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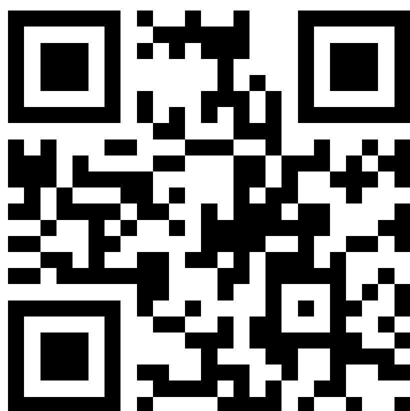

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# Isolation and Characterization of Anti-Sickling Bioactive Compounds from Seeds of Picralima Nitida Stapf (Apocynaceae)

Felix N. Osuala, Uchenna E. Odoh

**ABSTRACT**

**Aim:** To isolate and Characterize the anti-sickling bioactive compounds of Ethanol fraction of *Picralima nitida* seeds.

**Methods**: The fresh matured fruits (8 kg) of *P. nitida* were cut into transverse section to expose the seeds, The seeds were air-dried and then pulverized into a homogenous powder, The organoleptic properties (taste, colour, odor, texture) of the powdered *P. nitida* were determined using the sensory organs. A 2.5 kg pulverized seeds were extracted with methanol and concentrated in a rotary evaporator to yield a dried residue. The methanol extract of *Picralima nitida* was then defatted using n-hexane. The extract was successively fractionated with different solvents, chloroform, dichloromethane, ethyl acetate and 50% ethanol in increasing order of polarity to obtain the chloroform fraction (CF), dichloromethane fraction (DCMF), ethyl acetate fraction (EF) and ethanol fraction. Antisickling activity tests were carried out on all the fractions at the same time, comparing their antisickling activities with that of p-hydroxybenzoic acid (positive control). The Ethanol Fraction which has the greatest anti sickling activity was subjected to further fractionation using silica gel column chromatography. The fractions based on bioactivity-guide, were eluted with gradient mixtures followed by a preparative TLC using EtOAc:MeOH (2:3) as solvent system to get compounds 1 and 2.The structures of the isolated active compounds were elucidated using spectrophototropic analyses.

**Result:** The two compounds were identified as (19∝)-16, 17-didehydro-19-methyl-oxayohimbin-16-Carboxylic acid methyl ester (Ajmalicine) and (19∝)-19-methyl-2-oxoformosanan-16-carboxylic acid methyl ester (Ajmalicine Oxindole B). Ajmalicine showed significant antisickling effect (97 %) when compared to the standard drug (50 %). Ajmalicine Oxindole B Showed (90 %) antisickling effect at concentration 2.5 mg/ mL at 90 minutes incubation in HbSS blood.

**Conclusion** The seeds of *Picralima nitida* possesses antisickling effect and could serve as a lead to produce further antisickling drug.

**Key-words:** Picralima nitida, Ajmalicine, Ajmalicin oxindole B, Anti sickling compounds.

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**INTRODUCTION.**

Herbs have been used for several generations and their traditional knowledge is irreplaceable. The medicinal herbs are nature's remedies and have been placed there by the creator of the universe. They are God's remedial agents for afflicted humanity. Thus herbal treatment is designed to return body to its healthy state by stimulating natural metabolism and maintaining system functions. Being natural, they are less aggressive than synthetic ones and they have no significant side effects. (1). There is a belief by traditional healers, that the bioactive ingredients that have therapeutic activities in plants have holistic nature of treatment. Substances found in medicinal plant containing the healing property of the plant are known as the active principle. (2). It defers from plant to plant and examples of active principles can be anthraquinones, flavonoids, glycosides, saponins, tannins etc and other compounds such as morphine, atropine, codeine, steroids, lactones and volatile oils which possess medicinal value for the treatment of different diseases. (3). Due to the challenges faced by the scientists in developing countries, so many sources of constituents capable of ameliorating the sickle cell crises have been investigated with a view to contribute to search for substances that would be effective in solving the sickle cell disease problem. Based on this; the anti -sickling effects of different substances have been investigated. A recent WHO report estimated that around 2% of newborns in Nigeria were affected by sickle cell anaemia, giving a total of 150,000 affected children born every year in Nigeria alone. The carrier frequency ranges between 10% and 40% across equatorial Africa, decreasing to 1-2% on the North African coast and <1% in South Africa. (4). Thus, sickle cell disease is a significant health problem in Nigeria and its managements should go beyond conventional synthetic chemotherapy. The use of herbal medicine needs to be encouraged. *P.nitida*, a multi potent herb used in various disease management holds a strong promise as a native herb with potentials for sickle cell disease management. This is the premise of this study.

