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Full Length Research Paper

In vitro biochemical investigations of the effects of *Carica papaya* and *Fagara zanthoxyloides* on antioxidant status and sickle erythrocytes

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Various works have identified a number of herbal applications that have ameliorating effects on sickle cell disorders. The antisickling activities of dried *Carica papaya* leaves and roots of *Fagara zanthoxyloides* are being investigated in this study to determine the antioxidant properties of the plant extracts and their effects on homozygous sickle cell (SS) erythrocytes *in vitro*. The antisickling activity of both extracts were determined as well as analyses of hematological parameters, hemolysis of SS cells and formation of membrane associated denatured hemoglobin (MADH) used to measure the effects of plant extracts on the erythrocyte. Folin-C total phenol and beta-carotene methods of assay were used to determine antioxidant activity, while the effect of plant extracts on oxidative stress was measured by assaying for superoxide dismutase, catalase, gluthathione transferase levels and lipid peroxidation. Results confirmed the potent antisickling activity of both plants. The levels of the oxidative stress enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione (GST) and lipid peroxidation were reduced after blood samples had been incubated with the extracts. The extracts therefore protected membrane integrity resulting in a reduction of red blood cells (RBC) hemolysis without met-hemoglobin formation. Both plant extracts possess potent antioxidant activity which may be responsible for their observed antisickling action.

Key words: *Fagara*, *papaya*, oxidative stress enzymes, antioxidant activity, hemolysis.

INTRODUCTION

First-line clinical management of sickle cell anemia include use of Folic acid, amino acids (as nutritional supplements), Penicillin prophylaxis (helps prevent infection) and anti-malarial prophylaxis (helps prevent malaria attack) for example, Paludrine^{R} in varying doses in childhood, adulthood and pregnancy. The faulty 'S' gene is not eradicated in treatment, rather the condition is managed and synthesis of RBC induced to stabilize the patient's hemoglobin level. Further management and treatment of this disorder with compounds or techniques which directly affect the hemoglobin (Hb) molecule (for example, Hydroxyurea, Bone Marrow Transplantation and Blood Transfusion) are very expensive and out ofreach of the masses and besides expose the patient to mutagenicity, iron overload and other fatal risks (Brittain and Han, 2004; Steinberg, 2004; Amrolia, 2003; Sauntharajah and Maziarz, 2003; Nagel, 1998). It is acknowledged world-wide that traditional medicine can be explored and exploited to be used along-side synthetic pharmaceutical products for enhanced health management. Due to the high mortality rate of sickle cell patients, especially in children, and since chemotherapy has its adverse effects, there is need for rational drug development that must embrace not only synthetic drugs but also natural products (phytomedicines/herbal drugs),

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Abbreviations: SS, Sickle cell; **MADH,** membrane associated denatured hemoglobin; **GST,** glutathione; **SOD,** superoxide dismutase; **CAT,** catalase; **RBC,** red blood cells; **Hb,** hemoglobin; **EDTA,** ethylenediaminetetraacetic acid; **FRIN,** forestry research institute of Nigeria; **HbAA,** hemoglobin AA; **HbSS,** hemoglobin SS; **PHBA,** para-hydroxybenzoic acid; **TBARS,** thiobarbituric acid; **MCHC,** mean corpuscular hemoglobin concentration; **RDW,** red cell distribution width; **MDA,** malondiadehyde; **SCD,** sickle cell disease.

naturally occurring antisickling agents which can be obtained from our vast forest resources and can be used to effectively manage the sickle cell patient and treat the anemic condition accompanying this disorder.

Active constituents of medicinal plants and naturally occurring compounds, known as antisickling agents, which improve the health of sickle cell individuals are rich in aromatic amino acids, phenolic compounds and antioxidant nutrients (Abraham et al., 1991) which are thought to be responsible for their observed antisickling action. An herbal preparation of *Cajanus cajan* was found to contain phenylalanine, carjaminose and hydroxybenzoic acid as active constituents and are thought to be the reason for its antisickling effect (Ogoda et al., 2002). The antisickling activities of dried *C. papaya* leaves and roots of *F. zanthoxyloides* are being investigated in this study because some indigenes use their extracts as folk medicine therapies for sickle cell crisis and also there are recent scientific reports on the antisickling activity of dried leaves of *C. papaya* and roots of *F. zanthoxyloides* (Sofowora et al., 1975; Imaga et al., 2009). A previous study showed that unripe papaya fruit extract has antisickling activity (Oduola et al., 2006). Another study found *C. papaya* leaf extract to have an appreciable potent antisickling activity, greatly affecting the time course for sickling in a dose-dependent manner, the most effective doses being 5 and 10 mg/ml extract concentration (Imaga et al., 2009). The effect of varied concentrations of the plant extracts on erythrocyte membranes, analyzed using the osmotic fragility test, revealed appreciable membrane protective effects of the herb and an inhibitory action on hemolysis of red blood cells. The toxicity profile of the plants have also been assessed by histological and biochemical analyses and did not reveal any substantial toxicity of the plants (Sofowora et al., 1975; Oyedapo and Famurewa, 1995; Elekwa et al., 2005; Imaga et al., 2009). However, adequate biochemical studies concerning the mechanism of action of these antisickling agents are yet to be fully understood. This offers an opportunity through this work to evaluate the antioxidant properties of the plant extracts and asses their effects on homozygous SS erythrocytes.

MATERIALS AND METHODS

Chemicals

Chemicals used were of analytical grade obtained from Sigma Chemical Company and used without further purification.

Blood samples

5 ml venous blood samples were collected with full informed consent from SS individuals aged between 18 and 20 years, who came in for routine visits at the SS Out-Patients' Clinic of the Lagos University Teaching Hospital, Idi-araba, Lagos, Nigeria. The blood samples were collected in sodium Ethylenediaminetetraacetic acid (EDTA) bottles and used for the experiments.

Collection of plant material

Sun-dried *C. papaya* (fruit bearing) leaves and *F. zanthoxyloides* roots were collected during the dry season in December 2009 at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria. The plants were authenticated by Mr. T. K. Odewo of FRIN and samples kept at their herbarium.

Extraction of plant material

Organic extraction

C. papaya ground leaves were extracted using the methods of Ogoda et al. (2002), as follows: 400 g of dark brown, smooth textured powdered sample was extracted with 1.5 L of petroleum ether using a Soxhlet extractor for 6 h. The petroleum ether extract obtained was further evaporated to dryness using vacuum rotary evaporator. The marc was further extracted with 1.5 L of aqueous methanol (1:3 v/v) using the soxhlet extractor for another 6 h. The obtained methanolic extract was evaporated to dryness using vacuum rotary evaporator. Both extracts were freeze dried and stored at 4°C. This process was repeated to obtain petroleum ether and methanolic extracts for *F. zanthoxyloides*. Thus the herbal extracts were obtained via soxhlet extraction (with petroleum ether 60 to 80°C and aqueous methanol (1:3, 60 to 80°C) as solvents and stored at 4°C in freeze dried form.

