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Der Pharmacia Lettre, 2023, 15 (6): 01-27

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ISSN 0975-5071
USA CODEN: DPLEB4

In Vitro Antioxidant Characterization of Extracts of *Ficus Thonningii, Jatropha Tanjorensis* and *Justicea Carnea*: Implication on Sickle Cell Disorder

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***Received**: 01-Jul-2022, Manuscript No. DPL-22-63610; **Editor assigned**: 04-Jul-2022, PreQC No. DPL-22-63610 (PQ); **Reviewed**: 24-Jul-2022, QC No. DPL-22-63610; **Revised**: 21-Apr-2023, Manuscript No. DPL-22-63610 (R); **Published**: 28-Apr-2023, DOI: 10.37532/0975-5071.2023.15.1.*

ABSTRACT

Antioxidants has been utilized in the management of oxidative stress related pathologies specifically sickle cell disorder. Here we analyze the antioxidants potential of *Ficus thonningii*, *Jatropha tanjorensis* and *Justicia carnea* and their pure isolates using DPPH, hydroxyl radical scavenging activity, reducing power, FRAP and phosphomolybdemum antioxidant. The extracts were isolated and purified using the harbone method and the structures of each isolate were elucidated using NMR analysis. The results of our analysis validate these plants use as medicinal and nutraceuticals in the management of sickle cell patients in Ebonyi state. The results of our analysis indicate that all the analyzed plant extracts showed a variable antioxidant activity, whereas the pure isolates had better antioxidant activity than the crude extract and confirms the use of these plants extracts in the management of sickle cell disorder. Therefore, our findings suggest that *Ficus thonningii*, *Jatropha tanjorensis* and *Justicia carnea* and their pure isolates are potential source of natural antioxidant in preventing oxidative stress related degenerative diseases in sickle cell anemia patients.

Keywords: Antioxidant, Ascorbic acid, Sickle cell disorder, *Ficus thonningii*, *Jatropha tanjorensis*, *Justicia carnea*.

INTRODUCTION

One in every 600 black's people in the united states has cell anemia in addition hemoglobin C disease and sickle cell β-thalassemia, which are other common genotypes of sickle cell disease, together are as common as sickle cell anemia. Sickle cell hemoglobin (hemoglobin S, α_2 β_2S) accounts for over half the hemoglobin inpatients with these disorders. There are no documented records on the number of sickle cell patient in Nigeria [1].

Oxidative stress is an imbalance between production of reactive oxygen species and antioxidant defenses. The redox stress triggers the activation of immune cells which release pro-inflammatory cytokines, reactive oxygen and nitrogen species that causes damage to biological molecules and induces imbalances in physiological and pathological pathways. Epidemiological and *in vivo* studies have provided evidence that dietary intake of antioxidant and anti-inflammatory

compounds is a key strategy for health promotion by lowering oxidative stress and inflammation. Currently, synthetic antioxidants, such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), and Tert-Butylhydroquinone (TBHQ) are widely used in the food industry. However, restriction on the synthetic antioxidants is being imposed because of their toxicity to liver and carcinogenicity. Lack of a large, readily accessible population for clinical studies have contributed to inadequacy in data gathering and understanding of SCD pathophysiology, therefore, the development and utilization of more effective antioxidants of natural origins are desired [2].

Antioxidants are used in the treatment of many disorders notably neoplasia but also including Sickle Cell Anemia (SCA), in which a phase 3 clinical trial of L-glutamine has recently been carried out. An important feature of SCA is oxidative stress. Increased levels of reactive oxygen species are released from activated endothelial and white cells, from ischemia reperfusion injury and from within RBCs, as HbS is more unstable than HbA breaking down into hemichromes and free iron. It is also likely that antioxidant provision is reduced, both within RBCs and without circulating in plasma. For example, sickle red cells have a lower redox ratio with reduced levels of reduced nicotinamide adenine dinucleotide. Oxidant stress can result in lipid and protein damage with important pathogenic sequelae. It may also increase the solute permeability of RBCs, which is in itself problematical. It is not surprising; therefore, that the possible beneficial roles of antioxidant provision have received considerable attention provision of antioxidant therapy to ameliorate the complications of SCA has been extensively investigated. Antioxidants have been shown to have several beneficial effects, protecting against RBC lipid peroxidation and increasing levels of reduced Glutathione (GSH) while reducing levels of reactive oxygen species [3].

SCA affects millions of people worldwide, especially in sub-Saharan Africa and India, where it results in considerable morbidity and mortality, as well as economic impact. Blood transfusion, antibiotic therapy and pneumococcal vaccination all ameliorate complications; however, this treatment remains largely supportive. Whereas hydroxyl urea has emerged as a specific approved drug, it is not without problems, and therefore, new effective therapies are keenly sought.

Oxidants damage the RBC membrane and cytoskeleton. They affect RBC deformability and rheology. They have also been shown to stimulate solute loss and therefore increase RBC dehydration [4].

Therefore, in this assay, the antioxidants activities of the methanol extract of *F. thonningii*, *Jatropha tanjorensis* and *Justicia carnea* as well as their purified isolated were evaluated since the anti-sickling characterizations of *Ficus thonningii*, *Jatropha tanjorensis* and *Justicia carnea* have been previously reported and they exist a relationship between antioxidants and anti-sickling activity.

MATERIALS AND METHODS

Identification and authentication

The plant part used in this study was identified by Prof. S.S.C Onyekwelu and authenticated by Prof. S.C Eze both from the department of applied biology, Ebonyi state university [5-8].

Extraction

Sequential solvent extraction of the plant part was successively carried out separately with solvents of increasing polarity: n-Hexane, chloroform, methanol and methanol water mixture (4:1). 10 kg of the pulverized leaves of each plant part each were weighed and soaked in the appropriate solvents in order of increasing polarity for about 72 hours. The mixture was filtered, and the filtrate heated in a water bath to one-tenth (1/10) of the volume at temperature of about 40°C. Each dried extract was weighed and stored at -4^{o}C. This extract was thereafter subjected to column chromatography.

Chromatographic separation

The crude extract was separated by column chromatographic technique. The glass column (150 x 1.5cm. ID) was packed with two third (2/3) the length with silica gel (70-230 mesh). The glass column was plugged with cotton wool at the bottom and a Polytetraflouroethylene (PTFE) stop cork, 100 ml of chloroform and methanol mixture (80:5 v/v) was poured into the column and allowed to drain to the level of the gel bed in order to condition the system. In each 15 g of the crude extract was subjected to column chromatography and eluted with hexane ethyl acetate (80:20, 70:30, 60:40, 50:50.), ethyl acetate (100%) and methanol (100%) gradients. Slurry of silica gel 70-230 mesh (600g) was made with the eluting solvent and packed into the glass column. The tap was opened to allow excess solvent to run off. 15 g of the hexane leaf extract was dissolved in the eluting

solvent and packed on top of the silica gel slurry with a pipette. As soon as the cake began to form on the column, glass wool fiber was placed on top of the extract and the eluting solvent was added. Collection of the eluent was done with 50 mL and 100 mL conical flasks. Further elution was done with increasing concentration gradients.

For the methanol leaf crude extract, elution was carried out using dichloromethane ethyl acetate (80:20, 70:30), ethyl acetate (100%), ethyl acetate-methanol (50:50) and methanol (100%) gradients. For the fractionation of chloroform leaf crude extract, elution was done with hexane dichloromethane gradients (60:40, 50:50), ethyl acetate (100%), ethyl acetate-methanol (50:50), and finally with 100% methanol. Elution of methanol water leaf extract was carried out with dichloromethane ethyl acetate (80:20), ethyl acetate (100%), methanol (100%).

Fractions collected were monitored with spotting on Thin Layer Chromatographic (TLC) plates and viewed under the visible UV light (254 nm). Plates were also placed in iodine chroma tanks to view the spots. A spray of 0.5% vanillin and 10% sulphuric acid was used on the plates, and the plates were dried in hot air oven at 110^{o}C for 1 hour and colour changes observed.

The pure fractions were subjected to further separation and purification on a silica gel column chromatography and on flash chromatography. The pure isolates in chloroform leaf extract were FTH1, and FTH2, whereas FTH3 was isolated from the MeOH:H_2O while JTR1 and JTR3 were active fractions in methanol leaf extract, then JCN1, JCN3 and JCN4 were active fractions isolated from the n-Hexane extract [9,10].

Further purification of the pure fractions

Fractions from column chromatography were further purified using a solvent system of petroleum ether: Chloroform (4:1) as the mobile phase, this revealed one major spot with minor spots, these fractions were further purified using flash chromatography (silica gel, mesh 230-400, 30 g) prior to each analysis. Elution was carried out with varying proportions of petroleum ether: Chloroform. Elution with solvent mixture of petroleum ether: Chloroform (5:1) yielded a major single spot on TLC with some minor impurities at the origin. The process was repeated and similar for all fractions. Concentration, drying and washing of the fractions severally with methanol afforded a total of ten pure isolates of which eight labeled FTH1, FTH2, FTH3 for the *F.thonningii*, pure isolates, JTR1, JTR3 for the *Jatropha tanjorensis* pure isolate and JCN1, JCN3 and JCN4 for the *Justicia carnea* isolates from the three medicinal plants was used in this assay. The pure fractions were further recrystallized three times, and the pure compounds washed with methanol. The purity of all fractions was monitored using TLC.

Recrystallization of the fractions

The fractions were dissolved in 50 ml of methanol and heated slightly at a temperature of 40^{o}C for 15 minutes in a water bath. Then the fractions were removed and allowed to cool in a refrigerator and filtered using a filter paper the process was repeated three times and the melting point of each pure fraction was determined.

Melting point determination

The various fractions were placed in a thin walled capillary tube and closed at one end. The capillary tube, which contained the sample, were attached to a thermometer, and heated slowly. The temperature range over which the sample is observed to melt is taken as the melting point.

Nuclear Magnetic Resonance (^{1}H-NMR and ^{13}C-NMR)

The ^{1}H-NMR spectral analyses were carried out using bruker 500 MHz nuclear magnetic resonance spectrometer to determine types of protons in the sample using the chemical structures as well as the joules coupling constant. The ^{13}C-NMR analyses were carried out using the same bruker 500 MHz nuclear magnetic resonance spectrometer, the samples were dissolved in the appropriate solvent. This was used to determine the types and number of carbon atoms present in each sample. The structural elucidation was done using a combined NMR analysis of H-NMR, C-NMR, DEPT-135, TOCSY, COSY, HSQC and HMBC.

Scavenging activity of DPPH radical

The DPPH free radical scavenging assay was carried out for the evaluation of the antioxidant activity. A solution of varying concentration (1000, 500, 250, and 125 μg ml^{-1}) of each crude extracts, isolates and ascorbic acid was prepared and 1 ml of methanol solution of DPPH 0.1 mm was added. The mixture was kept in the dark at room temperature for 30 min and the absorbance was measured at 517 nm against a blank. The percentage radical scavenging activity was calculated using equation 1.

$$\text{Percentage DPPH radical scavenging activity} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)} \times 100}{\text{Absorbance (control)}} \quad (1)$$

The IC_{50} value (µg/mL) is the effective concentration at which DPPH radicals were scavenged by 50% and the value was obtained by interpolation from linear regression analysis.

Hydroxyl radical scavenging activity

The scavenging activity of the extract on hydroxyl radical was measured according to a previously described method. Various concentration (1000, 500, 250, and 125 µg mL^{-1}) of each crude extracts, isolates and ascorbic acid was prepared and 60 µL of $FeCl_3$ (1 mM), 90 µL of 1,10-phenanthroline (1 mM), 2.4 mL of 0.2 M phosphate buffer, pH 7.8 and 150 µL of H_2O_2 (0.17 M) was added respectively. The mixture was then homogenized and incubated at room temperature for 5 min. The absorbance was read at 560 nm against the blank. The percentage hydroxyl radical scavenging activity was calculated using equation 2 [11,12].

$$\text{Percentage OH radical scavenging activity} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)} \times 100}{\text{Absorbance (control)}} \quad (2)$$

The extract concentration providing 50% inhibition (IC_{50}) was calculated and obtained by interpolation from linear regression analysis.

