



Evaluation of an Undocumented Polyherbal (Faradin®) Used for the Treatment of Sickle Cell Disease in West Africa. Part I: Phytochemistry and Ex-vivo Anti-sickling Study

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Authors' contributions

This work was carried out in collaboration between all authors. Author MCA designed and supervised the study including the clinical protocol, wrote the original draft, reviewed and edited the final version. Author DAG co-designed the clinical protocol and edited the final version. Author LOF manufactured the formulation, provided the extracts and formulation, contributed to the review of the manuscript. Authors IR, NO, CR, BS and MB performed some of the anti-sickling study or phytochemical experiments. Authors RP and SM conducted the cell viability study. All authors read and approved the final manuscript.

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ABSTRACT

Background: An undocumented complementary and alternative medicine (CAM)- Faradin® - was screened for its phytochemicals, anti-sickling effect using a clinical protocol, and toxicity. Faradin is a polyherbal made up of *Zanthoxylum zanthoxyloides*, *Alnus glutinosa* and *Alchornea cordofolia*.

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Methods: The polyherbal and the individual components (coded V, M, and F) were separately screened for phytochemicals such as alkaloids, phenols, flavonoids, sterols and coumarins, using standard tests. Institutional Review Board approved clinical protocol involving 4 female de-identified sickle cell patients was used for the ex-vivo anti-sickling evaluation. The negative control was phosphate-buffered saline (PBS)-washed erythrocytes treated with 2% sodium metabisulfite (SMB) to cause a hypoxic state and result in HBSS polymerization or sickling. As positive control, 2% w/v p-hydroxy benzoic acid (PHBA) solution was used. The anti-sickling effect was studied by incubating the washed erythrocytes in various concentrations of Faradin - Faradin: water v/v ratios (2.5:1 – 10:1) - for 4 hours followed by further incubation with SMB for 1½ hrs. The respective samples were examined under an optical microscope and the number of sickled cells counted. Cell viability to Faradin was also done using human primary hepatocytes (HPP) and Cell Titer-Glo Luminescent assay.

Results: Faradin tested positive for flavonoids, phenols, coumarins, alkaloids and antioxidants. The V component tested positive for coumarins, alkaloids and antioxidants. The M component was positive for phenols, alkaloids and antioxidants while the F component tested positive for flavonoids, phenols and antioxidants. For the anti-sickling study, the negative control (SMB treated erythrocytes) showed 20-28% increase in sickling relative to the PBS-washed. The positive control caused a reversal of sickling by decreasing the percent sickled cells from 21% to 8%. There was a dose-dependent decrease in percent sickled cells; the highest dose reduced the percent sickled cells from 21% to 2%. The activity was likely due to the phenols, polyphenols, flavonoids and antioxidants in the CAM. Cell viability of Faradin® was greater than 85%.

Conclusions: The combined phytochemicals in the polyherbal contributed to the reversal of sickling that is similar to the positive control. Faradin has a high potential for clinical effectiveness in the management of SCD.

Keywords: Faradin; polyherbal; complementary alternative medicine; sickle cell disease; phytochemicals; ex-vivo; anti-sickling; cell viability.

1. INTRODUCTION

1.1 The Disease

Sickle cell disease (SCD) is a genetic disease of the erythrocytes in which the β -globin chain of the adult hemoglobin (HbA) produces a mutant form of homozygous inheritance of the hemoglobin known as sickle hemoglobin (HbSS). This is a result of replacement of the polar glutamic acid with non-polar valine at position 6 of the 146 amino acid β chain [1,2] – Fig. 1 - with resultant loss of negative charge that causes destabilization of the oxygenated hemoglobin. Consequently, there is a decrease in solubility of the deoxygenated hemoglobin and aggregation and polymerization tetramers into long multi-strand polymer fibers or sickled cells as shown in Fig. 2.

A person with sickle cell disease carries two abnormal sickle genes (one from each parent) and is referred to be only homozygous HbSS [3]. Other heterozygous groups such as HbSC, HbAS, HbAC carries only one sickle cell gene, and they are referred to as sickle cell trait (SCT). The C is for abnormal hemoglobin C. These variants of abnormal hemoglobins are collectively classified as sickle cell disease (SCD). Sickled

red blood cells are easily deoxygenated and in this state, tend to precipitate in solution and polymerizes from the normal discoid red cell shape into fibrils that result in sickled cell shape [4]. Unlike normal red blood cells (Fig. 3A), sickled cells do not flow well in the blood vessel and tend to block the blood vessel (Fig. 3B).

1.2 Co-morbidities

SCD is manifested by many clinical outcomes that include painful vaso-occlusive crises (VOC) triggered by sickled red blood cells, as well as leukocytes, platelets and the vascular endothelium, are elements that obstruct vessels [2,5]. The VOC causes intravascular hemolysis, RBCs rupturing and release of free hemoglobin into the plasma.

The free hemoglobin from the VOC has oxidant and inflammatory effects that results in endothelium dysfunction. The heme, hemoglobin and reactive oxygen species (ROS) then result in oxidative stress, decrease in vasodilator nitric oxide (NO) and further inflammation. The result of repeated episodes of these in the spleen is obstruction of blood vessels leading to the infarction of the organ and progressive shrinkage called functional asplenia that may be seen in

children under five years old. The subsequent loss of spleen function leads to susceptibility to bacterial infections, especially pneumococcal infections [6]. Other co-morbidities include stroke, priapism, pulmonary hypertension, acute

chest syndrome (ACS) and chronic organ injuries. Therefore, for effective treatment of SCD, the therapeutic agent should possess anti-infective, anti-sickling and anti-inflammatory properties.

