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EVIDENCE-BASED PREFERENTIAL *IN VITRO* ANTISICKLING MECHANISM OF THREE NATIVE NIGERIAN PLANTS USED IN THE MANAGEMENT OF SICKLE CELL DISEASE

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Abstract

Sickle cell anemia is a hereditary genetic disease caused by the substitution of glutamic acid by valine at beta six (β-6) position of the hemoglobin; the clinical implication is that the erythrocyte contains the hemoglobin polymerizes, leading to severe clinical consequences. Therefore, we investigated the antisickling mechanism of *Ficus thonningii* (FTH), *Jatropha tanjorensis* (JTR) and *Justicia carnae* (JCN) native to Southeast Nigeria used in the management of sickle cell disease. The experiment was designed to include erythrocyte fragility, erythrocyte reversibility and polymerization inhibition mechanisms. The results obtained were analyzed using one-way ANOVA and Tukey Posthoc test. From the results of the erythrocyte fragility test, it was observed that at 0.35% saline concentration, FTH, JTR and JCN extracts reduced hemolysis to 26.548±0.056%, 18.055±0.064%, and 20.217±0.035% respectively while hydroxyurea (control drug) reduced hemolysis to 14.459±0.040%. In the presence of the control, the percentage number of sickle cells was 91.001±0.170% whereas, hydroxyurea was 30.414±0.162% while, extracts of FTH, JTR and JCN reduced sickling to 49.818±0.082%, 41.001±0.413% and 33.957±0.062% respectively. Also, the results of the polymerization inhibition analysis showed that extracts of FTH, JTR and JCN had relative percentage polymerization inhibition of 76.888±0.042%, 48.723±0.113% and 75.447±0.063%, respectively, in comparison to hydroxyurea which inhibited sickle erythrocyte polymerization by 70.903±0.150%. The results of our findings suggest the preferential but variable antisickling mechanism of the studied extracts; hence, the leaves of the assayed plants contain potential antisickling phytochemicals and should be explored further for their antisickling benefits.

INTRODUCTION

Sickle cell disease (SCD) is a molecular disease characterized by abnormal rigid sickled shape of the erythrocyte and occurs due to the polymerization of the deoxygenated sickle hemoglobin. SCD affects over 50 million people in the world [1, 2] of which 75% of reported sickle cell anemia (SCA) cases worldwide is in Africa [3]. In a pilot study of 3603 newborn babies in Nigeria it was observed that homozygous sickle cell hemoglobin (HbSS) accounted for 1% birth while Heterozygous sickle cell hemoglobin (HbSC) accounts for < 1% birth [4], also in Nigeria, the estimated national average under-5 mortality for children with sickle cell disease born between 2003-2013 was 490 per 1000 livebirths, i.e. 4 times higher than children with Normal hemoglobin (HbAA) [5]. SCA is characterized by painful crisis (vaso-occlusive crisis), chronic anemia, asplenia, and organ damage [6]. Sickled erythrocytes are viscous and hinders normal oxygen circulation in blood vessels leading to ischemia and infarction [7, 8] hence, micro-vascular occlusion occurs which most times leads to severe consequences [9]. Because of hemolysis sickled

erythrocyte have short life span [8]. Blood transfusion and hydroxyurea (HU) are among numerous cares prescribed for acute management of SCD patients [10] while the use of multivitamins, folic acid, vitamin C, vitamin B-complex, zinc, pyridoxamine etc. are considered for long-term management of SCD patients. Research into ethnomedicinal use of herbs in the management of sickle cell disease has been profitable [11]. Antisickling characterization of different plant extract via different mechanism have been reported [12, 13, 14, 15]. Some indigenous Nigerian plants are already on clinical trials for use in the management of SCD [16, 17]. Although, they are limited *in vitro* and in vivo research on antisickling activity of native Nigerian plants however, the antisickling activity of JTR has been reported [18, 19] while, the effects of JTR aqueous leaf extracts on hematological parameters in Wister rats has been profiled [20]. The antisickling evaluation of JCN has been reported [21] while, its antianemic and antioxidant activities have been studied [22, 23, 24]. FTH has been reported to possess a broad-spectrum medicinal activity and ethnomedicinal survey suggest antisickling activity [12, 25]. Therefore, the specific objectives of this research was to corroborate the results of ethnomedicinal surveys validating the use of FTH, JTR and JCN extracts in the management of SCD in Southeast Nigeria using standard analytical tools geared towards understanding the mechanism of action of these plant extracts *in vitro*.

