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ANTISICKLING PROPERTIES OF THREE MEDICINAL PLANTS AND THEIR COMBINATION

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ABSTRACT: Background: Sickle cell anemia continues to be prevalent in the African population of which Nigeria alone has an epidemiology of 25% and only a few official medications are available, necessitating this current study. **Methods:** *Cnestis ferruginea* Vahl ex DC, Connaraceae, *Alchornea laxiflora* (Benth.) Pax & K. Hoffman Euphorbiaceae, and *Spathodea campanulata* Beauv., Bignoniaceae, which have various uses ethno medicinally in Nigeria were extracted using a cold method of extraction with absolute methanol. The abilities of the extracts individually and in combination, to inhibit or reverse sickling of erythrocytes *in-vitro* at low oxygen tensions according to the modified methodology of Dean and Schechter, 1978 were examined. **Results:** While *C. ferruginea* leaf gave 94.6% sickling reversal activity at 4 mg/ml, *S. campanulata* gave reversal activity of 89.6% at 4 mg/ml and *A. laxiflora* leaf gave the highest sickling inhibitory activity of 98.8% at 8 mg/ml. The combination of *A. laxiflora* leaf and *C. ferruginea* leaves when extracted together at ratio 1:2 gave an inhibition activity of 91.7% at 4mg/ml and 93.5% inhibition at ratio 2:1 at 8 mg/ml, these values were statistically significant at $P < 0.05$. **Conclusion:** This study shows that some of these plants were more active when tested individually than when in any combinations.

INTRODUCTION: Sickle cell disease is a general term that is used to describe a family of hemoglobinopathies (a condition of blood disorders, genetic in origin; which resulted from the alteration of the normal sequence arrangement of the amino acids of the globin gene of the hemoglobin).

Among these haemoglobinopathies, sickle-cell anemia (SCA) is the most severe accounting for over 60% of the world's major haemoglobinopathies and with respect to survival, individuals with SCA has a median survival nearly 20 years lower than the other hemoglobinopathies due to the differences in clinical severity observed in individuals with sickle cell anemia. Nigeria alone has an epidemiology of 25%¹ and only a few official medications are available, necessitating this current study.

Cnestis ferruginea Vahl ex DC, (Connaraceae) is a shrub, found in the Western tropical Africa majorly Senegal to West Camerouns and in other parts of

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tropical Africa, it has unipinnate compound leaf, that is alternately arranged. It is also referred to as; Horn-of-plenty (English), and “Akara-aje” “Gboyin-gboyin” Omu-aje (Yoruba), “Amunketa”, “Okpe-nketa”, or Okpe-isi-uketa (Igbo), “Utina bua” (Efik) and “Ukpe”, or “ibi-eka” (Bini) tribes of Nigeria. This plant ethnomedicinally have been used as abortifacient, laxative, wound healing, bronchitis, fever (iba) treatment, taken as a tonic, teeth-whitening, snake-bite treatment, walk stimulant in weak children in various parts of in Africa ^{2, 3}. Its analgesic, anti-inflammatory, hypoglycemic and antioxidant pharmacological activities have been reported ^{4,5}.

Alchornea laxiflora (Benth) Pax & K. Hoffm is a deciduous, erect to straggling shrub or small tree found in the Eastern part of Nigeria to Ethiopia and DR Congo and through East Africa to Zimbabwe, Mozambique, North-eastern part of South Africa and Swaziland ². It is also called Short pod, Alum plant (English), Ijàn, Ijàn funfun, Ijándú, Pèpè, Opoto (Yoruba), Uwenuwen, Ukpo-ubieka (Edo) Ububo, (Igbo), Urievwu (Urhobo) Fura amarya (Hausa) ⁶.

In Nigeria various parts of this plant have been used to treat inflammatory and infectious disease, herbal antimalarial preparations, preservation of kola nuts, fruits and vegetables, used as chew-sticks and treatment of poliomyelitis, stiff necks and measles ^{7, 8}. Antimicrobial, antimalarial, antioxidant and hepatoprotective activities have been reported in various parts of the plant ^{9, 10, 11, 12}. *Spathodea campanulata* Beauv is a medium sized tree native to the following tropical African countries: Angola, Ethiopia, Ghana, Kenya, Sudan, Tanzania, Uganda, Zambia ¹³. It is also called African tulip tree, Flame of the forest (English), Akoko, Amuju-tooro (Yoruba), Ogili-si, Ogirisi (Ibo), Aduruku (Hausa) tribes in Nigeria.