The plant contains alkaloids, glycosides and saponins in the root, stem barks, fruits, leaves, mature and immature seeds. (5). The barks and leaves yielded only amorphous alkaloids. (6). The first finding was the presence of alkaloids in seeds, leaves and stem. The plant contains 3.5% of total alkaloid. The seeds have been found to contain ten alkaloids, seven of which have been crystallized and characterized. The first alkaloidal isolation of seed was akuammine from the bark. Other alkaloids that were isolated include: akuammigine, pseudo-akuammigine, akuamimmenine, pseudo-akuammingine, Akuammine hydrate. A number of alkaloids have been isolated and re-isolated from the plant. Among the alkaloids isolated are picraline, picraphyline, picracine, picralicine, picratidine, picratine, burnamine and pericine (6, 7, 8). Pericine and pericalline were isolatede from the cell suspension culture.

Triterpenoid, Saponin, B-amyrin was isolated from the stem bark. (9). Most of these alkaloids were found not only in one morphological part the plant but they occurred in different parts of the plant at the same time. Three coumestan glycosides were isolated from the roots of *P. nitida.* (10). These glycosides afforded three coumestan derivatives. The cumastan glycosides are: 3-hydroxy-9-methoxy-2-[2' (E)-3'-methyl-4'-0 -β-D-3'-methyl-0-β-D –glucopyranosylbutenyl] -8 –[ 2" (E)- 3"- methyl- 4"- oxobutenyl] coumestan and 3-hydroxy-9-methoxy-4-[2' (E)-3'-methyl-4'-0 - β- D – 3' –methyl -0 - β -D-glucopyranosylbutenyl] -8 –[ 2" (E)- 3"- methyl-4"- oxobutenyl] coumestan. The coumestan glycosides derivatives are:

3-hydroxy-9-methoxy-2-[2'(E)-4'-hydroxy-3'-methylbutenyl]-8-isoprenylcoumestan;

3-hydroxy-9-methoxy-2-[2'(E)-4'-hydroxy-3'-methylbutenyl]-8-[2"(E)-3"-methyl-4" oxobutenyl]coumestan and

3-hydroxy-9-methoxy-4-[2"(E)-3"-methyl-4"-oxobutenyl]coumestan. This present study isolated the antisickling compounds of ethanol fraction indicated for the of *Picralima nitida*.

**MATERIALS AND METHODS.**

**Plant Materials, Collection and Preparation.**

Matured and riped fruits of *P. nitida* were collected in its fruit bearing season from Dim Anozie village in Isu Imo state in the month of May 2010. The plant was authenticated at the Botany Herbarium of the University of Nigeria Nsukka where Voucher specimen was maintained. The fresh matured fruits (8 kg) were cut into transverse section to expose the seeds, the pulp and the rinds. The seeds were air-dried for three weeks and then pulverized with laboratory hammer mill into a homogenous powder, and stored inside an airtight container until used.

**Extraction and fractionation of *P. nitida* seeds.**

A 2.5 kg pulverized seeds were extracted with methanol in a soxhlet apparatus The extract was concentrated in a rotary evaporator to yield a dried residue. The methanol extract of *Picralima nitida* was then defatted using

n-hexane. The defatted methanol extract (13.8 g) was mixed with dry silica gel at a ratio of 1:2, put into a tall column cylindrical tube packed with silica gel up to half the volume. A cotton fiber was packed at the bottom of the column to prevent the silica gel from coming out of the tap. The extract was successively fractionated with different solvents, chloroform, dichloromethane, ethyl acetate and 50% ethanol in increasing order of polarity to obtain the chloroform fraction (CF), dichloromethane fraction (DCMF), ethyl acetate fraction (EAF) and aqueous fraction. Antisickling activity tests were carried out on all the fractions at the same time, comparing their antisickling activities with that of p-hydroxybenzoic acid (positive control).

