80 ±4.52	76.80 ±5.12	95.90 ±9.18

Rutin: 20(µg/ml) =70.60±5.39, 40(µg/ml) =75.50±5.41, 80(µg/ml) = 78.90±5.46, 100(µg/ml) =80.50±5.57

Table 1b:
The reducing power of various fractions of the stem bark of *A.boonei*

Conc. (µg/ml)	ME	HF	CF	EF	MF
250	0.022_{d} ±0.001	0.046_{c} ±0.009	0.010_{e} ±0.004	0.090_{b} ±0.001	0.222_{a} ±0.003
500	0.039_{c} ±0.016	0.047_{c} ±0.006	0.036_{c} ±0.005	0.117_{b} ±0.026	0.321_{a} ±0.014
1000	0.059_{c} ±0.001	0.048_{c} ±0.002	0.089_{c} ±0.032	0.188_{b} ±0.022	0.517_{a} ±0.026
2000	0.077_{d} ±0.008	0.051_{d} ±0.003	0.152_{c} ±0.006	0.280_{b} ±0.002	1.311_{a} ±0.054

Ascorbic acid 20(µg/mL) = 0.193, 30 (µg/mL) = 0.220, 40 (µg/mL) = 0.476, 50 (µg/mL) = 0.693

Table 1c:
The percentage inhibition of DPPH radical by various fractions of the stem bark of *A.boonei*

Conc.(µg/ml)	ME	HF	CF	EF	MF
250	61.10_{c} ±0.025	36.80_{a} ±0.017	18.00_{a} ±0.014	72.00_{c} ±0.021	60.00_{a} ±0.01
500	64.00_{a} ±0.010	49.60_{a} ±0.010	47.00_{a} ±0.020	80.00_{c} ±0.125	65.00_{a} ±0.022
1000	67.30_{a} ±0.013	49.70_{a} ±0.005	56.00_{a} ±0.007	85.00_{c} ±0.012	90.00_{a} ±0.017
2000	87.30_{a} ±0.004	77.00_{ab} ±0.003	67.00_{ab} ±0.027	87.00_{bc} ±0.026	90.00_{c} ±0.010

Ascorbic acid: 10(µg/mL)=83.00±0.040; 20(µg/mL)=49.90±0.348; 40(µg/mL)=76.10±0.077; 80(µg/mL)= 84.50±0.030

Table 1d:
Percentage hydroxyl radical scavenging activities of the various solvent fraction of the stem bark of *A.boonei*

Conc.(µg/ml)	ME	HF	CF	EF	MF
10	75.66_{a} ±4.67	36.43_{b} ±4.81	75.50_{b} ±6.27	47.11_{c} ±2.39	71.19_{b} ±5.01
20	76.92_{a} ±5.18	45.66_{a} ±4.87	78.88_{a} ±6.76	49.84_{b} ±3.95	72.03_{b} ±4.98
30	77.20_{a} ±5.22	48.22_{a} ±4.92	79.30_{a} ±6.80	52.27_{a} ±4.00	75.66_{a} ±5.03
40	77.62_{a} ±5.43	56.36_{a} ±5.00	79.72_{a} ±6.84	53.49_{a} ±4.77	76.08_{a} ±5.01

Ascorbic acid: 1µg= 52.50±4.28; 2µg= 61.80±4.65; 3µg = 63.10±4.66; 4µg= 64.84±4.82

Table 1e:
Percentage inhibition of lipid peroxidation by various fraction of the stem bark of *A.boonei*

Conc. (µg/ml)	ME	HF	CF	EF	MF
5.0	73.70_{c} ±10.08	62.40_{c} ±9.17	75.20_{b} ±4.38	73.18_{c} ±9.93	49.37_{d} ±3.64
10.0	74.40_{c} ±5.32	66.96_{b} ±8.22	95.50_{a} ±11.26	83.70_{b} ±9.88	62.90_{c} ±7.43
15.0	83.90_{b} ±9.82	66.96_{b} ±7.32	96.49_{a} ±12.00	88.22_{a} ±8.43	72.93_{b} ±7.70
20.0	89.70_{a} ±9.80	79.84_{a} ±8.46	96.70_{a} ±12.44	90.72_{a} ±10.11	82.70_{a} ±9.70

