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Bioactivity-directed isolation of antisickling compounds from *Cnidoscolus acontifolius* (Mill.) I.M. Johnst leaf extract

[Aislamiento dirigido por bioactividad de compuestos anti-sicklémicos de extracto de hoja de *Cnidoscolus acontifolius* (Mill.) I.M. Johnst]

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Abstract

Context: There is continuous search for therapeutic agents from indigenous plants that can be employed in the treatment of sickle cell anemia.

Aims: To evaluate the antisickling potential of *Cnidoscolus aconitifolius* leaf extract, determine the most active fraction, and isolate the putative compounds.

Methods: Oven dried leaves of *C. aconitifolius* (CA) were extracted by maceration in ethanol for 72 h. The extract was fractionated into *n*-hexane, dichloromethane, ethyl acetate and methanol using vacuum liquid chromatography (VLC). The crude CA extract and fractions were subjected to inhibitory and reversal antisickling assays at 0.25-4.00 mg/mL concentration range. Bioactivity-directed fractionation of the most active fraction was done on repeated silica gel column chromatography, followed by preparative thin-layer chromatography, and their analyses on thin-layer chromatography. The isolated compounds were characterized using spectroscopic methods of ^{1}H- and ^{13}C- Nuclear Magnetic Resonance, COSY, HMBC, HSQC, and LC-MS.

Results: The results showed that CA had 80.4 ± 0.15% inhibitory and 56.0 ± 2.90% reversal effects at 4 mg/mL. The ethyl acetate fraction gave significantly higher ($p<0.05$) inhibitory (68.0 ± 4.32%) and reversal (61.4 ± 6.2%) activities at 4 mg/mL than the other VLC fractions. The positive control Ciklavit® had 59.8 ± 0.3% inhibitory and 56.6 ± 0.2% reversal properties. Two compounds, **T1** and **T2** were isolated from the ethyl acetate fraction and identified as tetramethyl bicosahydropicen-3-ol and 5β-pregnane, respectively. Compound **T1** demonstrated an inhibitory effect of 83.6 ± 0.11%.

Conclusions: The study concluded that the ethyl acetate fraction of the ethanol extract of *C. aconitifolius* has the highest antisickling property and identified tetramethylbicosahydropicen-3-ol as a potential antisickling agent.

Keywords: antisickling; *Cnidoscolus aconitifolius*; 5β-pregnane; sickle cell anemia; tetramethyl bicosahydropicen-3-ol.

Resumen

Contexto: Existe una búsqueda continua de agentes terapéuticos a partir de plantas autóctonas que puedan emplearse en el tratamiento de la anemia de células falciformes.

Objetivos: Evaluar el potencial anti-sicklémico del extracto de hoja de *Cnidoscolus aconitifolius*, determinar la fracción más activa y aislar los compuestos putativos.

Métodos: Se extrajeron hojas secadas al horno de *C. aconitifolius* (CA) mediante maceración en etanol durante 72 h. El extracto se fraccionó en n-hexano, diclorometano, acetato de etilo y metanol usando cromatografía líquida de vacío (VLC). El extracto crudo de CA y las fracciones se sometieron a ensayos anti-sicklémicos inhibidores y de reversión en un intervalo de concentración de 0,25-4,00 mg/mL. El fraccionamiento dirigido por bioactividad de la fracción más activa se realizó mediante cromatografía en columna de gel de sílice repetida, seguido de cromatografía en capa fina preparativa y sus análisis en cromatografía en capa fina. Los compuestos aislados se caracterizaron utilizando métodos espectroscópicos de resonancia magnética nuclear ^{1}H y ^{13}C, COSY, HMBC, HSQC y LC-MS.

Resultados: Los resultados mostraron que CA tuvo 80,4 ± 0,15% de inhibición y 56,0 ± 2.90% de reversión a 4 mg/mL. La fracción de acetato de etilo proporcionó actividades inhibidoras (68,0 ± 4,32%) y de inversión (61,4 ± 6,2%) a 4 mg/mL mayores que las otras fracciones de VLC ($p <0,05$). El control positivo Ciklavit® tuvo 59,8 ± 0,3% de inhibición y 56,6 ± 0,2% de reversión. Se aislaron dos compuestos, T1 y T2 de la fracción de acetato de etilo, y se identificaron como tetrametil bicosahidropicen-3-ol y 5β-pregnane, respectivamente. El compuesto T1 demostró un efecto inhibidor del 83,6 ± 0.11%.

Conclusiones: El estudio concluyó que la fracción de acetato de etilo del extracto etanólico de *C. aconitifolius* tiene la mayor propiedad anti-sicklémica e identificó al tetrametilbicosahidropicen-3-ol como un potencial agente anti-sicklémico.