MATERIALS AND METHODS

Identification and Authentication

The plants were identified and authenticated as *Ficus thonningii, Jatropha tanjorensis* and *Justicia Carnea* by a taxonomist at the department of applied biology Ebonyi State University Abakaliki, Nigeria.

Extraction of Crude Sample

The solvent used for extraction and phytochemical analysis were > 95% purity and were sigma Aldrich quality grade. Extraction of the leaves was carried out using methanol 100% (v/v). 10 kg of the pulverized leaves of each plant was weighed and soaked in methanol for 72 hours. The mixture was filtered using Whatman No. 42 filter paper, and the filtrate heated in a water bath (Remi, India) to one-tenth (1/10) of its initial volume at < 40 °C. Each dried extract was weighed using an analytical balance (Ohaus, USA) and stored at -4 ºC prior to analysis.

Qualitative and Quantitative Phytochemical Screening of the Crude Plant Extract

The phytochemical analysis of the crude extracts was performed using the methods described by Ijoma and Ajiwe, [26]. The phytochemical of interest for the qualitative phytochemical analysis includes acidic components (litmus paper test), flavonoids (aluminum chloride test), saponins (frothing Test), reducing sugars (Fehling test), carbohydrates (molish test), tannins (ferric chloride test), steroids (Liebermann Burchard test), terpenoids (Salkowski test), alkaloids (Mayer Test), Oils (spot/stain Test), cardiac glycosides (Keller-Killani Test), cyanogenic glycosides (sodium picrate test), proteins (million test), anthraquinones (Bontrager test) while those of quantitative phytochemical analysis includes alkaloids, flavonoid, phenolics, saponin, steroid, tannin, cardiac glycoside, terpenoid, protein, acidic component, carbohydrate, cyanogenic glycosides, oil and reducing sugar content**.**

Antisickling Characterizations

Collection of Blood Samples

Fresh venous blood samples (10 mL) were obtained by venipuncture of five confirmed SCD patients (aged 18-32 years) in steady state of the disease attending the hematology clinic. The samples were collected using EDTA anticoagulant tubes after appropriate written informed consent was obtained from the Research and Ethics Committee of Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Ebonyi State, Nigeria (AE-FUTHA/REC/VOL 3/2021/167). The SS status of the patients were confirmed using electrophoresis test and only one type of HbSS blood sample was used at a time for each experiment.

Osmotic Fragility Test

The protocol by Jaja *et al.,* [27] was used for the analysis. To 10 mL reaction vessels containing 4 mL of different concentrations (0.00 - 0.85%) of buffered saline (pH 7.4), 1 mL of hydroxyurea and each proposed antisickling agent (100 µg/mL) and 0.05 mL HbSS (hemoglobin SS) blood were added separately. The mixture was left in an incubator (Eppendorf, Germany) at room temperature (25 ºC) for 24 hours then, placed in centrifuge (LW Scientific, USA) at 3000 rpm for 15 mins. The supernatant was collected and using the V.2.30 version of UV-2500 PC series it was read at 540 nm against blank (0.85% buffered saline concentration) [11, 28, 29]. A control (CTR) experiment was performed in the absence of any antisickling agents. The mean corpuscular fragility was determined from the concentration of saline causing 50% hemolysis of the Red Blood Cell (RBC) obtained from a plot of% Hemolysis versus NaCl concentration. The analysis was done in triplicate.

$$\text{Percentage hemolysis} = \frac{\chi 1 \times 100}{\chi 2} \quad (1)$$

$\chi 1$ = Absorbance of supernatant in all tubes

$\chi 2$ = Absorbance of supernatant in tubes with zero conc. of NaCl

Results were presented graphically as percent hemolysis plotted against the concentration of NaCl.