Ascorbic Acid Conc: 5.0.(µg/ml)=43.48_{d}±1.23; 10.0.(µg/ml)= 54.14_{c}±2.88; 15.0.(µg/ml)= 74.93_{b}±5.63; 20.0.(µg/ml)=93.48_{a}±10.41

Table 1f: The total flavonoid content (µg/ml Quercetin equivalent) of the solvent fractions of the stem bark of *A. boonei*

Conc.(µg/ml)	ME	HF	CF	EF	MF
250	22.00_{cd} ±2.64	20.00_{cd} ±1.71	23.50_{b} ±1.46	36.00_{b} ±2.81	22.00_{bc} ±2.50
500	23.00_{bc} ±2.24	21.00_{bc} ±1.91	24.00_{b} ±2.62	44.00_{a} ±3.12	23.00_{bc} ±2.55
1000	23.00_{c} ±2.57	24.00_{cd} ±2.55	28.50_{b} ±2.87	53.50_{a} ±4.01	24.50_{c} ±2.61
2000	26.00_{cd} ±2.91	28.50_{cd} ±2.73	34.00_{b} ±2.75	60.00_{a} ±4.26	31.00_{c} ±2.64

Table 1g; Phenolic content (µg/ml) of the solvent fractions of the stem bark of *A.boonei*

Conc.(µg/ml)	ME	HF	CF	EF	MF
2000	742.03a ±8.06	50.20a ±2.13	1039.03 ±9.62	896.03 ±8.72	611.03 ±3.48

Gallic Acid Standard :250µg/ml=2114.70±11.21

Values are mean ± standard deviation of three determinations. Values with different superscript along the column are statistically significant (p<0.05). ME (Methanol Extract); HF (n-Hexane Fraction); CF (Chloroform Fraction); EF (Ethylacetate Fraction); MF (Methanol Fraction).

***In vivo* antioxidant assays:** The oral treatment of experimental animals with the graded doses of extract and fractions of *Alstonia boonei* caused a significant (p<0.001) increase in the concentration of reduced glutathione (GSH) dose dependently relative to control in ME, MF and HF groups. However, there was a hometic response in the CF group where the maximum response was significantly (p< 0.001) achieved at the 100 mg/kg dose. Interestingly, the response of the treated groups to the EF fraction showed that although there was a significant (p<0.001) increase in the GSH concentration relative to the control, there was an inverse response of the GSH concentration relative to the increasing dose (Figure 1a). Although, the level of malondialdehyde generated in all the treated groups was not significantly different from the control, the peroxidative products generated when 200 mg/kg dose was administered was higher than the control, buttressing the hometic response noticed in Figure 1b. Catalase activity increased dose dependently in the ME, MF and EF groups whereas it decreased dose dependently in the HF and CF groups. However in all the groups, the control had the highest catalase activity (Figure 2a). The superoxide dismutase activity increased significantly (p<0.001) in all the treated groups compared with the normal control (Figure 2b). Glutathione peroxidase (GPx) increased significantly (p<0.001) and dose dependently only in the EF groups but decreased dose dependently in the ME and HF groups. The

MF and CF significantly increased ($p<0.001$) maximally at the 100 mg/kg dose showing the hometic response as observed in Figure 3a. Glutathione S transferase (GST) activity increased significantly ($p<0.001$) at the highest dose in ME, MF and CF but maximally at 50mg/kg in the HF group (Figure 3b). There is no significant increase in GST activity at all doses in the EF groups relative to control.