Palabras Clave: anti-sicklémico; *Cnidoscolus aconitifolius*; 5β-pregnano; anemia falciforme; tetrametil bicosahidropicen-3-ol.

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INTRODUCTION

Medicinal plants contain organic as well as inorganic substances that can provide therapeutic effects. They are widely used as prophylaxis for, and treatment of, many diseases. A medicinal plant may possess a wide spectrum of effects due to the presence of various groups of chemical compounds and various microelements hence a preparation obtained from one plant can simultaneously be an analgesic, sedative, cardiotonic, anti-inflammatory, antimalarial, or anti-anemic. *Cnidoscolous aconitifolius* (Miller) I.M. Johnson (family *Euphorbiaceae*) is known as tree spinach found commonly growing in Southwestern Nigeria. It is an ornamental, evergreen plant, mostly referred to as a deciduous plant because of its known broad leaves. The large, 3 to 5 m tall, 32 cm long and 30 cm wide, palmate shaped leaves are arranged in alternate form. *C. aconitifolius* (CA) leaves are edible, commonly eaten as vegetable in Nigeria (Oyagbemi et al., 2008). CA shoot and leaves are used as diuretic, laxative, and as stimulants for blood circulation and lactation. It is also used for the treatment of diabetes, acne, kidney stones and eye problem in Nigeria (Musa et al., 2008).

Sickle cell anemia is a chronic hereditary anemia in which the red blood cells deform in its normal shape at low oxygen tension and become crescent shaped. It affects millions of people allover the world and found to be particularly common in Sub-Saharan Africa region. Based on a research done in the year 2013, it was estimated that 313,000 children are born each year with SCD, 75% of who live in Africa (Piel et al., 2013). SCD affects about 2 to 3% of the Nigerian population of more than 160 million (WHO, 2017). In South-South Nigeria, a large retrospective study in Benin-city revealed sickle cell disease prevalence of 2.39% and a carrier rate of about 23% (Nwogoh et al., 2012). Hemolysis, which is one of the symptoms of SCD, results from dehydrated dense sickle cells as they impair the microcirculation (Bartolucci et al., 2012).

In our earlier studies, we demonstrated that the ethanol extract of CA possessed inhibitory and reversal antisickling activities using ultra-pure nitrogen gas as deoxygenating agent (Cyril-Olutayo and Agbedahunsi, 2015). The phytochemical screening of the ethanol leaf extracts reported by Chikezie et al. (2016), revealed the presence of saponins, flavonoids, alkaloids, phlobatannins, steroids, anthraquinones and phenols; while the water extract showed the presence of tannins, oxalate and cyanogenic glycosides. Numerous flavonoid compounds most of which are kaempferol and quercetin glycosides have been isolated from the leaves. However, the most active antisickling fraction and the putative compound(s) of the ethanol extract have not been determined, hence, this study.

MATERIAL AND METHODS

Chemicals and reagents

Ethanol, *n*-hexane, dichloromethane, ethyl acetate and methanol (Sigma-Aldrich, St. Louis, Missouri, USA) were re-distilled before use. Thin-layer chromatography (TLC) plates were pre-coated Silica gel F_{254} (0.25 mm thickness, Darmstardt, Germany), while preparative TLC plates were pre-coated Silica gel G with binder (0.75 mm thickness, Darmstardt, Germany).

Plant material and extraction

Fresh leaves of *Cnidoscolus aconitifolius* were collected in April, 2017 at Obafemi Awolowo Junior staff Quarters Road 10 with latitude 7°31'6.4488" N and longitude 4°32'12.5484" E. The plant was identified and authenticated by the taxonomist at the IFE Herbarium, Botany Department, OAU, Ile-Ife. Herbarium specimen was deposited at the IFE Herbarium with voucher number IFE 17256. Dried leaves (1.85 kg) were extracted by maceration in absolute ethanol (5 L) at room temperature for 72 h. Extract was evaporated to dryness *in vacuo* on a Buchi rotavapor and kept in the refrigerator until when needed.

Collection of blood

Fresh blood samples collected from confirmed Hb SS individuals who attend routine check-ups at

the Hematology Department of the Obafemi Awolowo University Teaching Hospitals complex, Ile-Ife, Nigeria (OAUTHC) were used within 24 h of collection. Ethical clearance with reference number IRB/IEC/0004553 was obtained from the Ethical and Research committee of the OAUTHC, Ile-Ife.

Antisickling assay procedures

Inhibitory and reversal antisickling model

The assays were carried out using the method of Sofowora (1979) modified by Cyril-Olutayo et al. (2009). The CA extract was tested at concentrations 0.25, 0.5, 1, 2 and 4 mg/mL to determine the optimal concentration for the bioactivity directed isolation; fractions and sub-fractions were tested at 4 mg/mL concentration. Phosphate buffered saline (0.2 mL) was used as negative control and 0.2 mL Ciklavit® concentrate, a nutraceutical product used in the management of SCD was employed as the positive control.