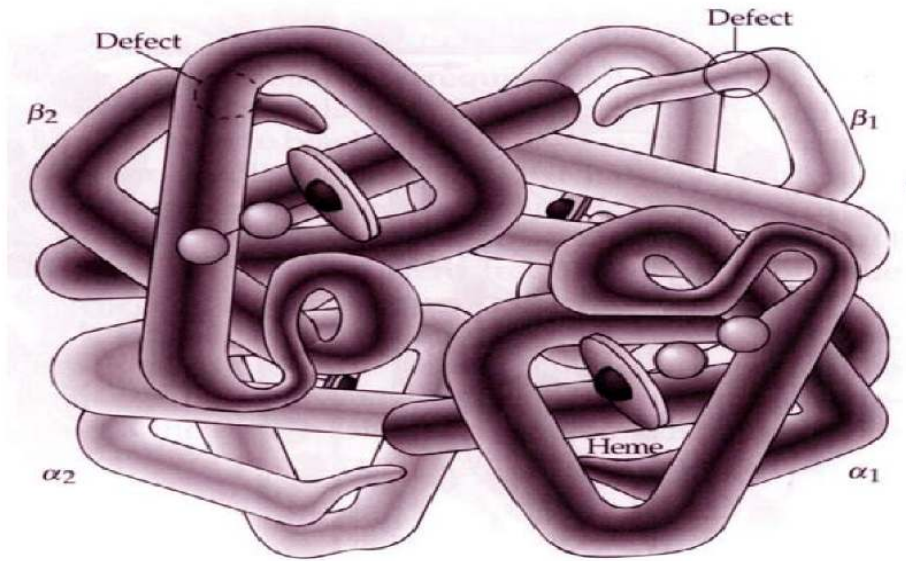


Fig. 1. Structure of HbS hemoglobin (1)

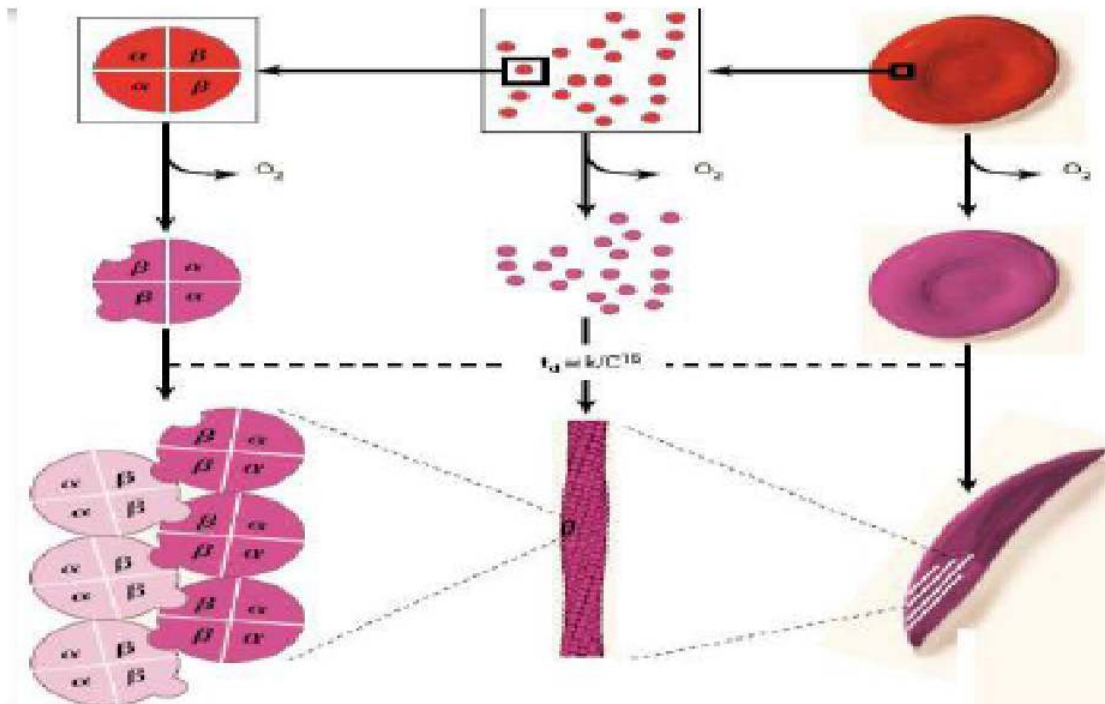


Fig. 2. Polymerization of deoxygenated HBS (2)