While the superoxide dismutase activity did not vary linearly with the dose in the HF groups, there was significant ($p<0.001$) linear, dose dependent response in the activity of this enzyme relative to control while the catalase activity significantly ($p<0.001$) reduced compared with the control (Figure 2). Xanthine oxidase activity (Figure 4) increased significantly ($p<0.001$) in ME, MF, HF and CF while there was no significant increase in the EF groups at all doses

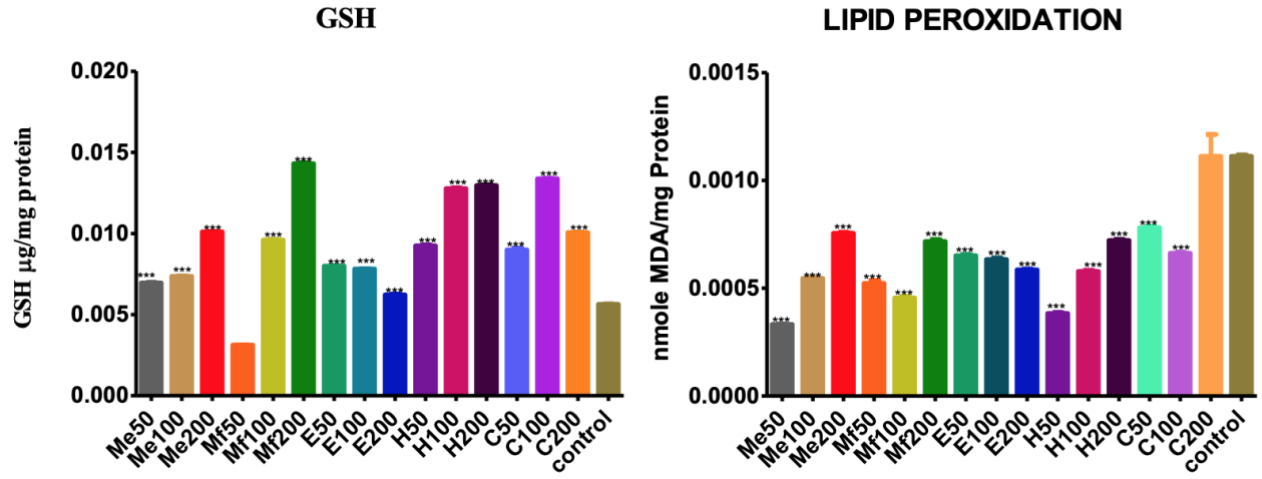

Figure 1:
Relative dose-dependent increase in reduced glutathione (Figure 1A) and evidence of decrease in malondaldehyde (Figure 1B)in all the groups treated with various fractions of *A. boonei* stem bark extract (***$p<0.001$ vs control in each case).