The median effective inhibitory concentration was the concentration of extract which reduces/inhibits the red blood cell sickling by 50% and is calculated according to the method of Finny (1971). Percentages of inhibition are transformed into probit values. The regression lines are drawn according to equation [1].

$$y = ax + b \quad [1]$$

Where: a= regression coefficient; b = constant; y = the probit; x = log10 of the concentrations (Djekoun, 2016).

Fractionation of CA leaf extract

Fractionation was carried out using Vacuum Liquid Chromatography method (Harborne, 1998; Hostettmann et al., 1998). Seventy-five grams of CA extract was adsorbed unto 75 g of silica gel and dry-packed on a 225 g silica gel Vacuum Liquid Chromatography (VLC) set up as stationary phase. Solvents (mobile phase) were introduced based on increasing polarity starting from n-hexane (3.45 L), dichloromethane (3.65 L), ethyl acetate (4.04 L) and lastly methanol (3.75 L). Fractions were concentrated to dryness *in vacuo* and evaluated for antisickling activities according to Cyril-Olutayo et al. (2009).

Column chromatography of ethyl acetate fraction

The bioactive ethyl acetate fraction of CA extract (9.0 g) was adsorbed unto 25 g of silica gel and dry-packed on a 122 g silica gel column (60 × 5 cm) as the stationary phase. The mobile phase used comprised binary solvent systems of increasing level of polarity, viz: *n*-hexane – DCM (8:2), (6:4), (1:9); DCM – EtOAc (9:1), (6:4), (2:8) and; EtOAc – MeOH (95:5). The eluates were bulked into seven sub-fractions (C1 - C7) based on their normal phase TLC profiles (Harborne, 1998; Hostettmann et al., 1998). They were concentrated to dryness *in vacuo* and evaluated for antisickling activities (Cyril-Olutayo et al., 2009).

Column chromatography of ethyl acetate sub-fraction C2

The active column bulked sub-fraction **C2** (4.0 g) was adsorbed unto 4.0 g silica gel and eluted on a 36 g silica gel column, using gradient solvent systems of increasing polarity such as: *n*-hexane – DCM (50:50), DCM (100%); DCM – MeOH (95:5), (90:10), (80:20), (50:50) and; MeOH (100%). The eluates were bulked into five sub-fractions **C2a – C2e**, based on TLC profile, using Hex-EtOAc (8:2). **C2c** formed solid deposits and was washed with 200 mL of MeOH (100%) (Harborne, 1998; Hostettmann et al., 1998).

Preparative TLC (PTLC) of sub-fraction C2b

Sub-fraction **C2b** (72 mg) was dissolved in 3 mL ethyl acetate (100%) and streaked on a 20 × 20 cm PTLC plate. It was air-dried for 15 minutes and developed in a TLC tank, using n-hexane: EtOAc (7:2) solvent system. The developed plate was dried and visualized under the UV light (254 nm and 366 nm). Five bands were separately scrapped, filtered with EtOAc (100%), and concentrated to dryness *in vacuo*. They were analysed on TLC using Hex-EtOAc (9:1) (Harborne, 1998; Hostettmann et al., 1998).

Spectroscopic analysis

The isolated compounds were characterized on a 400 MHz Agilent-NMR; 300 and 600 MHz Bruker NMR Spectrometer, where information on ^{1}H, ^{13}C, DEPT-135, COSY, HSQC, and HMBC were recorded (Elufioye et al., 2016).

Liquid Chromatography-Mass Spectrometry

Reversed-phase chromatography was performed on a Phenomenex Gemini-NX 5 µm C18 column (100 mm × 4.6 mm) using an Alliance HPLC system 2695 (Waters). The column temperature was set at 25°C and the variable wavelength UV-Vis detector was set at 220 nm. An elution gradient was applied with solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in MeOH). The initial mobile phase composition was 70% of A and 30% B at 0 min, then linear gradient to 100% of B over 30 min and held at that composition for 5 min (flow rate of 1 mL/min). The LC system was connected to a quadrupole time-of-flight (TOF) mass spectrometer (Waters Micromass LCT) having an electrospray ion source. The response was recorded in real time by the mass spectrometer data system (Waters MassLynx version 4.1) (Khan et al., 2018).

Statistical analysis

Each test was performed in triplicates and the results expressed as mean values and standard error of mean (SEM), using Microsoft Excel version 2007. Results were subjected to One-way analysis of variance (ANOVA) followed by Student-Newman-Keul's post-hoc test using GraphPad Prism version 5.0. The level of significance was set at $p<0.05$.

