Tropical Journal of Pharmaceutical Research November 2020; 19 (11): 2377-2383
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i11.19

Original Research Article

Anti-cancer and anti-trypanosomal properties of alkaloids from the root bark of *Zanthoxylum leprieurii* Guill and Perr

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Sent for review: 9 April 2020 *Revised accepted: 21 October 2020*

Abstract

Purpose: *To isolate the anti-cancer and anti-trypanosomal principles of Zanthoxylum leprieurii, a medicinally versatile wild tropical plant used for managing tumours, African trypanosomiasis, and inflammation in southeastern Nigeria.*
Methods: *The pure compounds were isolated using chromatographic methods. The structural elucidation of the pure compounds was based on their NMR (1D and 2D) and mass spectral data as well as chemical test results. Structure-activity relationships were based on the structural differences among the compounds. The cytotoxicity of the extracts and compounds (1, 2, 3, and 4) was evaluated in HeLa (human cervix adenocarcinoma) cell line while the trypanocidal activities were evaluated on Trypanosoma brucei brucei.*
Results: *Two acridone alkaloids, 1-hydroxy-3-methoxy-10-methylacridin-9 (10H)-one, named fabiocinine (1), and 1-hydroxy-2,3-dimethoxy-10-methylacridin-9 (10H)-one (arborinine, 2), together with a furoquinoline alkaloid, skimmianine (3), and a chelerythrine derivative, 6-acetonyl-5,6-dihydrochelerythrine (4) were isolated from the root bark of Zanthoxylum leprieurii. Skimmianine (3) exhibited cytotoxicity and anti-trypanosomal IC_{50} of 12.8 and 13.2 µg/mL respectively ($p < 0.05$). Compound (1) and arborinine (2) were selectively cytotoxic to HeLa cells with cytotoxicity IC_{50} of 28.49 and 62.71 µg/mL, respectively, while (4) did not show significant activity ($p < 0.05$).*
Conclusion: *Zanthoxylum leprieurii root bark contains cytotoxic and trypanocidal compounds, and is thus a potential source of anti-cancer and anti-trypanosomal leads.*

Keywords: *Zanthoxylum leprieuri, Root bark, Anti-cancer, Anti-trypanosomal, Cytotoxicity, Alkaloids*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Zanthoxylum leprieurii Guill. & Perr. (Rutaceae) (syn. *Fagara leprieurii*), with the common name of satinwood or prickly ash, is a wild tropical plant widely distributed in sub-Saharan Africa and used extensively in traditional medicine. It occurs commonly in the rain forest vegetation of southern Nigeria [1] and is locally known as *ukonta* or *ota* among the Igbo tribe of southeastern Nigeria.

Different parts of *Z. leprieurii* have been well utilized in ethno-medicinal applications. A decoction of the stem or root bark is usually administered for the treatment of malaria, toothache, rheumatic pain, arthritis, bacterial infections, and HIV/AIDS [2-3]. In Senegal, the powdered bark, together with the latex of *Baissea axillaris* (Benth.) Hua, is used on tumours [1]. In Cameroon, fruit infusion is taken to treat sickle cell anemia [4], bacterial and fungal infections [5]. It is used by communities in Uganda for the management of tuberculosis and cough-related infections [6]. In southeastern Nigeria, particularly among the rural dwellers in the Nsukka area of Enugu State, the root or stem bark is masticated, held in the mouth, and the juice gradually swallowed as an effective remedy for inflammations of the gum or gut.

Previous studies and some anecdotal evidence on *Z. leprieurii* indicate that the plant holds promise as a potential source of drug leads. Several acridone alkaloids, including 1-hydroxy-3,4-dimethoxy-*N*-methylacridone [7,8] have been isolated from the fruits of *Z. leprieurii* used as a food spice in Cameroon. These alkaloids have been reported to exhibit anti-oxidant, anti-malarial [4], or antibacterial activity [8,9]. Three anti-tubercular acridone alkaloids have also been isolated from the stem bark[3]. Essential oils from the fruits have been reported for antimicrobial properties [10,11]. Antibacterial and cytotoxic depsidones have been obtained from an endophytic fungus *Chaetomium* sp. associated with *Z. leprieurii* [12]. Nitidine isolated from *Zanthoxylum nitidum* and from *Zanthoxylum macrophylla* exhibited strong antileukemic activity against L1210 and P388 and showed growth inhibition of Lewis lung carcinoma and B 16 melanoma [13]. Fagaronine isolated from *Zanthoxylum zanthoxyloides* demonstrated strong antileukemic activity against both L-1210 and P-388 leukemia [13]. The benzophenanthridine alkaloids of the *Zanthoxylum* generally exhibit some anti-neoplastic properties, though their clinical use is limited by their high toxicity [4].