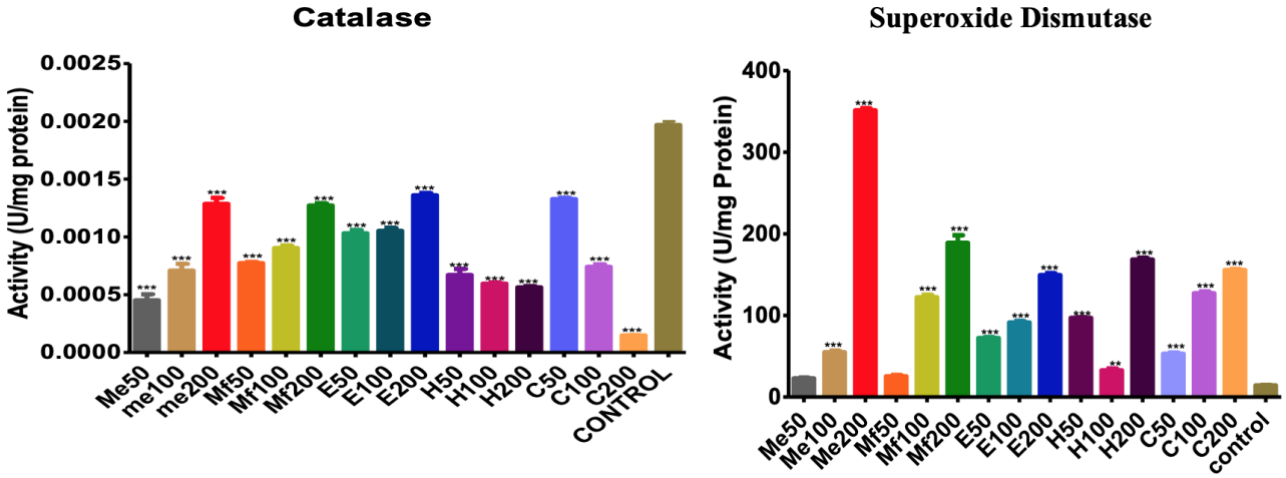

Figure 2:
Effects of oral administration of solvent extract and fractions of *Alstonia boonei* on the activities of catalase and superoxide dismutase. Graded doses of the extract and fractions of *Alstonia boonei* significantly ($p<0.001$) reduced catalase activity but at the same level of significance increased superoxide dismutase activity relative to control.

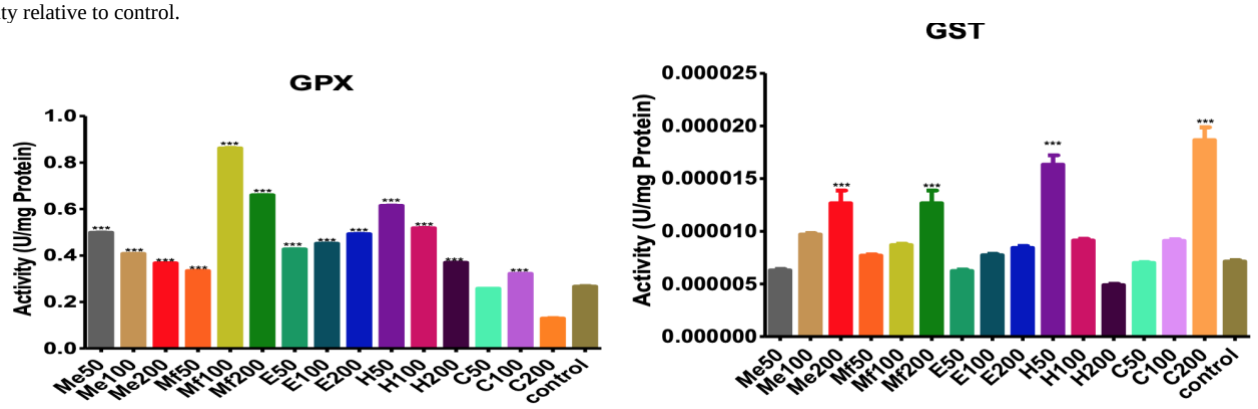

Figure 3:
Effects of oral administration of solvent extract and fractions of *Alstonia boonei* on the activities of glutathione peroxidase (GPx, A) and glutathione S transferase (GST, B). Graded doses of the extract and fractions of *Alstonia boonei* significantly ($p<0.001$) reduced GPx activity (except in the ethylacetate (EF) fraction) while there was increase in the activity of the GST at highest dose of the extract and fractions except in the n-hexane fraction that decreased as the dose increased.

Membrane stabilizing study

The membrane stabilizing effect of the solvent extract and fractions of the stem bark of *Alstonia boonei* is represented in Figure 5. The results obtained showed that at all concentrations, methanol extract, ethylacetate and methanol fractions of *Alstonia boonei* induced membrane damage in heat-induced model of membrane stabilisation.

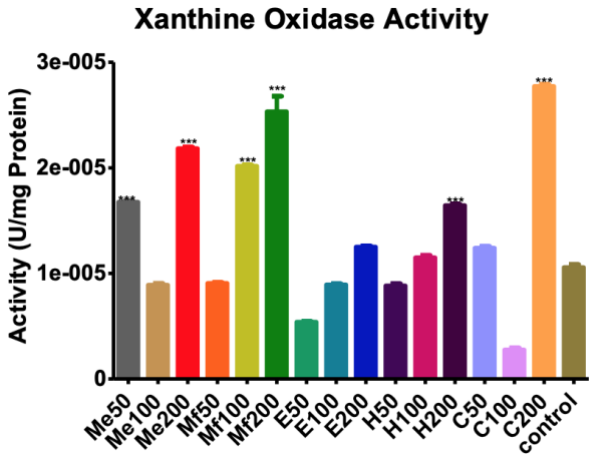

Figure 4:
Effects of oral administration of solvent extract and fractions of *Alstonia boonei* on the activities of Xanthine oxidase. The dosage groups especially of methanol extract, methanol, hexane and chloroform fractions increased significantly ($p<0.001$) higher than control.