RESULTS

Extraction of *Cnidoscolous aconitifolius* leaf

The dry weight of the starting plant material was 1.85 kg extracted with 9 L of ethanol by maceration. The procedure yielded 94.3 g of extract which is equivalent to 5.1% yield.

Antisickling properties of the *Cnidoscolus aconitifolius* crude extract and fractions

Concentration dependent inhibitory and reversal activities were observed, with the highest activities at 4 mg/mL, for CA crude extract on Hb SS red blood cells using sodium metabisulphite as deoxygenation agent (Table 1, Fig. 1). The IC_{50} of the crude extract of CA was 2.17 mg/mL for the inhibitory and 3.19 mg/mL for the reversal activities.

The ethyl acetate fraction of CA extract gave the highest inhibitory and reversal antisickling properties. These activities are significantly higher ($p<0.05$) than the other fractions and Ciklavit®, the positive control (Tables 2 - 3).

Antisickling properties of sub-fractions

Sub-fractions **C1 – C6** were tested for their antisickling properties and the activities were found to reside in sub-fraction C2 with 69.65 ± 1.02% inhibition and 82.75 ± 2.11% reversal. The antisickling activities of the six column sub-fractions at 4 mg/mL are presented in Table 4 and Fig. 2.

From C2 sub-fraction**,** C2b and C2c were eluted by column chromatography. A solid deposit was formed from C2c. This was washed with 200 mL of MeOH (100%), thus afforded an ash amorphous powder coded **T1** [(31 mg; R_f 0.75) and Hex-DCM 8:2 (R_f 0.46)]. It was UV active with purple coloration when sprayed with vanillin/H_2SO_4, which suggested an unsaturated terpenoidal compound. **T1** was tested for its antisickling property and was found to be highly active with 83.60 ± 0.11% inhibitory property (Fig. 3).

Sub-fraction C2b was purified using PTLC and the purest of the bands was coded **T2** [(12 mg; R_f 0.84) and Hex-DCM 8:2 (R_f 0.73)]. It was not UV active, but gave purple coloration with vanillin/H_2SO_4 spray, thus suggested a saturated terpenoidal compound.

Table 1. Inhibitory and reversal effects of the crude ethanol extract of *Cnidoscolus aconitifolius* on Hb SS blood cells.

Concentration (mg/mL)	% Inhibition	% Reversal
0.25	19.22 ± 9.75	19.69 ± 0.17
0.50	20.67 ± 3.58	21.37 ± 1.64
1.00	23.06 ± 0.21	22.60 ± 5.29
2.00	52.43 ± 0.61	45.00 ± 3.38
4.00	80.40 ± 0.15*	56.00 ± 2.90*
Ciklavit®	59.83 ± 0.30	56.57 ± 0.20

Results were shown as mean ± standard error of mean of the analysed values (n=3). *P<0.05 statistically significant differences with respect to the positive control (Ciklavit®, 0.2 mL).

Table 2. Inhibitory activities of the fractions of *C. aconitifolius* ethanol extract.

Concentration (mg/mL)	% Inhibition				
	n-Hexane	DCM	Ethyl acetate	Methanol	Ciklavit®
0.25	49.38 ± 1.51	16.15 ± 4.64	35.12 ± 0.34	18.54 ± 0.26	59.83 ± 0.30
0.50	53.74 ± 1.89	19.03 ± 2.92	46.29 ± 7.10	35.49 ± 2.25	-
1.00	55.29 ± 2.76	20.18 ± 3.19	58.41 ± 2.52	35.93 ± 7.95	-
2.00	57.09 ± 0.23	22.79 ± 9.27	59.89 ± 2.99	37.36 ± 3.64	-
4.00	57.56 ± 0.15	28.59 ± 1.37	68.03 ± 4.32*	40.06 ± 5.52	-

Results were shown as mean ± standard error of mean of the analyzed values (n=3). *P<0.05 statistically significant differences with respect to the positive control. (Ciklavit®, 0.2 mL).

Table 3. Reversal activities of the fractions of *C. aconitifolius* ethanol extract.

Concentration (mg/mL)	% Reversal				
	n-Hexane	DCM	Ethyl acetate	Methanol	Ciklavit®
0.25	25.39 ± 4.24	23.61 ± 1.71	25.36 ± 2.04	24.67 ± 0.20	56.57 ± 0.20
0.5	29.75 ± 3.34	23.98 ± 1.11	31.52 ± 0.38	25.99 ± 1.78	-
1.0	33.25 ± 0.78	26.43 ± 3.18	36.37 ± 1.83	30.04 ± 2.28	-
2.0	38.36 ± 1.70	28.65 ± 3.93	51.89 ± 2.15	30.35 ± 1.33	-
4.0	59.88 ± 0.21*	28.69 ± 2.68	61.42 ± 1.18*	32.60 ± 0.67	

Results were shown as mean ± standard error of mean of the analyzed values (n=3). The mean difference is significant at p<0.05. *=significant compared with the positive control (Ciklavit®, 0.2 mL).