**Purification and isolation**

The most active fraction (ethanol fraction) was subjected to bioassay guided fractionation and isolation using several chromatographic techniques. A 6 g portion of the ethanol fraction (EtOH F) was subjected to column chromatography over silica gel (230 – 400 mesh) eluting with n-hexane (100 %), n-hexane: ethyl acetate (2:1), n-hexane:ethylacetate (1:1) ethyl acetate 100 %, ethyl acetate:methanol (19-1), ethyl acetate: methanol (9:3) and ethyl acetate : methanol (4:2) in increasing order of polarity to obtain 32 sub-fractions of 100 ml each. The sub fractions were pooled based on their thin layer chromatographic (TLC) characteristics. A series of silica gel column chromatography was carried out of fractions 19-23 with mixture of EtOAc:MeOH in increasing order of polarity, followed by a preparative TLC using EtOAc:MeOH (2:3) as solvent system to get compounds 1 and 2. Fractions 30-34 were also subjected to the same purification process to obtain compound 3.

**Characterization of isolated compounds**

The isolated compounds were characterized by the following spectroscopic technicques: ESI-MS, UV, NMR. The mass Spectra was recorded on Bruker TOF Mass Spectrometer (Shimadzu, Japan) using electrospray ionization (ESI). The UV Spectra (ƛmax: nm) were recorded on Shimadzu UV- 2700 Spectrometer (Shimadzu, Japan) in MeOH. The $^{1}$H-NMR and $^{13}$C NMR spectra were recorded on a Bruker DPX- 400 NMR Spectrometer (Billerica, USA) (500 MHz for $^{1}$H and125 MHz for $^{13}$C-NMR) using $CDCl_3$ as solvents.

**Antisickling activity of crude methanol extract, fractions and isolates of *P. nitida***

The extract and fractions of *P. nitida* at different concentrations were screened for antisickling activity. The plant extract/fractions for sickling reversal activity was carried out according to the procedure. (11). A 0.5 ml volume of the washed erythrocytes was mixed with 0.5 ml of different concentrations of extract in the uncovered test tubes. A 0.5 ml volume of 2 % sodium metabisulphite was added to deoxygenate the blood sample. The system was mixed thoroughly and sealed with liquid paraffin. Samples were taken from different mixtures immediately at zero time and the remaining mixtures were incubated at 37 °C, in a shaking incubator. Samples were taken in triplicate subsequently at 30 minutes interval (0, 30, 60, 90, 120, 150 and 180 minutes) from the different mixtures. Thin film of each sample was made and examined under oil immersion light microscope 100 x magnification. Counting of 100 sickled and unsickled red blood cells of the sample was carried out and the percentage of unsickled cells determined. The same experimental procedure was repeated for both positive and negative controls i.e parahydroxybenzoic acid and normal saline.

**RESULTS**

**Table 1: Results of organoleptic tests of *P. nitida.***

| Parameter | Seed | Powdered seed |
|---|---|---|
| Colour | Light Brown | Light brown |
| Odour | Characteristic | Characteristic |
| Taste | Bitter | Strong bitter taste |
| Shape | Obovoid | Powder |
| Surface texture | Smooth | Fine grain |
| Solubility | Not soluble in water/ alcohol. | not soluble in water/ alcohol. |

**Macroscopic and microscopic analysis of the seeds** The powder contains parenchymatous cells. There were sclerenchymatous cells of the seed testa. Microscopical examination showed presence of calcium oxalate crystals (prisms and sphero types), epidermal cells with fat globules.

**Table 1:** Antisickling effect of Ethanol fraction of *P. nitida* on HbSS red blood cells

| time (min | Neg. cont N/S | Para hydroxy benzoic acid (mg/ml) % reversal of sickling | | | Ethanol fraction concentration (mg/ml) % reversal of sickling | | |
|---|---|---|---|---|---|---|---|
| | | 5 | 50 | 500 | 2.5 | 5 | 10 |
| 0 | 43 | 38 ± 0.80* | 40 ± 0.42* | 45 ± 0.60* | 30 ± 0.64* | 32 ± 0.63* | 45 ± 1.25* |
| 30 | 43 | 41± 1.22* | 58 ± 0.82* | 100 ±1.07* | 43 ± 0.34* | 44 ± 0.45* | 50 ± 0.92* |
| 60 | 43 | 44± 0.67* | 60 ± 1.84* | 100 ± 0.72* | 45 ± 0.89* | 58 ± 1.52* | 55 ± 0.62* |
| 90 | 43 | 50 ± 0.24* | 79 ± 0.32* | 100 ± 1.08* | 45 ± 0.63* | 64 ± 0.38* | 67 ± 2.06* |
| 120 | 43 | 56 ± 1.25* | 88 ± 0.45* | 100 ± 0.54* | 57 ± 0.22* | 67 ± 0.60* | 76 ± 1.76* |
| 150 | 43 | 56 ± 0.54* | 88 ± 0.86* | 100 ± 0.16* | 70 ± 1.44* | 72 ± 0.93* | 85 ± 0.45* |
| 180 | 43 | 58 ± 0.88* | 98 ± 0.63* | 100 ± 1.18* | 81 ± 0.78* | 83 ± 0.64* | 85± 0.82* |