Reducing power assay

Fe^{3+} reducing power of the crude extracts and isolated compound was determined according the method of Oyaizu. The crude extracts, isolated compounds and ascorbic acid (1 mL) of various concentrations (1000, 500, 250, and 125 µg mL^{-1}) were mixed with phosphate buffer (pH 6.6, 0.2 M, 2.5 mL) and potassium ferricyanide (1%, 2.5 mL) was added to the mixture. A portion of the resulting mixture was mixed with $FeCl_3$ (0.1%, 0.5 mL) and the absorbance measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated higher reductive potential of the sample [13,14].

Total antioxidant activity by Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP method was used to determine the total antioxidant activity which measures the reduction of ferric ion to the ferrous form in the presence of antioxidant compounds. The fresh FRAP reagent consist of 500 mL of acetate buffer (300 mM pH 3.6), 50 mL of 2, 4, 6-Tri (2-Pyridyl)-s-Triazin (TPTZ) (10 mM), and 50 mL of $FeCl_3 \bullet 6H_2O$ (50 mM). For the assay, 1000, 500, 250, and 125 µg mL^{-1} of each crude extract, isolated compounds and ascorbic acid was mixed with 2 mL of FRAP reagent and the optical density was read after 180 seconds at 593 nm against the blank.

Total antioxidant activity by phosphomolybdenum assay

The total antioxidant capacities of the crude extracts and isolated compounds were determined by phosphomolybdenum method according to the procedure described by Prieto, et al. using ascorbic acid as a standard. The assay is based on the reduction of Mo (VI) to Mo (V) by the crude extracts and isolated compounds and subsequent formation of green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.1 mL of various concentrations of crude extracts, isolated compounds and ascorbic acid (1000, 500, and 250 and 125 µg/mL) and DCMF (7, 3.5, and 1.75 mg/mL) was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95^{o}C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. A typical blank contained 1 mL of the reagent solution and the appropriate volume of the solvent (0.1 mL) and incubated under the same conditions. Ascorbic acid was used as standard. The antioxidant activity was expressed as Ascorbic Acid Equivalent (AAE).

Statistical analysis

Each test was performed in triplicate and the results expressed as mean ± standard error of the mean. The ANOVA analysis Student Newman Keuls (SNK) Posthoc test, ($P<0.05$), homogeneity of variance, and descriptive statistics was carried out on each data from each of the crude extracts using sigma plot version 14 for windows software. The IC_{50} was determined with the multiple regression analysis using Microsoft excel for windows [15].

RESULTS AND DISCUSSION

Results of structural elucidations

FTH1 (4-methoxy-7H-furo (3, 2-g) chromen-7-one): White crystalline solid; Rf 0.68; melting point 188-190^{o}C. ^{13}C (500 MHZ, $CDCl_3$): C-1 (105.09 ppm), C-2 (144.79 ppm), C-3 (112.58 ppm), C-4 (149.55 ppm), C-5 (106.32 ppm), C-6 (139.33 ppm), C-7 (112.48 ppm), C-8 (161.30 ppm), C-9 (152.67 ppm), C-10 (93.78 ppm), C-11 (158.36 ppm), C-12 (60.06 ppm).^{1}H (500 MHZ, $CDCl_3$): H-1 (7.02 ppm), H-2 (7.59 ppm), H-6 (8.16), H-7 (6.28 ppm), H-10 (7.13 ppm), H-12 (4.27 ppm). The ^{13}C, ^{1}H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for FTH1 matched those of 4-methoxy-7H-furo (3, 2-g) chromen-7-one and was thus assigned. Molar mass 216.18 g/mol (Figure 1).

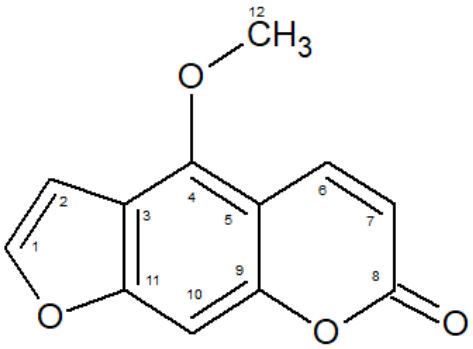

Figure 1: 4-methoxy-7H-furo (3, 2-g) chromen-7-one ($C_{12}H_8O_4$).

FTH2 (3, 4-dihydroxybenzeoic acid)

Gray crystalline solid; Rf 0.72; melting point 221-223^{o}C ^{13}C (500 MHZ, D_2O): C-1 (125.24 ppm), C-2 (119.63 ppm), C-3 (150.04 ppm), C-4 (146.07 ppm), C-5 (131.39 ppm), C-6 (118.12 ppm), C-7 (177.94 ppm). ^{1}H (500 MHZ, D_2O): H-2 (7.42 ppm), H-5 (6.92 ppm), C-6 (7.39 ppm). The ^{13}C, ^{1}H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for FTH2 matched those of 3, 4-dihydroxybenzeoic acid and was thus assigned. Molecular weight of 154.12 g/mol (Figure 2).

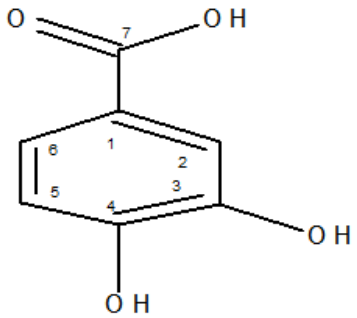

Figure 2: 3, 4-dihydroxybenzeoic acid ($C_7H_6O_4$).

FTH3 (Methyl (2E)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enoate)

Yellowish brown crystal; Rf 0.83; melting point 63^{o}C-65^{o}C ^{13}C (500 MHZ, DMSO-d6): C-1 (125.55), C-2 (111.29), C-3 (147.91), C-4 (149.36), C-5 (114.19), C-6 (123.12), C-7 (145.10), C-8 (115.50), C-9 (167.09), 9-OCH_3 (51.22), 3-OCH_3 (55.71). ^{1}H (500 MHZ, DMSO-d6): H-1 (7.12), H-5 (6.80), H-6 (7.32), H-7 (7.57), H-8 (6.50), 9-OCH_3 (3.70), 3-OCH_3 (3.81), 4-OH (6.46). The ^{13}C, ^{1}H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for FTH2 matched those of methyl (2E)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enoate and was thus assigned. Molecular weight 208.21 g/mol (Figure 3).

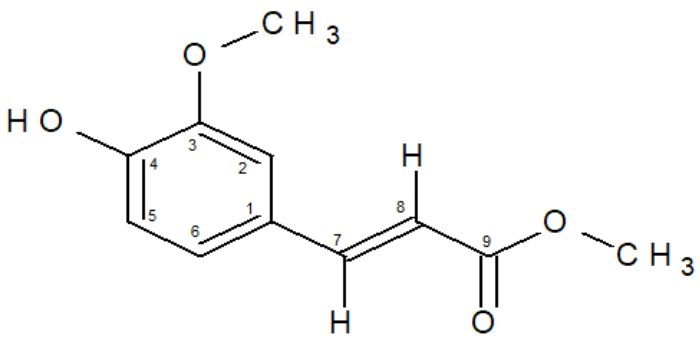

Figure 3: Methyl (2*E*)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enoate ($C_{11}H_{12}O_4$).

JTR1: α-tocopherol

Yellowish brown oil; Rf 0.66; melting point 2°C-3°C; ^{1}H-NR (500 MHz, $CDCl_3$): δH 7.18 ppm (6-OH), 2.38 ppm (H-4), 1.55 ppm (H-3'), 2.16 ppm (H-1'), 2.11 ppm (H-2'), 1.55 ppm (H-3), 1.49 ppm (H-11) 1.24-1.39 (H-12, H-14, H-18), 1.20-1.24 ppm (H-13, H-15, H-16, H-20), 1.10 ppm (H-4'), 1.04-1.08 (H-17, H-19, H-21, H-22), 0.73-0.76 (H-5', H-6', H-7', H-23). 13C-NMR (500 MHz, $CDCl_3$): δ C 145.52 ppm (C-6), 145.54 ppm (C-9), 122.61 ppm (C-8), 117. 35 ppm (C-10), 121.00 ppm (C-7), 118.46 ppm (C-5), 74.52 ppm (C-2), 39.87 ppm (C-11), 37.46 ppm (C-13), 37.39 ppm (C-15), 37.29 ppm (C-17), 32.79 ppm (C-19), 39.37 ppm (C-21), 32.70 ppm (C-14), 31.54 ppm (C-18), 19.69 ppm (C-4), 27.98 ppm (C-22), 24.82 ppm (C-20), 27.98 ppm (C-22), 22.64 ppm (C-23), 20.77 ppm (C-12), 19.65 ppm (C-5'), 19.60 ppm (C-6'), 31.48 ppm (C-3), 11.29 ppm (C-1'), 12.22 ppm (C-2'), 11.79 ppm (C-3'), 23.80 ppm (C-4'), 22.74 ppm (C-7'). Combination of ^{1}H, ^{13}C, DEPT-135, TOCSY, COSY, HSQC and HMBC confirmed the structure as alpha tocopherol 430.71g/mol (Figure 4).

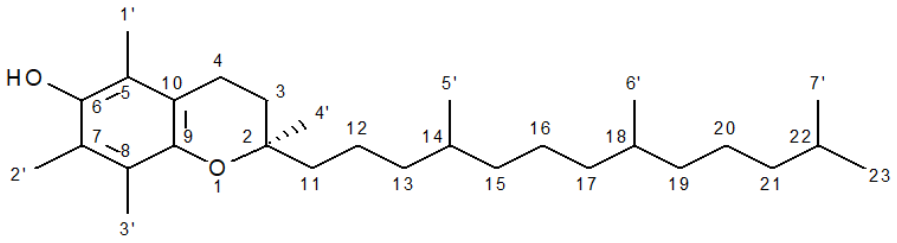

Figure 4: α-tocopherol ($C_{29}H_{50}O_2$).

JTR 3: (2S)-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one ($C_{15}H_{12}O_5$)

White solid; Rf 0.81; melting point 253-255°C ^{13}C (500 MHZ, DMSO-d6): C-2 (80.51 ppm), C-3 (42.06 ppm), C-4 (197.83 ppm), C-5 (165.50 ppm), C-6 (97.06 ppm), C-7 (165.50 ppm), C-8 (96.18 ppm), C-9 (168.36 ppm), C-10 (103.36 ppm), C-1' (131.09 ppm), C-2' (129.10 ppm), C-3' (116.33 PPM), C-4' (159.06 PPM), C-5' (116.32), C-6' (129.32). ^{1}H (500 MHZ, DMSO-d6): H-2 (5.30 ppm), H-3 (α-3.10, β-2.69), H-6 (5.90 ppm), H-8 (5.89 ppm), H-2' (7.30 ppm), H-3' (6.80 ppm), H-5' (6.83 ppm), H-6' (7.33 ppm). The ^{13}C, ^{1}H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for JTR3 matched those of (2S)-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one and was thus assigned. Molecular mass of 272.25278 g/mol (Figure 5).

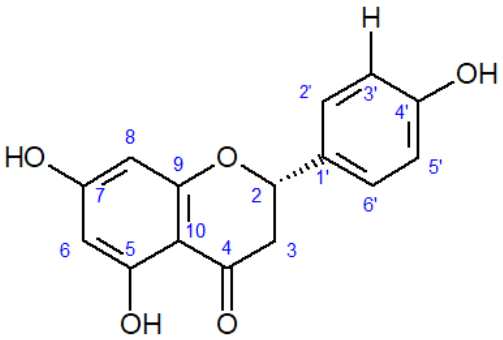

Figure 5: (2S)-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one ($C_{15}H_{12}O_5$).

JCN1: (1S,3R,4R,5R)-3-(((2E)-3-(3,4-dihydroxyphenyl) prop-2-enoyl) oxy)-1,4,5-trihydroxycyclohexanecarboxylic acid)

White powdered solid; Rf 0.73; melting point 206-208^oC ^{13}C (500 MHZ, D_2O): C-1 (129.44 ppm), C-2 (118.89 ppm), C-3 (148.84 ppm), C-4 (147.08 ppm), C-5 (117.70 ppm), C-6 (125.47 ppm), C-7 (150.17 ppm), C-8 (117.04 ppm), C-9 (171.87 ppm), C-1' (79.62 ppm), C-2' (41.20 ppm), C-3' (75.64 ppm), C-4' (73.86 ppm), C-5' (73.48 ppm), C-6' (40.04 ppm), C-7' (183.68 ppm). ^1H (500 MHZ, D_2O): H-2 (7.01 ppm), H-5 (6.87 ppm), H-6 (6.88 ppm), H-7 (7.54 ppm), H-8 (6.27 ppm), H-2' (2.22 ppm), H-2' (5.34 ppm), H-4' (3.85 ppm), H-5'(4.25 ppm), H-6' (2.00 ppm). The ^{13}C, ^1H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for JCN1 matched those of 1S,3R,4R,5R)-3-(((2E)-3-(3,4-dihydroxyphenyl) prop-2-enoyl] oxy)-1,4,5 trihydroxycyclohexanecarboxylic acid (chlorogenic acid) and was thus assigned. 354.31 g/mol and a molecular formula of $C_{16}H_{18}O_6$ (Figure 6).