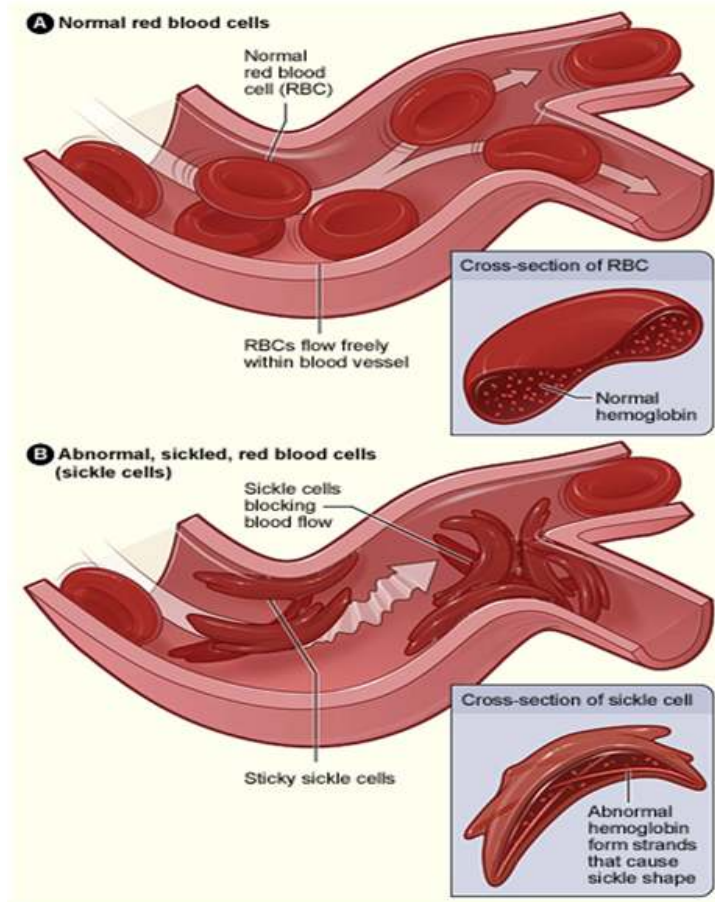


Fig. 3. Normal and Sickled red blood Cells (3)

1.3 Current Treatment of SCD

Currently, hydroxyurea is recommended for treatment of children with SCD. Hydroxyurea is an anti-neoplastic agent for treatment of chronic myelogenous leukemia, melanoma and carcinoma of the ovary. However, toxicities such as myelosuppression or decreased ability of the bone marrow to make red blood and white blood cells, and platelets, neutropenia and reticulopenia have been reported. Thus the patient is expected to visit the clinic every month for blood count monitoring [7,8], which could be a burden for many patients. The precise mechanism by which it produces its antineoplastic effect cannot be described. It was reported to be mutagenic (ability to change genetic material), teratogenic (could cause birth defects) and carcinogenic. Other potential therapeutics for treatment of SCD are 5-hydroxymethyl-2-furfural (5HMF), Aes-103 that is in Phase II clinical trial, hydroxycarbamide and a

newly approved modified heparin drug product – Sevuparin® [9].

1.4 Complementary and Alternative Medicines

A number of CAMs are currently used in Africa for SCD, including Niprisan, Dioscovite, Nicosan, Hildi, Sicklervite and Faradin®. Faradin®, is a liquid polyherbal (three herbal plants) that have been found anecdotally to be effective for treatment of SCD (and with no reported toxicity) in Nigeria and few other West African countries. However, very little investigation has been conducted or published on Faradin [10-12]. It was approved by the Nigerian Agency for Food and Drug Administration and Control (NAFDAC) as a supplement (Certificate number 4-0077L). According to the label of the product, the components of Faradin® are *Zanthoxylum zanthoxyloides*, *Alnus glutinosa* and *Alchornea cordofolia*.

The Nigerian *Zanthoxylum* has eleven species and is commonly found in rain forest vegetation of Southern Nigeria) [13]. A few species are also found in the savannah and dry forest vegetation of South-western Nigeria. The *Zanthoxylum* species belong to the sub-Family Rutoideae; tribe, Zanthoxyleae in the family Rutaceae. It was reported that the species have close similarities and characteristics [14]. *Zanthoxylum zanthoxyloides* or *Fagara zanthoxyloides* has been reported to contain several bioactive compounds that possess medicinal properties. These bioactives include alkaloids, phenolic alkaloids [15,16] that have been reported to have medicinal properties such as antibacterial [17] anti-hypertensive [18] and anti-neoplastic. *Zanthoxylum zanthoxyloides* has amides that are reported to have anesthetic properties [19,20,21, 22]. *Zanthoxylum zanthoxyloides* also reported to contain coumarins that are known to have anti-bacterial, anti-tumor, vasodilatory (in coronary vessels) and anti-coagulant effects [23]. Odebiyi and Sofowora also reported that benzophenanthridine, chelerythrine and aporphine, berberine and phenolic canthine-6-one isolated from *Zanthoxylum zanthoxyloides* exhibited antimicrobial activity. In addition, the root of *Z. zanthoxyloides* also possess anti-sickling effects that have been alluded to presence of bioactives such as 2-hydroxymethyl benzoic acid and vanillic acid [23-26].

Alnus glutinosa (Family: Betulaceae) is known as black alder or 'european alder' and it is found in many North African countries, Asia and Europe. It is one of about 30 species of the trees and shrubs of the genus *Alnus* [27,28]. *Alnus glutinosa* is reported to possess antibacterial and antioxidant properties [27]. Bioactives found in *Alnus glutinosa* include flavonoid, glycosides, terpenoids and xanthenes [29]. *Alnus glutinosa* has also been reported to have anti-inflammatory, emetic and hemostatic effects [30].

Alchornea cordofolia (Family: Euphorbiaceae) is a shrub found in many African countries where it is usually distributed in moist, marshy places and can grow very tall. It is used as herbal medicine for treating various diseases such as stomach ulcers, conjunctivitis, venereal diseases and toothache [31-36], chest pain and anemia [37,38]. The secondary plant metabolites found in *Alchornea cordofolia* include flavonoids, alkaloids, tannins, inulin and alchornine. In the investigation of Adeshina et al. (2012), it was reported that the antibacterial activity could be due to the secondary metabolites.

The rationale for the use of a polyherbal is to treat or manage a disease state holistically, i.e., target the disease and other co-morbidities or reduce unwanted side effects that could have been observed in a single herbal plant. Faradin® has been observed anecdotally to be very effective in the management of sickle cell disease, reducing VOC remarkably and improving the quality of life of the patients. There is a possibility that the polyherbal could also be effective for management of the co-morbidities that include the pain crisis, inflammation and infection. Therefore, the goal of the study is to first evaluate the anti-sickling effect of the polyherbal Faradin® using an ex-vivo method from four de-identified pediatric patients in an approved clinical protocol. An initial investigation of the cell viability of the polyherbal will also be conducted to examine the toxicity of Faradin. Since SCD is a disease with co-morbidities, a report of the antibacterial effects will be presented in future publications.