Values are Mean ± SEM, $*p < 0.05$, n =3

Compound 1**,** was obtained as white powder. Its molecular formula was derived as $C_{21}H_{24}N_2O_3$ by the high resolution Shimadzu IT-TOF spectrum, showing an [M] + ion at ***m/z*** **=** 353.1867. The expected mass was 353.1860, error 0.7mDa, 1.98ppm. The M.p. 253-254 ºC., UV/ (MeOH) λmax : 257 nm (4.07), 1H-NMR (Measured in $CDCL_3$) and $^{13}$C-NMR (Measured in $CDCL_3$). Based on the spectral analysis (UV, chemical shifts, coupling constant of the 1H-NMR and $^{13}$C-NMR spectrum experiments) the complete structural elucidation of compound 1 was derived as (19∝)-16,17-didehydro-19-methyl-oxayohimbin-16-Carboxylic acid methyl ester (Ajamalicine). It showed the best anti-sickling activity (100 % reversal of sickling at 120 min) and comparable to that of the standard drug parahydroxybenzoic acid at 500 mg/ml (100 % reversal of sickling at 30 min).

Compound **2,** was obtained as light yellow powder. Its molecular formula was derived as $C_{21}H_{24}N_2O_4$ by the high resolution Shimadzu IT-TOF spectrum, showing an [M] + ion at *m/z* **=** 369.1815.The expected mass 369.1809, error 0.6mDa, 1.63ppm, mass about 16 heavier than ajmalicine. The M.p. 265-266 ºC., UV/ (MeOH) λmax: 257 nm (4.35), 1H-NMR (Measured in $CDCL_3$) and $^{13}$C-NMR (Measured in $CDCL_3$) . The fragmentation does show a loss of 32.0249Da (to 337.1566); this is appropriate for MeOH (expected neutral mass 32.0262Da), which could account for the esterified methyl group on -COOMe). The structure of Compound 2 was finally concluded as (19∝)-19-methyl-2-oxoformosanan-16-carboxylic acid methyl ester (Ajmalicine Oxindole B).

Compound 3 is a cream coloured amorphous powder yet uncharacterazed but has anti-sickling activity more than compound 2. The anti –sickling activity of the compounds is in the order CP1 > CP3 > CP2 at concentration 2.5 mg/ml at 120 min (Table 2).

**Table 2: The** Antisickling effect of isolated pure compounds from *P. nitida* on HbSS red blood cells.

| **Incubation time** | **N/saline** | **Parahydroxybenzoic acid Concentrations (mg/ml) % reversal of sickling** | | | **Pure compounds % reversal of sickling, concentrations (mg/ml)** **Compound 1** | | | **Compound 2** | | | **Compound 3** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5 | 50 | 500 | 2.5 | 5 | 10 | 2.5 | 5 | 10 | 2.5 | 5 | 10 |
| 0 | 43 | 38 ± 0.62* | 40± 0.44* | 45 ± 0.73* | 73± 0.29* | 84±0.2* | - | 72 ± 0.17* | 79± 0.83* | -- | 73 ± 0.26* | 86 ± 0.23* | -- |
| 30 | 43 | 41 ± 0.41* | 58± 0.57* | 100 ± 0.61* | 95± 0*.55 | - | - | 78 ± 0.85 | 79± 0.51* | -- | 86± 0.14* | 90 ± 0.38* | -- |
| 60 | 43 | 44 ± 0.88* | 60± 0.63* | 100 ± 0.70* | 97± 0.39* | - | - | 91± 0.77* | 92± 0.68* | -- | 92± 0.37* | 95 ± 0.74* | -- |
| 90 | 43 | 50± 0.47* | 79± 0.19* | 100 ± 0.03* | 97 ± 0.13 | - | -- | 90 ± 0.45 | 92± 0.59* | -- | 92± 0.50* | 97 ± 0.18* | -- |
| 120 | 43 | 56 ± 0.16* | 88± 0.68* | 100 ± 0.28* | 100±0.36* | - | - | 92± 0.31* | 94± 0.48* | -- | 95± 0.98* | 97 ± 0.32* | -- |
| 150 | 43 | 56 ± 0.53* | 88± 0.48* | 100 ± 0.60* | 100±0.84* | - | - | 93 ± 0.49 | 97± 0.11* | -- | 97 ± 0.31* | 97 ± 0.67* | -- |
| 180 | 43 | 58 ± 0.71* | 98± 0.29* | 100 ± 0.15* | -- | - | - | 100±0.27* | 100±0.66* | -- | 100 ± 9.44* | 100 ± 0.49 | -- |