Furthermore, chloroquine and artesunate significantly ($p<0.001$) induced membrane damage while *n*-hexane and chloroform fractions of *Alstonia boonei* significantly maintained erythrocyte membrane integrity and prevented the red blood cells from hemolysis. In the hypotonic solution-induced model, similar protective effects of the *n* -hexane and chloroform fractions were evident. Although, the artesunate, chloroquine, methanol extract ethylacetate and methanol fractions did not induce hemolysis in the hypotonic solution model of the red cell membrane stability, the stabilization of the red cell membranes by these extract and fractions of *Alstonia boonei* were significantly lower ($p<0.001$) when compared with acetylsalicylic acid

Proteinase inhibitory study

The use of proteinase inhibition as an index of inflammatory marker in this study have shown that the *n*-hexane and chloroform fractions of *Alstonia boonei* significantly ($p<0.001$) inhibited proteinase activity *in vitro*. Although, other fractions and methanol extract showed inhibition of proteinase activity, there was a concentration dependent decrease in proteinase inhibition as the concentration increased (Figure 6). Therefore, lower concentrations of the methanol extract and the ethylacetate and methanol fractions of *Alstonia boonei* possessed proteinase inhibitory effect indicating a note of caution in their indiscriminate use

DISCUSSION

Oxidative stress occurs as a result of imbalance in the levels of prooxidants and antioxidants. Detail studies of the mechanism of action of many medicinal plants have attributed their curative and preventive potentials to their antioxidant capacities. In this study, it has been observed that *Alstonia boonei* stem bark contains phytochemicals capable of mitigating the damaging effects of prooxidants in biological systems. *In vitro* antioxidant screenings of *Alstonia boonei* using nitric oxide scavenging capacity, ferric reducing power, DPPH and hydroxyl radical scavenging activities, lipid peroxidation total flavonoid and phenolic contents were employed in this study.

Nitric oxides are reactive free radicals produced by the body cells to give more reactive species such as peroxynitrite which are unstable and be decomposed to form hydroxyl radicals. At a concentration of 400 µg/ml, the methanol fraction of *Alstonia boonei* had a mitigating effect against sodium nitroprusside-induced nitric oxide generation that was not statistically significant from the effect of rutin at 100 µg/ml concentration. The ferric reducing power of the stem bark extract and fractions of *Alstonia boonei* was evaluated by measuring the transformation of the ferricyanide complex (Fe^{3+}) to the ferrous (Fe^{2+}) form. From our results, the ferric reducing power of the MF was significantly the highest.

The reducing ability of plant extracts and fractions of different solvent systems to transform Fe^{2+} to Fe^{3+} could be as a result of its antioxidant effect. This reaction is necessary and needful in biological systems because ferrous iron via Haber Weiss and Fenton reactions account for the generation of highly reactive HO in biological systems (Kehrer, 2000). The DPPH antioxidant assay is based on the capability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolourise in the presence of antioxidants (Amudha and Rani, 2016). The result obtained in this study showed that the MF and EF significantly inhibited the free radical generation from DPPH in a concentration dependent manner. The effect of antioxidants on DPPH radical could be as a result of their hydrogen donating ability (Chebouat *et al.*, 2011).