Aqueous extraction

Powdered *C. papaya* (400 g) was weighed into a clean bowl and 2.5 L of hot distilled water was poured in and the bowl covered. This was allowed to infuse for 3 h and then cooled at room temperature. The mixture was sieved with a clean muslin cloth. The resultant aqueous extract was freeze-dried and stored in the refrigerator at 4°C till use. This process was repeated to obtain aqueous extracts for the ground sample of *F. zanthoxyloides*.

In vitro hemoglobin studies

Antisickling activity

5 ml blood samples obtained from patients were centrifuged at 5,000 rpm for 10 min in phosphate buffered saline thrice to obtain the RBC which were then resuspended in normal saline and used for the analysis according to earlier described methods of Imaga et al. (2009). The aqueous and methanol extracts of *C. papaya* leaves and *F. zanthoxyloides* roots were used in this experiment, with para-hydroxybenzoic acid as the chemical standard. 1 ml SS blood cell suspensions were pre-incubated with 0 to 10 mg/ml concentrations of the extracts in the presence of 2% sodium metabisulphite solution and the time course of the effect of varied concentrations of extracts on the sickling of SS erythrocytes was microscopically analyzed. A plot of percentage sickling inhibition against extract concentration was analyzed for possible explanation of the observed antisickling effect.

Determination of haematological parameters, MADH

The effects of the *papaya* and *Fagara* aqueous-methanol extracts on hematological parameters, hemolysis of SS cells and formation of (MADH)/met-hemoglobin in SS blood cells were determined using the methods of Ekeke and Shode (1985), Iyamu et al. (2002) and Abdulmalik et al. (2005).

Determination of complete blood count

Blood samples were collected from hemoglobin AA (HbAA) and hemoglobin SS (HbSS) individuals. Complete blood cell count (full hematological parameters) was determined using Sysmex 21 KXN. The HbSS blood samples were incubated with papaya extract or *Fagara* extract as follows: 1 g each of methanol and aqueous extracts of papaya and *Fagara* was dissolved in 100 ml normal saline. The solution was filtered and 0.5 ml of the filtrate was added to 1 ml of HbSS blood sample. This was incubated for 30 min after which complete blood cell count was determined using Sysmex 21 KXN. HbAA blood sample served as positive control and HbSS blood sample not incubated with plant extract served as the negative control.

Hemolysis of SS cells

The time-course of hemolysis as a function of the *papaya* and *Fagara* concentrations was examined over a 4 h period at plant extract concentration of 5 mg/ml. Blood samples collected from HbSS individuals were used for this experiment. The red blood cells were washed twice in physiological saline by centrifugation at 5000 rpm for 2 min and resuspended in normal saline. Iml of the solution was incubated in 5 mg/ml extract each of *papaya* and *Fagara* in the ratio 1:1 for 4 h. I ml of the solution was also incubated in para-hydroxybenzoic acid (PHBA) in the same ratio for the same duration (as the control). At 10 min intervals, an aliquot of mixture was removed and absorbance read at 415 nm.

Formation of methemoglobin and membrane associated (denatured) hemoglobin (MADH)

Aliquots (0.5 ml) of SS blood were diluted in 1.5 ml normal saline in each of four tubes. A reagent blank was put in the first tube- contained all reagents except the HbSS blood sample. 1 ml of PHBA was added to the second tube while1ml of *Fagara* extract was added to the third tube. To the fourth tube was added 1ml of *C. papaya* extract. The tubes were incubated for 4 h. At 10 min interval, 0.5 ml of each of the mixtures was lysed with hemolysing solution (phosphate buffer pH 8) and absorbance was read at 630 nm.

Antioxidant assay

Tests for the presence of phenolic compounds (indicative of antioxidant activity) contained in the plant extracts was done according to the methods of Wilson and Walker (2001), Padma et al. (2006) and Canini et al. (2007).

Estimation of total phenolic content

0, 1, 2, 3, 5 and 10 ml of prepared garlic extract solution (the control) were pipetted into different 100 ml volumetric flasks and diluted to volume with distilled water. This was done to prepare a calibration curve with each volumetric flask having: 0, 50, 100, 150, 250 and 500 µg garlic extract stock. 7.9 ml of distilled water was pipetted into different tubes; 6 for the standards and 5 for the extracts. 0.1ml of each calibration solution sample was pipetted into the separate tubes. To the resulting solution, 0.5 ml of Folin-Ciocalteau reagent and 1.5 ml of Na_2CO_3 were added. In place of the standard (Garlic stock) solution, the individual papaya and *Fagara* aqueous and organic extracts were added into test tubes T_7 – T_{11}. All the test tubes were incubated in a thermostatic water-bath at 40°C for 30 min. Their absorbance readings were then taken at 765 nm using a UV-VIS GENESYS 8 spectrophotometer.

Total antioxidant activity (β–carotene assay)

β-carotene (2 mg) was dissolved in 10 ml of chloroform, after which the chloroform was removed under vacuum. From the solution, 2 ml was pipetted into a 100 ml round bottom flask. 0.5 ml of purified oleic acid, 5 ml of tween 20 emulsifier and 100 ml of distilled water were added to the mixture in the round bottom flask, then mixed vigorously. 4.8 ml of the emulsion formed was pipetted into each test tube containing different concentrations of the extracts (that is, 2, 4, 6, 8, 10 µg/ml). For comparison purposes, standard antioxidant vitamins, vitamin C (Ascorbic acid) and Vitamin E (α-tocopherol) were used. As soon as the emulsion was added to each tube, zero time absorbance was measured on a UV-VIS spectrophotometer at 470 nm. The tubes were then placed in a water bath at 50°C and the measurement of their absorbance was continued for 2 h. Graphs of absorbance against time were plotted for the different concentrations of extracts. A control devoid of the extracts but containing all other reagents used was also prepared for the assay as a standard.

Effect of the extracts on antioxidant /oxidative stress enzymes (SOD and Catalase), Lipid peroxidation (MDA) and Glutathione transferase (GST)

These give the measure of the extracts protective anti-oxidative effects on the RBC membrane. The antioxidant enzyme assays were done in 3 stages: (a) With AA blood serum alone; (b) With SS blood serum alone (c) with AA blood serum + SS blood serum + prepared extract (that is, vitamin C, *C. papaya* and *F. zanthoxyloides* extracts) where vitamin C served as a positive control, being a known antioxidant. Vitamin C solution was prepared by dissolving 1g of vitamin C in 100 ml of distilled water.

