Regular paper

Evaluation of antioxidant, antimutagenic, and lipid peroxidation inhibitory activities of selected fractions of *Holarrhena floribunda* (G. Don) leaves

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Exposure to environmental pollutants often leads to an upsurge in the production of reactive oxygen species (ROS). ROS oxidize cellular fatty acids to produce lipid peroxyl radicals, subsequently transformed into lipid peroxides, which decrease membrane fluidity and increase the activity of various enzymes implicated in degenerative diseases and cancer formation. Edible plants that contain exogenous compounds like curcumeroid, β-carotene, turmeric, and so on, protect the aerobic cells from oxidation of free radicals. This study thus evaluates antioxidant and antimutagenic activities of ethyl acetate, aqueous and methanolic fractions of *Holarrhena floribunda* leaves. Inhibitory activities of the ethyl acetate fraction on Fe^{2+}-induced lipid peroxidation in hen egg yolk; rat liver and brain tissues were also evaluated. The *Allium cepa* root assay was used to evaluate antimutagenic activity. Results showed that the ethyl acetate scavenged DPPH, $OH^\cdot$, and $\cdot O_2^-$ much stronger than other fractions, as evidenced by its lowest respective IC_{50} values. All the fractions displayed antimutagenic activities against cyclophosphamide-induced chromosomal aberrations. Likewise, all the fractions induced a reduction in mitotic index, a hallmark of cytotoxicity in the root meristem of *Allium cepa*. The decrease in mitotic index was most profound for the ethyl acetate fraction, which also demonstrated a significant lipid peroxidation inhibitory activity in the liver and brain homogenates, but not in egg yolk, compared with the ascorbic acid standard. In general, the results suggest that the ethyl acetate fraction might contain beneficial phytochemicals that should be explored as novel candidates for preclinical drug development.

Key words: *Holarrhena floribunda,* ethyl acetate, aqueous, methanol, fractions, aberrations

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INTRODUCTION

Free radicals generated during normal cellular metabolism are not lethal to cells that constitutively express antioxidant enzymes, which clear them from the system. However, exposure to environmental pollutants such as heavy metals and ultra violet light leads to an upsurge in reactive oxygen species (ROS) (Rao *et al.*, 2001) production and inability of cells to cope with the accumulation. Therefore, cellular fatty acids are readily oxidized by ROS to produce lipid peroxyl radicals that are subsequently transformed into lipid peroxides in the form of malondialdehyde (Rao *et al.*, 2001; Rice-Evans & Burdon, 1993; Bachowski *et al.*, 1997). Lipid peroxidation decreases membrane fluidity and exacerbates the activity of various enzymes implicated in degenerative diseases and cancer (Cerutti, 1991; Finkel & Holbrook, 2000). ROS oxidizes deoxyribose sugar and base residues of DNA causing single strand breaks, which eventually lead to double strand breaks (Breen & Murphy, 1995). Consequently, the inability of the DNA repair mechanisms to restore the breaks leads to the formation of chromosomal aberration.

Some exogenous compounds obtained from food and vegetables such as curcumeroid, β-carotene, turmeric, and so on, do effectively protect the aerobic cells from oxidation of free radicals (Aqil *et al.*, 2006). These phytochemicals have revealed both antigenotoxic and significant antioxidant potential. Furthermore, many ailments that afflict humans such as aging, arthritis, coronary heart disease, Alzheimer's disease, cataract, and cancer are known to be ROS-mediated and can be neutralized by the use of antioxidants (Devasagayam *et al.*, 2004). These have thus led to an intensive search for natural plant products with antioxidant properties. *Holarrhena floribunda* (HF) leaves have a history of folklore uses for several diseases including malaria and diabetes. It has been reported to contain saponins, tannins, and cardiac glycoside. Also, antioxidant activity against radicals like $OH^\cdot$, DPPH, $NO^\cdot_2$, and Lipid peroxidation inhibition of the methanolic leaf extract of HF, have been reported (Badmus *et al.*, 2010).

The present study sought to evaluate antioxidant, antimutagenic, and lipid peroxidation inhibition of some fractions of HF leaves against ascorbic acid as standard.