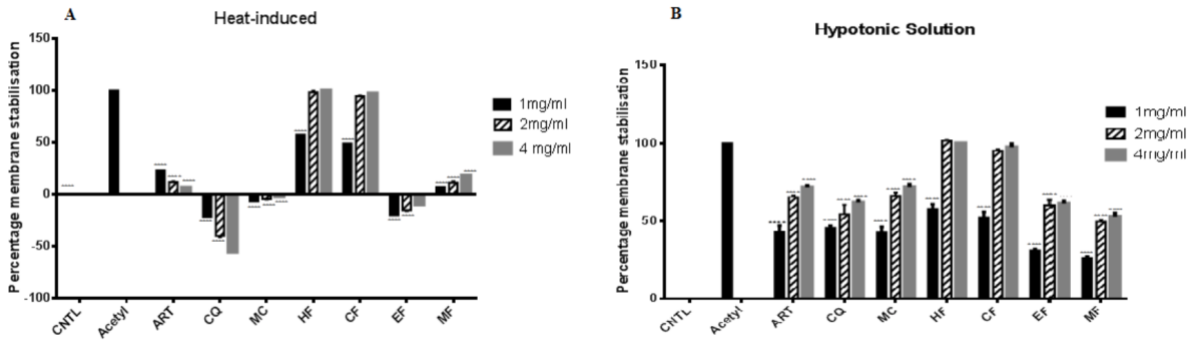

Figure 5:
Effects of extract and solvent fractions of the stem bark extract of Alstonia boonei on membrane stabilization. Abbreviations carry the usualnotation.

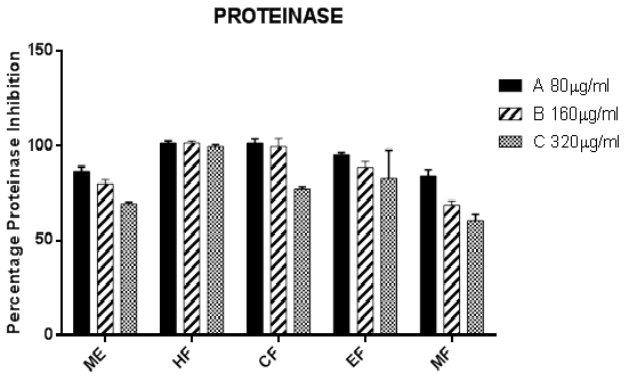

Figure 6:
Inhibitory effect of extract and solvent fractions of Alstonia boonei stem bark extract on proteinase. Abbreviations carry the usualnotation

The hydroxyl radical (HO) is the most reactive of the reactive oxygen species and it has the shortest half-life when compared with the other reactive oxygen species (Amudha and Rani, 2016). It is capable of causing oxidative damage to DNA, proteins and lipids. The CF had significant effect in scavenging hydroxyl radicals thus protecting membrane lipids, proteins and DNA from oxidative damage. Peroxidation of membrane lipids is one of the hallmarks of membrane compromise leading to cell death.

The CF and EF had the highest inhibitory effect on lipid peroxidation at the highest concentration used (20 µg/ml) thus they can be used to protect unsaturated fatty acids that are highly susceptible to peroxidation in cell membranes. Specifically, membranes are rich in unsaturated fatty acids such as linoleic and arachidonic acids that are highly susceptible to oxidative processes (Nebavi *et al.*, 2009).
Polyphenols exhibit antioxidant properties due to their redox properties by donating protons to highly reactive oxygen species (Zheng and Wang, 2001). The EF and the CF of *Alstonia boonei* had the highest concentrations of flavonoid and phenolic contents respectively. The redox reaction of polyphenols is important because of their ability to adsorb and neutralize free radicals, degrade peroxides to their harmless forms and also quench the activities of reactive oxygen species (Akinpelu *et al.*, 2010).

In this study, we observed significant increase in the level of reduced glutathione. It is well documented in literature that reduced glutathione is the end product of glutathione biosynthesis (Lu, 1999; Meierjohann, *et al.*, 2002) thus rendering it available for proton donation to reduce pro-oxidants. Interestingly, the extract and fractions of *Alstonia boonei* were found to increase the activities of GPx and GST at higher doses. Glutathione S-transferases represent a group of detoxification enzymes and they catalyse the conjugation of xenobiotics that contain electrophilic center with reduced glutathione through the formation of a thioester bond (Dzoyem *et al.*, 2014). Increased activities of GPx and GST at higher doses implied that reduced glutathione usage increased via GPx and GST thus affecting glutathione cycle. Glutathione peroxidase catalyses the reduction of hydrogen peroxides by GSH (Flohe, 1971).