Table 4. Percentage inhibition and reversal activities of sub-fractions C1 – C6.

Fraction code	% Inhibition	% Reversal
C1	40.93 ± 0.03[b]	25.99 ± 3.01[b]
C2	69.65 ± 1.02[d]	82.75 ± 2.11[d]
C3	52.98 ± 3 02[c]	29.22 ± 1.12[b]
C4	49.94 ± 3.06[c]	52.24 ± 3.36[c]
C5	40.11 ± 2.31[b]	53.70 ± 1.08[c]
C6	24.44 ± 3.12[a]	17.54 ± 0.09[a]

Data are expressed as mean ± SEM (n=3), analyzed using One-way ANOVA, followed by Student-Newman-Keul's posthoc test. Values with different alphabets in superscripts are significant ($p<0.05$). C1-C6 are column chromatography bulked fractions.

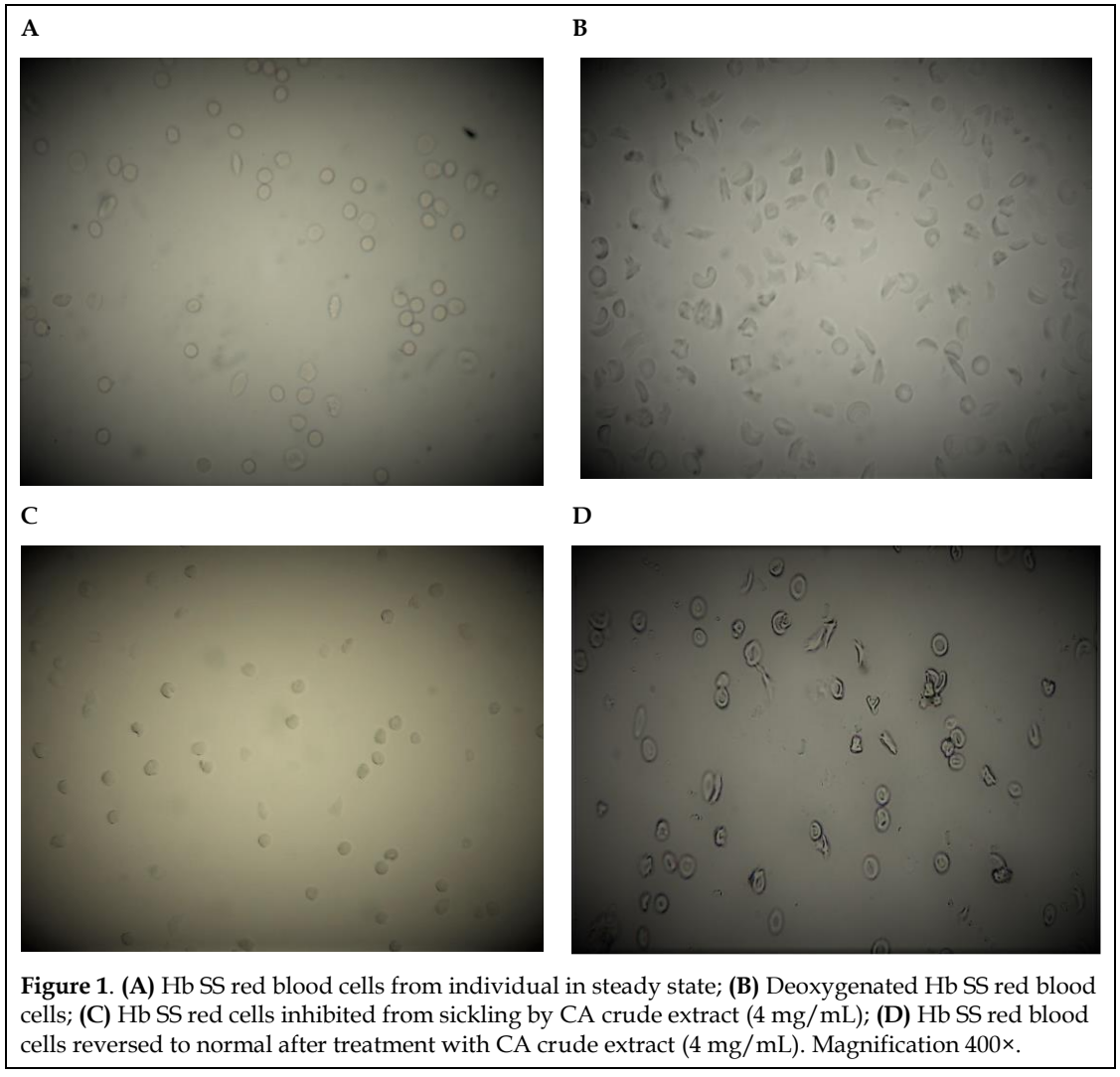

Figure 1. **(A)** Hb SS red blood cells from individual in steady state; **(B)** Deoxygenated Hb SS red blood cells; **(C)** Hb SS red cells inhibited from sickling by CA crude extract (4 mg/mL); **(D)** Hb SS red blood cells reversed to normal after treatment with CA crude extract (4 mg/mL). Magnification 400×.

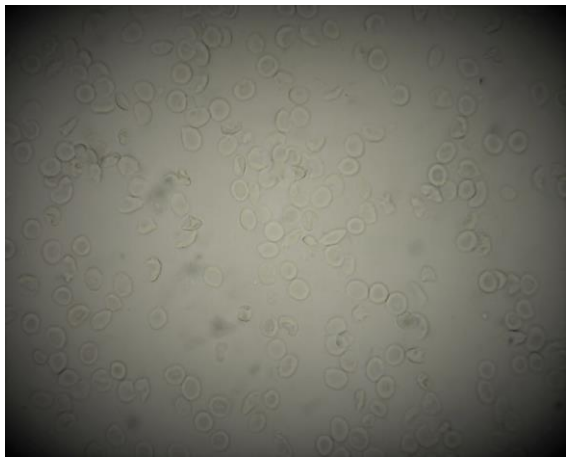

Figure 2. (A) inhibition and **(B)** reversal of Hb SS red blood cells by C2 subfraction of CA extract (4 mg/mL). Magnification 400×.

Figure 3. Inhibitory effect of pure compound T1 (4 mg/mL) on Hb SS red bloods cells. Magnification 400×.

Structure elucidation of isolated compounds T1 and T2

Compound T1

TOF MS ES+ (m/z, % abundance): 369.6360 $[M]^+$ (28%) consistent with the molecular formula $C_{26}H_{42}O$ (exact mass = 370.32), m/z 312.6073 $[M-58]^+$ (60%), m/z 274.5071 $[M^+]$ (100%), m/z 222.3815 $[M - 148]^+$ (19%), m/z 182.2201 $[M - 188]^+$ (28%), m/z 167.3177 $[M - 203]^+$ (10%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.68 (3H, *s*, H-25), 0.85 (3H, *bd*, H-23), 0.92 (3H, *d*, H-26), 1.02 (3H, *s*, H-24), 2.01, (H, *t*, H-4), 3.51 (H-OH, *m*, H-3), 5.33 (H, *brs*, H-12).

^{13}C NMR (100 MHz, $CDCl_3$) δ ppm : 37.24 (C-1), 31.66 (C-2), 71.81 (C-3), 42.32 (C-4), 140.75 (C-5), 121.72 (C-6), 29.17 (C-7), 39.77 (C-8), 56.06 (C-9), 36.14 (C-10), 24.29 (C-11), 33.93 (C-12), 45.83 (C-13), 56.76 (C-14), 19.06 (C-15), 23.07 (C-16), 36.49 (C-17), 50.13 (C-18), 18.81 (C-19), 31.90 (C-20), 28.24 (C-21), 26.08 (C-22), 21.08 (C-23), 19.44 (C-24), 19.34 (C-25), 11.96 (C-26).

Compound T2

Molecular formula: $C_{21}H_{36}$

TOF MS ES+ (m/z, % abundance): 289 $[M+H]^+$ (5%) consistent with the molecular formula $C_{21}H_{36}$, m/z 274.5071 (M^+) (100%, base peak), m/z

222.3815 [M – 148]$^+$ (19%), m/z 182.2201 [M – 188]$^+$ (28%), m/z 167.3177 [M – 203]$^+$ (10%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.85 (3H, *t*, H-19), 0.98 (3H, *s*, H-20), 1.10 (3H, *s*, H-21)

^{13}C NMR (400 MHz, $CDCl_3$) δ ppm: 37.29 (C-1), 24.47 (C-2), 29.25 (C-3), 29.45 (C-4), 25.20 (C-5), 29.70 (C-6), 32.68 (C-7), 22.73 (C-8), 27.98 (C-9), 36.50 (C-10), 22.63 (C-11), 39.37 (C-12), 42.40 (C-13), 33.66 (C-14), 25.20 (C-15), 29.93 (C-16), 31.93 (C-17), 24.87 (C-18), 14.12 (C-19), 21.40 (C-20), 19.75 (C-21).