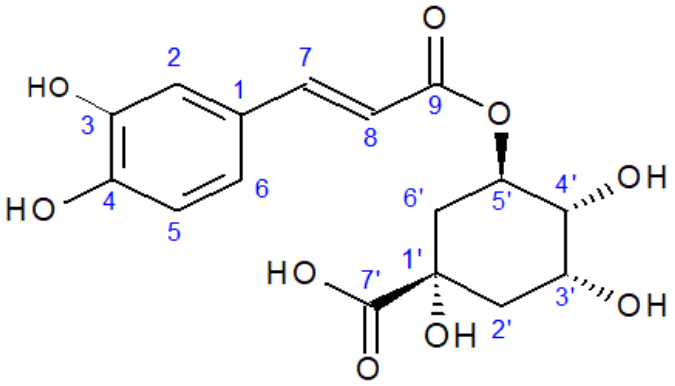

Figure 6: 1*S*,3*R*,4*R*,5*R*)-3-(((2*E*)-3-(3,4-dihydroxyphenyl) prop-2-enoyl) oxy)-1,4,5 trihydroxycyclohexanecarboxylic acid ($C_{16}H_{18}O_6$).

JCN3 (Quinoline-2-carboxylic acid)

Light brown crystalline solid; Rf 0.77; mp 155-156^oC ^{13}C (500 MHZ, D_2O): C-2 (156.89 ppm), C-3 (123.04 ppm), C-4 (140.96 ppm), C-5 (130.85 ppm), C-6 (130.62 ppm), C-7 (130.43 ppm), C-8 (131.15 ppm), C-4a (133.15 ppm), C-9 (175.78 ppm), C-8a (148.78 ppm). ^1H (500 MHZ, D_2O): H-8 (8.05 ppm), H-7 (7.61 ppm), H-6 (7.59 ppm), H-5 (7.78 ppm), H-4 (8.03 ppm), H-3 (7.59 ppm), H-2 (8.31 ppm) [16-18]. The ^{13}C, ^1H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for JCN1 matched those of quinoline-2-carboxylic acid and was thus assigned. $C_{10}H_7NO_2$ and a molecular mass of 173.17 g/mol (Figure 7).

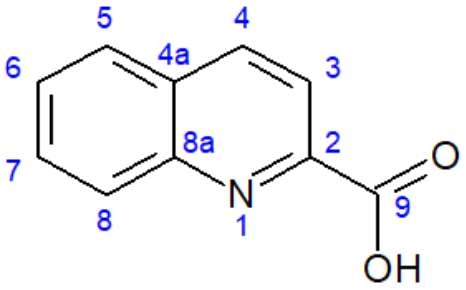

Figure 7: Quinoline-2-carboxylic acid ($C_{10}H_7NO_2$)

JCN4 ((9Z)-octadec-9-enoic acid)

Yellow liquid; Rf 0.86; melting point 14-16^oC (500 MHZ, MeOH): C-1 (177.60 ppm), C-2 (35.11 ppm), C-3 (26.25 ppm), C-4 (30.43 ppm), C-5 (30.40 ppm), C-6 (30.86 ppm), C-7 (33.22 ppm), C-8 (30.18 ppm), C-9 (131.62 ppm), C-10 (131.72 ppm), C-11 (30.37 ppm), C-12 (33.75 ppm), C-13 (30.75 ppm), C-14 (30.93 ppm), C-15 (30.64 ppm), C-16 (33.79 ppm), C-17 (23.91 ppm), C-18 (14.62 ppm). ^1H (500 MHZ, MeOH): H-2 (2.29 ppm), H-3 (1.55 ppm), H-4 (1.29 ppm), H-5 (1.29 ppm), H-6 (1.29 ppm), H-7 (1.32 ppm), H-8 (1.99 ppm), H-9 (5.39 ppm), H-10 (5.38 ppm), H-11 (1.99 ppm), H-12 (1.29 ppm), H-13 (1.29 ppm), H-14 (1.29 ppm), H-15 (1.32 ppm), H-16 (1.32 ppm), H-17 (1.29 ppm), H-18 (0.88 ppm) [19,20]. The ^{13}C, 1H, DEPT-135, TOCSY, COSY, HSQC and HMBC spectra for JCN1 matched those of (9Z)-octadec-9-enoic acid ($C_{18}H_{34}O_2$) and was thus assigned (Figure 8).

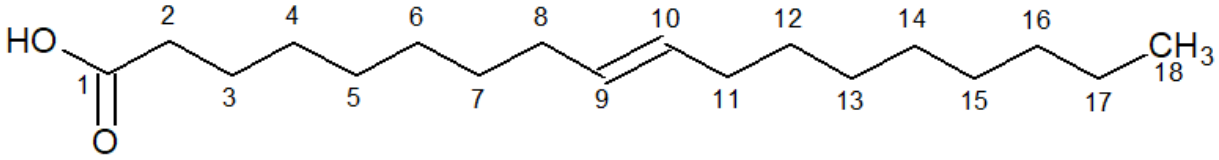

Figure 8: (9Z)-octadec-9-enoic acid ($C_{18}H_{34}O_2$)

Results of antioxidant analysis for crude isolates

DPPH scavenging activity: DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate radical scavenging activity. In DPPH radical scavenging assay, antioxidants react with DPPH (deep violet color) and convert it to yellow coloured α, α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the radical scavenging potential of the antioxidant. The extract neutralized the DPPH free radicals *via* hydrogen donating activity.

The IC_{50} of FTH, JTR and JCN were 892.258 ± 2.983 µg/mL (y=8.8836ln(x)-10.353; R^2=0.3552), 10.490 ± 0.002 µg/mL (y=4.0865ln(x)+40.395; R^2=0.3839) and 207.649 ± 3.695 µg/mL (y=16.82ln(x)-39.749: R^2=0.8298) respectively while those of ascorbic acid was 114.140 ± 5.135 µg/mL (y=24.456ln (x)-65.83; R^2=0.9082). The DPPH scavenging activity for JCN and ascorbic acid was increased in a concentration dependent manner while those of FTH and JTR were concentration independent (figure 9), the extracts however inhibited the formation of DPPH radical in a concentration dependent manner ($P<0.001$). The dose independent response of FTH and JTR were attributed to the presence of interfering impurities (analytes) at different concentration due to the unfractionated nature of the samples into uniform pure components to ensure homogeneity of components as obtained in pharmaceutical drugs. The % inhibition of JTR (10.490 ± 0.0320 µg/mL) was very significantly ($P<0.001$) higher than those of the standard drug (Ascorbic acid) while those of FTH and JCN were very significantly ($P<0.001$) lower than those of ascorbic acid.

A lower IC_{50} value portends higher activity *in vitro*. The maximum % inhibition for FTH, JTR and JCN were 54.433 ± 0.001%, 70.690 ± 0.013% and 79.475 ± 0.003% respectively (Figure 9). Although ascorbic acid showed a higher % inhibition than JTR, the IC_{50} of JTR was lower than those of the ascorbic acid; this is because IC_{50} is a cumulative effect of a drug over a given range of concentration under study and not just the effect at one or two unique concentrations.

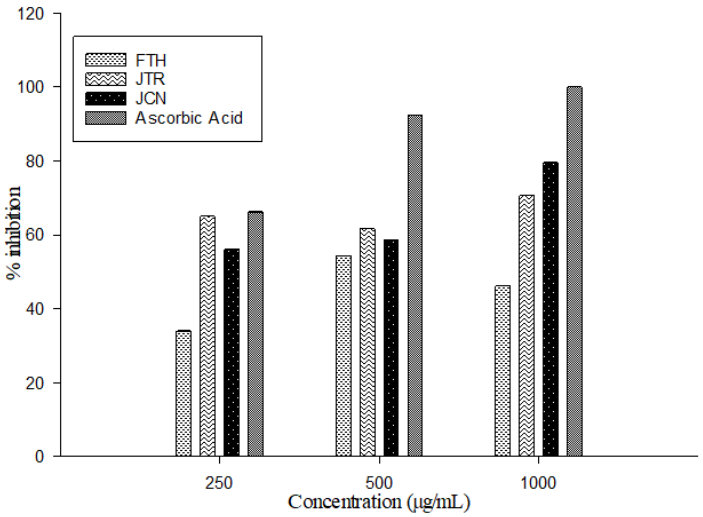

Figure 9: % inhibition of DPPH by FTH, JTR JCN and ascorbic acid

The antioxidant activity of the natural plant extracts is generally attributed to their hydrogen donating ability. It is well known that free radicals cause auto-oxidation of unsaturated lipids in food. Antioxidants are believed to intercept the free radical chain of oxidation by donating hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate oxidation of the lipids. At all the concentration determined, there were significant difference ($P<0.001$) between the extract and the standard drug (ascorbic acid).

Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. This radical has the capacity to conjugate with nucleotides in DNA and cause strand breakage, having far reaching implications leading ultimately to carcinogenesis, mutagenesis and cytotoxicity. In addition, these species are considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids of which lipid per oxidation has been implicated in sickle cell disorder.

From Figure 10, the estimated IC_{50} for FTH (IC_{50}=523.095 ± 13.629; y=67.854ln(x)-374.75; R^2=0.9924) and JCN (IC_{50}=503.733 ± 7.562; y=37.785ln(x)-185.1; R^2=0.9995) while those of Ascorbic acid (IC_{50}=298.989 ± 14.462; y=66.797ln(x)-330.77; R^2=0.8541). The IC_{50} for JTR extract was not determined because at maximum concentration the observed % inhibition was 35.751 ± 0.002% and further dose variations did not yield an inhibition greater than 50%. The % inhibition of hydroxyl radical for ascorbic acid was very significantly ($P<0.001$) higher than those of the extracts at all concentration determined.

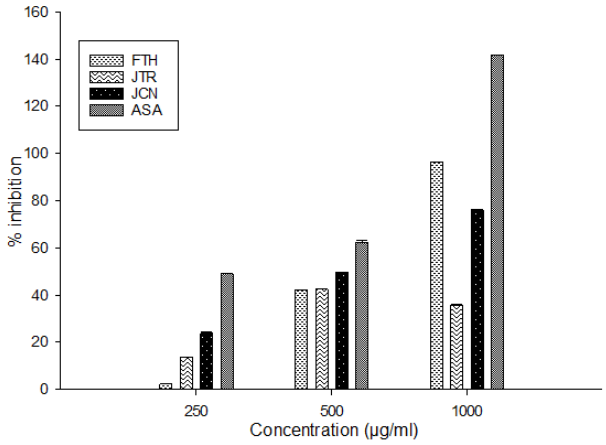

Figure 10: % Inhibition by hydroxyl radical scavenging of FTH, JTR JCN and ascorbic acid

Circulating sickle cells are prone to oxidative damage due to altered membrane structure, exposure to high oxygen tension and the presence of excess serum iron and since iron is a potent promoter of potent hydroxyl radical formation, depleting chain breaking antioxidants and thereby potentiating tissue damage and increasing erythrocyte fragility therefore the use *F. thonningii*, *Jatropha tanjorensis* and *Justicia carnea* as natural sources of antioxidant can mitigate processes that trigger erythrocyte fragility and inhibit as well as scavenge hydroxyl radical as evidenced from the results of the hydroxy radical scavenging activity.

Reducing power

The reducing ability of a compound generally depends on the presence of reductants which have been exhibiting anti-oxidative potential by breaking the free radical chain and donating a hydrogen atom. The presence of reductants in *F. thonningii*, *Jatropha tanjorensis* and *Justicia carnea* extract causes the reduction of the Fe^{3+}/ferricynide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Purssian blue at 700 nm. From Figure 11, 12 and 13 it was observed that the results of the reducing power of the analyzed extracts (as shown by their absorbance at 700 nm) correlates with increasing concentrations. There was a very significant difference ($P<0.001$) between the reducing power of the isolates and those of the standard drug, hence, the reducing power of ascorbic acid was significantly higher than those of the extracts.

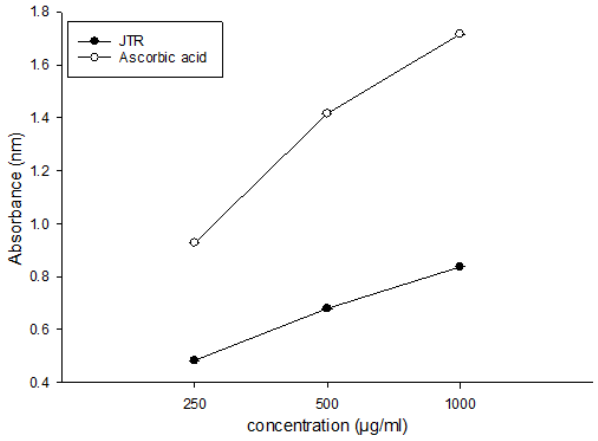

Figure 11: Reducing power assay of MeOH extract of JTR in comparison to ascorbic acid.

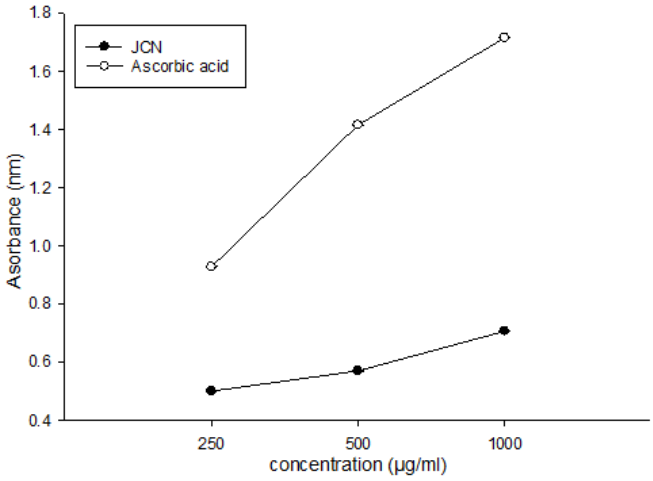

Figure 12: Reducing power assay of MeOH extract of JCN in comparison to ascorbic acid.

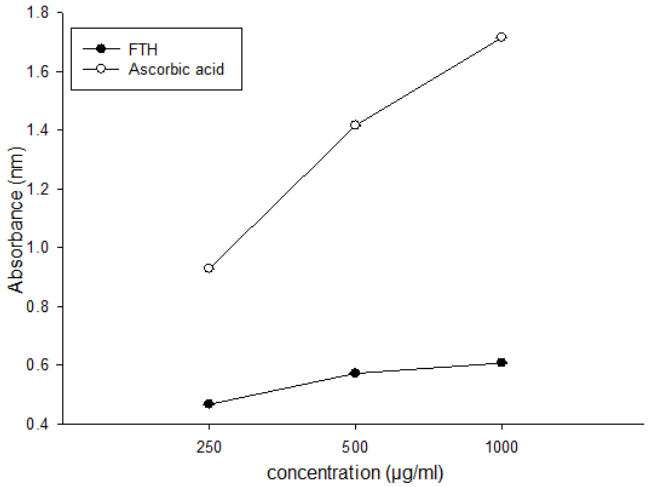

Figure 13: Reducing power Assay of MeOH extract of FTH in comparison to the standard drug ascorbic acid.

Ferric Reducing Antioxidant Power (FRAP)

It has been shown that a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species. Basically, the presences of compounds that possess the reducing properties apply their effect by donating a hydrogen atom that results in interruption of the free radical chain.

In FRAP assay, the antioxidants presence in the sample act as a reductant in a redox linked colorimetric reaction. Most commonly known reservoir of natural antioxidants is plants, which includes polyphenols, ascorbate, tocopherols, and terpenoids. To provide the body with a constant supply of antioxidants through dietary supplements is essential although the body has effective defense mechanisms that protect it against oxidative stress because the ability of these defensive mechanisms decreases as one ages. Several biomolecules such as proteins, lipoproteins, lipids and DNA are easily damaged due to the actions of ROS. A research has shown that the consumption of antioxidants, such as vitamin C, lowers the chances of cardiovascular diseases, cancers and neurodegenerative diseases. The observed increase in absorbance for FRAP assay correlates well with increase in concentration (Figure 14). The FRAP value at 1000 µg/mL for FTH, JTR and JCN was 365.411 ± 0.791 mg AAE/gm, 406.328 ± 1.524 mg AAE/gm and 335.0904 ± 0.791 mg AAE/gm respectively. The FRAP value of standard drug (ascorbic acid) was very significantly ($P<0.001$) higher than those of the extracts at various concentration analyzed.

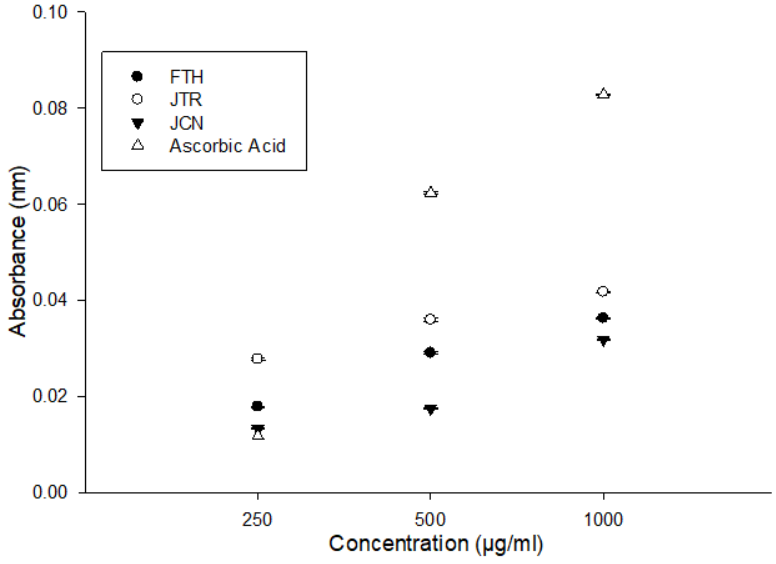

Figure 14: FRAP Assay of MeOH extract of FTH in comparison to the standard drug.

Phosphomolybdenum assay

Phosphomolybdenum assay is based on the reduction of phosphate-Mo (VI) to phosphate-Mo (V) by the analyte and subsequent formation of a bluish green colored phosphate/Mo (V) complex at acid pH. The phosphomolybdenum method is routinely applied in the laboratory to evaluate the total antioxidant capacity of plant extracts (Figures 15 and 16).

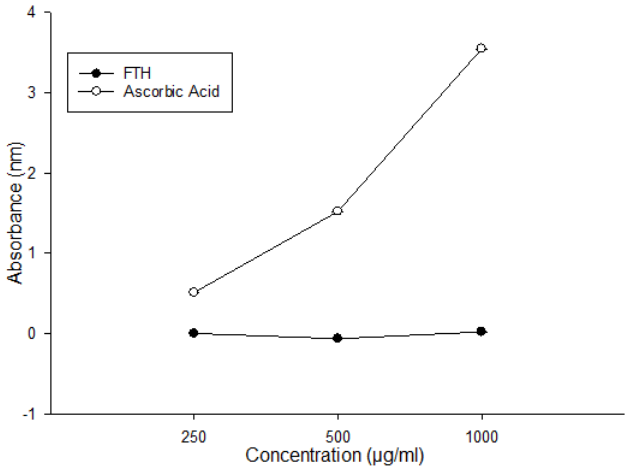

Figure 15: Phosphomolybdenum assay of FTH extract in comparison to the standard drug.

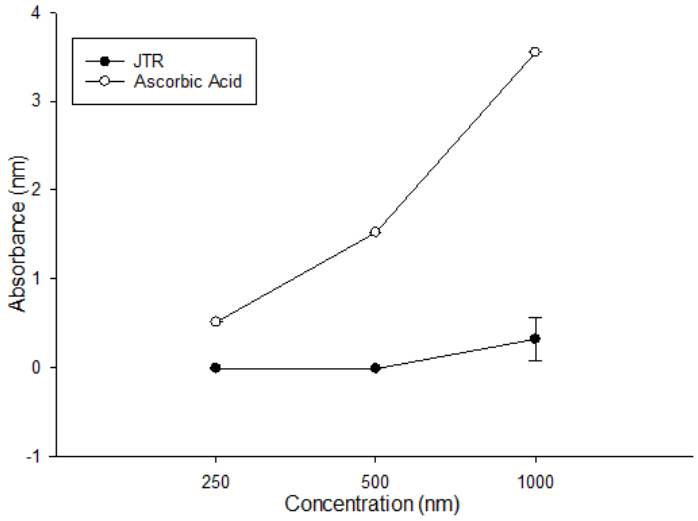

Figure 16: Phosphomolybdenum assay of JTR extract in comparison to the standard drug.

The phosphomolybdenum assay for Ascorbic acid was very significantly higher ($P<0.001$) than those of the extracts. The phosphomolybdenum assay at maximum concentration for FTH, JTR and JCN was calculated as 10.617 ± 0.111 mg AAE/gm, 26.759 ± 0.0535 mg AAE/gm and 18.179 ± 0.0535 mg AAE/gm respectively. The result of the phosphomolybdenum assay shows that JTR extract had better antioxidant capacity. The observed increase in absorbance for phosphomolybdenum assay didn't correlates well with increase in concentration, this was attributed to the interference of impurities at varying concentration (figure 1-3) and thus affirms the need for purification of these extracts before use (Figure 17).

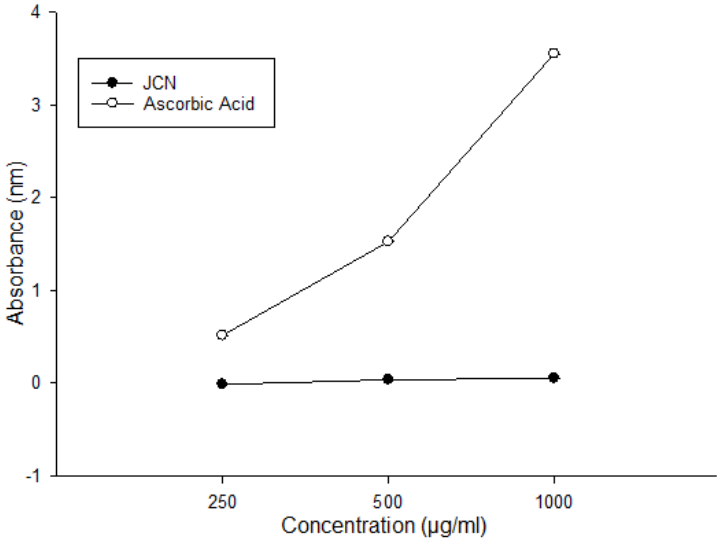

Figure 17: Phosphomolybdenum assay of JCN extract in comparison to the standard drug

Sickle cell disease is characterized by recurrent vaso occlusive and hemolytic anemia, and involves decreased vitamin E antioxidant capacity that is capable of scavenging free radicals and hence stabilizing the membrane bi-layer, therefore, plants rich in antioxidant can supply SCD patients with needed antioxidants and since the extracts assayed herein has shown to be strong antioxidants, they can as well serve as a nutraceutical for the management of SCD patients and thus proves the use of these plants in the management of SCD patients.

Antioxidant analysis of pure isolates from FTH, JTR and JCN

DPPH scavenging activity results: In a separate experiment, the antioxidant characterizations of the pure isolates from FTH, JTR and JCN were analyzed and compared with the standard drug (ascorbic acid). From the results, it was shown that the IC_{50} for FTH1, FTH2 and FTH3 were 49.944 ± 5.395 µg/mL, 24.286 ± 0.0003 µg/mL and 48.988 ± 0.0949 µg/mL, respectively whereas the IC_{50} for JTR1 and JTR3 were 10.174 ± 0.000 µg/mL and 14.531 ± 0.001 µg/mL respectively (Figures 18 and 19).