Ethnomedicinally, various parts of this plant have been used as diuretic, anti-inflammatory, urethra inflammation treatment, antidote against animal venoms, treatment of convulsing children, treatment of fungal skin infection, herpes, stomach ache, diarrhea, dyspepsia, peptic ulcer, toothaches, diabetics, wound healing in various parts of Africa ¹⁴. The analgesic, anti-inflammatory, antibacterial, antifungal, antioxidant, hypoglycemic, larvicidal,

pupicidal and wound healing activities have been reported in various parts of the plant ¹⁵⁻²⁰.

Since only a few medications are officially available for the management sickle cell disease, the aim of this research was to test the ability of *Cnestis ferruginea*, *Alchornea laxiflora*, and *Spathodea campanulata* singly and in combination to inhibit or reverse sickling or polymerization of erythrocytes *in-vitro* by using a reducing agent to create low oxygen condition similar to what triggers sickle cell crisis ²¹, some of these plants were selected due to their bright red colorations in fruit (*C. ferruginea*), and flower (*S. campanulata*) which is suggestive of high presence of anthocyanins, a flavonoidal compound which have established pharmacological activity as antioxidants, and anti-sickling ²².

MATERIALS AND METHODS:

Collection of Plant Materials: The root, leaf and fruit of *Cnestis ferruginea* Vahl ex DC (Connaraceae), leaf of *Alchornea laxiflora* (Benth.) Pax & K. Hoffman (Euphorbiaceae) and the flower of *Spathodea campanulata* Beauv. (Bignoniaceae) were all collected from different locations within the campus of Obafemi Awolowo University, Ile-Ife, Nigeria. Each plant was identified by Mr. Ogunowo, I.I. from Department of Pharmacognosy herbarium and Mr. Ademoriyo from the Department of Botany herbarium both in Obafemi Awolowo University, Ile-Ife, and voucher specimens were deposited at the Department of Botany herbarium.

The flower of *S. Campanulata*, the fresh leaf samples of *A. laxiflora* and *C. ferruginea* were air-dried at room temperature while the fruit and root of *C. ferruginea* were oven-dried at 60 °C, after which all the dried plant materials were powdered separately using the grinding machine.

Extraction of Plant Material: Powdered plant material (200 g) of the root, leaf and fruit of *Cnestis ferruginea*, leaf of *A. laxiflora* and the flower of *S. campanulata* were separately macerated with cold methanol and shaken in a mechanical shaker at regular intervals for 72 h, filtered and the filtrate concentrated to dryness *in-vacuo*, to yield their respective methanolic dried extracts.

Solvent Partitioning: The methanolic extract (3 g) of *Alchornea laxiflora* leaf was suspended in 100 ml distilled water and successively partitioned into n-hexane (750 ml), dichloromethane (750 ml) and ethyl acetate (700 ml), each fraction was concentrated *in-vacuo* to dryness, to give n-hexane (0.3 g), dichloromethane (1.44 g), ethyl acetate (0.63 g), and the aqueous layer was also concentrated to dryness to give (0.15 g). This partitioning experiment was repeated for *C. ferruginea* leaf extract (3 g), suspended in 100 ml distilled water and successively partitioned into n-hexane (750 ml), dichloromethane (750 ml) and ethyl acetate (700 ml) each fraction was concentrated *in-vacuo* to dryness, to give the n-hexane (0.66g), dichloromethane (0.35 g), ethyl acetate (0.05 g) and the aqueous layer was also concentrated to dryness to give (0.15 g) partitioned fraction. The eight (8) partitioned fraction solid residues were then reserved for reversal and inhibitory antisickling experiments respectively.