2. METHODOLOGY

2.1 Materials

2.1.1 Reagents

Gallic acid, quercetin, p-hydroxybenzoic acid, sodium metabisulfite and other chemicals were purchased from Sigma-Aldrich (USA). All other reagents were of analytical grade.

2.1.2 Faradin and plant materials

The manufacturer, Atipo Ventures of Nigeria supplied Faradin. The component plants were first collected in 1995 and authenticated at the National Institute for Pharmaceutical Research and Development, Nigeria. The fresh plants were re-authenticated in 2017 at the herbarium of Ladoke Akintola University of Technology, Ogbomosho, Nigeria.

2.2 Methods

2.2.1 Preparation of faradin

The root of *Zanthoxylum zanthoxyloides*, the bark of *Alnus glutinosa* and the ripe leaves of *Alchornea cordofolia* were harvested, washed with water and sun-dried. Each was individually milled and all were mixed together and extracted by percolation in boiling water. After the extract was allowed to cool, followed by the addition of

sugar (as sweetener) and benzoic acid and alcohol as the preservatives.

2.2.2 Phytochemistry

2.2.2.1 Qualitative

Phytochemical profiles of Faradin and the individual components (coded F, M and V), were investigated by using standard published tests [39] for various bioactive components as stated below. Faradin was used as received for the phytochemistry and all other tests in the study. It is a dark brownish liquid formulation containing about 12% of sugar.

2.2.2.1.1 Flavonoid test

Three drops of concentrated hydrochloric acid and small amount of zinc (25 mg) was added to 1 ml of the percolated extract. A pink or red coloration that disappear on standing (3 min) is an indication of the presence of flavonoids.

2.2.2.1.2 Alkaloid test- Mayer's test

Mayer's reagent was prepared by separately dissolving 1.36 gm of mercuric chloride in 60ml and 5gm of potassium iodide in 10 ml of distilled water. The two solvents were mixed and diluted to 100 ml using distilled water. Acidic aqueous solution of Faradin was prepared by dissolving 300 mg Faradin 3 ml 10% hydrochloric acid and filtered. Few drops of Mayer's reagent will then be added to 1 ml of acidic aqueous solution of samples. A yellow-white precipitate will indicate the presence of the alkaloidal base.

2.2.2.1.3 Phenolic compound test

One milliliter of alcoholic solution of sample was added to 2 ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution. A change of the solution to a dark blue color upon the addition indicates the presence of phenols.

2.2.2.1.4 Tannins test

Ethanol extract of Faradin (1 ml) was added to 2 ml of water in a test tube. 3 drops of diluted ferric chloride solution will then be added. If a deep blue-black color is observed, the presence of gallic tannins (polyphenols) is indicated.

2.2.2.1.5 Steroids/terpenoids test

A small portion of extract was dissolved in 1 ml of chloroform and filtered. To the filtrate on ice, 1 ml of acetic acid was added and then a few drops of

concentrated sulfuric acid were slowly added to the side of the test tube. The appearance of a pink or pinkish-brown ring/color is indicative of the presence of terpenoids. The appearance of blue, bluish-green or a rapid change from pink to blue colors will indicate the presence of steroids and a combination of pink and these colors indicates the presence of both steroids and terpenoids.

2.2.2.1.6 Coumarins

Five ml of ethanolic solution of Faradin was evaporated and the residue dissolved in 1-2 ml of hot distilled water. The mixture was divided into two parts. Half of the volume served as control while to the other half was added 0.5 ml 10% NH₄OH. The two spots were placed on a filter paper and examined under UV light. Intense fluorescence of the treated sample is indicative of the presence of coumarins.

2.2.2.2 Semi-quantitative

Faradin and the components, F, M and V were further examined semi-quantitatively for the total phenol, total flavonoid contents and the antioxidant activity.

2.2.2.2.1 Total phenolic content

This was determined by using Folin-Ciocalteu reagent [40]. Gallic acid, a phenol, was used as a reference standard for plotting calibration curve. The absorbance of triplicate concentrations of 50, 40, 20, 10, 5 and 1 µg/ml at 765 nm λ_{max} was recorded to obtain a standard calibration curve. 200 µL of Faradin or single extract was added to 1mL of Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water). The mixture was neutralized, after 4 min, with 800 µL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 2 hours after which the absorbance was measured at the stated wavelength using double beam UV-VIS spectrophotometer (DU 730 Beckman Coulter). The total phenolic contents were determined from the linear equation of a standard curve. The content of total phenolic compounds was expressed as µg/mg gallic acid equivalent (GAE).

2.2.2.2.2 Total flavonoid content

It was measured with the aluminum chloride colorimetric assay [41]. Aliquots of 1 ml standard quercetin solution (0.29, 0.43, 0.57, 0.71, 0.86 and 1.00 mg/mL) was placed in test tubes and

4ml of distilled water and 0.3 ml of 5% sodium nitrite solution was added into each. After 5 minutes, 0.3 ml of 10% aluminum chloride was added. This was followed by addition of 2 ml of 1 M sodium hydroxide at the 6th minute. Finally, the volume was made up to 10 ml with distilled water and mixed well. The absorbance was determined at 510 nm using UV-Visible spectrophotometer (DU 730 Beckman Coulter). The samples were performed in triplicates. The calibration curve was plotted and linearity was determined. The flavonoid content was expressed as mg/mg quercetin equivalent (QE).