Whereas there are reports on the anti-malarial, antibacterial and antioxidant properties of the fruits and stem bark of the *Z. leprieurii*, no part of the plant, or its compound, has been previously evaluated for anti-trypanosomal property and cytotoxic activity on HeLa cell line. Incidentally, the root of this plant is the most efficacious and the most commonly used part in Nigerian herbal medicine but has not been sufficiently studied scientifically. Although the compound, 6-acetonyl-5, 6-dihydrochelerythrine, has been isolated from *Zanthoxylum rhetsa* [14], it has not been isolated from any part of *Z. leprieurii*.

Hence, the purpose of this study was to evaluate the root bark of *Z. leprieurii* for anti-trypanosomal and cytotoxic properties and isolate the compounds responsible for these activities. This could lead to development of new anti-trypanosomal and anti-cancer 'leads'.

EXPERIMENTAL

Plant collection and preparation

The root bark of *Z. leprieurii* was obtained in April from a forest in Ihe/Owerre community in Nsukka, Nigeria. The plant was validated at the International Centre for Ethno-medicine and Drug Development, InterCEDD, Nsukka where a voucher specimen (voucher no: InterCEDD/16017) was kept. The plant materials were cleaned, dried under the shade and crushed to tiny chips before solvent extraction.

Equipment and experimental procedures

With respect to NMR experiments, ^{1}H, ^{13}C, DEPT-135, COSY, HSQC and HMBC were conducted for each compound for structural elucidation. All the NMR samples were prepared in $CDCl_3$ and the spectra recorded on Brucker Avance II 400 MHz spectrometer run by Topspin package. The chemical shifts were given in ppm with solvent reference peaks at δ_H 7.26 and δ_C77.2. MS spectra were obtained from a Bruker Daltonics Compact QTOF mass spectrometer using the positive mode of an electrospray ionisation probe. UV-Visible spectra were recorded on Perkin Elmer UV Winlab, Lamda 25. Analytical and preparative TLC experiments were performed on pre-coated plates with silica gel 60 F_{254} (Merck, Germany, 0.2mm and 1mm respectively) eluted with various solvent systems and the developed chromatograms viewed under UV light (254 and 365nm). All the melting points were measured using Electrothermal® melting point apparatus fitted with a thermometer (0-360 ^{o}C range).

Solvent extraction

The prepared plant materials (500g) were extracted with 1 litre of 90% aqueous methanol for three consecutive times using the cold maceration method at room temperature within 96 h and the combined filtrates concentrated in a rotary evaporator at 40 ºC under reduced pressure to yield 96 g (19.2 %) crude extract, (ZL). The crude extract was re-dissolved in 90 % methanol (500 mL) and subjected to solvent

fractionation by partitioning the solution sequentially with n-hexane and ethyl acetate (EtOAc) (500 mL each) four consecutive times each to yield n-hexane fraction (ZHF) - 5.1 g (5.3 %) and EtOAc fraction (ZEF 7.4 g) (7.7 %). The EtOAc marc was consecutively extracted with absolute methanol and water to yield methanol fraction (ZMF) - 48.0 g (50 %) and water fraction (ZWF) - 17.5 g (18.2 %) respectively, leaving an insoluble residue (ZMK), 17.4 g (18.1 %). The yield of the crude extract was given as a percentage of the weight of the plant materials and yields of the fractions were expressed as a percentage of the crude extract.