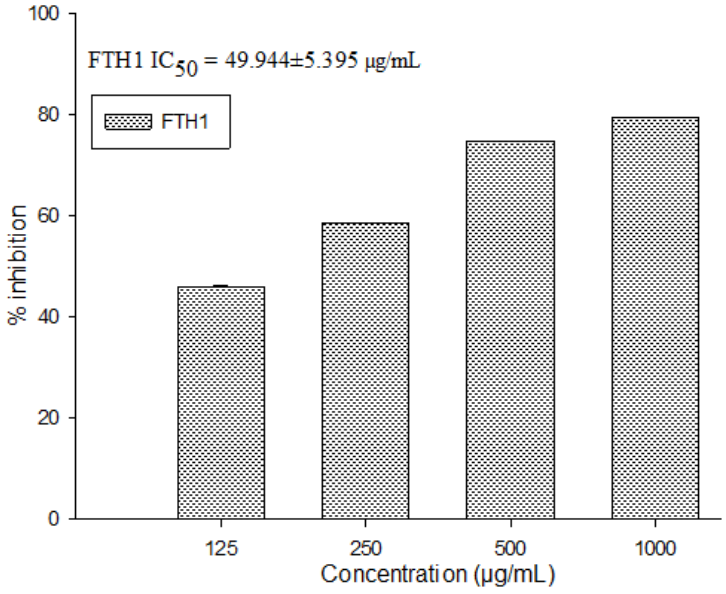

Figure 18: Graph of % inhibition of DPPH by FTH1

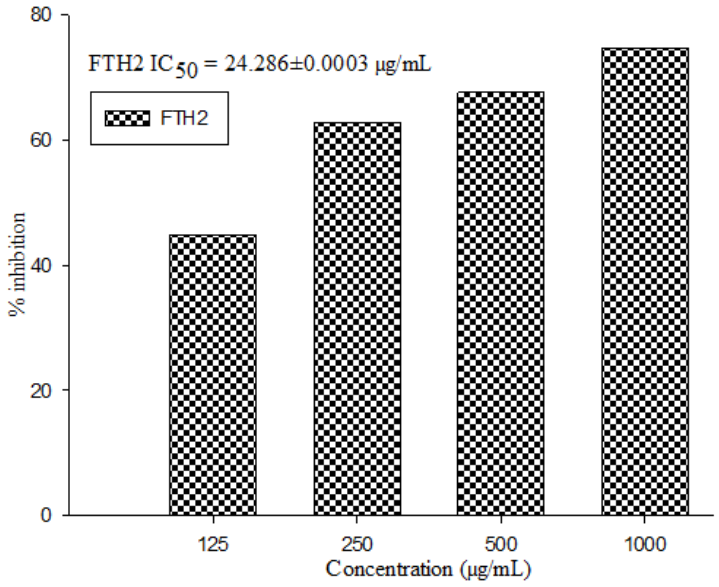

Figure 19: Graph of % inhibition of DPPH by FTH2

The DPPH IC_{50} for JCN1, JCN3 and JCN4 were calculated as 6.636 ± 0.004 µg/mL (Figure 23), 74.532 ± 7.022 µg/mL, 148.086 ± 6.949 µg/mL respectively whereas those of the standard drug ascorbic acid was 8.124 ± 0.006 µg/mL (Figure 26) The results of the DPPH radical scavenging activity indicates that the isolates and the control drug inhibited the formation of free radical in a dose dependent manner (P<0.050); also, JCN1 (6.636 ± 0.004 µg/mL), JTR1 (10.174 ± 0.000 µg/mL), and JTR3 (14.531 ± 0.001 µg/mL) offered better antioxidant characterization in the DPPH assay overall; which were comparable to those of ascorbic acid (8.124 ± 0.006 µg/mL). There was a very significant difference (P<0.0010) between the isolates and Ascorbic acid at various concentrations determined

The DPPH results assay for JTR1, JTR3. JCN1, FTH1, FTH2 and FTH3 matched those from literature with slight differences attributed to solvent used in sample preparation. The IC50 or EC50 are usually used to determine antioxidant capacity of a sample compared to standard; samples that has IC_{50} or EC_{50} less than 50 µg/ml are considered a very strong antioxidant, 50-100 µg/ml are strong antioxidant, 101-150 µg/ml are medium antioxidant, while IC_{50} or EC_{50} greater than 150 µg/ml are weak antioxidant therefore, from the DPPH assay the pure isolates have shown very strong antioxidant capacity (Figures 20 and 21).

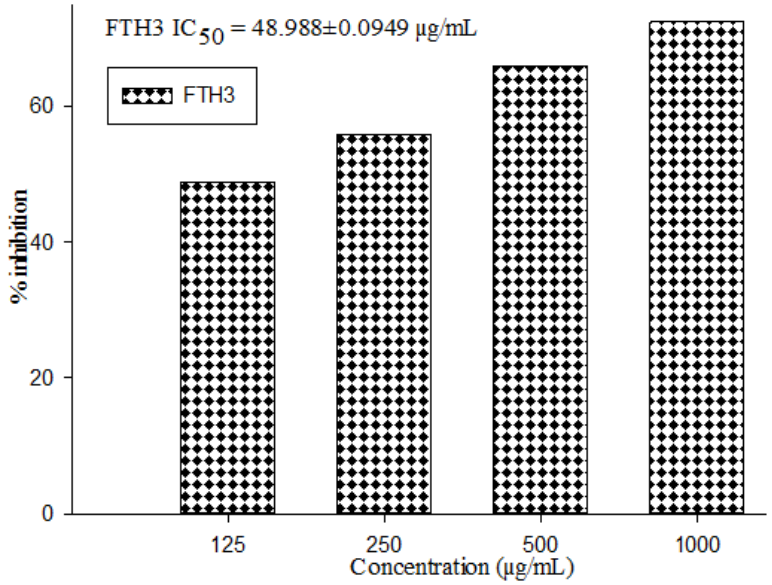

Figure 20: Graph of % inhibition of DPPH by FTH3.

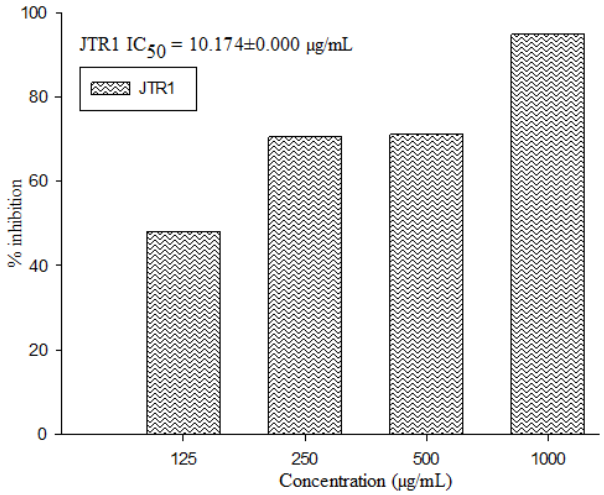

Figure 21: Graph of % inhibition of DPPH by JTR1.

The results of the DPPH assay depend on the structural conformation and stereochemistry of these isolates of which the hydroxyl group of the flavonoids were highly favorable. The anti-sickling characterization of these isolates were similarly attributed in part to their antioxidant properties since a synergy exist between anti-sickling agents and antioxidants properties; moreover, previous reports has shown that these isolates are potential anti-sickling agents. The DPPH scavenging activity of the isolates corroborated with the concentration of the extracts with strong significant positive correlation (R^2) thus the calculated R^2 were; ascorbic acid (R^2=0.7264), FTH1 (R^2=0.8009), FTH2 (R^2=0.7274), and FTH3 (R^2=0.8991), while JTR1 (R^2=0.8677) and JTR3 (R^2=0.9318). Similarly, those of JCN1 (R^2=0.8619), JCN3 (R^2=0.8605) and JCN4 (R^2=0.6308) (Figures 22 and 23).

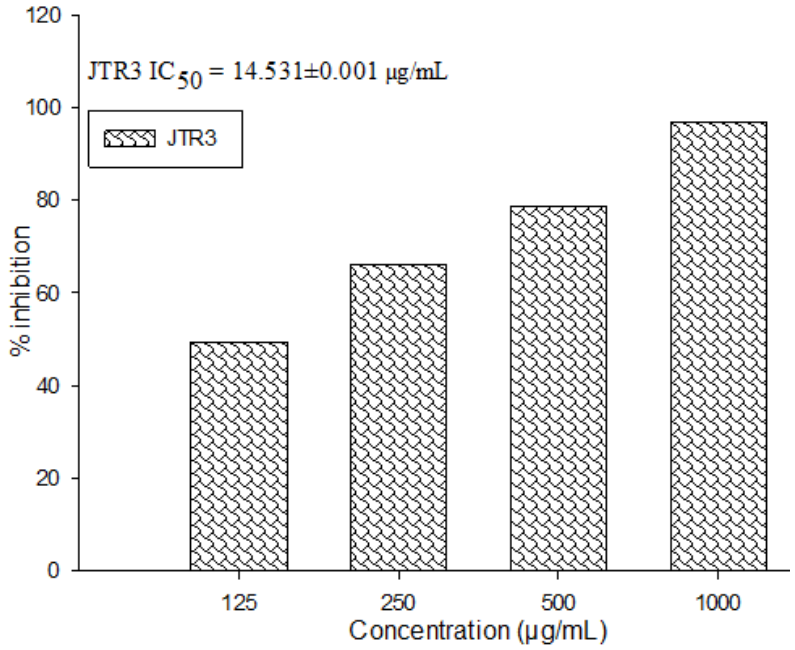

Figure 22: Graph of % inhibition of DPPH by JTR3.

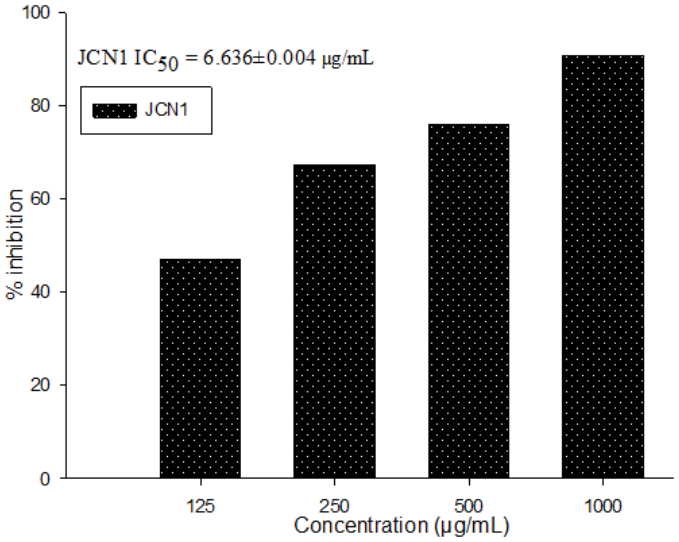

Figure 23: Graph of % inhibition of DPPH by JCN1.

As earlier suggested the increase in the percentage inhibition and subsequent lowering of the IC_{50} for the isolates were attributed to purity or absence of interfering pro-oxidants. Since sickle cell anemia is emerging as an important model of oxidative stress and since in disorders with abnormal haemoglobin, such as sickle cell, the haemoglobin stabilizing capacity is impaired, making the RBCs more vulnerable to oxidative stress, this may overwhelm the antioxidant defense system, therefore, the presence of antioxidant such as those isolated herein has shown that *F. thonningii*, *Jatropha tanjorensis* and *Justicia carnea* are good natural sources of antioxidant and consequently can ameliorate/delay processes that trigger sickle hemoglobin gelation. Antioxidant intake protects individuals from oxidative damage of *in vivo* lipids and proteins. Antioxidants (scavengers of free radicals) are believed to be major components of anti-sickling agents that add to their potential. The higher antioxidant potential of a compound, the higher its possible anti-sickling effect as this enables it to reduce oxidative stress that contributes to sickle cell crisis. Increased antioxidant intake may be beneficial for SCD clients as they support cell turnover and RBC formation. Hence our results have shown in part the importance and role of these plant parts and their isolates in the management of sickle cell patients and thus validates its use to ameliorate the occurrence of vaso occlusive crisis in sickle cell disorder (Figures 24 and 25).