Sickle Reversal Studies

The ability of the plant extracts to reverse the sickling state of the RBCs was performed by a previously described procedure by Pauline *et al.* [11]. One mL HbSS blood sample was washed twice in five volumes of phosphate buffered saline (PBS) with pH 7.4 by centrifugation at 5000 rpm. Into a clean Eppendorf tube, 100 µL of the washed red blood cells and 100 µL of freshly prepared 2% sodium metabisulfite ($Na_2S_2O_5$) was added and incubated for two hours at 37 ºC. Then 100 µL of antisickling agent (1000 µg/mL) was added and incubated for another two hours at 37 ºC. Ten microliters (10 µL) of the incubated cells was diluted 100 times. A drop was taken and transferred to a hemocytometer (LW Scientific, USA) and the cells were viewed and counted microscopically. A control test was performed by replacing 100 µL drug and proposed antisickling agents with 100 µL of PBS. The cells were classified as normal or sickled by observing their shapes. Biconcave or disc-like shapes were taken to be normal while the elongated, star-like, or wrinkled shapes were considered sickled. The percentage sickled cells were calculated using the formula [29]. The experiment was done in triplicate. The percentage sickle cell was calculated using equation 2,

$$\text{Percentage Sickling (\%)} = \frac{\Upsilon \times 100}{\Sigma C} \qquad (2)$$

Υ = Number of sickled cells
ΣC = Total number of counted cells

Polymerization Inhibition

The polymerization inhibition test was carried out following the method of Nwaoguikpe *et al.,* [30]. The procedure involved the measurement of the turbidity of the polymerizing solution of RBCs at wavelength of 700 nm at 26 ºC. Freshly prepared 2% Sodium metabisulfite (0.88 mL) was transferred into a cuvette followed by 0.1 mL PBS, 0.02 mL HbSS blood and then 0.1 mL of distilled water. Absorbance was read at 700 nm immediately and at every 2 minutes for 30 minutes. This served as control test. For the inhibition test, 0.1 mL of distilled water was replaced by 0.1 mL equivalent to 1% w/v of hydroxyurea and proposed antisickling agents. The analysis was done in triplicate. The rate of polymerization in percentage was calculated using equation 3,

$$\text{Rate of polymerization (Rp)} = \frac{\lambda f - \lambda i}{tm - to} \qquad (3)$$

λf = Final absorbance at maximum time (t_m)
λi = Initial absorbance at time zero (t_o)

The rate was expressed as relative percentage polymerization with respect to the control.

Statistical Analysis

Each test was performed in triplicate and the results expressed as mean ± standard error of the mean. Descriptive statistics, homogeneity of variance, ANOVA and Tukey posthoc test were carried out on each data set using the software SPSS version 20. The results were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Results of Percentage Yield

Table 1 showed the% yield of methanol extracts of FTH, JTR and JCN. The result showed that the% yield was in the order JTR > JCN> FTH.

Table 1. Percentage yield of methanol extracts of FTH, JTR and JCN

	FTH	JTR	JCN
Yield (g)	104.44	127.36	116.48
% Yield	1.04	1.27	1.16

Results of Phytochemical Analysis

Results of Qualitative Phytochemical Analysis

Table 2 indicated results of qualitative phytochemical analysis of FTH, JTR and JCN methanol extracts. Antisickling bioactive plants are known to contain phytochemicals [31, 32, 33, 34]. Active constituents of antisickling agents are rich in aromatic amino acids, phenolic compounds and antioxidant nutrients [35] which are thought to be responsible for their observed antisickling action. Phytochemical examination of antisickling plant extracts suggest that they contained anthraquinones derivatives, steroidal glycosides and cardiac glycosides. In contrary to our findings, alkaloids and tannins were completely absent in extracts of *C. populnea* that showed antisickling activity [36] suggesting that other classes of secondary metabolites possess antisickling potentials too.