Acute toxicity test

The acute toxicity profile of the crude extract was determined based on the Lorke's technique [15]. Both male and female Swiss albino mice, (weighing 21-34 g) were maintained in good conditions and used for the experiment. Food and water were withdrawn from the animals 12 h prior to the test in order to achieve constant and uniform hydration. The test procedure was sanctioned by the ethics committee of the University of Nigeria, Nsukka, as registered by the National Health Research Ethics Committee of Nigeria (ref no. NHREC/05/01/2008B). The animal studies were carried out according to the globally established standard for handling experimental animals (NIH publication no. 85-23, revised 1985).

Qualitative phytochemical analysis

The qualitative phytochemical compositions of the extracts, fractions and pure compounds were determined according to established procedures [16,17].

Isolation of bioactive compounds

Based on the bioassay and results of phytochemical analysis, the ZEF fraction (1 g) was chromatographed in a column packed with 100 g silica gel 60 (60Á, 70-230 mesh) eluted sequentially with 200 ml each of diethylether (DEE), diethylether:dichloromethane (DEE:DCM 5:1 volume rate), diethylether:ethylacetate (DEE:ETOAc - 5:1), DCM:ETOAc (1:1) and ETOAc which afforded 13 sub-fractions ($ZEFC_1$-$ZEFC_{13}$). The sub-fractions $ZEFC_6$ and $ZEFC_8$ were obtained in good yield, and contained alkaloids only. Their components also gave the best separation on analytical TLC. $ZEFC_6$ (300 mg) was purified further on silica gel (80 g) eluted with 600 mL DCM: DEE (4:1) mixture to give ZEF-05(**1,** 121 mg) and ZEF-06 (**4**, 37 mg).

The $ZEFC_8$ fraction (400 mg) was also purified further on silica gel (70 g) column eluted with 600 mL DCM:DEE (4:1) to afford two compounds which were analyzed for spectral purity, viz; ZEF-02 **(2,** 152 mg**)**and ZEF-04 (**3,** 56 mg). The ZEF fraction was also subjected to preparative TLC on pre-coated 1 mm silica gel plates eluted with DEE/DCM (5:1) to yield a chromatogram with about twelve fairly distinct bands, B_1-B_8 (Table 2, Figure 2). After recovering and purifying the bands, B_2, B_4, B_5 and B_6 were identified as compounds **2**, **3, 1** and **4** respectively. Qualitative phytochemical analysis was also carried out on the TLC bands after recovery.

Cytotoxicity studies

The cytotoxicities of the test extracts, fractions and compounds were assessed based on the reduction of resazurin to resorufin by living cells [18-20]. A 50 µg/mL solution of each of the test samples was prepared in pure DMSO and incubated with HeLa cells in 96-well plates at a temperature of 37 ^oC for 2 days in an environment of 5% carbon dioxide. The HeLa cells were cultured and maintained in DMEM. To ascertain the number of HeLa cells that survived the drug interaction, a resazurin-containing reagent was added to the mixture at the end of the incubation period and the resorufin fluorescence was measured in a multiwell plate reader. This fluorescence is a measure of the number of enduring cells.

Results were given as percentage cell viability. Triplicate measurement was made for each compound and a standard deviation (SD) obtained for each. Emetine and pure DMSO were used as positive and negative controls respectively. The IC_{50}'s of samples, which drastically annihilated the cells to percentage viabilities below 25 %, were further determined from dose-response curves using non-linear regression [18-20].

Determination of anti-trypanosomal activity

The trypanocidal effects of the test samples were determined based on the conversion of resazurin to resorufin by viable living cells. Resorufin is a fluorescent material (560 nm excitation/590 nm emission) [18-20].

Trypanosoma brucei brucei (s427) cells, cultured in a medium of HM111, were exposed to 50 µg/mL solution of each of the test samples and incubated in a 96-well plate at 37^oC for 2 days in a 5% carbondioxide environment. After the incubation, a resazurin-containing reagent was added to the mixture for reduction to resorufin by

the surviving cells. The resorufin fluorescence was measured as a function of the viable cells and the result given as percentage cell viability. Pentamidine and pure DMSO were used as positive and negative controls respectively. The IC_{50}'s of non-cytotoxic samples, which gave percentage viabilities below 20 %, were further determined from dose response curves using non-linear regression.