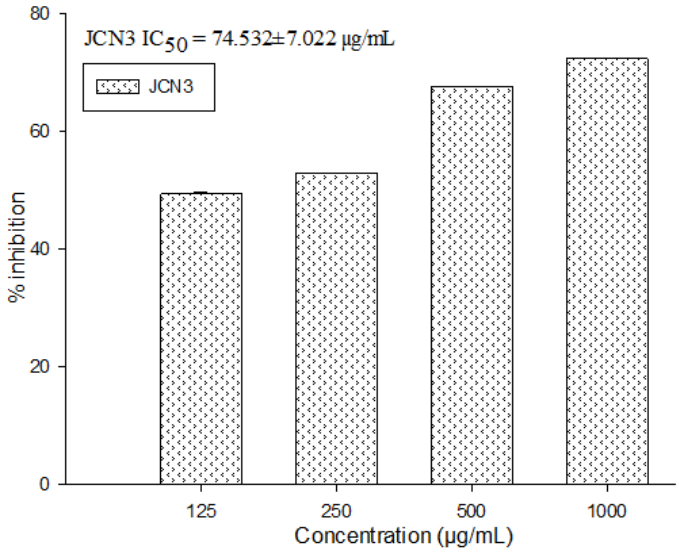

Figure 24: Graph of % inhibition of DPPH by JCN3

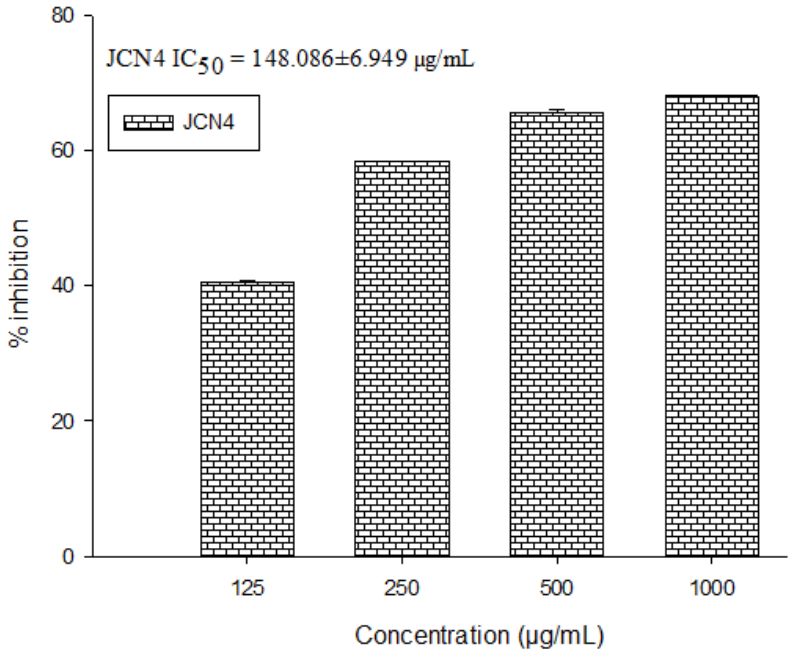

Figure 25: Graph of % inhibition of DPPH by JCN4

Compounds derived from secondary metabolism, specifically phenolic compounds, play a fundamental role against oxidative stress. These compounds are known to act as antioxidants not only for their ability to donate hydrogen or electrons but also because they are stable radical intermediates. Phenolic compounds also have protective effects on humans when the plants are consumed as food. Generally, the antioxidant capacity of phenols in plant extracts is effective at low concentrations, and in humans, it is associated with the prevention of cardiovascular disease, cancer and sickle cell anemia (Figure 26).

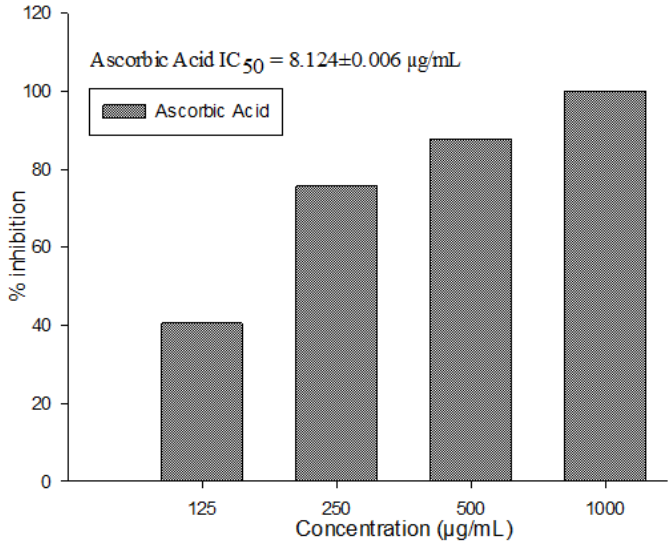

Figure 26: Graph of % inhibition of DPPH by ascorbic acid.

Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. Hydroxyl radical species are one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids of which lipid per oxidation has been implicated in sickle cell disorder. It has been shown that sickle RBCs produce greater

quantities of superoxide radical, hydrogen peroxide (H_2O_2) and hydroxyl radical than do normal RBCs (Figures 27 and 28).

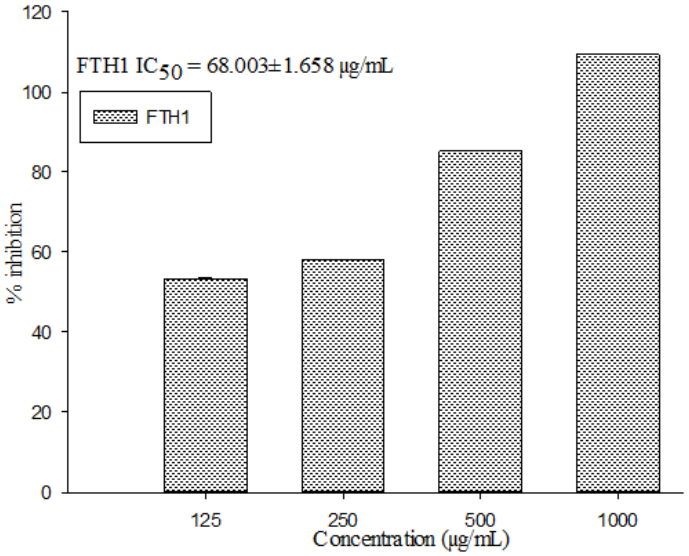

Figure 27: Graph of % inhibition of HO· radical by FTH1.

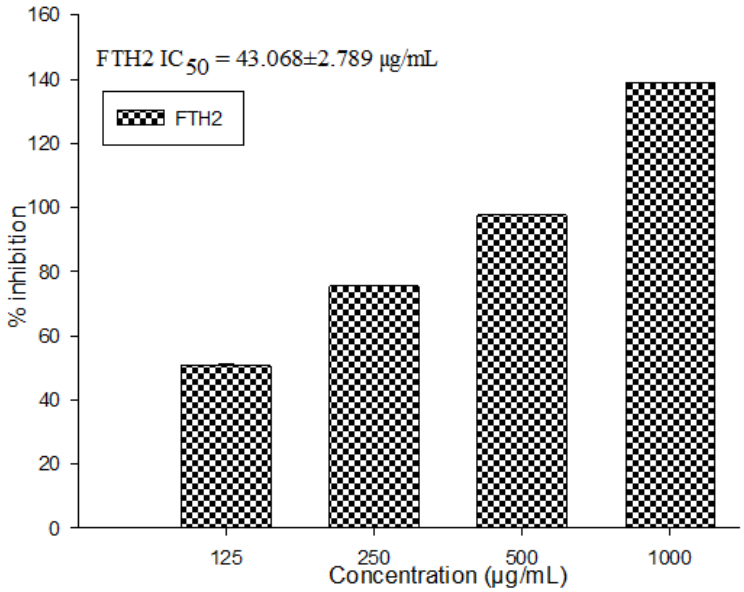

Figure 28: Graph of % inhibition of HO radical by FTH2.

From the results analyzed *in vitro*, it was observed that FTH1, FTH2 and FTH3 had IC_{50} and R^2 in the hydroxyl radical scavenging assay of 68.003 ± 1.658 µg/mL, R^2=0.9678, 43.068 ± 2.789 µg/mL, R^2=0.9747, 53.605 ± 3.255, R^2=0.9657 respectively whereas those of JTR1 and JTR3 were 16.947 ± 1.141 µg/mL, R^2=0.977 and 15.247 ± 1.003 µg/mL, R^2=0.9882 respectively. Also, the calculated IC_{50} for JCN1, JCN3 and JCN4 were 13.863 ± 1.141 µg/mL, R^2=0.9869, 56.765 ± 0.436 µg/mL, R^2=0.977; R^2=0.9822 and 153.944 ± 0.679 µg/mL, R^2=0.9535 respectively while the standard drug (ascorbic acid) had an IC_{50} of 20.798±2.370 µg/mL, R^2=0.9936 (Figures 29 and 30).

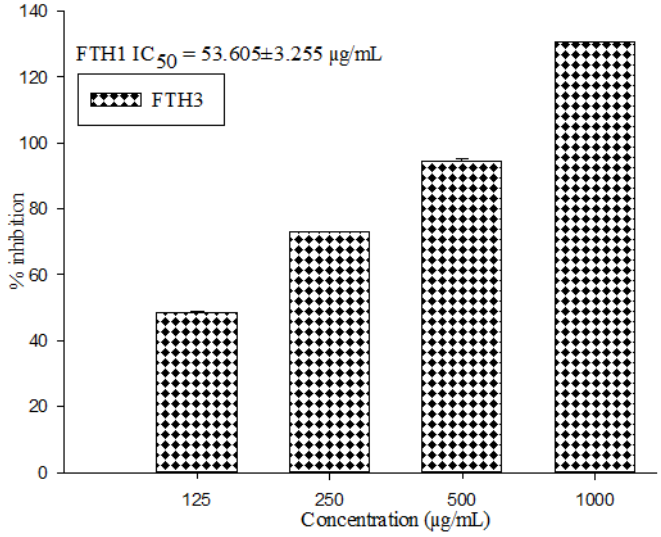

Figure 29: Graph of % inhibition of HO· radical by FTH3.

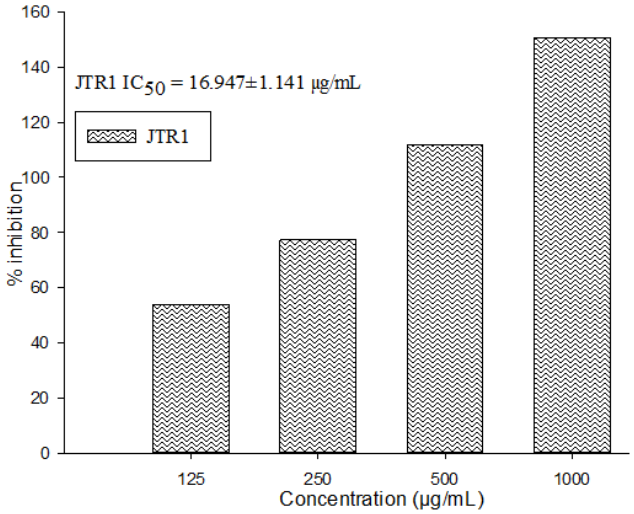

Figure 30: Graph of % inhibition of HO radical by JTR1.

The hydroxyl radical scavenging activity results shows that JCN1 (IC_{50}=13.863 ± 1.141 µg/mL), JTR3 (IC_{50}=15.247 ± 1.003 µg/mL), and JTR1 (IC_{50}=16.947 ± 1.141 µg/mL), had better hydroxyl radical scavenging activity. Similarly, it was observed from the SNK ANOVA analysis that there was a very significant difference (P<0.001) between the hydroxyl radical scavenging activity of the isolates and the standard drug (ascorbic acid) at various concentration determined. Also, the results indicate that the isolates significantly (P<0.050) inhibited hydroxyl radicals in a dose dependent manne (Figures 31 and 32).

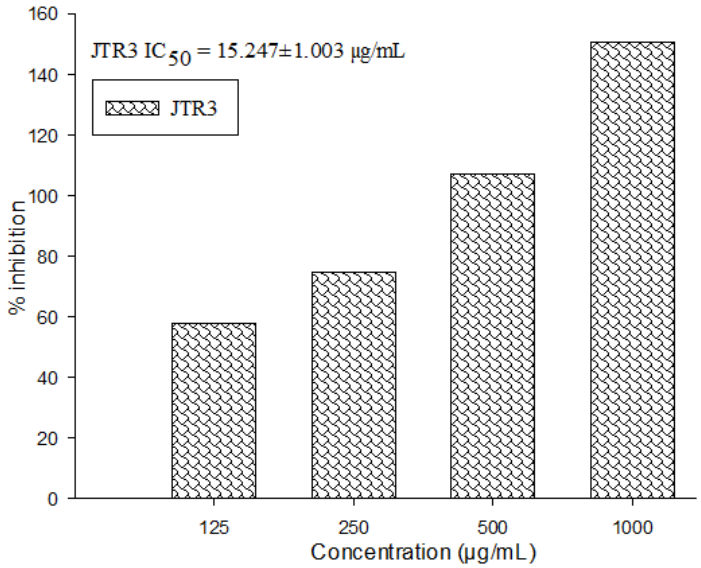

Figure 31: Graph of % inhibition of HO· radical by JTR3.