Table 2. Results of qualitative phytochemical screening of the plant leaf crude methanol extract

S/N	Phytochemical	Reagent/Test	*F. thonningii*	*Jatropha tanjorensis*	*Justicia carnea*
1	acid component	Litmus paper	+	++	+
2	Carbohydrate	Molish	++	+	+
3	Oil	Stain/spot	+	++	+
4	Protein	Million	+	+	++
5	Resin	Turbidity	+	+	ND
6	Saponin	Frothing	++	++	+
7	Reducing sugar	Fehling	+	+++	++
8	Tannins	Ferric chloride	+	+	+
9	Alkaloids	Mayer	+	+++	++
10	Flavonoid	Aluminium chloride	+	+++	+++
11	Terpenoid	Salkowski test	+	++	+
12	Cardiac glycosides	Keller Killiani Test	+	+	++
13	Cyanogenic glycosides	Sodium picrate paper	ND	ND	+
14	Steroids	Lieberman Burchard	+	ND	ND
15	Anthraquinone	Bontrager	ND	++	+++

ND: not detected, +: present in low concentration, ++: present in moderate concentration, +++: present in very high concentration

Results of Quantitative Phytochemical Analysis

Table 3 showed the results of quantitative phytochemical evaluations of FTH, JTR and JCN methanol extracts. The results of the analysis of each phytochemical quantified confirmed the presence of the phytochemicals assayed. Presence of phytochemical such as flavonoids and terpenes portend bioactivities for instance *S. dulcis* antisickling activity was attributed to the rich presence of flavonoids and terpenes [37,38, 39, 40, 41]. Thus, the presence of flavonoids may portend antisickling activity for FTH, JCN and JTR extracts. Structural elucidation of some phytochemicals from antisickling plants identified 5-hydrooxymethyl-2-furfural (5HMF) [42], epigallocatechin gallate [43], 2-dihdroxybenzoic acid, vanillic acid, p-hydroxy benzoic acid and p-fluoro benzoic acid [44, 45, 46], furanoditerpene [47] as potential antisickling compounds. These phytocompounds functions via different antisickling pathways/mechanisms even when present in very low concentrations.

Table 3. Results of quantitative phytochemical screening of the plant leaf crude methanol extract

S/N	Phytochemical (%)	*F. thonningii*	*Jatropha tanjorensis*	*Justicia carnea*
1	acid components	0.210±0.05	0.110±0.03	1.32±0.01
2	Carbohydrate	1.270±0.03	6.727±0.08	4.082±0.09
3	Oil	3.421±0.08	11.730±0.03	0.221±0.01
4	Protein	3.706±0.10	6.991±0.04	2.651±0.02
5	Saponin	0.257±0.03	1.361±0.07	1.151±0.01
6	Reducing sugar	2.540±0.003	8.982±0.02	5.863±0.30
7	Tannins	1.061±0.03	0.350±0.03	0.694±0.00
8	Alkaloids	2.920±0.03	8.385±0.05	5.812±0.04
9	Flavonoid	2.764±0.01	15.581±0.06	7.127±0.05
10	Terpenoid	0.211±0.01	1.044±0.00	0.363±0.04
11	Cardiac glycosides	5.441±0.07	7.544±0.09	5.446±0.49
12	Cyanogenic glycosides	0.133±0.02	0.272±0.01	0.072±0.02
13	Steroids	0.897±0.06	0.131±0.09	0.007±0.03
14	Phenols	0.232±0.02	0.065±0.05	5.789±0.15

Results of Antisickling Analysis

Osmotic Fragility

Erythrocyte fragility measures the membrane stability effect of the extracts in osmotic stress/ hypotonic lysis after 30 minutes incubation. Figure 1 showed a shift to the left of the control indicating a significant resistance to hemolysis. At 0.35% buffered saline concentration the osmotic fragiliogram showed significant decrease in the number of hemolyzed cell for FTH extract (0.184±0.000), JTR extract (0.121±0.000) and JCN extract (0.137±000) which corresponds to a percentage decrease in hemolysis of 26.548±0.056%, 18.055±0.064%, and 20.217±0.035% respectively. The fragiliogram indicated that HU offered better resistance to hemolysis when compared to the extracts and control. HU showed the lowest number of hemolyzed cell (0.091±000) at 0.35% buffered saline concentration equivalent to 14.459±0.040% reduction in hemolysis.