Catalase activity assay

This was determined by the method of Cohen and Dembiec (1970); by measuring the decrease in H_2O_2 concentration and reading the absorbance value at 240 nm. To 0.2 ml (200 µl) of serum or serum-extract sample (for both AA and SS blood), 1.8 ml of 30 mM H_2O_2 was added. Reagent buffer was used as the blank and their absorbance readings were taken at 240 nm, at 60 s intervals for 5 min.

Superoxide dismutase (SOD) activity

SOD enzyme activity was determined according to the method described by Sun and Zigman (1978). Here, the SOD enzyme assay determines the difference between superoxide anion decomposition and production that is, its ability to inhibit the autoxidation of epinephrine. The assay was performed in 3.0 ml of 50 mM Na_2CO_3 buffer (in 2 different test tubes) to which 0.02 ml of each serum sample was added. 0.03 ml of the epinephrine stock solution was then added to the above before taking absorbance readings at 480 nm for 3 to 5 min.

Determination of glutathione-s-transferase (GST) activity

The activity of GST was determined according to the method of Habig et al. (1974). 0.1 ml of CDNB solution was pipetted into a conical flask before adding 1ml of phosphate buffer and 1.7 ml of distilled water. Next, the mixture was incubated at 37°C for 5 min. After the incubation, 0.1 ml of the serum sample and also 0.1 ml of GSH solution (using an automatic micropipette) were added. A blank devoid of the serum was prepared for background correction.

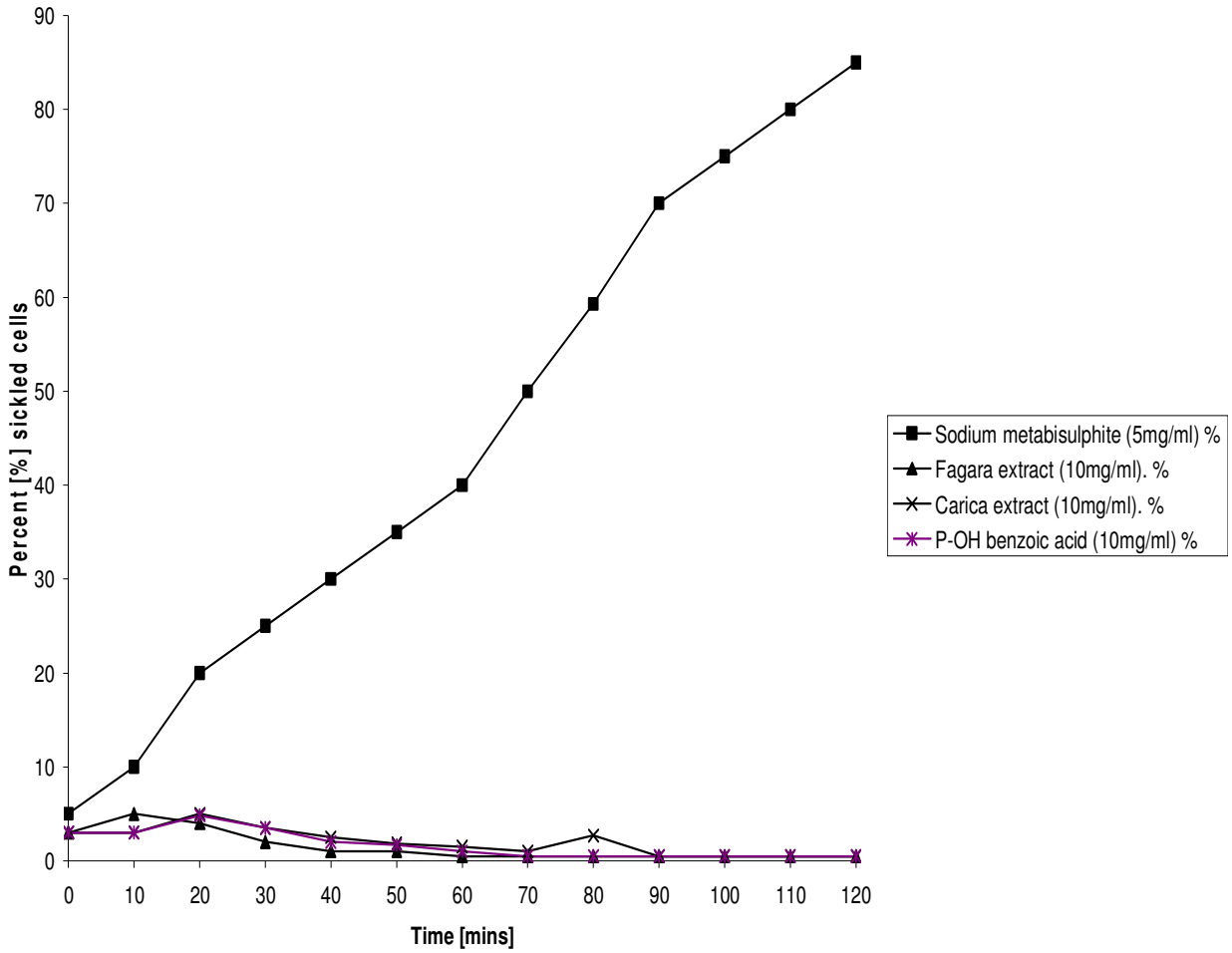

Figure 1. Timecourse of *Papaya* and *Fagara* extracts on sickling. Sickle cell suspensions pre-incubated with extracts prior to exposure to 2% sodium metabisulphite solution.

Absorbance readings at 340 nm were taken for 5 min at 60 s interval using a GENESYS 8 UV-VIS spectrophotometer.

Lipid peroxidation analysis

The Thiobarbituric acid (TBARS) assay method was used for the lipid peroxidation analysis. It is used to measure total free radical damage in a biological system; where Malondialdehyde, the end product of lipid peroxidation serves as a convenient index and can be measured spectrophotometrically at 535 nm to assay for the extent of lipid peroxidation in a sample. 1ml of serum sample was added to 2 ml of TCA-TBA-HCl reagent (1:1:1) in a test tube and mixed thoroughly. The mixture was then heated for 15 min in a boiling water bath and cooled. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 min. The absorbance of the sample was then determined at 535 nm against a blank.

Statistical analyses

Data from the various studies are presented as mean ± SEM (standard error of mean). The results were analyzed for statistical significance using Microsoft Word© software systems, (2007). Students't-test and satterwhaites' method of one way analysis of variance were used to compare mean values between groups. $p<0.05$ was taken to indicate a statistical significance.