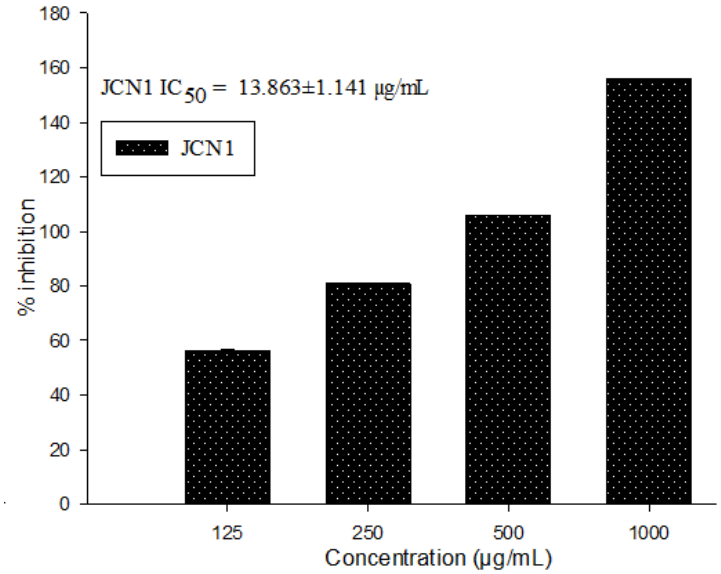

Figure 32: Graph of % inhibition of HO radical by JCN1.

The need to purify these extracts for optimum activity was evident from the results of the crude extracts which showed no hydroxyl radical scavenging activity for JTR extract but on isolation and purification, the isolates (JTR1 and JTR3) showed potential hydroxyl radical scavenging activity thus validating our initial claim of interference of impurities or pro-oxidants that inhibits its activity. The % inhibition of hydroxyl radical of ascorbic acid were very significantly ($P<0.001$) higher than those of FTH1, FTH3, JCN3 and JCN4 at various concentrations analyzed. The induced inhibition of hydroxyl radicals by the isolates were dose dependent ($P<0.050$) and were attributed to purity and absence of pro-oxidants (Figures 33 and 34).

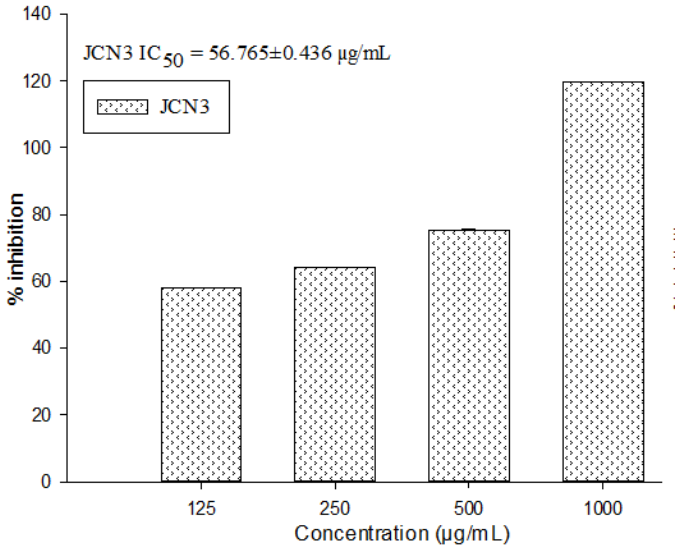

Figure 33: Graph of % inhibition of HO· radical by JCN3

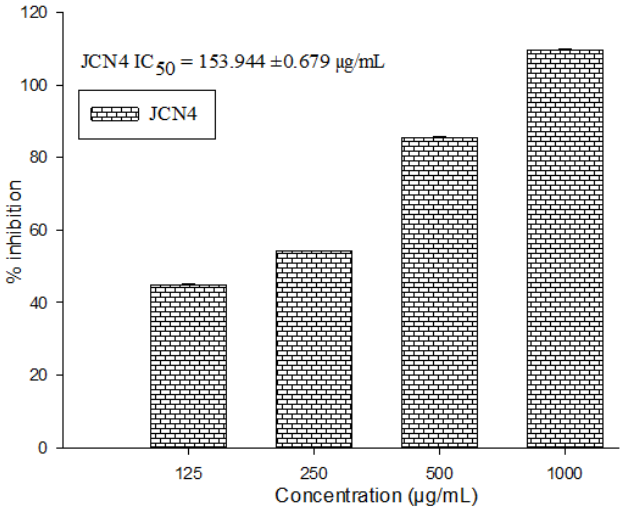

Figure 34: Graph of % inhibition of HO radical by JCN4.

In plants, the main compounds with antioxidant activity are phenols, as they have an aromatic ring that allows the stabilization and relocation of the unpaired electrons of their structure, thus facilitating the donation of hydrogen atoms and electrons from their hydroxyl groups. Secondary metabolites of plants, specifically phenols, can adjust the concentration of Reactive Oxygen Species (ROS), thus activating a network of biochemical events to increase tolerance, hence the importance of studying the antioxidant activity of typical plant species (Figure 35).

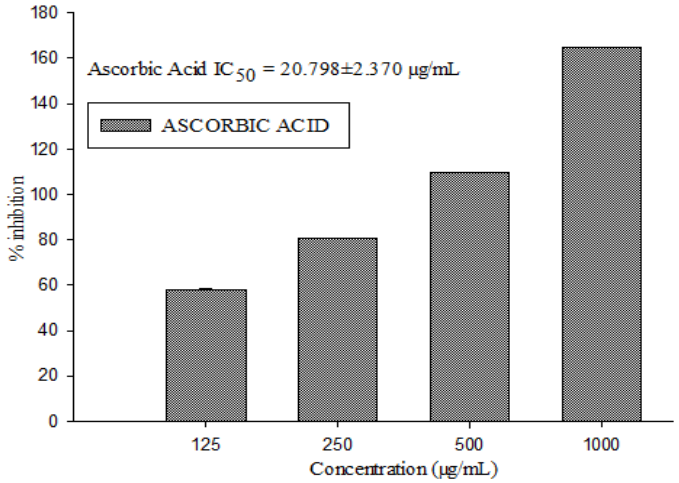

Figure 35: Graph of % inhibition of HO radical by ascorbic acid

The antioxidant properties of polyphenols and phenolic acids such as FTH2, FTH3, JTR1, JTR3, and JCN1 has been studied and variations in antioxidant activity related to the stereochemistry of hydroxyl, methoxy and carboxylic acid groups, as well as the solvent system (reaction system). Carboxylic acid groups affect the antioxidant activity of phenolic acids according to their electron-donating ability. In phenolic acids the number and position of phenolic hydroxyl groups are directly related to the free radical scavenging ability. When the number of phenolic hydroxyl groups on the benzene ring is less than 4, the antioxidant activity of phenolic acids is proportional to the number of phenolic hydroxyl groups. Moreover, because phenolic hydroxyl groups are electron donor groups they can enhance the antioxidant activity of another phenolic hydroxyl. Similarly, under the same mother nucleus structure, the more the number of the Methoxyl groups, the higher is the antioxidant activity of phenolic acids.

The HbS releases its heme easily; the released heme undergoes autoxidation 1.7 times faster than HbA forming meth-HbS that precipitates onto the inner leaflet of the red cell membrane. Free heme within the cytosol repels deoxy-HbS driving them toward each other and accelerating polymer formation Chelated and membrane-bound Fe^{3+} acts as a Fenton's reagent and together with hydrogen peroxide generates hydroxyl and peroxides, the Hydroxyl radical scavenging activity correlates positively with metal chelating ability of an antioxidant, thus these extracts can inhibit or mob up Hydroxyl radicals and also can consequently reduce the rate of heme autoxidation thereby reducing the rate and frequency of sickle cell crises.

Reducing power: The results from reducing power assay was determined and compared with Standard drug (ascorbic acid). The reducing power of iron was found to be higher in JCN1, JTR1, and JTR3 than in other isolates. The reducing power of the isolate were relatively more prominent indicating that the isolates are potential reducing agents. Iron causes lipid peroxidation and decomposes the lipid hydroxide into peroxyl and alkoxyl radicals that can lead the chain reactions these isolates acts as reductants and thus are responsible for the reducing capacity of the plants assayed, and similarly reductants are known to prevent chain initiation, binding of metal ions, decomposition of peroxides, and radical scavenging.

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. In this study, the Reducing power of isolates were compared with a reference agent ascorbic acid, the reducing power of the isolates were concentration dependent ($P<0.050$). At all concentration determined, there were very significant difference ($P<0.001$) between the reducing power of the isolates and those of the standard drug (ascorbic acid).

From ongoing intravascular hemolysis, sickle erythrocytes liberate up to 30 g of cell free Hb daily. This liberated Hb generates superoxide, peroxide, hydroxyl radical, and hydrogen peroxide about thirty times the amount generated by HbAA individuals. Increases in concentration of reactive oxygen species (ROS) increase malondialdehyde (a reactive naturally occurring organic compound used as a marker for oxidative stress), decrease glutathione levels, reduce levels of enzyme cofactors such as zinc and copper, and promote lipid peroxidation therefore antioxidant supplementations can delay the sickle hemoglobin gelation by mitigating these processes that trigger sickle erythrocyte polymerization as well as scavenge ROS generated in vivo, and consequently improve glutathione levels and inhibit/decrease lipid peroxidation (Figure 36).

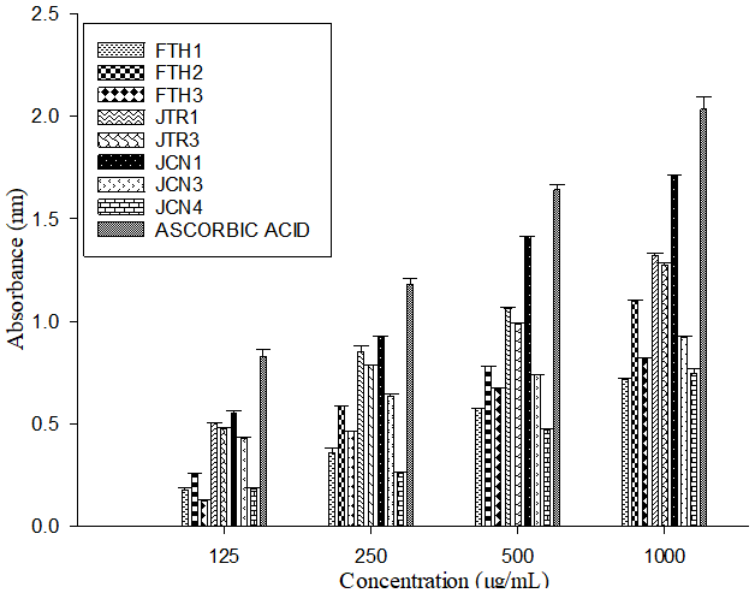

Figure 36: Reducing power of isolates in comparison with standard drug

The red cell is not the only source of ROS in SCA. In SCA, a chronic inflammatory state occurs from a constant recruitment and activation of leukocytes and monocytes. Neutrophils on activation demonstrate an increased oxidant production *in vivo* thus necessitating a subsequent increase in antioxidant supplementation to prevent damages to cells, organs and vaso occlusive crisis, hence validating the nutraceutical supplementation and use of *F. thonnigii*, *Jatropha tanjorensis* and *Justicia carnea* isolates in the management of SCA

Phosphomolybdenum assay

Phosphomolybdenum assay is a quantitative method to evaluate water soluble and fat soluble antioxidant capacity (total antioxidant capacity), from the results, it was evident that the isolates of JTR1, JTR3 and JCN1 as well as FTH2 had greater electron donating capacity than other isolates and thus, may act as a great radical chain terminator, transforming reactive free radical species into more stable non-reactive products as supported by Dorman, et al., and free radicals has been implicated in sickle cell disorder similarly, antioxidant plants has been shown to increase erythrocyte solubility and thus may prevent sickle polymerization, since a synergy exist between anti-sickling and antioxidant properties. They were very significant difference ($P<0.001$) between the various isolates and the standard drug (ascorbic acid) at various concentration tested, also the absorbance (nm) of the isolates were concentration dependent (Figure 37).