Although, HU reduced the number of hemolyzed cells to the barest minimum, most of the extracts were compared to the control (0.212±0.000, 29.733±0.057%) in which no antisickling agent was added this showed that both HU and the ethnomedicinal extracts significantly ($P < 0.05$) reduced osmotic fragility and offered significant resistance to hemolysis.

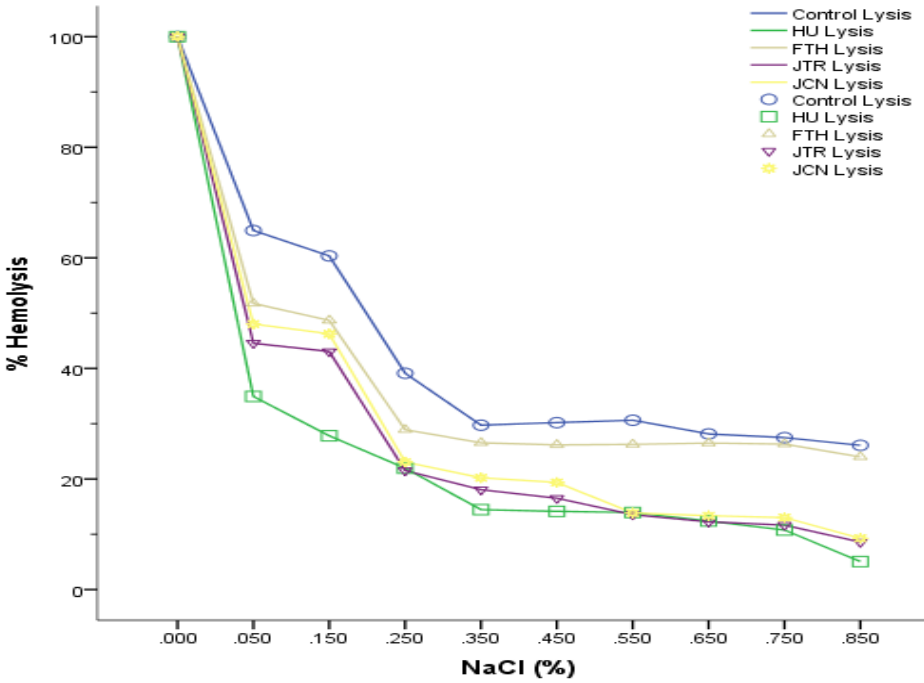

Figure 1. Osmotic fragiliogram of control, HU and crude extracts. *Statistical significance ($p < 0.05$) between groups (FTH: F = 103438.805, K = 3.239. JTR: F = 69097.918, K = 2.261. JCN: F = 68567.975, K = 3.736. Control: F = 124704.344, K = 4,039. HU: F = 135051.504, K = 2.773). F = F-Statistics, K = Levine Statistics. Each line plot and its corresponding line marker as obtained from SPSS represents a plot of % hemolysis Vs % NaCl for control, HU, FTH, JTR and JCN

Sickle Reversal Analysis

Figure 2 showed the results of the sickle reversal analysis of the methanol extracts of FTH, JTR and JCN in comparison with HU and Control. To quantitatively evaluate the antisickling characterization of the ethnomedicinal antisickling agents with respect to their ability to reverse sickling *in vitro*, we carried out an experiment in comparison with the control (RBC's in PBS) and HU. The percentage sickle cell for the control was 91.001±0.170% while, HU reduced the number of sickle cell to 30.414±0.162%. In comparison with the control, the extracts of FTH, JTR and JCN reduced sickling to 49.818±0.082%, 41.001±0.413% and 33.957±0.062% respectively (Figure 2). Although, all extracts showed significant ($P < 0.05$) reduction in the number of sickle cell when compared to the control, the slightly higher potential of HU may be attributed to purity and its synthetic nature because, as a synthetic drug there is little or no interference with other components in its action [29].