RESULTS

Antisickling activity of plant extracts

Antisickling data was obtained from a typical three independent experiments performed in duplicate using blood samples from SS individuals. Results showed that the time course for 60% sickling was 40 min for the control (SS blood without extract). *F. zanthoxyloides* showed higher antisickling activity after 40 min of incubation compared to *C. papaya* and chemical standards used (Figure 1).

Effect of extract on the blood cell components

In order to study the potential adverse effects of *C.*

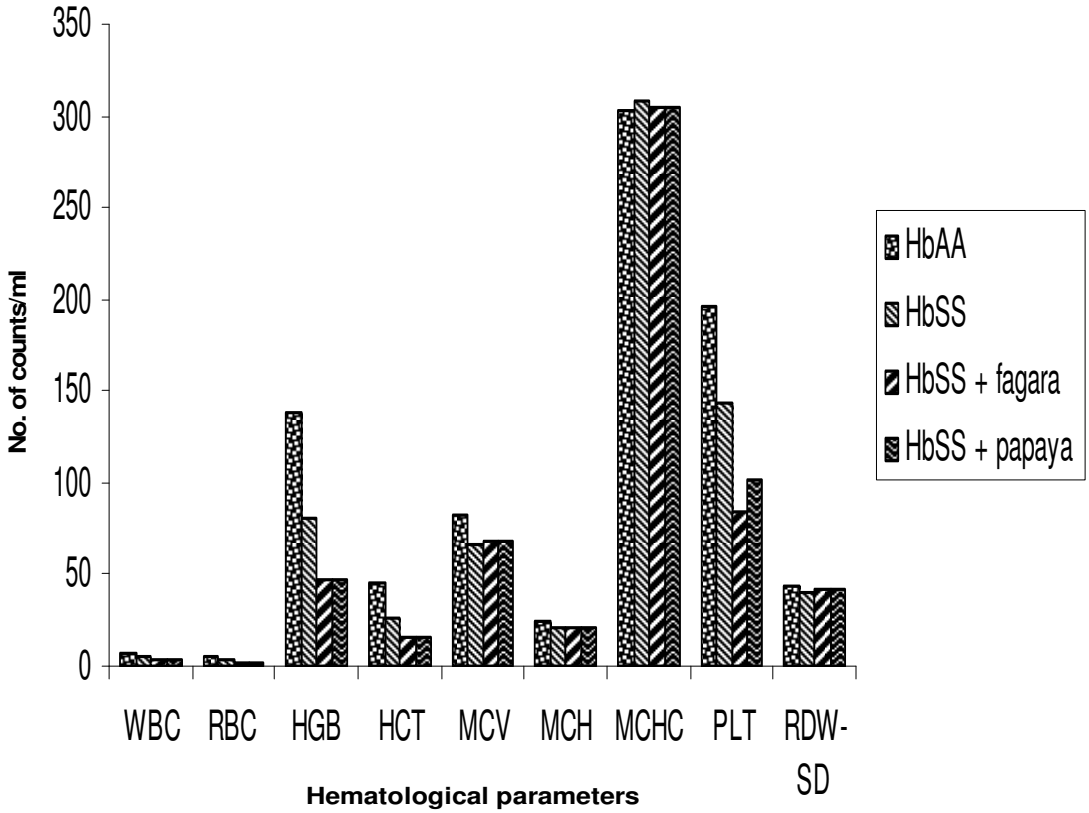

Figure 2. Effect of *Papaya* and *Fagara* extracts on some hematological parameters.

papaya on the blood cells, SS cells were incubated in the presence of 5 mg/ml concentration of extract. A full blood count, rate of hemolysis of SS cells and formation of met-hemoglobin was done. The mean corpuscular hemoglobin concentration (MCHC) and the red cell distribution width (RDW) indicated no statistically significant differences between extract-treated and control samples (Figure 2). Though there were marked differences in the Platelet count, PLT and blood volume, HCT (hematocrit/ Packed Cell Volume) as shown, results obtained are not statistically significant.

Effect of extract on the rate of RBC hemolysis

The rate of hemolysis of blood samples incubated with plant extract was determined. The results indicated after 2 h incubation with the blood samples that both papaya and control PHBA lowered the incidence of RBC lysis (Figure 3), unlike the *Fagara* extract.

Effect of extract on the inhibition of met-hemoglobin formation

The rate of formation of met Hb (indirectly the level of MADH formed) was also determined. The results indicated after 2 h incubation with the blood samples that both *C. papaya* and control PHBA decreased the formation of met-Hb better than *Fagara* extract (Figure 4).

Total phenolic compounds present in *Papaya* and *Fagara* extracts

The total phenol compounds present in the plant extracts was analyzed using the Folin-C method of assay. As shown, aqueous *C. papaya* extract had the highest total phenol (antioxidant) concentration of 540 mg/L, (Figure 5) followed by aqueous *F. zanthoxyloides* with a concentration of 500 mg/L when compared with garlic extracts (a strong phenolic compound).

Effect on total antioxidant status

The plant extracts showed high total antioxidant properties. Figure 6 shows that aqueous *C. papaya* had the highest inhibition of beta carotene bleaching activity over the methanol and petroleum ether extracts and thus the highest antioxidant property at a concentration of 4 µg/ml, when compared with vitamin A positive control. Aqueous extracts of *C. papaya* and *F. zanthoxyloides* showed high total antioxidant properties. Aqueous extract

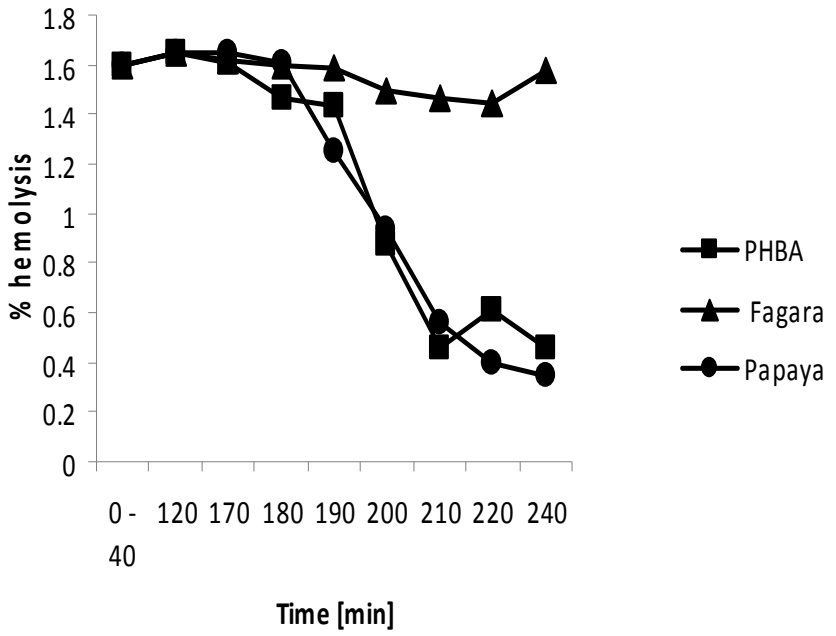

Figure 3. Effect of *Papaya* and *Fagara* extracts on RBC hemolysis.