Values are Mean ± SEM, $*p < 0.05$, n =3

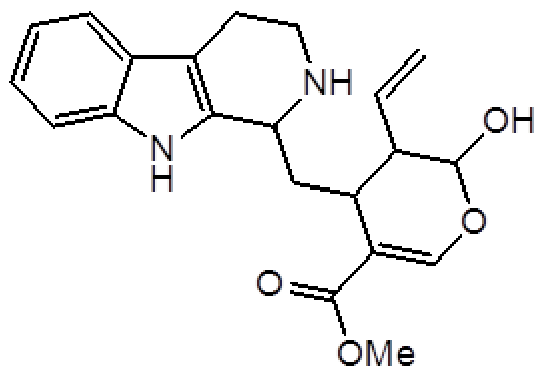
**Physical and Spectroscopic Data of Compounds 1 and 2**

**Table 3:** H[1]-NMR ($\delta_H$ in ppm, 500MHz) Data of Compound 1 (Ajmaciline) and compound 2 (Ajmaciline oxindole B)

| | Compound 1 | Compound 2 |
|---|---|---|
| Position | $\delta_H$ | $\delta_H$ |
| 1 | 1.60 (1H, m) | 0.82 (1H, s, *J*.1.8 ) |
| 2 | - | - |
| 3 | 3.20 (1H,dd, *J*.2.1) | 3.49(1H, td, *J*.5.5, 11.1) |
| 4 | - | - |
| 5 | 0.95(2H, s) | 2.27(2H, m) |
| 6 | 1.38 (2H, d, *J*. 7.3) | 5.33(2H,d, *J*, 5.1) |
| 7 | - | - |
| 8 | - | - |
| 9 | 1.95 (1H, s) | 0.80 (1H, d) |
| 10 | 1.91(1H, d, *J*.6.5) | 1.64(1H,d, *J*.11.5) |
| 11 | 1.87(1H, dd, *J*, 1.57 | 1.25(1H, dd, *J*.7.3, 14.7) |
| 12 | 5.16 (1H,t, *J*.3.6) | 1.16(1H,m) |
| 13 | - | - |
| 14 | 0.97 (2H, s) | 1.97( 2H, s) |
| 15 | 1.50(1H, s, *J*.1.88 | 2.21(1H, s) |
| 16 | - | - |
| 17 | 1.11(1H, s *J*.2.9) | 0.90 (1H, s, *J*.6.5) |
| 18 | 0.81 (4H, d, *J*.3.4) | 0.67 (4H, d, *J*.9.1 |
| 19 | 0.77 (3H,d, *J*.12.5) | 0.99 (3H,d, *J*.6.5) |
| 20 | - | - |
| 21 | 0.85 (3H,s, *J*. 7.2) | 1.83(3H, d, *J*.3.8, 9.8) |

**Table 4:** C[13]-NMR ($\delta_C$ in ppm, 125MHz) Data of Compounds 1(Ajmaciline) and 2 (Ajmaciline oxindole B)

| | Compound 1 | Compound 2 |
|---|---|---|
| Position | $\delta_C$ | $\delta_C$ |
| 1 | 38.90 | 32.15 |
| 2 | 28.31 (C) | 29.43(C) |
| 3 | 81.18(CH) | 72.05(CH) |
| 4 | 39.95 | 40.04 |
| 5 | 55.48($CH_2$) | 140.97($CH_2$) |
| 6 | 18.43($CH_2$) | 122.15($CH_2$) |
| 7 | 32.72(C) | 32.15(C) |
| 8 | 40.27(CH) | 31.90(CH) |
| 9 | 47.89(CH) | 50.37(CH) |
| 10 | 37.34(CH) | 34.19(CH) |
| 11 | 23.87(CH) | 21.32(CH) |
| 12 | 124.56(CH) | 37.49(CH) |
| 13 | 139.90(CH) | 42.53 (CH) |
| 14 | 42.31($CH_2$) | 57.01($CH_2$) |
| 15 | 28.31(CH) | 24.53(CH0 |
| 16 | 23.62(C) | 28.46(C) |
| 17 | 33.08(CH) | 56.40(CH) |
| 18 | 59.30($CH_3$) | 12.10($CH_3$) |
| 19 | 39.97(CH) | 20.03(CH) |
| 20 | 31.42($CH_2$) | 36.78($CH_2$) |
| 21 | 37.03($CH_3$) | 19.62($CH_3$) |