MATERIALS AND METHODS

Chemicals. Trichloroacetic acid (TCA), ferrous sulphate ($FeSO_4$), thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), folin-ciocalteus, glacial acetic acid,

ascorbic acid, 1,1 diphenyl-2-picrylhydrazyl (DPPH), and Orcein salt were procured from Sigma Co (St. Louis, MO) USA. Cyclophosphamide was purchased from Candila Healthcare Limited, Germany. All other reagents used were of analytical grades.

Extraction procedure. The powdered leaves of HF (1.5 kg) were exhaustively extracted with 4.5 liters of 70% methanol. The methanolic filtrate was concentrated under reduced pressure to yield a methanolic extract. A fraction of this extract was suspended in distilled water and partitioned with *n*-hexane, chloroform, and ethyl acetate. Each fraction was concentrated under reduced pressure to yield their respective fractions. The methanolic, ethyl acetate, and aqueous fractions were used for the evaluations.

***In-vitro* Antioxidant assay**. The DPPH scavenging potential was estimated as previously described (Mensor *et al.*, 2001). One ml of a 0.3 mM DPPH methanol solution was added to a 2.5 ml solution of each fraction and allowed to react at room temperature in the dark for 30 min. The absorbance of the resulting mixture was measured at 517 nm.

The hydroxyl radical scavenging ability of the fractions was evaluated as previously described (Halliwell *et al.*, 1987). The reaction mixtures used contain ascorbic acid (200 μM), $FeCl_3$ (200 μM), EDTA (200 μM), H_2O_2 (20 mM), deoxyribose (5 mM) with different concentrations of the fractions in 1 ml of PBS (20 mM, pH 7.4). The mixture was incubated at 37°C for 1 h and then 1 ml of 2.8% TCA (w/v in water) and 1 ml of 0.8% TBA (w/v) were added. The mixture was heated in a boiling water bath for 15 min. It was cooled and absorbance was measured at 532 nm.

Superoxide anion scavenging ability was assessed as previously described (Beauchamp & Fridovich, 1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 μg riboflavin, 12 mM EDTA, NBT (0.1 mg/3 ml) added in that order. The reaction was initiated by illuminating the reaction mixture using fluorescent lamps (20 W) with different concentrations of fractions for 150 s. The absorbance was measured at 590 nm immediately after illumination.

The absorbance of the entire assay was converted to percentage antioxidant activity (AA%), using the formula;

$$AA\% = [(Abs_{blank} — Abs_{sample}) \times 100]$$

Allium cepa assay. Healthy onion bulbs (*Allium cepa* L. 2n = 16) of similar size were purchased from Wazo market at Ogbomoso, Oyo state, Nigeria. The onions were sun dried for three weeks so as to reduce the moisture content and facilitate growth. The dried outer scales were carefully removed leaving the ring of primordial roots intact. These were used for bioassay according to the standard procedure. Ten onion bulbs were used for each fraction and controls. Tap water was used as negative control, cyclophosphamide as positive control, and ascorbic acid as a standard control. The base of each of the bulbs was suspended on the extract inside 100 ml capacity beaker. Test extracts and control media were changed daily. The experiment was performed in the dark at 25 ± 1°C. Five onion bulbs were harvested from each group after 48 h of growth for microscopic evaluation. Onion root tips were prepared for microscopic evaluation as previously described (Akinboro & Bakare, 2007; Lateef *et al.*, 2007). A number of diving cells and chromosomal aberrations in 5000 cells per concentration were analyzed. Mitotic index (MI), mitotic inhibition (MH) and frequency of aberrant cells (FOA) were calculated (Akinboro & Bakare, 2007).

Mitotic index (MI) = number of dividing cells in the treatment ×100.

Total number of cells. Mitotic inhibition (MH) = mitotic index of control — mitotic index of treatment ×100.

Mitotic index of control. Frequency of aberrant cells (FOA) = number of aberrant cells in the treatment.

Total number of dividing cells in the treatment. Dose selection and experimental protocol. The dose of the fractions used for antimutagenic activity in the *A. cepa* assay was based on different concentrations (1:1, 1:10, 1:100 and 1:1000) of fractions tested on the growth of onion roots (results not presented here). The choice of 1:1000 concentration was based on the fact that at least no fractions of HF support less than 45% root growth.