2.2.2.2.3 Antioxidant activity of Faradin

1, 1--diphenyl--2--picrylhydrazyl (DPPH) free radical scavenging assay was used [42]. A calibration curve was generated using quercetin as the standard and the following concentrations - 500 µg/ml, 50 µg/ml, 5 µg/ml, 500 ng/ml, 50 ng/ml and 5 ng/ml. Diluted solutions (1 mL each) was mixed with DPPH (1 mL) and allowed to stand for 30 min for any reaction to occur. DPPH contains an odd electron that becomes paired off in the presence of antioxidant compounds. In the stable free radical form, DPPH is purple and when in contact with antioxidant compounds, it becomes yellow due to the scavenging activity. The resulting decolorization is stoichiometric with respect to the concentration of antioxidant. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was recorded for each concentration.

2.2.3 Ex-vivo clinical protocol

2.2.3.1 Institutional Review Board (IRB) approvals

The IRB approvals were obtained from - Roosevelt University (RU 2014-103) and Texas Baylor College of Medicine/Children's Hospital (H-35374) before the commencement of the study.

2.2.3.2 Clinical protocol criteria

The inclusion criteria were as follows: sickle cell anemia patients, ages 1 - 17 years, and both genders. The exclusion criteria were 1) prior exposure to any sickle cell anemia drug, e.g., hydroxyurea, 2) co-morbidities - HIV/AIDS, tuberculosis, severe renal, liver and heart diseases, 3) no recent blood transfusion within three months prior to sampling and 4) pregnant and breastfeeding females.

2.2.3.3 Informed consent

It was obtained from the blood donors in compliance with the Baylor College of Medicine IRB's protocols. Blood samples were collected from de-identified patients.

2.2.3.4 Collection of blood samples

Five ml of fresh whole blood sample was collected by venepuncture into ethylene diamine tetra-acetic acid (EDTA) vials and used within 48 hours.

Four SCD de-identified female patients (ages 10 months -11 years) were the subjects. A material transfer agreement was signed by both Baylor College of Medicine and Roosevelt University prior to shipping of samples. Each time, the blood was mailed overnight immediately after collection (from Texas Children's Hematology Center to Roosevelt University) and used within 48 hours for the anti-sickling study.

2.2.3.5 Sickling and inhibition studies using De-alcoholized Faradin

2.2.3.5.1 De-alcoholization of Faradin

Prior to the study, the CAM was de-alcoholized with rotary evaporator (using a pre-determined time) to remove the alcohol that was used as preservative in the traditional medicine. The individual extracts were not used for the ex-vivo anti-sickling study.

2.2.3.5.2 Negative and positive controls

The erythrocytes, washed in phosphate-buffered saline (PBS) were treated with 2% sodium metabisulfite or SMB (that causes a hypoxic state and result in HBSS polymerization or sickling) was used as the negative control. .0.5% w/v p-hydroxy benzoic acid (PHBA) solution was used as a positive control according to the report of Ogunyemi et al. [43].

2.2.3.5.3 Treatment of blood samples with Faradin

The washed cells sample was incubated in various concentrations of Faradin - Faradin: water v/v ratios (2.5:1 - 10:1) - for 4 hours followed by further incubation with SMB for 1½ hours. The sample was then placed in 5% buffered formalin solution and kept until the percent sickling was determined by counting under an optical microscope (40X).

2.2.4 Cell viability study

As a preliminary evaluation of the toxicity of Faradin, Cell Titer-Glo Luminescent cell viability study was performed pending future whole animal study. The 96- well white plates were seeded with the human primary hepatocytes (HPH) for a period of 24 hours, along with control wells containing medium without cells to obtain a value for background luminescence. 2 ul of the test compounds Faradin, Extract F, Extract M or Extract V in the ratio of 2.5:1 with deionized water were incubated for a period of 30 min, 1 h and 2 h. The plate was then equilibrated at room temperature for 30 minutes. An equal volume of the cell Titer-Glo reagent to the volume of cell culture medium present in each well was added. The contents were mixed on an orbital shaker for 2 minutes to induce cell lysis. The plate was allowed to stabilize at room temperature before recording the luminescence that was measured using DTX 880 Multimode Detectors from Beckman Coulter (Fullerton, CA). An average percentage of cell viability to that of the normal cells was calculated to determine the action of the test compounds on the HPH based on the assumption that the luminescence recorded would be proportional to the viable cells.

2.3 Statistical Analysis

The statistical analyses for the anti-sickling effect of Faradin were carried out using one-way analysis of variance (ANOVA) single factor where P value < 0.05. Similarly, ANOVA was used for the cell viability differences between the polyherbal Faradin and individual herbal components F, M and V and the effect of time on the cell viability of Faradin.

3. RESULTS AND DISCUSSION

3.1 Phytochemistry

3.1.1 Qualitative tests

Faradin exhibited presence of flavonoids, phenolic compounds, coumarins, alkaloids, flavonoids and antioxidants (Table 1). The phenolic compounds and coumarins are from *Zanthoxylum zanthoxyloides* component and these could be contributing bioactives for the anti-sickling activity. The *Alnus glutinosa* component had phenolic compounds and alkaloid whereas the *Alchornea cordofolia* possessed flavonoids as well as phenols. All the components showed presence of antioxidants.

The qualitative tests indicated no presence of steroids/terpenoids. The single components, F, M and V showed the characteristic differences in bioactive components that made the summative presence of the secondary metabolites in Faradin.

3.1.2 Semi-quantitative tests

3.1.2.1 Total phenolic content

The total phenolic contents in Faradin, F and M and V are presented in Fig. 4. Using the calibration curve and the dry weight of the sample, the contents for Faradin, extract F and M were similar, 769.3, 758.0 and 796.3 ng/mg GAE respectively. The V extract had the lowest phenolic compounds, 310 ng/mg GAE. In the qualitative assay, there was a negative observation of phenols but the latter test showed better sensitivity where the phenolic content could be detected.