Statistical analysis

Data obtained were analyzed using Graph Pad Prism 7.0 and subjected to Dunnett's post hoc test. The results were expressed as mean ± standard deviation (SD) with $p < 0.05$ considered statistically significant.

RESULTS

Spectral data

Fabiocinine, Compound 1(1-hydroxy-3-methoxy-10-methylacridin-9 (10H)-one)*:* Yellow powder (MP 172 °C; ^{1}H NMR ($CDCl_3$, 400MHz) and ^{13}C NMR ($CDCl_3$,100 MHz) See supporting data; HRESIMS *m/z* 256.1060 $[M + H]^+$ (calculated $C_{15}H_{14}NO_3^+$: 256.0970).

Arborinine, Compound 2 (1-hydroxy-2,3-methoxy-10-methylacridin-9 (10H)-one): Yellow crystals, $C_{16}H_{15}NO_4$; MP~177°C; ^{1}H NMR ($CDCl_3$, 400MHz) and ^{13}C NMR ($CDCl_3$,100 MHz) see supporting data; HR-ESI-MS *m/z*286.1176 $[M + H]^+$ (calcd for $C_{16}H_{16}NO_4^+$, 286.1075).

Skimmianine, Compound 3 (4,7,8-trimethoxyfuro[2,3-b]quinoline): Pale yellow amorphous powder; $C_{14}H_{13}NO_4$; MP~175ºC; ^{1}H NMR ($CDCl_3$, 400MHz) and ^{13}C NMR ($CDCl_3$,100 MHz) See supporting data; HR-ESI-MS *m/z* 260.1017 $[M + H]^+$ (calcd for $C_{14}H_{14}NO_4^+$, 260.0919).

6-*Acetonyl-5, 6-dihydrochelerythrine, Compound 4:* 1-(7, 8-dimethoxy-5-methyl-6H-[1,3]benzo dioxolophenanthridin-6-yl)propan-2-one; pale yellow solid; $C_{24}H_{23}NO_5$; M.P ~188ºC; ^{1}H NMR ($CDCl_3$, 400MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) See supporting data; HR-ESI-MS *m/z* 406.1830 $[M + H]^+$ (calculated for $C_{24}H_{24}NO_5^+$, 406. 1648).

Qualitative phytochemical profile

The phytochemical constituents of the different fractions of *Z. leprieurii* root bark are given in Table 1 and those of ethyl acetate TLC bands in Table 2. The TLC chromatogram for the ethyl acetate fraction, ZEF, is given in Figure 2. The ethylacetate fraction contains mainly alkaloids and terpenoids. Almost all the root bark extracts and compounds were yellow in colour and yet flavonoids were present only in limited quantities.

Table 1: Phytochemical constituents of the different fractions of *Z. leprieurii*

Fraction	Alkaloids	Steroids	Terpenes	Saponins	Flavonoids	Glycosides
ZHF	++	++	**+++**	-	-	-
ZEF	+++	+	**++**	-	-	-
ZMF	+++	-	**-**	+++	+	++
ZWF	+	-	**-**	+++	+	++

+ Trace, ++ moderate, +++ very abundant, - not detected, ZHF, ZEF, ZMF and ZWF represents hexane, EtOAc, methanol and water fractions, respectively

Table 2: Preparative TLC bands of ethylacetate fraction of *Z. leprieurii* root bark

	Band code	R_f (cm)	Colour (white light)	Colour under UV 365 nm	Effect on fluorescence	Phytochemical class[#]
1	*B_1	0.00	Brown	Light green	quenched	Alkaloid, terpenoid
2	1B	0.31	Colourless	Blue	none	Alkaloid
3	1BB	0.31	Colourless	Yellow	none	-
4	B_2	0.38	Yellow; turns green	Brown	quenched	Alkaloid
5	B_3	0.44	Colourless	Yellow	blue	-
6	B_4	0.50	Pale yellow	Blue	none	Alkaloid
7	4B	0.53	Colourless	Yellow	quenched	-
8	B_5	0.63	Yellow; turns red	Yark red	quenched	Alkaloid
9	B_6	0.75	Brownish-yellow	Yellow	none	Alkaloid, terpenoid
10	6B	0.78	Light yellow	Brown	quenched	steroid
11	B_7	0.81	Yellow	Yellow	yellow	terpenoid
12	B_8	0.88	Yellow	Brown	yellow	terpenoid