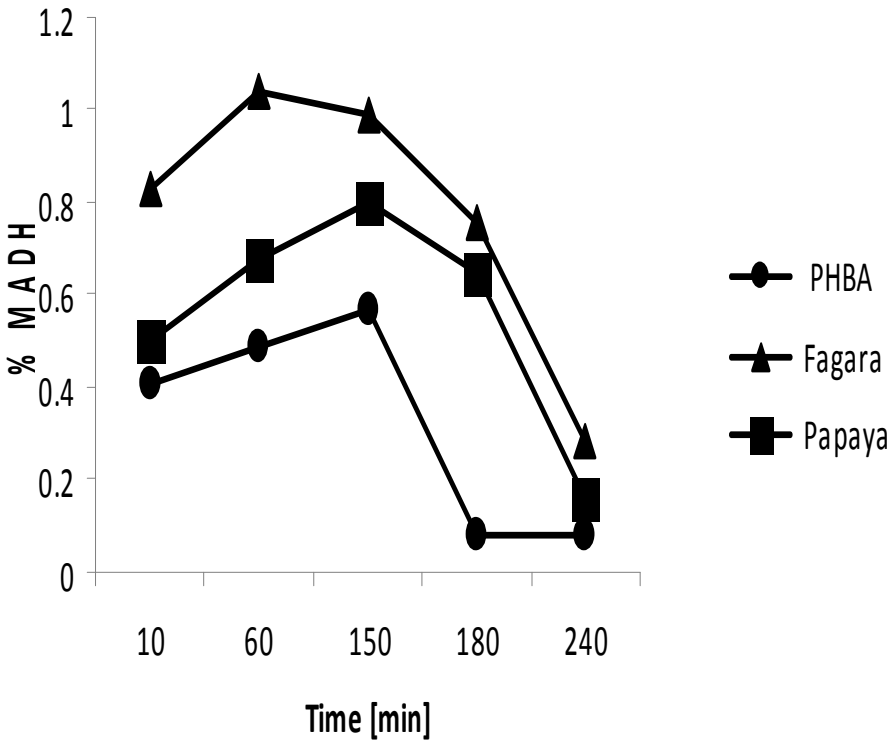

Figure 4. Effect of *Papaya* and *Fagara* extracts on Met-Hb formation (% MADH).

of papaya exhibited a higher and more stable antioxidant activity than the aqueous extract of *Fagara*, but both extracts had higher activity over vitamins A and E at 4 µg/ml concentration as shown in Figure 7. Methanol extracts of *C.papaya* and *F. zanthoxyloides* also showed high total antioxidant properties. Methanol extract of *Fagara* exhibited a higher antioxidant activity than the methanol extract of papaya. Both extracts had higher activity over vitamins A and E but gave unstable activity as shown in Figure 8.

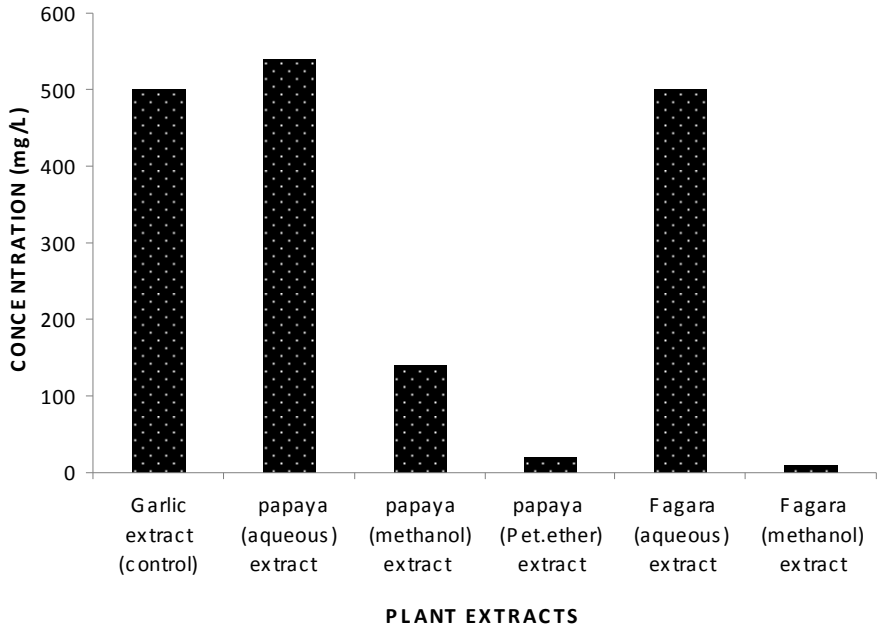

Figure 5. Total phenolic compounds present in *Papaya* and *Fagara* extracts.

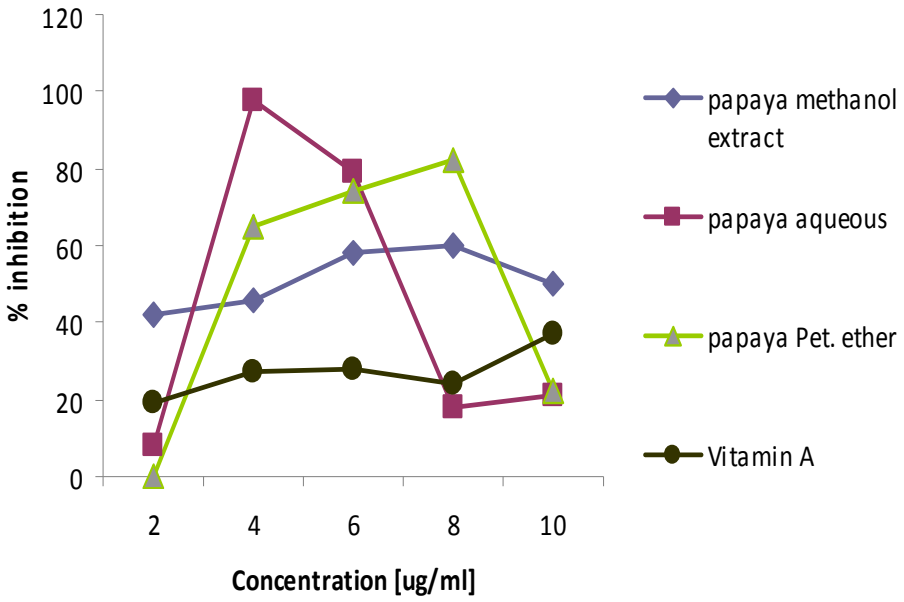

Figure 6. Antioxidant effects of *C. papaya* extracts via beta-carotene inhibition.