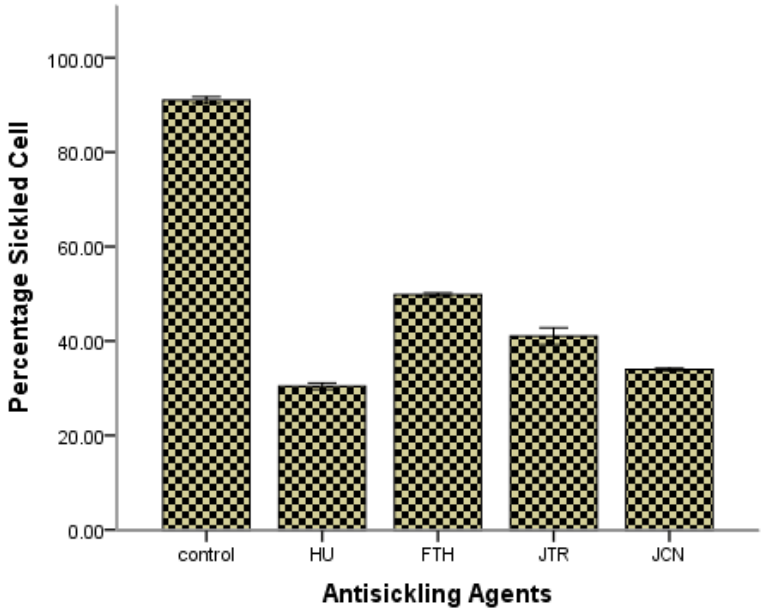

Figure 2. Graph of percentage sickle cell of control, HU and crude extracts. *Statistical significance ($p < 0.05$) between groups ($F = 12680.855$, $K = 5.164$). F = F-Statistics, K = Levine Statistics. Each bar represents the percentage sickle cell in the presence of the control, HU, FTH, JTR and JCN

Polymerization Inhibition Analysis

The underlying principle of sickle hemoglobin polymerization inhibition is that HbSS molecules undergo gelation when deprived of oxygen thus, sodium thiosulphate ($Na_2S_2O_3$) was used as a reductant i.e. deoxygenating agent [48, 49] in this assay.

Figure 3 showed that the extracts of FTH, JTR and JCN inhibited sickle erythrocyte polymerization *in vitro*. Extract of FTH (0.097±0.000) had the least rate of polymerization which indicated that it contained phytocompounds that interacts with the RBC membrane or any of the amino acids involved in sickling, such interactions can perturb the processes that trigger sickle erythrocyte gelation thereby inhibiting sickling.

Similarly, the extracts of JTR (0.216±0.000), JCN (0.104±0.000) and HU (0.123±0.000) also reduced the rate of sickle erythrocyte polymerization when compared with the control in which no antisickling agent was added. Also, there were significant difference ($P < 0.05$) in the rate of polymerization of the antisickling agents assayed.

Figure 4 showed the relative percentage polymerization inhibition of each of the extracts in comparison to the control. The results showed that extracts of FTH, JTR and JCN had percentage polymerization inhibition of 76.888±0.042%, 48.723±0.113% and 75.447±0.063% respectively in comparison to those of HU (70.903±0.150%).

The ability of the extracts to access the studied mechanisms correlates to the phytochemicals responsible for their antisickling activity. Comparatively, for the erythrocyte fragility assay the order of activity for the extracts, HU and control was HU > JTR > JCN > FTH > CTR while, for the sickle reversal assay the order was HU > JCN > JTR > FTH > CTR similarly, the polymerization inhibition assay had the following order of activity FTH > JCN > HU > JTR > CTR. This showed that among the crude extracts, JTR extract preferentially conferred stability to the erythrocyte cell wall while, JCN extract preferentially reduced the number of sickle cells then, FTH extract preferentially inhibited/delayed the processes that trigger the formation of sickle erythrocyte. The presence of phytochemicals even in very low concentration in the plant part studied suggest that these plant part possess antisickling properties since the activity of medicinal plants is attributed to the presence of appropriate phytochemical [3, 12, 16, 17, 47, 50]. Hence, the results of the phytochemical analysis reviewed the presence of phytocompounds which was in line with those reported for the antisickling characterization of plants extracts thus, these phytochemicals may be responsible for the observed antisickling properties of the plant extracts.