*Remnant of the spots. – Not determined. [#]The bands were recovered before analysis

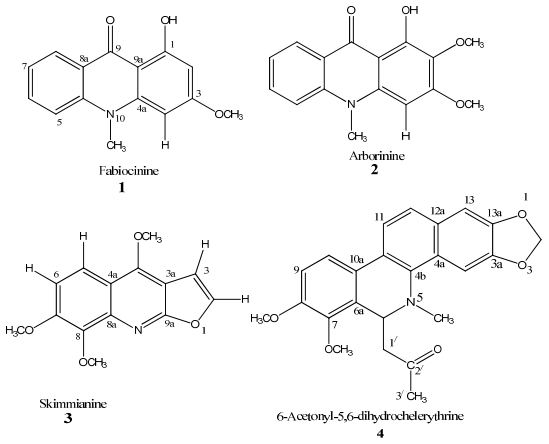

Figure 1: Chemical structures of the isolated compounds

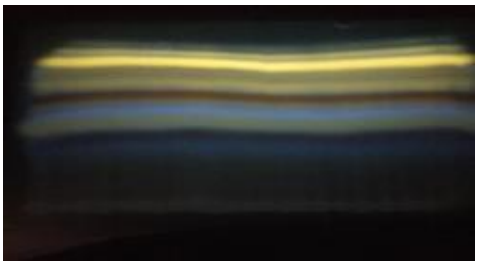

Figure 2: TLC Chromatogram of ethyl acetate fraction (ZEF) under UV Light at 365 nm

Acute toxicity profile

The acute toxicity test on the crude extract, ZL, showed no adverse effect or mortality in the animals up to 12,000 mg/kg oral dose during 24 h monitoring period. The LD_{50} is, therefore, greater than 10, 000 mg/kg indicating high safety margin.

Cytotoxicity study

The cytotoxicity (IC_{50} values) of the fractions and compounds are displayed in Table 3. The TLC fraction, B_1, gave the best activity of all the test samples followed by compound 2.

Table 3: Cytotoxicity profile of the plant fractions and compounds

Sample at 50µg/mL	IC_{50} (µg/mL) for samples, µM for Emetine
B_1	4.36
ZEF 02 (**2**)	62.71
ZEF 04 (**3**)	12.80
ZEF 05 (**1**)	28.49
ZEF 06 (**4**)	-
ZEF	25.76
ZMF	47.65
Emetine(1µg/ml)	0.026

Anti-trypanosomal activity

The results of anti-trypanosomal IC_{50} of the samples, which were not toxic to HeLa cells but drastically annihilated the parasites below 20% viability, are displayed in Table 4 and the percentage viabilities of all the test samples, at 50 µg/mL, in Fig. 4. The viability of the cells, after exposing them to the test drugs, is inversely proportional to the biological activity.

Table 4: Anti-*trypanosoma* activity of isolated compounds

Compound	IC_{50}(µg/mL) for samples, uM for pentamidine)
1B	3.47
B_8	4.66
ZEF 04 (**2**)	13.22
Pentamidine	0.004363

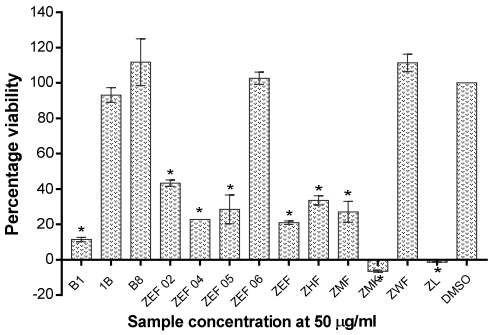

Figure 3: Cytotoxicity single concentration screen. Values are mean ± SD, n = 3, *p < 0.05, significant different compared with control (DMSO)