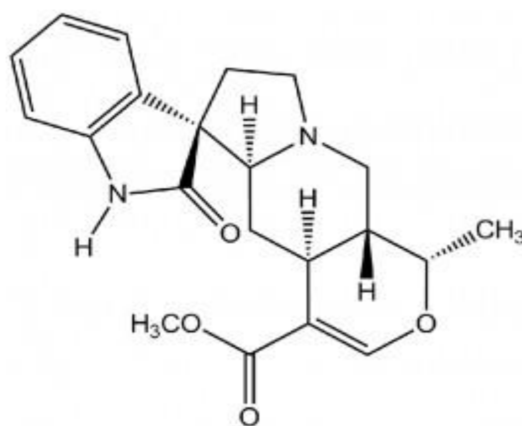




Fig 1: Structure of Compound 1-Ajmaciline Fig 2: Structure of Compound 2-Ajmaciline oxindole B

**DISCUSSION**

Phytochemical screening revealed that the extract was rich in secondary metabolites and they are the active principles. Studies have proved that these active principles: alkaloids, flavonoids, glycosides, saponins, tannins etc and other compounds such as morphine, atropine, codeine, steroids, lactones and volatile oils possess medicinal value for the treatment of different diseases. (3). The main alkaloid in *P. nitida* is akuammine has local anaesthei action. Its action can be compared to the anaesthetic action of cocaine. (12). The anti-sickling activities of the extracts of the roots of a plant *Cissus populnea* L. (CPK) (a major constituent of a herbal formula Ajawaron HF used in the management of sickle cell disease in south-west Nigeria) has been examined and the phytochemical examination of the extract showed the presence of anthraquinone derivatives, steroidal glycosides and cardiac glycosides. (13), the glycosides in combination with other active principles were responsible for antisickling activity of the plant. Phytochemical and antisickling activities of *Entandrophragma utile, Chenopodium ambrosioides* and *Petiveria alliacea* as investigated (14) revealed the presence of alkaloids, tannins and saponins while free and combined anthraquinones were absent. This result is similar to that reported (15) for *Plumbago zeylanica* and *Uvaria chamae* recipes used to manage sickle cell disease (SCD) in south-west Nigeria. In a related development, (16) reported that saponins, in addition to carboxylic acids and flavonoids may be responsible for the antisickling activity of *H.acida* leaves. Additionally, alkaloids are nerve stimulants, convulsants and muscle relaxants (17) hence; the presence of alkaloids in the investigated plant parts is an indication that they may be useful in alleviating some of the symptoms associated with pains. Phytochemical screening of *P. nitida,* showed the presence of those secondary metabolites. The results of antisickling assay of the extract/fractions of *P.nitida* in the present study showed that they exhibited substantial antisickling activity. This may give a rational explanation for the use of the plant in managing sickle cell disease (SCD) by traditional healers.*Picralima nitida* is known as a rich source of alkaloids**.** Some of them have been reported to show biological activities (18, 19, 20 and 21) . The use of the seeds of *P. nitida* as antisickling agent may be explained by the presence of these antisickling alkaloids compound 1 (ajmacline); compound 2 (ajmaciline oxindole B) and compound 3 (uncharacterize).

**CONCLUSION**

This study has established the anti-sickling constituents *of P. nitida* seeds. The separated products are alkaloids. The isolated compounds ajmalicine (19∝)-16,17-didehydro-19-methyl-oxayohimbin-16-carboxylic acid methyl ester.(1) and ajmalicine Oxindole B (19∝)-19-methyl-2-oxoformosanan-16-carboxylic acid methyl ester (2) were responsible for the antisickling effect in *P. nitida..*This could serve as a lead in the development of potent antisickling agents.

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