FTH1, FTH2, and FTH3 at maximum concentration had 506.623 ± 14.540 µg AAE/mg sample, 751.650 ± 1.756 µg AAE/mg sample, 641.395 ± 0.105 µg AAE/mg sample, while those of JTR1 and JTR3 were 1206.171 ± 0.446 µg AAE/mg sample and 1112.278 ± 1.728 µg AAE/mg sample respectively. Similarly, for JCN1, JCN3 and JCN4 were 1281.309 ± 0.0381 µg AAE/mg sample, 505.294 ± 0.0216 µg AAE/mg sample, 464.016 ± 1.756 µg AAE/mg sample respectively in the phosphomolybdenum assay. Free radicals or oxidative injury now appears to be the fundamental mechanism underlying several human neurologic and other disorders. Oxygen free radicals can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and play a role in the long-term complications of several diseases including sickle cell anemia hence the total antioxidant assay of the isolates shows that they can serve as potential antioxidant and since a correlation exist between antioxidant and anti-sickling characterization the isolates reported herein also may serve as potential anti-sickling agent capable of interfering with processes that trigger sickle hemoglobin gelation since high number of antioxidants are known anti-sickling agents.

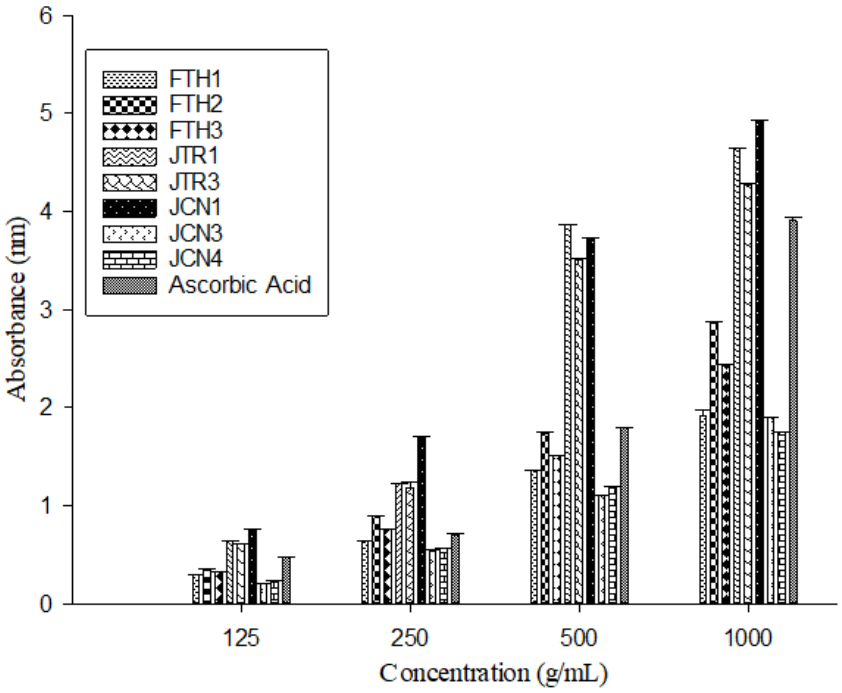

Figure 37: Results of phosmolybdenum assay

There is a significant direct relationship between total antioxidant status, Hb concentration, and hematocrit. Al-Naama, et al. explained this phenomenon by stating that intravascular hemolysis (which result in anemia) induces oxidative stress through the release of cell free Hb. Antioxidants are known to inhibit free radical formation, reduce free radical availability and thus reduce oxidative stress and by implication will reduce intravascular hemolysis.

The cell free Hb forms a stable complex with nitric oxide and iron which is a fenton's reagent involved in fenton's reactions. Reticuloendothelial macrophages also increase their erythrophagocytosis as a response to oxidative stress, thus reducing the hematocrit/Hb concentration thus antioxidants can improve the hematocrit/Hb concentration by reducing hemolysis. The isolate from *Ficus thonningii, Jatropha tanjorensis* and *Justicea carnea* have shown to be very strong antioxidant and from our present research has shown to possess the prerequisite for natural antioxidants since they are readily available, and its bioavailability abounds.

Similarly, Al-Naama, et al. and Fasola, et al. observed that the serum total antioxidant status of the respondents with a worse clinical course was significantly lower than those with a better clinical course using frequency of crises as the indicator. However, modifiers may ameliorate clinical symptoms of the SCA to a larger extent.

Ferric Reducing Antioxidant Power Assay (FRAP)

Three key antioxidant mechanisms involved in the process of quenching free radicals are Hydrogen Atom Transfer (HAT), Single Electron Transfer Followed by Proton Transfer (SET-PT), and Sequential Proton Loss Electron Transfer (SPLET). HAT is a one step reaction related to O-H Bond Dissociation Enthalpy (BDE), whereas SET-PT and SPLET are two-step reactions, the former is related to ionization Potential (IP) and Proton Dissociation Enthalpy (PDE), and the latter is related to Proton Affinity (PA) and Electron Transfer Enthalpy (ETE). These reaction mechanisms under different micro environments may occur independently or simultaneously at different rates therefore the variations in the antioxidant capacity of each of the plants pure isolate was attributed to Structure Activity Relationship (SAR), stereochemistry and the reaction environment (solvent used in carrying out the antioxidant analysis) *in vitro*.

The FRAP assay of the pure isolates was concentration dependent ($P<0.050$), the absorbance of all the isolates at the various concentration measured was very significantly ($P<0.001$) different from those of the standard drug, similarly, the FRAP of JTR1, JTR3 and JCN1 were significantly ($P<0.001$) higher than those of the standard drug (Figure 38).

FRAP is a simple and direct test of antioxidant capacity, in this study, the FRAP of the isolates were compared with a reference standard ascorbic acid and demonstrates that the isolate of JTR3 (1066.638 ± 0.137 µg AAE/mg sample), JTR1 (1094.518 ± 0.130 µg AAE/mg sample), JCN1 (1191.746 ± 1.572 µg AAE/mg sample), FTH2 (848.700 ± 6.939 µg AAE/mg sample) and FTH3 (716.478 ± 2.222 µg AAE/mg sample) had excellent status of FRAP than those of FTH1 (622.969 ± 0.478 µg AAE/mg sample), JCN3 (569.823 ± 6.910 µg AAE/mg sample),and JCN4 (526.478 ± 1.111 µg AAE/mg sample) which had a very good FRAP status. The results of the FRAP assay suggest that the pure isolates had very good antioxidant potentials and thus validates the use of *F. thonningii*, *Jatropha tanjorensis*, and *Justicia carnea* leaf as medicinal and nutraceutical in the management of sickle cell patients.

There is a significant inverse relationship between total antioxidant status, white cell, and lymphocyte count. Similarly, Fasola, et al. found a significant inverse relationship between total antioxidant status and total white cell count (total antioxidant status reduces as white cell counts increase). Neutrophils generate an oxidative burst, which produces free radicals that are required to destroy microbes. Leukocytosis will increase free radicals formed thus outstrip the body's capacity to neutralize them hence the antioxidant characteristics of these plant isolates has shown that they are potential antioxidants, moreover white cell count of SCA patients are always higher than those of HbA patients, by implications the HbS patients experience increased amount of free radicals than HbA patients and since free radicals has been implicated in sickle cell crisis therefore the ability of these isolates to scavenge free radicals generated by the white cells will in turn reduce the frequency of vaso occlusive crisis.

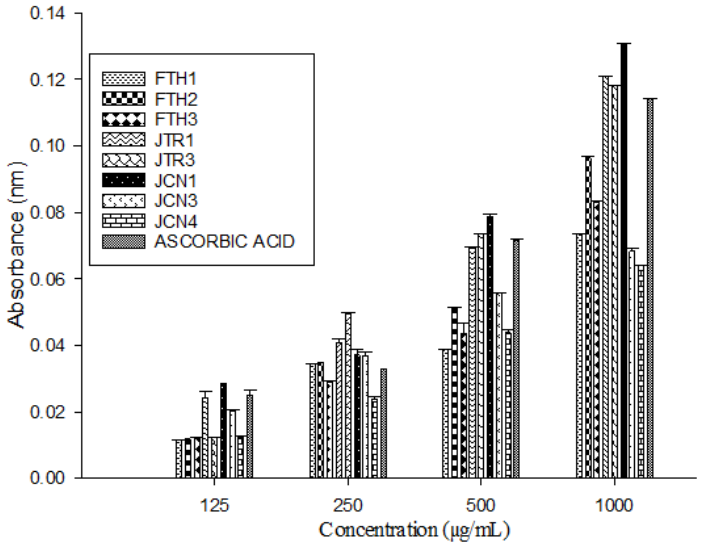

Figure 38: Graph of FRAP assay of isolates in comparison with standard drug.

Some vitamins like vitamin E has been shown to play an essential role in the antioxidant defense system and have a protective role against oxidative membrane attack. Several studies have shown that vitamin E level was significantly lower in the sickle cell patients compared to normal ones. This finding probably indicates the relative severity of oxidative stress in relation to relative severity of sickle cell disorder validating the role of antioxidant in reducing the relative frequency and severity of vaso occlusive crisis and cell damage in SCD patients.

JTR3 a variant of vitamin E isolated from *Jatropha tanjorensis* has shown antioxidant potentials thus validating initial claims by Ilondu and Enwa, and Mpiana, et al. on anti-sickling properties of *Jatropha* spp and supported by our present observations. Similarly, The *Azadirachta indica J*. seed oil contains an important quantity of vitamin E that explain its anti-sickling, and antioxidant properties. Vitamin E is a chain breaking antioxidant with a membrane protective role in almost all cells. Thus, in view of the evidence of multiple membrane defects in this condition (sickle cell disorder), it is possible that membrane damage might be a critical factor in this disorder and since the isolates reported herein possesses strong antioxidant activity, it implies that these isolates or foods rich in these isolates can be explored for their antioxidant benefits for SCD patients.

Interestingly, oxidatively modified membrane associated proteins are currently implicated in the formation of irreversible sickle cells, which is leading to a paradigm shift from the older crosslinking theory. In a small trial of patients with sickle haemoglobinopathy, vitamin E supplementation was shown to reduce sickling. On closer observation of the data, it becomes

evident that the level of difference in vitamin E in heterozygous cases compared to the respective controls was less than the corresponding difference obtained in homozygous cases thus, validating the role of antioxidants in the management of sickle cell patients.

CONCLUSION

Many plants constituents have been investigated for their anti-oxidative properties. Antioxidant intake protects individuals from oxidative damage of *in vivo* lipids and proteins. Antioxidants (scavengers of free radicals) are believed to be major components of these anti-sickling agents that add to their potential. The stronger the antioxidant capacity of an isolate/extract, the higher its possible anti-sickling effect as this enables it to reduce oxidative stress that contributes to sickle cell crisis. Increased antioxidant intake may be beneficial for SCD clients as they support cell turnover and RBC formation. Phenolic compounds are important components in vegetable foods, infusions, and teas for their beneficial effects on human health.

Vitamin E, beta-carotene, and vitamin C, as well as the isolates from this assay are all powerful natural antioxidants. Many clinical trials have shown that antioxidants such as vitamin E improve hemolysis, by increasing erythrocyte lifespan; in elevated hemoglobin level. Treatment with high doses of antioxidants reduces oxidative stress induced erythrocyte injury. Antioxidants decrease lipid peroxidation and improve erythrocyte membrane stability. Also, the use of Oleic acid and vitamin E as nutraceutical in the management of SCD has been reported

The study of antioxidants especially in various anti-sickling agents is of great significance because anti-sickling agents vary in their degree of efficacy. Antioxidants constitute a major component of these anti-sickling agents; thus, it is believed that the higher the antioxidant property of an anti-sickling agent, the higher its possible anti-sickling and therapeutic effect. Therefore, reducing oxidative stress may ameliorate sickle cell crisis.

The results of all antioxidant methods assayed indicates that *Jatropha tanjorensis*, *F. thonningii* and *Justicia carnea*, including all their isolates analyzed herein are all strong powerful antioxidant and supports in part the use of these plant part as nutraceutical in the management of sickle cell anemia patients in Ebonyi state

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