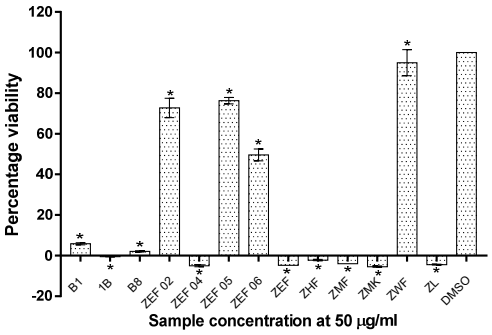

Figure 4: Anti-trypanosomal single concentration screen. Values are mean ± SD, n = 3, *p < 0.05, significant different compared with control (DMSO)

DISCUSSION

The crude extract, Z, gave LD_{50}> 10,000 mg/kg. No adverse effect or mortality was recorded in the animals up to 12,000 mg/kg oral dose within the 24 h period of monitoring. This indicates a wide margin of safety. The result of the

phytochemical analysis (Table 1) shows an abundance of alkaloids in the root bark. This conforms with literature reports on the phytochemical composition of the *Zanthoxylum* species [4,21].

From the results of the cytotoxicity assay (Table 3), compounds **1**, **2**, **3** and a TLC fraction **B_1** gave cytotoxicity IC_{50} of 28.49, 62.71, 12.80and 4.36 µg/mL respectively while the emetine standard gave 0.026 µM. Though emetine showed better activity, its high toxicity to the myocardium is worrisome. Numerous cytotoxic alkaloids from the *Zanthoxylum* species have been reported [4,8,12,13]. The acridone class of alkaloids has been investigated with particular attention to the anti-cancer evaluation of acronycine. The broad spectrum anti-tumour activity as well as the toxicity of acronycine has been reported [22]. Compounds **1**, **2** and **3,** at 50 µg/mL, gave anti-trypanosomal percentage viabilities of 76.35, 72.74 and -5.02 % respectively (Figure 4). Percentage viability is inversely proportional to biological activity. This shows that **1** and **2** are not appreciably active against *Transpanosoma brucei brucei* and hence are more selectively cytotoxic to HeLa cells than **3** which might be toxic to all cells**.** This implies that **1** and **2** have good selectivity indices and can serve as lead compounds in the development of anti-cancer drugs.

Substitution of C-2 with methoxy group in compound (**2**) relative to (**1**) leads to a decrease in cytotoxic activity, as evident in their IC_{50} values (Table 3), but a slight increase in antitrypanosomal activity (Figure 4). The cytotoxicities of compounds (**1**) and (**2**) are consistent with literature reports on acridone alkaloids as potential anti-cancer candidates [29]. Compound (**3**) is structurally similar to (**1**) and (**2**) in that each possesses a 3-ring conjugated aromatic system with nitrogen in the middle ring. This arrangement tends to favour cytotoxic activity since (**3**) also has very good activity while (**4**) shows no activity.

Two non-cytotoxic TLC fractions, **1B**(alkaloid**)** and **B_8**(oily terpenoid) showed anti-trypanosomal IC_{50} of 3.47 and 4.66 µg/mL respectively, lending credence to *Z. leprieurii* as a potential source of safe anti-trypanosomal compounds. Upon further purification and structural elucidation of **1B** and **B_8**, newer potent anti-trypanosomal compounds with good selectivity indices could be obtained.

CONCLUSION

The root bark of *Zanthoxylum leprieurii*, in addition to other bioactive compounds, contains fabiocinine, skimmianine, and as well as anti-trypanosomal terpenoids. Fabiocinine and arborinine exhibit selective cytotoxicity against HeLa (human cervix adenocarcinoma) cell line while skimmianine exhibits strong cytotoxicity against HeLa cell and mild anti-trypanosomal activity against *Trypanosoma brucei brucei*. This suggests that the root bark of *Zanthoxylum leprieurii* is a potential source of 'lead' compounds for the development of new and safer anti-cancer and anti-trypanosomal drugs with good selectivity index. It also accounts for the ethnopharmacological use of the plant in alleviating tumours and protozoal infections.

DECLARATIONS

Acknowledgement

This work was sponsored by the Nigeria Tertiary Education Trust Fund (TETFund), and supported by the South African Medical Research Council (MRC) with funds from National Treasury under its Economic Competitiveness and Support Package, and Rhodes University SandisaImbewu. Pharmabridge, Geneva, