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ANTISICKLING PROPERTIES OF THREE MEDICINAL PLANTS AND THEIR COMBINATIONS

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Keywords:

Sickle cell anemia, *Cnestis ferruginea*, *Alchornea. laxiflora*, *Spathodea campanulata*, Reversal, Inhibitory

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ABSTRACT: Background: Sickle cell anemia continues to be prevalent in the African population of which Nigeria alone has epidemiology of 25%, and only a few official medications are available, necessitating this current study. **Methods:** *Cnestis ferruginea* Vahl ex DC., Connaraceae, *Alcornea laxiflora* (Benth.) Pax & K. Hoffman Euphorbiaceae, and *Spathodea campanulata* Beauv., Bignoniaceae, which have various uses ethnomedicinally 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).

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Among these hemoglobinopathies, sickle-cell anemia (SCA) is the most severe accounting for over 60% of the world's major hemoglobin-opathies and concerning 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 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 Western tropical Africa majorly Senegal to West Cameroons and in other parts of

tropical Africa, it has a 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 has been used as abortifacient, laxative, wound healing, bronchitis, fever treatment, taken as a tonic, teeth-whitening, snake-bite treatment, walk stimulant in weak children in various parts of Africa [2, 3]. Its analgesic, anti-inflammatory, hypoglycemic and antioxidant activities have been reported [4, 5].

*Alchornea laxiflora (*Benth) Pax & K. Hoffm., Euphorbiaceae 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 , Ijàndú, Pẹ́pẹ́, 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 diseases, for 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 (Bignoniaceae) 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, the flame of the forest (English), "Akoko," "Amoju-tooro" (Yoruba), "Ogili-si," "Ogirisi" (Ibo), "Aduruku" (Hausa) tribes in Nigeria. Ethnomedicinally, various parts of this plant have been used as a 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, anti-fungal, 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 of 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 the sickle shapes of their fruits (*C. ferruginea*, *A. laxiflora* and *S. campanulata*) or blood-red coloration of the flower (*S. campanulata*) and of the fruit (*C. ferruginea*) which is suggestive of blood-related bioactivity and high concentration of anthocyanins, the flavonoidal compounds which have been reported as antioxidant and anti-sickling agents [22].

MATERIALS AND METHODS:

Plant Materials: The root, leaf and fruit *C. ferruginea* Vahl ex DC, (Connaraceae), leaf of *Alchornea laxiflora* (Benth.) Pax & K. Hoffman (Euphorbiaceae) and the flower of *S. 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.

Cnestis ferruginea (Leaf, fruit, and root); Voucher Specimen Number: IFE-17639. *Alchornea laxiflora* (Leaf); Voucher Specimen Number: IFE-17640. *Spathodea campanulata* (Flower); Voucher Specimen Number: IFE-17641.

Other Materials: Methanol (Fluka), *n*-hexane, dichloromethane, ethyl acetate (BDH),

formaldehyde, liquid paraffin, phosphate buffered saline pellets (7.0 pH), polysorbate 80 commercially known as tween 80, sodium metabisulphite (Hopkins and Williams), para-hydroxybenzoic acid (PHBA), vanillic acid, Ciklavit®, anti-sickling herbal product (marketed by Neimeth Pharmaceuticals PLC Lagos, Nigeria). All solvents were distilled before use, and all solutions were prepared with distilled water.

Extraction of Plant Material: Powdered plant material (200 g each) 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 Different Concentrations 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 for the concentration-response 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. An aliquot of 0.1% polysorbate 80 (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;

Inhibitory Antisickling Assay: The inhibitory antisickling assay was investigated using the modified method of Dean and Schechter [21], where 0.6 ml of freshly prepared 2% sodium metabisulphite solution was used to induce sickling of HbSS whole blood after initial 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 × 100 objective × 10 eye-piece); five fields of view were counted per slide from the triplicate experiments and the average % inhibitory activity calculated. Phosphate buffer 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 antisickling reversal 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 HbSS whole blood before treatment 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 × 100 objectives × 10 eye-piece); five fields of view were counted per slide from the triplicate experiments and the average % reversal activity calculated.

Phosphate buffer 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: The Ethical Clearance protocol for this research was submitted to the Ethics and Research Committee (ERC) of the Obafemi Awolowo University Teaching Hospitals Complex, and approved with Ethical Clearance Certificate No. ERC/2018/08/01.