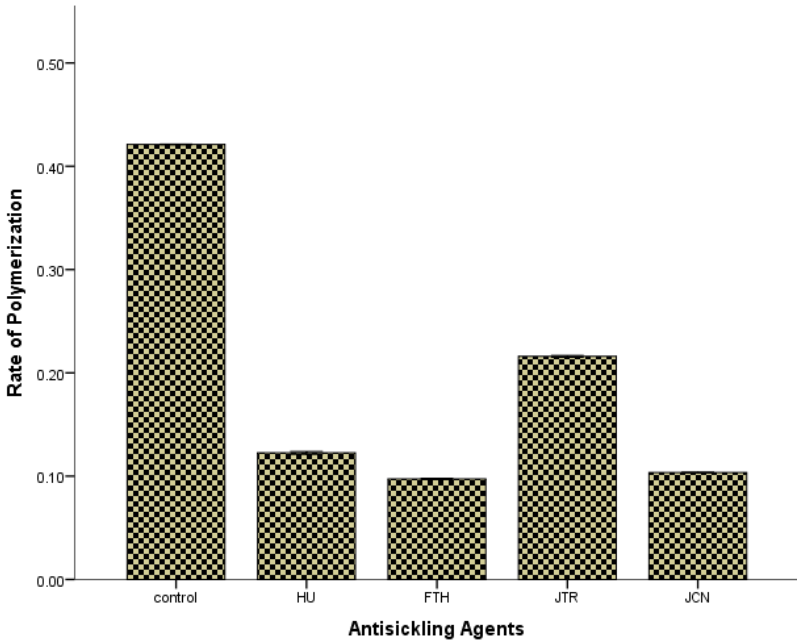

Figure 3. Graph of Rate of polymerization of control, HU and crude extracts. *Statistical significance ($p < 0.05$) between groups ($F = 126901.527$, $K = 4.022$). F = F-Statistics, K = Levine Statistics. Each bar represents the rate of polymerization of sickle erythrocyte in the presence of the control, HU, FTH, JTR and JCN

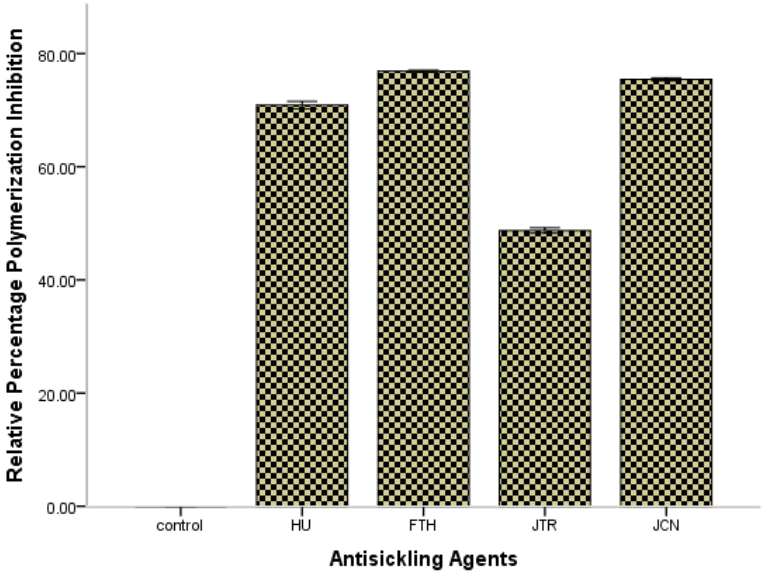

Figure 4. Graph of Relative percentage polymerization inhibition of HU and crude extracts. *Statistical significance ($p < 0.05$) between groups ($F = 128822.394$, $K = 3.622$). F = F-Statistics, K = Levine Statistics. Each bar represents the relative % polymerization of HU, FTH, JTR and JCN

CONCLUSION

The *in vitro* antisickling mechanism of methanol leaves extracts of *Ficus thonningii, Jatropha tanjorensis* and *Justicia carnea* using osmotic fragility test, sickle reversal analysis and polymerization inhibition analysis showed that the methanol extracts of these plant part contains potential antisickling phytochemicals and should further be explored as potential ameliorating alternative and complimentary medicine for the management of sickle cell disease. Consequently, evidenced based in vivo experiments to further confirm the antisickling potential of these extracts

should be considered afterwards, clinical trials of the assayed plant leaves extracts should be researched.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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