Preparation of the Concentration of Test Samples for Anti-Sickling Activities: For each of the various test samples, a stock solution of 10 mg/ml was prepared by dissolving 100 mg of the extract residue in 10 ml of distilled water while dilutions were made from the stock solution serially to obtain the following concentrations: 8 mg/ml, 6 mg/ml, 4 mg/ml, and 2 mg/ml in order to investigate the concentration-response relationship in antisickling experiments. For the partitioned fraction residues, a solution of 4 mg/ml was prepared by reconstituting 40 mg of the residue in 10 ml of distilled water and used for antisickling experiments. A 0.1% volume of Polysorbate 80, commercially known as Tween 80, was used to solubilize the partitioned fraction residues in distilled water.

Antisickling Assay Procedures: The inhibitory and reversal antisickling assay methods are described below;

RESULTS:

TABLE 1: PERCENTAGE ANTISICKLING ACTIVITIES OF COLD EXTRACTION OF THE THREE PLANTS AT DIFFERENT CONCENTRATIONS

Plant extract	Reversal Activity \pm SEM 2 mg/ml	Inhibitory Activity \pm SEM 2 mg/ml	Reversal Activity \pm SEM 4 mg/ml	Inhibitory Activity \pm SEM 4 mg/ml	Reversal Activity \pm SEM 6 mg/ml	Inhibitory Activity \pm SEM 6 mg/ml	Reversal Activity \pm SEM 8 mg/ml	Inhibitory Activity \pm SEM 8 mg/ml	Reversal Activity \pm SEM 10 mg/ml	Inhibitory Activity \pm SEM 10 mg/ml
<i>C. ferruginea</i> (L)	10 $\pm 0.1^*$	37.09 $\pm 0.2^*$	94.62 $\pm 0.3^*$	50.94 ± 1.2	88.3 $\pm 1.6^*$	12.40 $\pm 0.4^*$	64.6 $\pm 6.6^*$	11.40 $\pm 0.3^*$	54.6 ± 6.6	9.40 $\pm 0.3^*$

Inhibitory Antisickling Assay: The inhibitory antisickling assay was investigated using the modified method of ¹⁸ Dean and Schechter (1978), where 0.6 ml of freshly prepared 2% sodium metabisulphite solution was used to induce sickling on HbSS whole blood before treatment with the test sample. At the end of the experiment, both sickled and unsickled cells were counted using a light microscope at a magnification of 1000 (using $\times 100$ objective $\times 10$ eye-pieces); five fields of view were counted per slide from the triplicate experiments and the average % inhibitory activity calculated.

Phosphate buffered solution (0.2 ml) or 0.2 ml polysorbate tween-80 was used in place of the test sample to serve as negative control while vanillic acid solution and Ciklavit[®] (0.2 ml) were used as positive controls for the inhibitory activity experiments.

Reversal Antisickling Assay: The reversal antisickling assay was investigated using the modified method of Dean and Schechter (1978), where 0.6 ml of freshly prepared 2% sodium metabisulphite solution was used to induce sickling on HbSS whole blood after adding the test sample to HbSS whole blood. At the end of the experiment, both sickled and unsickled cells were counted using a light microscope at a magnification of 1000 (using $\times 100$ objective $\times 10$ eye-pieces); five fields of view were counted per slide from the triplicate experiments and the average % reversal activity calculated.

Phosphate buffered solution (0.2 ml) or 0.2 ml polysorbate tween-80 was used in place of the test sample to serve as negative control while para-hydroxyl benzoic acid solution (PHBA) and Ciklavit[®] (0.2 ml) were used as positive controls for the reversal activity experiments (Ethical clearance protocol number for this research is: ERC/2018/08/01).