RESULTS:

TABLE 1: PERCENTAGE ANTISICKLING ACTIVITIES OF THE THREE PLANT EXTRACTS AT DIFFERENT CONCENTRATIONS

Plant extract	Reversal Activity ± SEM 2 mg/ml	Inhibitory Activity± SEM 2 mg/ml	Reversal Activity ± SEM 4 mg/ml	Inhibitory Activity ± SEM 4 mg/ml	Reversal Activity ± SEM 6 mg/ml	Inhibitory Activity ± SEM 6 mg/ml	Reversal Activity ± SEM 8 mg/ml	Inhibitory Activity ± SEM 8 mg/ml	Reversal Activity ± SEM 10 mg/ml	Inhibitory Activity ± SEM 10 mg/ml
C. ferruginea (L)	10 ±0.1*	37.09 ±0.2*	94.62 ±0.3*	50.94 ±1.2	88.3 ±1.6*	12.40 ±0.4*	64.6 ±6.6*	11.40 ±0.3*	54.6 ±6.6	9.40 ±0.3*
C. ferruginea (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*
C. ferrruginea ®	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*
S. campanulata (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*
A. laxiflora (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*
Ciklavit®	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: (L) = leaf; (P) = fruit; (R) = root; (F) = flower; (*S. campanulata* (F))- *Spathodea campanulata* Flower, (*A. laxiflora* (L))- *Alchornea laxiflora* Leaf, Positive controls = Ciklavit, Vanillic acid and Para hydroxyl benzoic acid (PHBA). 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	Inhibition 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*	82.8± 2.57*
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	79.5± 4.2*
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*	85.2± 1.0*
Ciklavit®	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	64.5± 2.1
Vanillic acid	-	44.2 ±2.1	-	65.0 ± 4.1	-	56.1 ±1.3	-	45.2 ±2.3*	-	52.0± 1.5*
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 = Ciklavit®, 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
A. laxiflora (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*
C. ferruginea (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*
Ciklavit®		62.0 ± 0.5	54.7 ± 3.3
Vanillic acid		-	60.0 ± 3.1
PHBA		43.5 ± 2.1*	-

Keys: (L) = leaf; PHBA (p-hydroxybenzoic acid), Ciklavit(R) and vanillic acid were used as positive controls. Results are expressed as mean ± SEM. Significant results at $p<0.05$ are asterisked*

DISCUSSION AND CONCLUSION:

Antisickling Assays: The ability of each plant extract; 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-methoxy benzoic acid), a derivative of p-hydroxybenzoic acid, was used as a standard positive control for anti-sickling inhibitory activity and p-hydroxybenzoic acid was used as a standard positive control for antisickling reversal activity [23]. 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 assays [24].

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 of 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 time [5]. 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. [17] 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 $FeCl_3$ which interfered with its antioxidant activity as demonstrated by Heim *et al*., it could, therefore, be inferred that *S. campanulata* possessed 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 hematological parameters where it was found to be hepatotoxic 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 [17], *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 vanillic acid (65.0 %, 56.0 %, 45.2 % and 56.0 %) at the respective concentrations. Adeloye *et al.*, have isolated two flavonoidal compounds namely: quercitrin and taxifolin glycosides from *A. laxiflora* leaf and these compounds were found to exhibit high antioxidant activities [28]. 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 and extracted to produce Combo A extract. Thus, the mixture of *A. laxiflora* leaf plus *C. ferruginea* leaf, gave consistent and high inhibitory antisickling activities of 91.6% at 4 mg/ml, 97.9 % at 6 mg/ml, 98.8 % at 8 mg/ml and 93.7% 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 added value.

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 preventing of the sickling process in the first place is more advantageous because they could serve as prophylactic treatment. Consequently, the repeated process the process of sickling and un-sickling of erythrocytes which could lead to a high-density population of irreversibly sickled cells and red blood cell damage will be reduced.

CONCLUSION: In this study, the potentials of *C. ferruginea* leaf to reverse sickled erythrocytes at low oxygen tension *in-vitro* is reported for the first time. Similarly, the finding that *A. laxiflora* leaf selectively possessed such a potent inhibitory activity but somehow lacked reversal antisickling activities is also reported for the first time. The study further showed that all the three plants were more active when tested individually than when in any combinations. 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 antisickling drug products of the future.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest in this work.

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