3.1.2.2 Flavonoid test

The total flavonoid was expressed as mg/mg quercetin equivalent using the standard curve. The flavonoid contents of Faradin, F, M and V extracts varied 77.9, 126.7 and 93.1 mg/mg. M extract had the highest flavonoids followed by F and Faradin (Fig. 5). The V extract had the least flavonoids. This reflects the negative observation of the qualitative assay that is less sensitive.

3.1.2.3 Antioxidant or scavenging activity

The scavenging profiles of Faradin and the single extracts M and V were similar for 1:10 and 1:20 v/v dilution of Faradin:water. The activities were not greater than 10%. However, at higher concentrations (1:1 and 1:2), the scavenging activities increased rapidly to greater than 70%. F extract had an overall relatively lower activity (Fig. 6). It had similar low activity at lower concentrations but increased at higher concentration to an activity that was not greater than 50%.

Phenolic compounds, coumarins, flavonoids and antioxidants are reported to have anti-bacterial and anti-inflammatory actions respectively as stated above. Being a polyherbal, the Faradin reflects the phytochemicals of one or more of the three components. The contributory effects of each component is needed for treatment of SCD that is usually accompanied by co-morbidities such as asplenia that can lead to bacterial infections especially in younger children.

Table 1. Phytochemistry profiles of faradin and the constituents extracts – F, M and V

Phytochemicals	Faradin	M	F	V
Flavonoid	+	-	+	-
Phenolic Compounds/ Tannins	+	+	+	-
Coumarins	+	-	-	+
Alkaloids	+	++	-	+
Steroids/ Terpenoids	-	-	-	-
Antioxidant	+	+	+	+

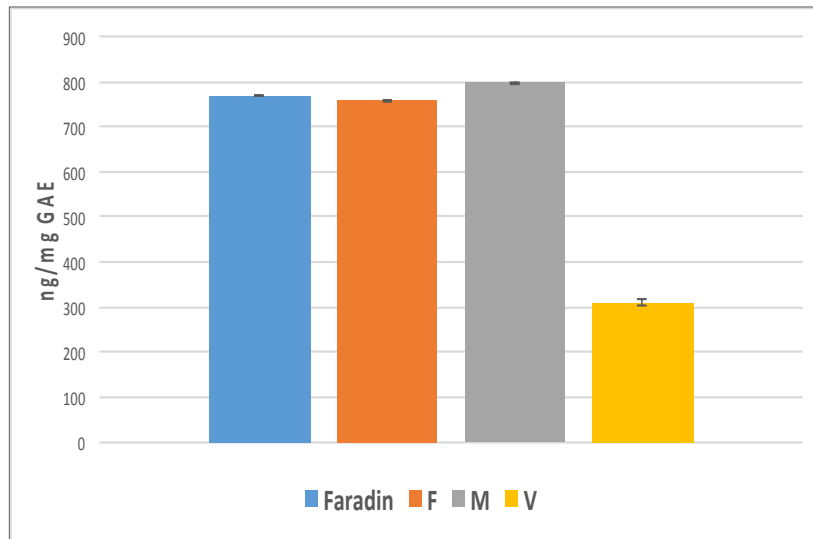


Fig. 4. Total Phenols in Faradin and the individual extracts F, M, V

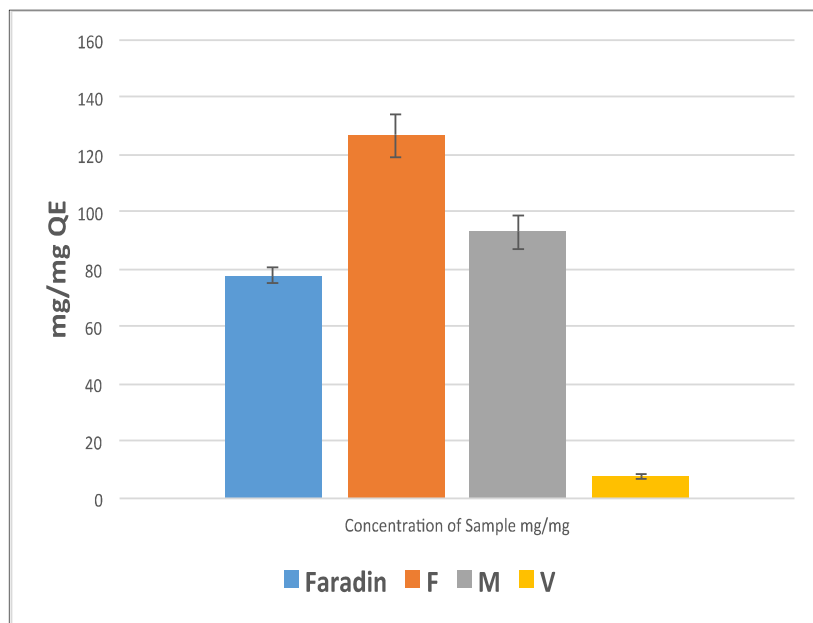


Fig. 5. Flavonoid contents in Faradin and the individual extracts F, M, V

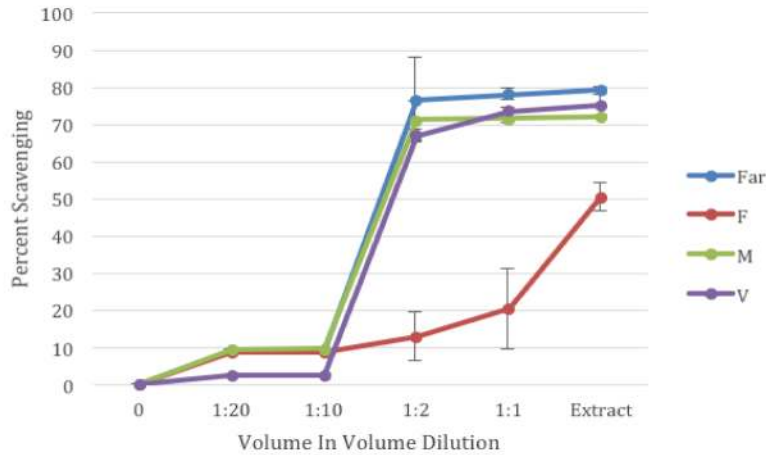


Fig. 6. Scavenging activities of faradin and the individual extracts, F, M and V

3.2 Ex-vivo Anti-sickling Studies

3.2.1 Sickling of the erythrocytes

The PBS washed cells had 21% sickled cells (Fig. 7). The SMB treated cells (negative control) had about 27% sickled cells, an indication that SMB increased the number of sickled cells by 28% as a result of polymerization.

3.2.2 Sickling inhibition or reversal of sickling studies

The positive control (PHBA) showed 8% sickled cells compared to the 21% in the sample that was washed with PBS (Figs. 8 and 9). Microscopic image of cells treated with 10:1 ratio of Faradin is shown in Fig. 10.

As seen in both the positive control and the treatments, the anti-sickling was not reversible upon addition of SMB to the cells samples that were incubated with Faradin for 1 1/2 hours.

There was a dose-dependent reversal of sickling using the different concentrations of Faradin - Faradin: water ratios 2.5:1, 5:1, 7.5:1 and 10:1 volume ratios (Fig. 11). The highest dose reduced the percent sickled cells from 21% to 2%, i.e., Faradin caused approximately 95% reversal of sickling at the highest dose.

The mechanism of anti-sickling activity could be due to inhibition of reactive oxygen species (ROS) generation by the antioxidants or polyphenols in Faradin®, leading to quenching of free radicals and alteration of the intra-cellular redox potential. The effect could also be due to interaction of antioxidants or polyphenols in

Faradin® with various inflammatory mediators such as cytokines and blocking of inflammatory signaling, and interfering with chemokines. The effect could also be due to interaction of antioxidants or polyphenols in Faradin® with various inflammatory mediators such as cytokines and blocking of inflammatory signaling, and interfering with chemokines. This will be further investigated in future studies.

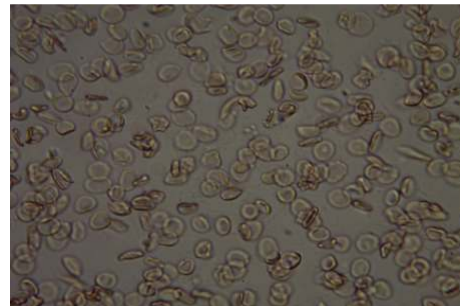


Fig. 7. Sickled Red Blood Cells (RBCs) washed with PBS (negative control)

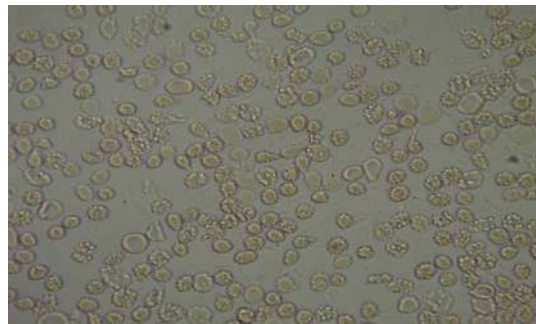


Fig. 8. Sickled RBCs treated with 0.5% PHBA (positive control)

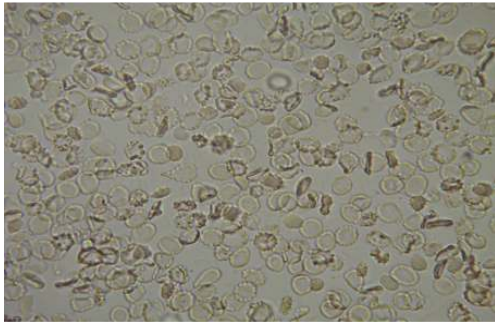


Fig. 9. Typical Sickled RBCs treated with SMB (negative control)

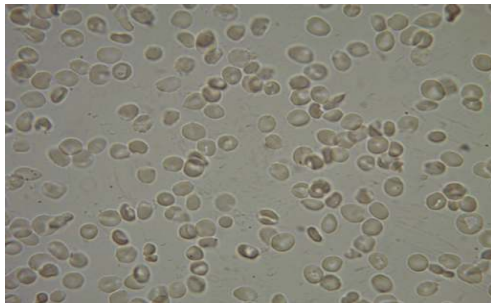


Fig. 10. Reversal of sickled RBCs incubated with 10:1 volume ratio of Faradin:Water

The anti-sickling effect of the Faradin® is due to the phytochemical activity of Faradin contributed by the components F, V and M and it is suggestive of effectiveness of the polyherbal in the treatment of SCD compared to the single component. The confirmed anti-sickling or

reversal of sickling, and similarity in action to the positive control makes the CAM, (reported to have no side effects), a highly relevant clinical candidate for treatment of SCD.

3.3 Cell Viability Study

Cell viability of Faradin and the individual components Faradin, F, M and V using 2.5:1 v/v ratio was time dependent. Faradin had 98%, 88% and 88% cell viability at 30 min, 1h and 2h respectively after treatment. Among the single extracts, F had the lowest cell viability – 88%, 61% and 49% - at the respective treatment times. M and V extracts had the cell viability > 96% at all treatment times. (Fig. 12A, B, C). Faradin, being a polyherbal showed an additive effect of the combination of the plants in modulating cell viability.

The cell viability of HPH showed no significant difference between polyherbal Faradin and individual herbal components F, M and V at the end of 30 minutes. No significant differences were noted at the end of 1 hour and 2-hour exposure period, except between Faradin and Extract F. There was no significant difference in cell viability for Faradin at 30 minutes, 1 hour and 2-hour time periods, having a range of 88%-98% cell viability ($p < 0.05$). More future studies will be done using whole animal model and higher levels of Faradin. Being a polyherbal, a combination of the three herbal plants, is able to maintain appreciable cell viability within the period.

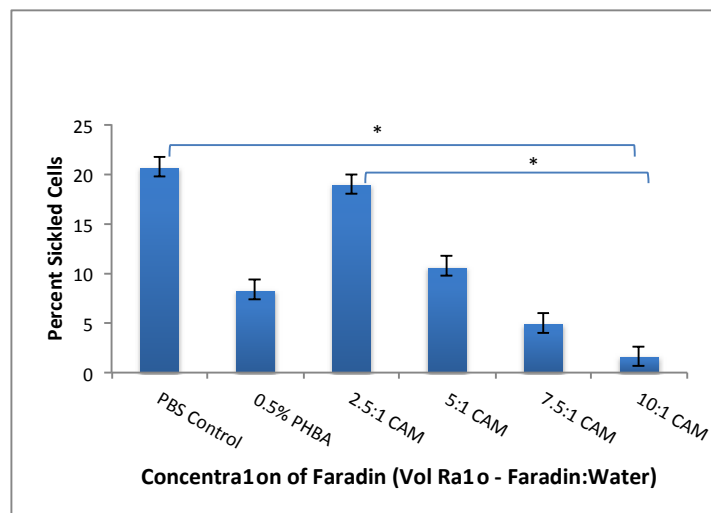


Fig. 11. Anti-sickling effect of Faradin® on Sickled erythrocytes from SCD patients
(* $p < 0.05$)

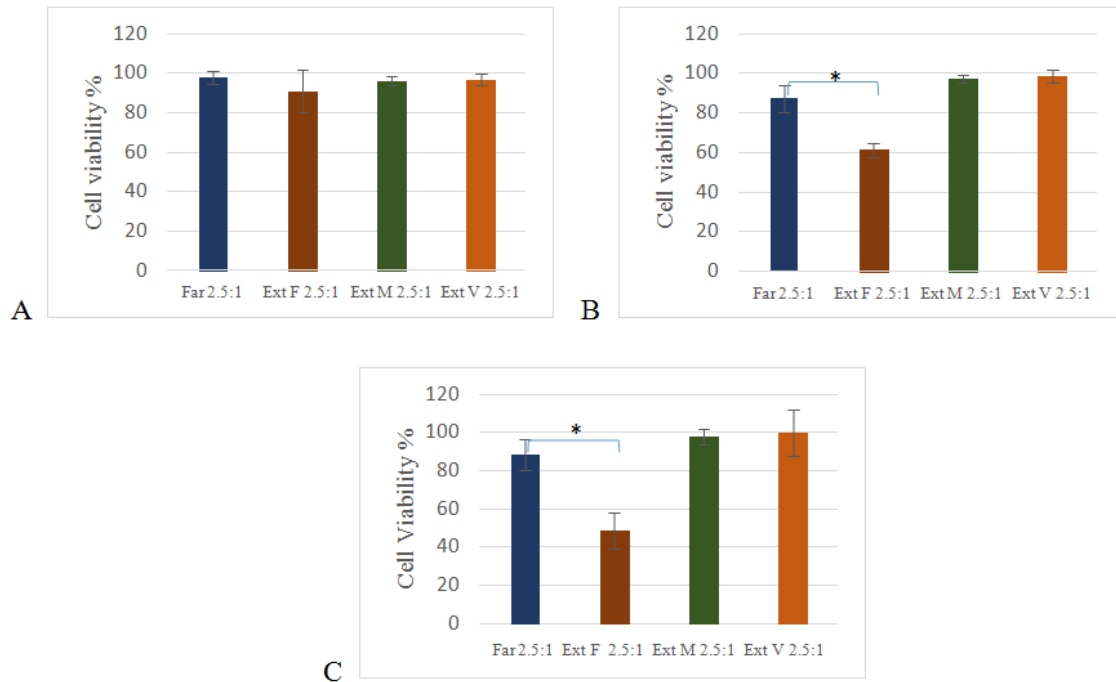


Fig. 12. Cell Viability of human primary hepatocytes exposed to polyherbal Faradin, individual herbal components F, M and V at 30 min (A), 1 h (B) and 2 h (C) Using CellTiter-Glo luminescent cell viability reagent
 (* $p < 0.05$)

4. CONCLUSIONS

The bioactives from the three herbal plant components of Faradin contributed to the observed anti-sickling effect of the polyherbal. The antioxidant properties of all the plants could have also been effective in cell protection and in anti-sickling combination. This makes the CAM, (anecdotally known clinically to have no side effects), a highly relevant clinical candidate for management of SCD. Further elucidation of the chemicals in the product that could be responsible for the anti-sickling effects is ongoing in our lab. Future research will include determination of anti-sickling mechanism of action, potential anti-inflammatory activity, elucidation of bioactive chemicals in the polyherbal using HPLC, LC-MS or other techniques, and clinical efficacy studies.

ETHICAL APPROVAL AND CONSENT

Declared under the Institutional Review Board (IRB) Approvals and Informed Consent, in compliance to the international standard or university standard.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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