DISCUSSION

The antisickling assays results showed that, the crude ethanol extract of *Cnidoscolus aconitifolius* was very active with 80.40 ± 0.15% inhibitory and 56.0 ± 2.9% reversal activity at 4 mg/mL (Table 1 and Fig. 1) with sodium metabisulphite as deoxygenating agent. This finding is in accordance with our earlier study where deoxygenation was induced by nitrogen gas (Cyril-Olutayo and Agbedahunsi, 2015). The ethyl acetate fraction among the other fractions was the most active with 68.03 ± 4.32% inhibition and 61.42 ± 6.18% reversal. The activity was concentration dependent as activity increased with increased concentration. The antisickling properties of the ethyl acetate fraction was significantly higher than that of Ciklavit® (positive control) (Tables 2 and 3). This suggested that the antisickling compounds are moderately polar. The antisickling effect of CA confirmed the report by Sarmiento-Franco et al. (2003) of the ameliorative effects of various extracts of *C. aconitifolius* on anemia and osmotic fragility. The crude extract of CA had been reported to contain phytochemicals such as alkaloids, carbohydrates, aminoacids, saponins, tannins, flavonoids, terpenoids, glycosides, steroids (Orji et al., 2016), amino acids such as glutamic acid, lysine, histidine, alanine, aspartic acids amongst others were reported to be present in the leaf extracts of CA, some of which have been implicated in antisickling activities (Markus et al., 2016). Tannins have anti-inflammatory properties and its presence in the plant could quicken the healing of wounds. Saponins have both hemolytic and cholesterol binding activities (Stray, 1998) while the presence of phenol in the plant suggests the ability to block specific enzymes that cause inflammation (Okwu, 2001). Phenolic compounds are also known for their antisickling properties. Flavonoids are antioxidants and free radical scavengers and also show anti-allergic and anti-inflammatory, and antimicrobial properties (Owolagba et al., 2009).

Bioactivity-guided fractionation of the most active ethyl acetate fraction yielded sub-fractions C1-C6. C2 with the highest antisickling properties (Table 4 and Fig. 2) led to the isolation of two compounds, **T1** and **T2** (see spectra in supplementary data).

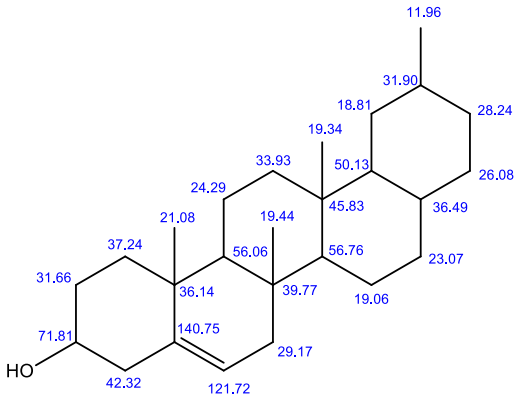

Figure 4. Structure of compound **T1** isolated from the ethyl acetate (C2 column) fraction of *C. aconitifolius*. Chemical Formula: $C_{26}H_{42}O$. Exact Mass: 370.32.
6a,11,12b,14b-Tetramethyl-1,2,3,4,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14,14a,14b-icosahydropicen-3-ol

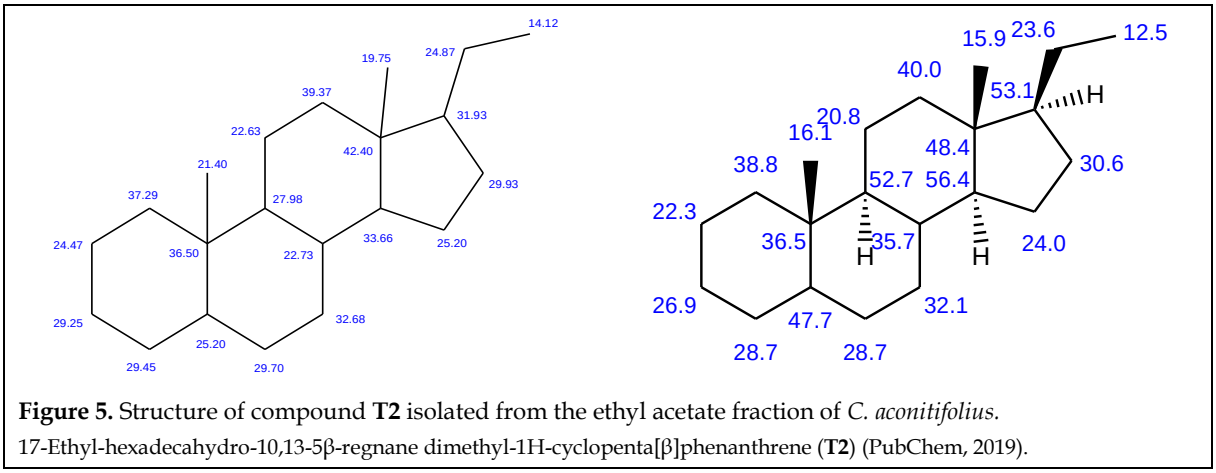

Figure 5. Structure of compound **T2** isolated from the ethyl acetate fraction of *C. aconitifolius*.
17-Ethyl-hexadecahydro-10,13-5β-regnane dimethyl-1H-cyclopenta[β]phenanthrene (**T2**) (PubChem, 2019).