Antioxidant property of papaya and Fagara extract: Effect on oxidative stress and lipid peroxidation

The methanol extracts of both *C. papaya* and *F. zanthoxyloides* also showed considerably good antioxidant effects with regards to their effects on oxidative stress enzymes (SOD, CAT and GST) and lipid peroxidation in SS serum samples. From Figure 9, it can be seen that both *F. zanthoxyloides* and *C. papaya* methanol extracts inhibited oxidative stress appreciably in HbSS samples when compared with vitamin C extracts as shown by the decreased level of SOD enzymes after supplementation and in the absence of any extract. *Papaya* lowered SOD activity more than *Fagara*. The methanol extract of *F. zanthoxyloides* had stronger antioxidant activity and resisted the effect of oxidative stress as seen by CAT enzymes levels when compared with methanol extract of *C. papaya.* It was however also observed that both methanol plant extracts did not give substantial resistance to oxidative stress when compared

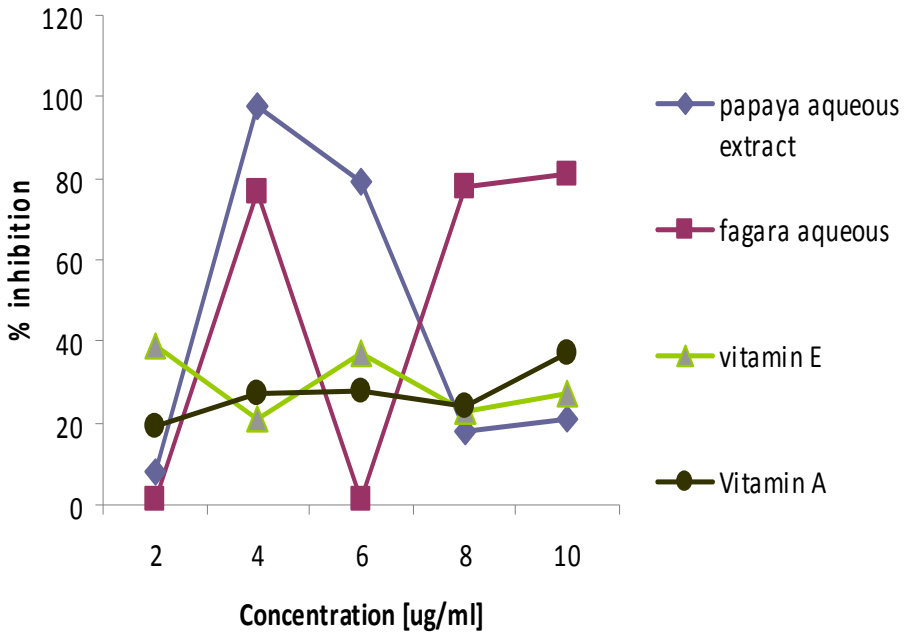

Figure 7. Antioxidant effects of aqueous extracts of *Papaya* and *Fagara* via beta-carotene inhibition.

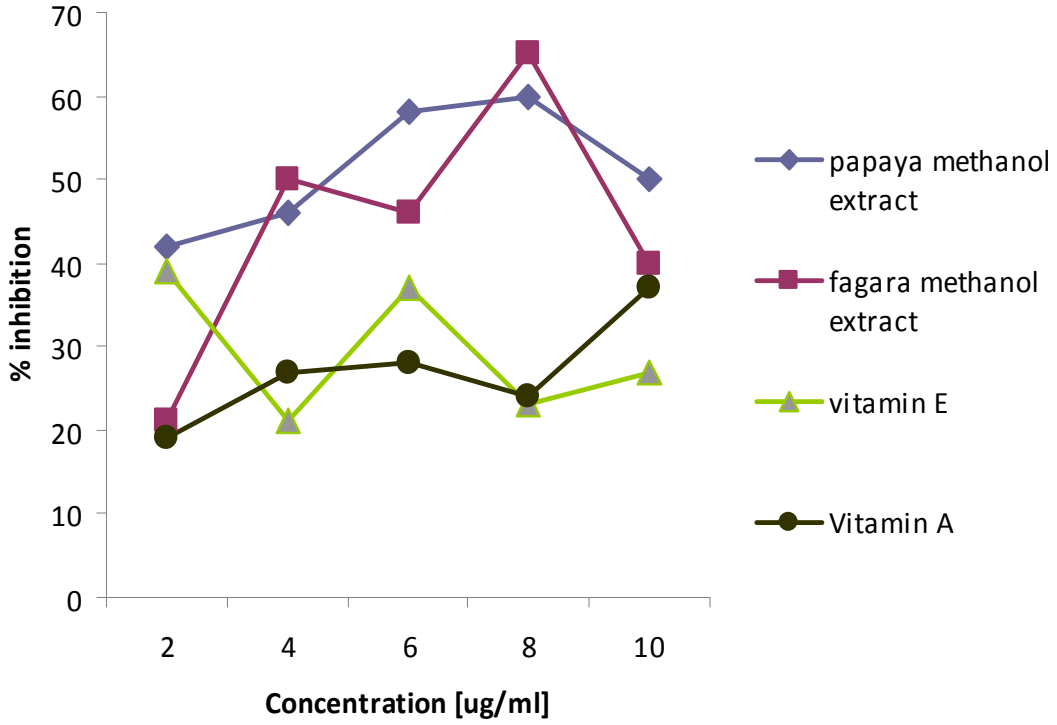

Figure 8. Antioxidant effects of methanol extracts of *Papaya* and *Fagara* via beta- carotene inhibition.

with vitamin C extract and in the absence of extracts as shown in Figures 10 and 11 shows that the methanol extract of *F. zanthoxyloides* induced higher resistance to oxidative stress in SS blood sample when compared with the papaya and vitamin C extracts. Figure 12 shows that the production of malondiadehyde (MDA) was reduced in SS serum sample incubated with *F. zanthoxyloides* and *C. papaya* methanol plant extracts (when compared with that of blood serum sample alone) hence reduction in lipid peroxidation activity.