<i>C. ferruginea</i> (P)	10 ±0.3*	10 ±0.4*	81.47 ±2.5*	8.24 ±2.2*	91.1 ±1.8*	52.21 ±0.3	41.7 ±8.0	42.21 ±0.2*	64.6 ±6.6	40.21 ±0.3*
<i>C. ferruginea</i> ®	10 ±0.2*	10 ±2.1*	10 ±2.4*	10 ±3.3*	10 ±3.2*	10 ±2.6*	10 ±0.5*	10 ±3.2*	10 ±3.1*	10 ±1.1*
<i>S. campanulata</i> (F)	10 ±0.5*	10 ±3.2*	89.62 ±2.1*	38.47 ±3.3*	10 ±3.1*	10 ±1.1*	10 ±0.1*	48.51 ±0.2*	30 ±2.5*	37.92 ±1.3*
<i>A. laxiflora</i> (L)	10 ±0.1*	48.51 ±0.2	48.66 ±1.1*	91.61 ±1.2*	30 ±2.5*	97.92 ±1.3*	25 ±2.5*	98.8 ±0.4*	27 ±2.5*	93.7 ±1.3*
Ciklavıt®	46.0 ±2.4	46.0 ±2.4	62.0 ±0.5	55.7 ±3.3	56.0 ±2.7	55.7 ±3.6	46.0 ±2.4	67.4 ±1.4*	65.0 ±4.5	68.5 ±2.1
Vanillic acid	-	45.2 ±2.1*	-	65.0 ±4.5	-	56.0 ±1.5	-	45.2 ±2.1*	-	56.0 ±1.5*
PHBA	43.3 ±2.1	-	43.5 ±2.1*	-	52.3 ±2.1	-	50.1 ±1.2	-	65.3 ±3.3	-

Keys: (*C. ferruginea* (L))-*Cnestis ferruginea* Leaf, (*C. ferruginea* (P))-*Cnestis ferruginea* Fruit, (*C. ferruginea* ®)- *Cnestis ferruginea* Root, (*S. campanulata* (F))- *Spathodea campanulata* Flower, (*A. laxiflora* (L))- *Alchornea laxiflora* Leaf, PHBA- Para hydroxyl benzoic acid, Positive controls = Ciklavıt®, Vanillic acid and Para hydroxyl benzoic acid. Results are expressed as mean ± SEM. Significant results at p<0.05 are asterisked

TABLE 2: PERCENTAGE ANTISICKLING ACTIVITIES OF A. LAXIFLORA LEAF PLUS C. FERRUGINEA LEAF WHEN EXTRACTED TOGETHER

Extract of the combined leaves and Ratios	Reversal Activity ± SEM 2 mg/ml	Inhibition Activity ± SEM 2 mg/ml	Reversal Activity ± SEM 4 mg/ml	Inhibition Activity ± SEM 4 mg/ml	Reversal Activity ± SEM 6 mg/ml	Inhibition Activity ± SEM 6 mg/ml	Reversal Activity ± SEM 8 mg/ml	Inhibition Activity ± SEM 8 mg/ml	Reversal Activity ± SEM 10 mg/ml
ALL+CFL (1:1)	18.6 ±6.6*	27.4 ±3.1*	15.2 ±4.1*	31.8 ±4.0*	13.4 ±3.3*	90.3 ±1.2*	81.7 ±3.1*	91.4 ±1.2*	20.2 ±2.2*
ALL+CFL (1:2)	8.4 ±3.1*	52.8 ±7.0	14.4 ±1.1*	91.7 ±2.9*	22.5 ±1.1*	24.3 ±3.8*	55.1 ±4.43	66.5 ±3.3	28.4 ±3.1
ALL+CFL (2:1)	13.4 ±3.1*	51.4 ±5.3	38.4 ±4.35*	28.2 ±3.8*	19.4 ±3.4*	17.1 ±2.1*	16.6 ±4.4*	93.5 ±1.01*	63.7 ±1.88*
Ciklavıt®	40.0 ±1.4	49.0 ±1.4	66.0 ±0.5	57.7 ±3.3	58.0 ±2.8	57.7 ±3.5	46.5 ±2.3	69.4 ±1.4	65.0 ±4.5
Vanillic acid	-	44.2 ±2.1	-	65.0 ±4.1	-	56.1 ±1.3	-	45.2 ±2.3*	-
PHBA	33.3 ±2.1	-	45.5 ±2.1*	-	56.3 ±2.2	-	50.3 ±1.1	-	67.3 ±3.3

Keys: (ALL)- *Alchornea laxiflora* Leaf, (CFL)-*Cnestis ferruginea* Leaf, PHBA- Para hydroxyl benzoic acid
Positive controls = Ciklavıt®, Vanillic acid and Para hydroxyl benzoic acid. Results are expressed as mean ± SEM Significant results at p<0.05 are asterisked*

TABLE 3: PERCENTAGE ANTISICKLING ACTIVITIES OF THE PARTITIONED FRACTIONS FROM A. LAXIFLORA LEAF AND C. FERRUGINEA LEAF EXTRACTS

Plant material	Partitioned fractions	Reversal Activity ± SEM 4 mg/ml	Inhibition Activity ± SEM 4 mg/ml
<i>A. laxiflora</i> (L)	n-Hexane	14.3.8 ± 0.95*	45.8 ± 0.85*
	DCM	14.1.6 ± 0.38*	50.3 ± 4.33*
	Ethyl acetate	50.0 ± 0.33	83.9 ± 1.70*
	Aqueous	48.6 ± 0.31*	62.2 ± 1.01*
<i>C. ferruginea</i> (L)	n-Hexane	10.3 ± 1.5*	19.23 ± 1.82*
	DCM	41.5 ± 0.90*	10.4 ± 2.1*
	Ethyl acetate	89.5 ± 1.1*	13. ± 3.3*
	Aqueous	52.1 ± 0.2*	10.5 ± 1.1*
Ciklavıt®		62.0 ± 0.5	54.7 ± 3.3
Vanillic acid		-	60.0 ± 3.1
PHBA		43.5 ± 2.1*	-

Keys: (*A. laxiflora* (L))- *Alchornea laxiflora* Leaf, (*C. ferruginea* (L))-*Cnestis ferruginea* Leaf, PHBA- Para hydroxyl benzoic acid.
Positive controls = Ciklavıt®, Vanillic acid and Para hydroxyl benzoic acid. Results are expressed as mean ± SEM. Significant results at p<0.05 are asterisked*

DISCUSSION AND CONCLUSION:

Antisickling Assays: The ability of each of the test extracts; singly and in combination, to prevent or reverse the sickling process was tested using 2%

w/v of sodium metabisulphite; a physiologically compatible reducing agent to induce sickling while vanillic acid (4-hydroxy-3-methoxybenzoic acid), a derivative of p-hydroxy benzoic acid was used as

a standard positive control for antisickling inhibitory activity and p-hydroxy benzoic acid was used as a standard positive control for antisickling reversal activity²³. Ciklavit®, an aqueous decoction of the seed of *Cajanus cajan*, already available in the Nigerian market and used effectively in the management of sickle cell anemia was used as a positive reference standard for both inhibitory and reversal assays²⁴.

However, in this study, it was observed that none of the plant materials tested either singly or as combinations exhibited both inhibitory and reversal activities to acceptable extents in the same plant. Thus, the activities observed were either inhibitory or reversal. This observation is buttressed by several studies which have established that a biomolecule could inhibit or reverse *in-vitro* polymerization of red blood cells either by binding to the complimentary site of deoxygenated HbS monomers and modify the amino acid residue that contribute to the three-dimensional structures of HbS contact region or the stabilization of the relaxed state of HbS molecule^{25,26}.

Reversal Anti-Sickling Assay: The methanolic extracts of *C. ferruginea* leaf and fruit, as well as that of *S. campanulata* flower showed significant reversal activities on sodium metabisulphite-induced sickled erythrocytes as follow; 94.6, 81.5, and 89.6%, respectively at 4 mg/ml. These values were higher than the positive control (PHBA, 43.5%) but comparable to Ciklavit® (62%). The reversal activity of *C. ferruginea* fruit gave 91.1% at 6 mg/kg.

The methanolic extract of the leaf of *C. ferruginea* had also been found to decrease the rate hemoglobin glycosylation in a manner comparable to quercetin when hemoglobin was exposed to varying amounts of glucose over time⁵. Since the assessment of antioxidant activity of a test compound involves the ability of the substance to inhibit peroxidation of membranes such as erythrocytes which can trigger pathological conditions such as sickle cell anemia because the phospholipids bilayers of cellular and subcellular membranes are major targets for free radicals therefore, *C. ferruginea* leaf is highly promising in the chemotherapy of sickle cell anemia. Likewise, the ethanol extract of *S. campanulata* flower had

been shown to demonstrate antioxidant activity on lipid peroxidation of liver microsome *in-vitro*, although there was a resultant activity loss as a result of iron complex formation due to a previous incubation of the test compound with FeCl₃ which interfered with its antioxidant activity as demonstrated by¹⁷, it could therefore be inferred that *S. campanulata* possesses an unstable antioxidant activity which could be the reason for the inability of the extract to exhibit anti-sickling activity beyond 6 mg/ml as observed in this study.

However, *A. laxiflora* leaf extract had a close to marginal reversal activity of 48% at 4 mg/ml while *C. ferruginea* root extract exhibited the least reversal antisickling activity at all the tested concentrations. This observation could be in tandem with the results from previous studies carried out on the effect of *C. ferruginea* root on haematological parameters where it was found to be haematotoxic and also have anemic inducing potential when a significant reduction in red blood cell and packed cell volume count was observed in the experimental animals on which it was administered^{4,27}.

Inhibitory Anti-Sickling Assay: *C. ferruginea* leaf showed a marginal (50.94%) antisickling inhibitory activity at 4 mg/ml while *C. ferruginea* fruit, *C. ferruginea* root and *S. campanulata* flower all had inhibitory activity less than 50% at all the concentrations tested. However amongst all the plant materials tested for antisickling activity, *A. laxiflora* leaf extract singly demonstrated profound and consistent antisickling inhibitory activities at 4 mg/ml, 6 mg/ml, 8 mg/ml and 10 mg/ml being statistically significant at p<0.05 when compared with the positive control.²⁸ Adeloje *et al.*, (2005) have isolated two flavonoidal compounds namely: quercetrin and taxifolin glycosides from *A. laxiflora* leaf and these compounds were found to exhibit high antioxidant activities.

Their presence in *A. laxiflora* leaf could be responsible for the consistent inhibitory antisickling activities observed in this study since pharmacological agents with antioxidant potentials play a role in the maintenance of balance, stability and integrity of erythrocyte membranes thus inhibiting sickling processes.

Extract Combinations: The plant extracts which showed the highest inhibitory or reversal antisickling activities when tested individually were mixed together and extracted to produce Combo A extract. Thus, the mixture of *A. laxiflora* leaf plus *C. ferruginea* leaf, gave consistent inhibitory antisickling activities of 91.7% at 4 mg/ml in ratio 1:2, 90.3% at 6 mg/ml in ratio 1:1, 91.4 and 93% at 8 mg/ml when combined in ratio 1:1 and 2:1 respectively, and 82.8, 79.5 and 85.2 % at 10 mg/ml when combined in ratios 1:1, 1:2 and 2:1 respectively.

The highest inhibitory activity was observed when combined in ratio 2:1 at 8 mg/ml (93.5%) higher than the positive control (45.2%) but comparable with Ciklavit® at 69.4%, since these effects were lower than when the extract of *A. laxiflora* was tested as alone (98%), it could be inferred that the mixture was of no significance.

It was observed from the plant combination antisickling assay results as shown in this study, that the antisickling activity culminated at inhibiting the sickling of erythrocytes. Pharmacological agents that are capable of inhibition or stopping of sickling process in the first place is more advantageous because they could serve as prophylactives; thereby avoiding the process of sickling and un-sickling of erythrocytes which could lead to the formation of high density population of irreversible sickled cells and red blood cell damage.

CONCLUSION: In this study, potential of *C. ferruginea* leaf to reverse sickled erythrocytes at low oxygen tension *in-vitro* is reported for the first time. This study also showed that *A. laxiflora* leaf is better at prevention or inhibition of sickled erythrocytes than its possible reversal antisickling activity. The study further showed that all the three plants were more active when tested individually than when in any combinations. The TLC fingerprint revealed the presence of medicinally important constituents such as phenolics, and perhaps flavonoids present in the most active plants.

From the results of this investigation *A. laxiflora* leaf for antisickling inhibitory activities and *C. ferruginea* leaf for reversal activities should qualify

for further work on their potentials as drug products for the future.

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CONFLICT OF INTEREST: Nil

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