TLC profile of **T1** showed it was UV-active and turned purple with vanillin/H_2SO_4 spray, which suggested it to be an unsaturated terpenoidal compound. **T2** also gave purple colouration but was UV-inactive, which suggested a saturated terpenoidal compound. **T1** exhibited an 83.6 ± 0.11% inhibitory effect (Fig. 3). It is an antisickling agent which can be suitable as a drug or a lead compound in the synthesis of antisickling drugs.

The ^{1}H NMR spectrum of **T1** showed four shielded methyl protons (CH_3), which comprised three singlets and one doublet signals at δ_H 0.68, 0.85, 1.02 and 0.92 ppm respectively, thus depicts an α-amyrin class of terpene (Pavia et al., 2001). The broad singlet signal at δ_H 5.33 ppm confirmed the presence of a terminal olefinic proton (i.e. **H – C** = C – X). While the multiplet signal at δ_H 3.51 ppm confirmed the presence of a methine proton (CH) directly attached to a hydroxyl (OH) group.

The ^{13}C NMR spectrum of **T1** showed twenty-six signals representing the number of carbon atoms. These comprised four shielded methyl groups at δ_C 11.96, 19.34, 19.44 and 21.08 ppm. The methylene carbon atoms resonated between δ_C 18.81-37.24 ppm, while the signals between δ_C 31.90-71.81 ppm represents the methine carbons. All of these gave an indication that the isolate is a terpenoid. The deshielded carbon signal at δ_C 71.81 ppm was due to an attachment of hydroxyl group. While the two further deshielded signals at δ_C 121.72 and 140.75 ppm was due to an unsaturation (double bond) of a cyclic ring caused by a *pi* electron-rich carbon (olefinic) (Silverstein and Webster, 1998; Pavia et al., 2001). The NMR spectra (^{1}H and ^{13}C) of **T1** were compared with campesterol reported by Choi et al. (2007).

The mass spectrometry of **T1** showed a molecular ion peak at m/z 369.6360 $[M]^+$ (28%) consistent with the molecular formula $C_{26}H_{42}O$. Thus, **T1** was identified as tetramethyl icosahydropicen-3-ol (see spectra in supplementary data).

The ^{1}H NMR spectrum of **T2** showed three shielded methyl groups which comprised one triplet at δ_H 0.85 ppm and two singlet signals at δ_H 0.98 ppm and 1.10 ppm. ^{13}C NMR spectrum of **T2** showed a total twenty-one signals, which represented twenty-one carbon atoms. The DEPT 135 experiment showed eleven CH_2, and eight CH and CH_3 signals. The absence of two carbon signals at δ_C 36.50 ppm and 42.40 ppm on the DEPT 135 spectrum confirmed two quaternary carbon atoms. The general pattern observed in the DEPT 135 experiment suggested a pregnane skeleton (see spectra in supplementary data) (PubChem, 2019).

The mass spectrometry of **T2** showed a molecular ion peak at m/z 289 consistent with the molecular formula $C_{21}H_{36}$. A possible loss of methyl group at C-21 position was accounted for by the largest fragment ion at m/z 274.5071 (see spectra in Annex), which represented the base peak (M^+). Upon consideration of the above spectra data and in comparison with literature report (PubChem, 2019), **T2** was characterize as 5β-pregnane. This marks the first report of the two compounds in *C. aconitifolius* leaf.

CONCLUSIONS

The study has established the ethyl acetate fraction of *C. aconitifolius* leaves as the most active antisickling fraction. Bioactivity-guided fractionation led to the isolation of two compounds **T1** and **T2** identified as tetramethylicosahydropicen-3-ol and 5β-pregnane. **T1** exhibited an 83.6 ± 0.11% inhibitory effect against the production of sickle cells *in vitro*, hence, could be lead compound in the search for candidate drugs for the management of sickle cell disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found at http://jppres.com/jppres/pdf/vol8/jppres20.864_8.6.580.suppl.pdf

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Contribution	Cyril-Olutayo MC	Adeyemo TA	Oriola AO	Agbedahunsi JM
Concepts or ideas	x			
Design	x			x
Definition of intellectual content	x		x	x
Literature search		x		
Experimental studies	x	x	x	
Data acquisition	x	x		
Data analysis		x	x	
Statistical analysis		x		
Manuscript preparation		x		
Manuscript editing	x		x	x
Manuscript review	x	x	x	x

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