Superoxide dismutase is the first enzyme encountered in the detoxification of superoxides and other reactive oxygen species. The final detoxification of mitochondrial superoxides and other superoxides occur through the activities of catalase (at high concentration of H_2O_2) and GPx (at low concentration of H_2O_2). We observed also in this study that the corresponding increase in the activity of catalase and superoxide dismutase would cause a decrease in oxidative pressure on membrane lipids thus reducing the malondialdehyde levels in all the treated groups except CF at 200 mg/kg.

Xanthine oxiodase (XO) is a superoxide-producing enzyme found normally in the serum during severe liver damage. It is interesting to note that XO increased significantly in all the treated groups only at the highest dose (200 mg/kg). This suggests that the extract and fractions of *Alstonia boonei* are hepatoprotective only at lower doses after which toxicity occurred. Again, the dose-response pattern of the extract and fractions of *Alstonia boonei* has shown that the experimental animals expressed different response to similar dose. For example, while ME, EF and MF increased the activities of CAT and SOD, there was a dose-dependent decrease in the activities of GPx and GST by HF. In addition, the activity of GPx in treated animals had hormetic response to MF and CF. Put together; this shows that the phytochemicals present in the extract and fractions of *Alstonia boonei* may vary in their antioxidant strength and properties. The biphasic response of GPx to CF and MF had shown that highest dose of these fractions (200 mg/kg) elicit an opposite response (probably toxicity) compared with the lowest dose (50 mg/kg) and that maximum activity was observed at 100 mg/kg.

The protection and maintenance of the red cell membrane integrity is paramount for the safety of the cells to avoid secondary damage through free radical inducing lipid peroxidation. Interestingly, the extract and fractions of *Alstonia boonei* showed varying degree of antioxidant activity both *in vitro* and *in vivo*. We compared extract and fractions of *Alstonia boonei* with chloroquine and artesunate because these orthodox drugs and *Alstonia boonei* are used in West Africa for the treatment of uncomplicated malarial infection. Antimalarial properties and *in vivo* antipyretic and analgesic properties of various solvent extract of *Alstonia boonei* are documented (Olajide *et al.*, 2000; Adotey *et al.*, 2012). Although, the chloroform and ethylacetate fractions that showed significant antioxidant activities but decreasing membrane stabilizing and proteinase inhibitory effects, it could be that the higher concentrations used may the cause for such observation as natural antioxidants are known for such different response at both lower and higher doses and concentrations. Natural products from medicinal plants have shown such hormetic response in some studies (Bao *et al.*, 2014; Teschke *et al.*, 2018). This effect further showed the reason why medicinal plants and natural products' use should be regulated for desired results.

Proteolytic enzymes are protein digesting enzymes with both helpful and deleterious effects. While they are essential for cell division, blood clotting, immune function and protein recycling processes, they are also useful to improve the digestion and absorption of dietary protein. They are abundant in living cells (plants and animals) and play important roles in intracellular proteolysis. They are the main targets for the investigation of many diseases including those that are related to cell death (Oliva and Sampaio, 2009). In this study, the *n*-hexane fraction inhibited proteinase activity, as evidently seen

in its effect against red cell membrane stabilization which is an index of eryptosis. This clearly showed that the n-hexane fraction of *Alstonia boonei* is capable of being used as chemopreventive agent (Magee *et al.*, 2012).

In conclusion, we have been able to provide evidence that extract and fractions of *Alstonia boonei* have both *in vitro* and in *vivo* antioxidant activities, prevent heat induced and hypotonic solution-induced erythrocyte membrane distabilisation and at the same time modulate the activities of protease enzymes. Diverse functions of the extract and fractions of this plant show that this plant contains array of different phytochemicals that can be of benefit to mankind when they are used with caution

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