Antimutagenic effects of fractions were evaluated by pregrowing the *A. cepa* in the medium containing the individual

Table 1. DPPH scavenging ability of methanolic, aqueous, and ethyl acetate fractions of *Holarrhena floribunda* leaves and ascorbic acid.

Sample	Concentration (μg/ml)	% inhibition (n=5)	Regression equation	IC$_{50}$ (μg/ml)
Methanolic	10	6.30±0.011	Y=1.295X –9.410	45.90
	20	9.90±0.026	(r^2=0.9568)	
	30	34.30±0.011		
	40	41.40±0.008		
	50	55.30±0.014		
Aqueous	50	51.20±0.005	Y=0.0834X+48.85	15.00*
	100	58.10±0.004	(r^2=0.9403)	
	150	63.50±0.005		
	200	65.80±0.005		
	250	68.20±0.009		
Ethyl acetate	5	51.10±0.007	Y=0.0834X+48.85	3.60*
	10	57.10±0.006	(r^2=0.9987)	
	15	62.30±0.006		
	20	67.20±0.013		
	25	72.50±0.010		
Ascorbic acid	5	54.20±0.007	Y=0.8580X+49.51	0.57*
	10	56.90±0.006	(r^2=0.9850)	
	15	63.00±0.006		
	20	67.40±0.013		
	25	70.40±0.006		

Data were expressed as means ±S.D. of five parallel measurements.*IC$_{50}$ was calculated from the regression equation.

Table 2. Hydroxyl radical scavenging ability of methanolic, aqueous, and ethyl acetate fractions of *Holarrhena floribunda* leaves and ascorbic acid.

Sample	Concentration (µg/ml)	% inhibition (n=5)	Regression equation	IC_{50} (µg/ml)
Methanolic	400	45.30±0.007	Y=0.0139X+39.86	700.0
	500	47.50±0.002	(r²=0.9808)	
	600	48.80±0.002		
	800	51.30±0.001		
Aqueous	12.5	38.80±0.003	Y=0.143X+37.18	90.1
	25.0	40.60±0.003	(r²=0.9901)	
	50.0	44.50±0.006		
	75.0	48.70±0.002		
	100.0	50.90±0.003		
Ethyl acetate	10	45.20±0.004	Y=0.2058X+43.4	32.5
	20	47.90±0.003	(r²=0.9694)	
	30	49.90±0.004		
	50	52.50±0.002		
	60	56.50±0.004		
Quercetin	10	20.20±0.004	Y=0.6320X–9.680	49.5
	20	23.00±0.006	(r² = 0.6618)	
	30	23.70±0.004		
	40	26.00±0.003		
	50	50.30±0.004		

Data were expressed as means±S.D. of five parallel measurements.

Table 3. Superoxide anion scavenging ability of methanolic, aqueous, and ethyl acetate fractions of *Holarrhena floribunda* leaves and ascorbic acid.

Sample	Concentration (µg/ml)	% inhibition (n=5)	Regression equation	IC_{50} (µg/ml)
Methanolic	3.3	25.80±0.007	Y=3.345X+16.45	10.05
	6.7	42.70±0.012	(r²=0.9594)	
	10.0	50.20±0.010		
	13.3	55.70±0.012		
	16.7	75.10±0.007		
Aqueous	3.3	28.50±0.012	Y=4.372X+22.38	6.35
	6.7	57.70±0.010	(r²=0.9111)	
	10.0	72.40±0.006		
	13.3	84.10±0.003		
	16.7	88.30±0.001		
Ethyl acetate	1.33	04.50±0.004	Y=14.08X–13.07	4.60
	2.67	22.50±0.017	(r²=0.9787)	
	4.00	45.50±0.015		
	5.33	68.20±0.013		
	6.67	75.60±0.003		
Ascorbic acid	0.67	11.30±0.005	Y=26.61X–7.78	2.17
	1.33	22.60±0.023	(r²=0.9794)	
	2.00	48.50±0.031		
	2.67	67.40±0.017		
	3.30	76.60±0.003		

Data were expressed as means ±S.D. of five parallel measurements.

fraction and ascorbic acid (control) for 24 h and then transferred to cyclophosphamide medium for another 24 h before analysis (pre-treatment). This procedure was reversed for the second group, where *A. cepa* were first grown in cyclophosphamide medium for 24 h before being transferred to growth media of different fractions (post-treatment).

Inhibition of lipid peroxidation. Inhibitions of lipid peroxidation in the egg of hen, liver and brain homogenates of rat were determined using a modified (Ohkawa *et al.*, 1979) thiobarbituric acid-reactive species (TBARS) assay as previously described (Ruberto *et al.*, 2000). Egg homogenate (0.5 ml, 10% in distilled water, v/v) and 0.1 ml of each fraction were mixed separately in a test tube and the volume was made up to 1 ml, by adding distilled water. Finally, 0.05 ml $FeSO_4$ (0.07 M) was added to the above mixture and incubated for 30 min, to induce lipid peroxidation. Thereafter, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA (w/v) in 1.1% sodium dodecyl sulfate (SDS) and 0.05 ml 20% TCA was added, vortexed and then heated in a boiling water bath for 60 min. After cooling, 5.0 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Also, 10% of liver and brain homogenates obtained from rat were also used in place of egg homogenate for the evaluation of lipid peroxidation.

Statistical analysis. Data were expressed as mean ± standard deviation (SD) from five different experiments. Results were analyzed statistically using one-way analysis of variance (ANOVA) followed by Dunnett's test. The regression curve analysis was used to calculate IC_{50} using Graph Pad Prism version 5.02 statistical software. IC_{50} value is a concentration of extract required to scavenge 50% free radical and is inversely proportional to the activity of the extract.

Table 4. The cytogenotoxic effects of *H. floribunda* leaves fractions on *Allium cepa* root cells in the presence and absence of standard (cyclophosphamide).

Conc. 1:1000 (ppt)	No. of dividing cell	Mean of dividing cells (X̄±S.E)	% of Mitotic index	% of Mitotic inhibition	No. of prophase	No. of Metaphase	No. of Anaphase	No. of Telophase	VM	AB	FC	TAC	% FOA
Negative control	143	28.6±0.06	2.86		71	32	10	30	nil	nil	nil	nil	–
Cyclophosphamide	81	16.6±0.07	1.66	41.96	40	12	13	12	2	1	1	4	0.08
methanolic	106	21.2±0.03	2.12	25.87	55	23	14	14	nil	nil	nil	nil	Nil
Ethyl acetate	87	17.6±0.06	1.76	38.46	33	26	11	17	nil	nil	nil	nil	Nil
Aqueous	113	22.6±0.04	2.26	20.98	55	19	17	22	nil	nil	nil	nil	Nil
Ascorbic acid	105	21.2±0.06	2.12	25.87	35	26	19	25	nil	nil	nil	nil	nil
Methanolic + Cyclophosphamide	67	13.4±0.06	1.34	53.15	57	25	19	20	nil	nil	nil	nil	nil
Ethyl acetate + Cyclophosphamide	67	13.4±0.06	1.34	53.15	28	13	10	16	nil	nil	nil	nil	nil
Aqueous + Cyclophosphamide	77	15.4±0.04	1.54	46.15	32	13	17	15	nil	nil	nil	nil	nil
Ascorbic acid + Cyclophosphamide	59	11.8±0.06	1.80	58.74	21	18	7	13	nil	nil	nil	nil	nil
Cyclophosphamide + Methanolic	54	10.8±0.03	1.08	51.05	26	09	07	11	nil	nil	nil	nil	nil
Cyclophosphamide + Ethyl acetate	75	15.0±0.07	1.50	47.55	19	21	14	21	nil	nil	nil	nil	nil
Cyclophosphamide + Aqueous	49	9.8±0.05	0.98	65.73	27	10	5	07	nil	nil	nil	nil	nil
Cyclophosphamide + Ascorbic acid	68	14.0±0.06	1.40	51.05	26	14	14	14	nil	nil	nil	nil	nil

VM, Vagrant metaphase; TAC, Total aberrant cell; Conc, Concentration; AB, Anaphase bridge; FOA, Frequency of aberration; FC, Fragmented chromosome

RESULTS

DPPH radical scavenging

IC_{50} values of the fractions and ascorbic acid were calculated using the regression equation as stated in Table 1. Ethyl acetate fraction showed significant DPPH scavenging activity with IC_{50} of 3.60 µg/ml with a regression equation of $Y = 0.0834X + 48.85$ and $r^2 = 0.9987$. This was followed by the aqueous fraction ($IC_{50} = 15.00$ µg/ml, $Y = 0, 0834X + 48.85$ and $r^2 = 0.9403$), methanolic fraction ($IC_{50} = 45.90$ µg/ml, $Y = 1. 295X - 9.410$ and $r^2 = 0.9568$). Ascorbic acid used as standard showed significant scavenging of DPPH with $IC_{50} = 0.57$ µg/ml.

Hydroxyl radical scavenging

All the fractions showed concentration dependent hydroxyl radical scavenging activities compared with the standard quercetin. Methanolic fraction scavenged hydroxyl radical with IC_{50} of 700.0 µg/ml, scavenging regression equation of $Y = 0.0139X + 39.86$ and $r^2 = 0.9808$. Aqueous fraction had IC_{50} of 90.1 µg/ml with a regression equation $Y = 0.143X + 37.18$ and $r^2 = 0.9901$. Ethyl acetate showed IC_{50} of 32.5 µg/ml with a regression equation of $Y = 0.2058X + 43.40$ and $r^2 = 0.9694$. Quercetin had IC_{50} of 49.5 µg/ml with a regression equation of $Y = 0.6320X - 9.680$ and $r^2 = 0.6618$. This result shows that ethyl acetate had highest scavenging potential against hydroxyl radical compared with other fractions and standard quercetin (Table 2).
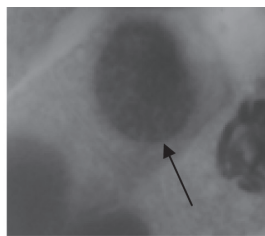
Superoxide anion radical scavenging

All the fractions showed strong scavenging and concentration dependent activities against superoxide anion generated using NBT/Riboflavin/illumination system as presented in (Table 3). Methanolic fraction had 10.05 µg/ml with a regression equation of $Y = 3.345X + 16.41$ and $r^2 = 0.9594$. Aqueous showed IC_{50} of 6.35 µg/ml, regression equation $Y = 4.372X + 22.38$ and $r^2 = 0.9111$. Ethyl acetate fraction had IC_{50} of 4.60 µg/ml, regression equation of $Y = 14.08X - 13.07$ and $r^2 = 0.9787$. Ascorbic acid had IC_{50} of 2.17 µg/ml, regression equation of $Y = 26.61X - 7.78$ and $r^2 = 0.9794$. This result shows that scavenging ability followed this descending order; Ascorbic > ethyl acetate > aqueous > methanolic fraction.

Antimutagenicity evaluation

The effect of methanolic, aqueous, ethyl acetate fractions of HF, and ascorbic acid on cell division, mitotic index and chromosomal aberrations are presented in Table 4. The number of dividing cells and mitotic index in the control group were higher compared to treated groups. Chromosomal aberrations were observed in the root tip cells of *Allium cepa* treated with cyclophosphamide with an aberration frequency of 0.08. Chromosomal aberrations observed were vagrant metaphase, anaphase bridge, and fragmented chromosome (Fig. 1).
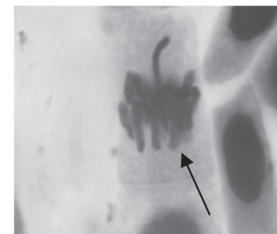
Post and pre-treatment of cyclophosphamide treated *Allium cepa roots* with the fractions of HF and ascorbic acid had additive effects on the reduction of mitotic index. Post treatment revealed that mitotic index reduction was more pronounced with ethyl acetate fraction while at pretreatment, similar mitotic re-
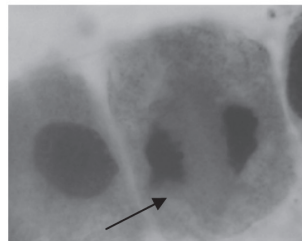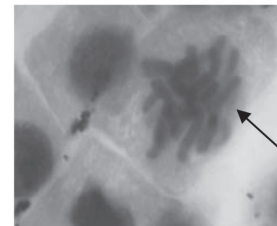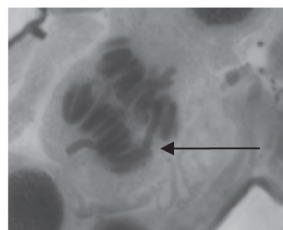

a: Interphone
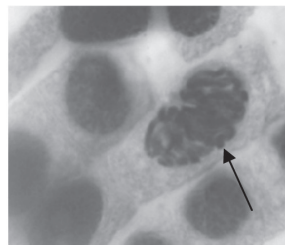
b: Prophase
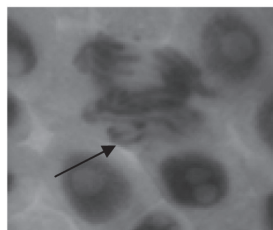

c: Metaphase

d: Anaphase

e: Telophase

f: Vagrant Metaphase

g: Anaphase bridge

h: Fragmented chromosome

Figure 1a–h. Normal stages of cell division and the observed chromosomal aberrations in cylophosphamide and *H. floribunda* treated *Allium cepa*.

duction effects were observed in the methanolic and ethyl acetate fractions. Chromosomal aberrations were observed in the cyclophosphamide treated *Allium cepa* roots, whereas no aberrant cell was observed in the HF fractions, ascorbic acid and at both the post and pretreatment combination of fractions and cyclophosphamide.

Inhibition of lipid peroxidation

Ethyl acetate fraction had higher lipid peroxidation inhibition in the egg yolk with IC_{50} of 41.9 μg/ml, regression equation $Y = 1.006X + 7.959$ with $r^2 = 0.9156$ compared with standard ascorbic acid with IC_{50} of 45.9 μg/ml, regression equation $Y = 0.7322X + 11.90$ with $r^2 = 0.9222$ (Table 5).

Table 5 shows the results of inhibition of lipid peroxidation in brain homogenate. Ethyl acetate had IC_{50} of 22.6 μg/ml, regression equation $Y = 1.825X + 8.658$ and $r^2 = 0.9838$ compared with ascorbic acid which had IC_{50} of 33.9 μg/ml, regression equation $Y = 1.428X + 1.649$ and $r^2 = 0.9916$. Ethyl acetate showed significant inhibition of lipid peroxidation in brain compared with ascorbic acid.

Inhibition of Fe^{2+}-induced lipid peroxidation in liver homogenate was significantly stronger than ascorbic acid. Ethyl acetate inhibited the lipid peroxidation with IC_{50} of 22.7 μg/ml, regression equation $Y = 2.224X - 0.537$ with $r^2 = 0.9903$ compared with ascorbic acid with IC_{50} of 40.8 μg/ml, regression equation $Y = 1.4863X - 10.64$ and $r^2 = 0.9516$ as presented in Table 5.

Table 5. Effects of the ethyl acetate fraction and ascorbic acid on the Fe^{2+}-induced lipid peroxidation in hen's egg yolk, rat brain and liver

Sample	Concentration (μg/ml)	% inhibition (n = 5)	Regression equation	IC_{50} (μg/ml)
Egg yolk Ethyl acetate	09.50	11.70 ± 0.007	Y = 1.006X + 7.959	41.9
	19.00	33.10 ± 0.013	(r^2 = 0.9156)	
	28.57	39.30 ± 0.012		
	38.00	46.10 ± 0.007		
	47.62	53.10 ± 0.019		
Ascorbic acid	09.50	20.60 ± 0.012	Y = 0.7322X + 11.90	45.9
	19.00	26.10 ± 0.003	(r^2 = 0.9222)	
	28.57	31.10 ± 0.014		
	38.00	35.40 ± 0.004		
	47.62	50.80 ± 0.015		
Brain Ethyl acetate	09.50	28.00 ± 0.010	Y = 1.825X + 8.658	22.6
	14.29	32.00 ± 0.006	(r^2 = 0.9838)	
	19.00	43.00 ± 0.011		
	23.81	53.00 ± 0.012		
	28.57	61.00 ± 0.008		
Ascorbic acid	09.50	13.00 ± 0.022	Y = 1.428X + 1.649	33.9
	19.00	30.00 ± 0.030	(r^2 = 0.9916)	
	28.57	45.00 ± 0.025		
	38.00	56.00 ± 0.006		
	47.62	68.00 ± 0.012		
Liver Ethyl acetate	09.50	22.00 ± 0.016	Y = 2.224X – 0.537	22.7
	14.29	31.00 ± 0.019	(r^2 = 0.9903)	
	19.00	40.00 ± 0.021		
	23.81	51.00 ± 0.021		
	28.57	65.00 ± 0.007		
Ascorbic acid	09.50	07.40 ± 0.012	Y = 1.4863X – 10.64	40.8
	19.00	12.90 ± 0.030	(r^2 = 0.9516)	
	28.57	37.50 ± 0.040		
	38.00	41.80 ± 0.052		
	47.62	65.90 ± 0.009		

Data were expressed as means ± S.D. of five parallel measurements.

DISCUSSION

The importance of searching for alternative plant products against a number of diseases associated with ROS continues to be popular because of limited toxicity associated with it as against synthetic products. Epidemiological studies have revealed that diets rich in fruit and vegetables correlate with a reduced risk of chronic diseases such as cardiovascular disease and some forms of cancer (Liu *et al.*, 2000; Gheldof *et al.*, 2003). However, a volume of work has been done to unravel some inherent benefits of some natural products. Consequently, this has led to the isolation of bioactive agents for clinical candidates. Furthermore, a large gap is still needed to be filled because there are a lot of potentially beneficial plants that are yet to be studied.

The antioxidant potential of methanolic extract of *Holarrhena floribunda* has been previously reported (Badmus *et al.*, 2010). In the continuous effort to evaluate potential medicinal values of the leaves of HF, the present research was conducted to investigate antioxidant and antimutagenic activities of methanolic, ethyl acetate, and aqueous extracts of HF. Lipid peroxidation inhibition of ethyl acetate fraction in the rat liver, brain, and hen's egg yolk homogenates were also assessed.

DPPH is a stable, free radical that reacts with a compound that has an H-donating ability. Its reduction to diphenylpicrylhydrazine after the reaction changes the colour from dark-purple to yellow and it is detected at wavelength of 517 nm. DPPH method is easy, ac-

curate, and reproducible, and it is comparable to other free radical scavenging systems (Gil *et al.*, 2000). All the fractions show dose dependent DPPH scavenging ability. This result shows that the ethyl acetate fraction can release H atom to stabilize the DPPH radical better than other fractions tested except Ascorbic acid.

Hydroxyl radical is the main reactive oxygen species involved in the induction of lipid peroxidation in the biological membrane by abstracting hydrogen atom from unsaturated fatty acids (Aruoma *et al.*, 1987). This radical also has the ability to initiate carcinogenesis, mutagenesis, and cytotoxicity (Esmaeili & Sonboli, 2010). This result shows that ethyl acetate prevents the Fenton reaction generated hydroxyl radical from oxidizing ribose sugar better than other tested fractions and the standard. The implication of this result is that ethyl acetate can protect the cell membrane from the oxidizing potential of hydroxyl radical better than any other tested fractions. Superoxide anion is known to play roles in initiating other reactive oxygen species such as H_2O_2, OH^-, and O^{2-} (Pietta, 2000). Its reaction with nitric oxide forms peroxylnitrite anion ($ONOO^-$), which leads to an increase in the nitric oxide toxicity (Huie & Padmaja, 1993). All the fractions show significant scavenging activity against riboflavin/NBT/illumination induced SO_2^-. Ethyl acetate had the highest activity with IC_{50} value of 4.47 µg/ml, followed by aqueous (6.13 µg/ml) and methanolic extract (9.83 µg/ml). However, this implies that the fractions have the ability to protect the cell against various deleterious effects of SO_2^-.

Allium cepa test is a fast, inexpensive, and sensitive method. It has been validated in international collaborative studies under the United Nations Environmental Program (UNEP), World Health Organization (WHO), and US Environmental Protection Agency (USEPA) as an efficient test for genetic monitoring of environmental Pollutant (Grant, 1999). Mitotic index (MI) is a parameter used to estimate the frequency of cell division in *Allium cepa* root (Turkoglu, 2008). Reduction of MI was observed in cyclophosphamide, *Holarrhena floribunda* fractions, and Vitamin C separately treated roots. Reductions of MI in ethyl acetate treated roots were higher when compared to other fractions and very close to that of cyclophosphamide treated roots. Also, ethyl acetate showed the highest reduction of MI in pre-treatment of the fractions before cyclophosphamide treated *Allium cepa* roots. The lowest reduction of MI was observed in post-treatment of ethyl acetate fraction when compared to other fractions and ascorbic acid. Reduction of mitotic index has been attributed to the inhibition of DNA synthesis and blocking of G2 phase of the cell cycle, thereby preventing the cell from entering mitosis (Van't Hof, 1968; Sudhakar *et al.*, 2001). The reduction of MI value lower than individual control may suggest the level of cytotoxicity that substance inflicts on meristematic cells (Akinboro & Bakare, 2007). From the present results, the ethyl acetate fraction is more cytotoxic than any other fraction and ascorbic acid. This could be attributed to the possible presence of phytochemicals such as alkaloids, tannins, and cardiac glycoside. These compounds have been shown to initiate cytotoxicity, antimutagenic, and antioxidant activities (Talib & Mahasneh, 2010; Park *et al.*, 2009).

Improper or un-repair oxidation of DNA deoxyribose sugar and a nitrogenous base of ROS lead to double strand break, which contributes to chromosomal aberrations (Breen & Murphy, 1995; Maluszynska & Juchimiuk, 2005). The results presented in Table 4 and Figure 1 (f–h) showed that cyclophosphamide induced vagrant metaphase, anaphase bridge, and fragmented chromosomes. Induction of vagrant has been shown to lead to the formation of daughter cells with unequal sized or irregularly shaped nuclei at interphase because of unequal numbers of chromosomes in the daughter cells (El-Ghamery *et al.*, 2003). Formation of bridges also could be attributed to chromosome breaks, stickiness and breakage/ reunion of broken ends (Yildiz *et al.*, 2009). All these aberrations were absent in all the fractions, either treated alone or in combination with cyclophosphamide (Post or pre) treated *A. cepa* roots. This shows that all the fractions have the ability to protect *A. cepa* meristemic cells from cyclophosphamide induced mutagenesis.

Polyunsaturated lipid contents of the biological membrane are susceptible to oxidative reaction of free radicals, which lead to lipid peroxidation. Products of lipid peroxidation such as malondialdeyde (MDA), 4-hydroxyl 2-nonenal, and some other alkanes react with cell macromolecules to form adducts with significant irreversible effects on cellular functions and biochemistry (Onyema *et al.*, 2003; Tuma & Casey, 2003). Consequently, the formation of adduct leads to membrane permeability, oxidative nucleic acid damage, and eventually to mutation and cancer (Brawn & Fridovich, 1981; Halliwell & Gutteridge, 1984). However, on the basis of antioxidant and antimutagenic activities the most active fraction was tested for lipid peroxidation inhibition. Lipid peroxidation was induced in three media of different lipid contents and characteristics (liver, brain and egg yolk) using ferric chloride. Inhibitions of lipid peroxidation in liver and brain homogenates by ethyl acetate were found to be significantly stronger when compared to ascorbic acid. Inhibition of lipid peroxidation in egg yolk by ethyl acetate was not significantly different when compared with ascorbic acid. These results showed that most of the number of reported phytochemicals of *H. floribunda* such as tannins, saponins, alkaloid, cardiac glycosides, and phenolic compound (Badmus *et al.*, 2010) could be higher in ethyl acetate fraction.

CONCLUSION

The present investigation shows that *Holarrhena floribunda* leaves are rich in phytochemicals that have antioxidant, antimutagenic and lipid peroxidation inhibition activities. However, the activities exhibited by the ethyl acetate fraction indicate that it contains more of these phytochemicals. Further research is required to elucidate the phytochemical contents of this fraction as well as its cytotoxic and antimutagenic properties.

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