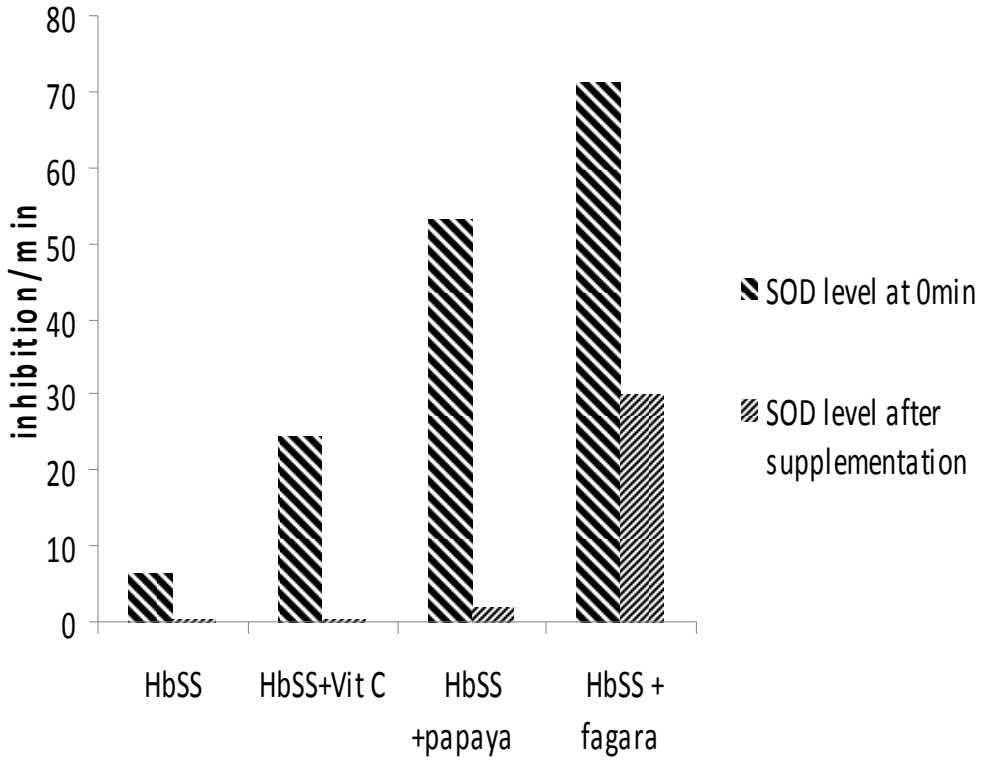

Figure 9. Effect of *Papaya* and *Fagara* extracts on SOD activity in HbSS samples.

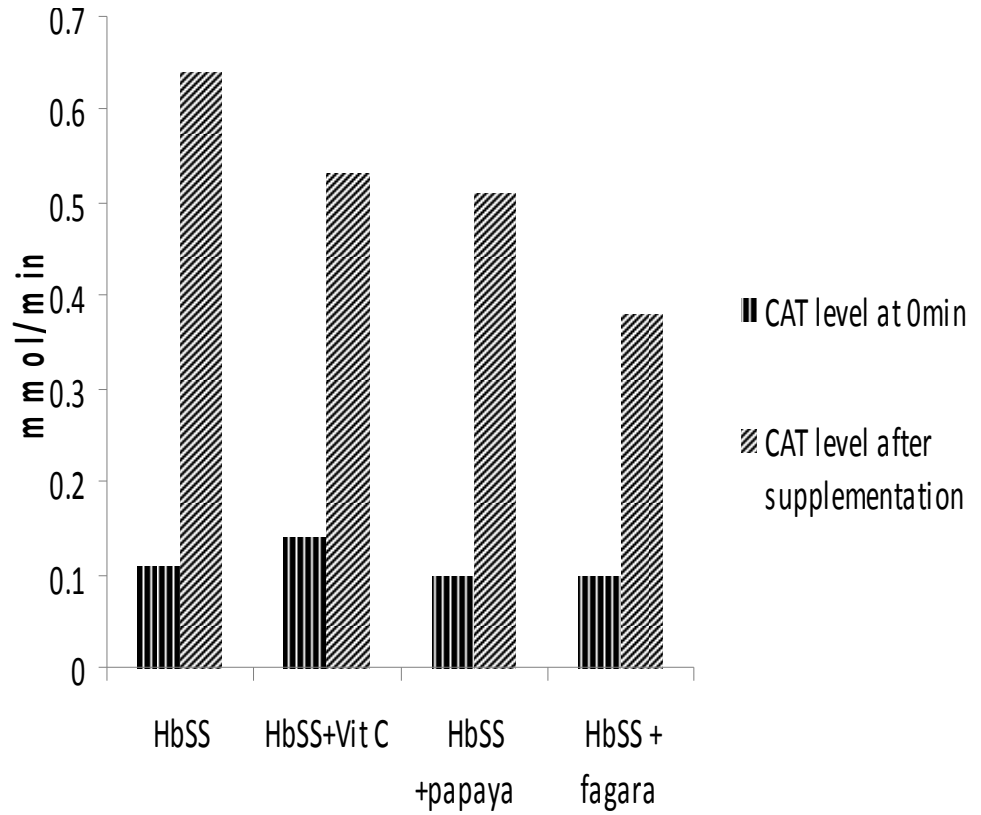

Figure 10. Effect of *Papaya* and *Fagara* extracts on catalase activity in HbSS samples.

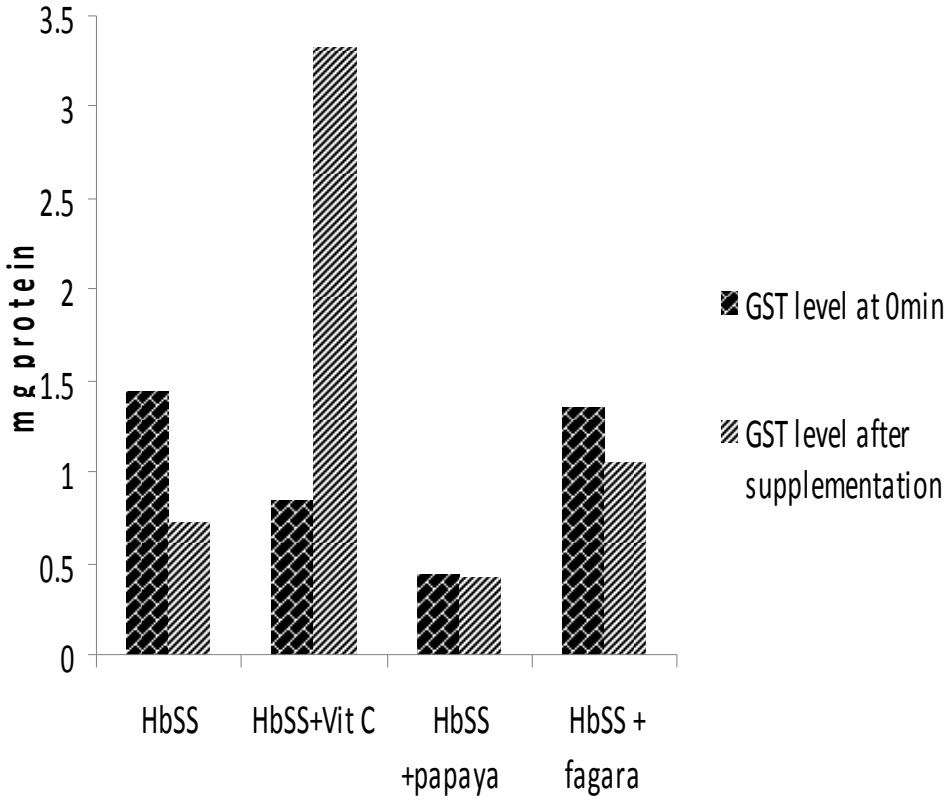

Figure 11. Effect of *Papaya* and *Fagara* extracts on glutathione activity in HbSS.

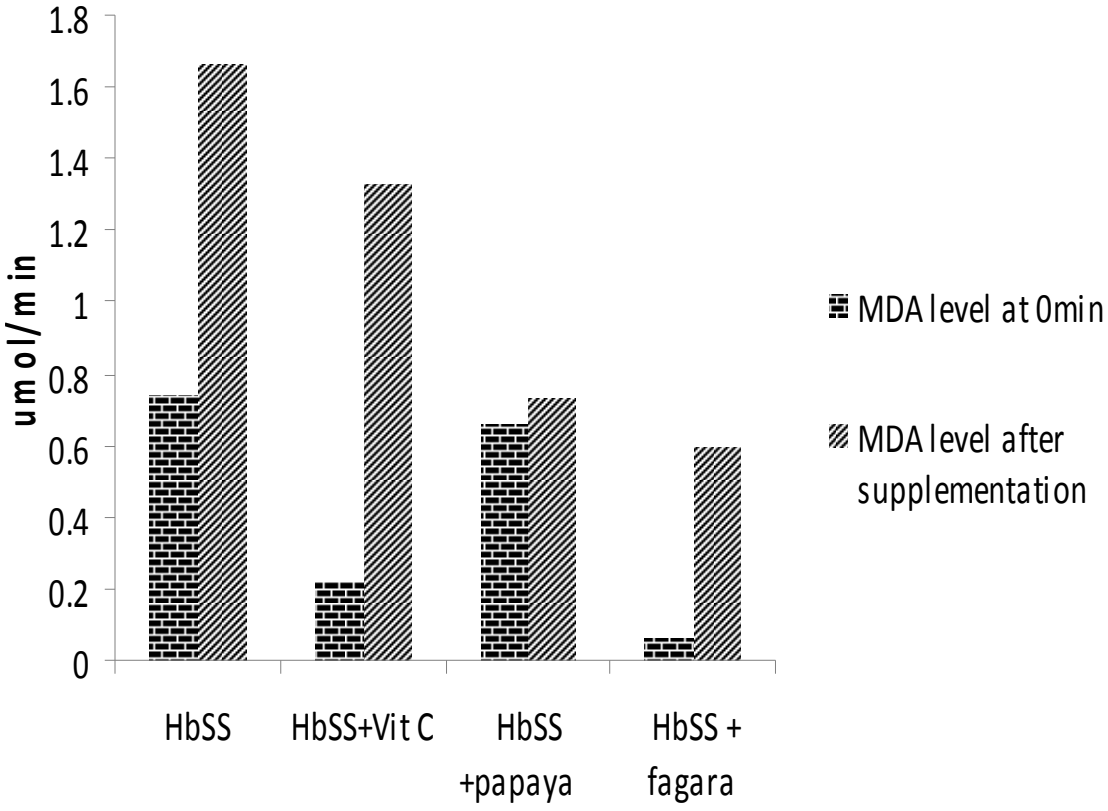

Figure 12. Effect of *Papaya* and *Fagara* extracts on lipid peroxidation [level of MDA] in HbSS.

DISCUSSION

For sickle cell disease (SCD), the study of antioxidants especially in various antisickling agents is of great importance because different antisickling agents have different degrees of effect. Antioxidants (scavengers of free radicals) are believed to be major components of these antisickling agents that add to their potential (Tatum and Chow, 1996). Thus, it is believed that the higher the antioxidant property of an antisickling agent, the higher its possible antisickling effect, as this enables it reduce oxidative stress that contributes to sickle cell crisis. In this research, the *C. papaya* leaf and *F. zanthoxyloides* root extracts induced membrane stabilization in dense sickle and normal red cells without deleterious effects on the erythrocytes. Aqueous extracts of *C. papaya* and *F. zanthoxyloides* have shown high total antioxidant properties (via β-carotene bleaching assay) and higher phenolic properties than garlic acid. This might explain why decoctions of these plants (used locally) over the years give relief to various oxidative stress associated diseases. The levels of the oxidative stress enzymes (SOD, CAT and GST) were reduced after blood samples had been incubated with the extracts. Lipid peroxidation, measured indirectly by the percentage of MDA inhibited by plant extract, was also reduced by the extracts.

These findings further confirm the antioxidant activity inherent in the plant extracts. HbSS individuals already in distress during oxidative stress-induced RBC membrane lysis do not need this situation aggravated by a plant extract that causes more oxidative stress to the erythrocyte membrane. The observed low levels of the oxidative stress enzymes show that papaya and *Fagara* extracts can quickly mop up free-radicals produced during sickle cell crisis and thus help preserve the integrity of the membrane. The extracts protected membrane integrity resulting in a reduction of RBC hemolysis during osmotic and oxidative stress without met-hemoglobin formation. This membrane stabilization effect is an additional benefit of the antisickling plants and confirms earlier reports on the membrane-stabilizing properties of the extracts on red blood cells as measured by their osmotic fragility (Imaga et al., 2009). The plant extracts' drastic antisickling effects could have been potentiated by their antioxidant activity and ability to maintain RBC membrane integrity under osmotic or oxidative stress.

Conclusion

C. papaya dried leaf extract and *F. zanthoxyloides* roots when used in 1 to 10 mg/ml concentrations *in vitro*, have no deleterious effects on erythrocytes and possess potent antioxidant activity which may be responsible for their observed antisickling action.

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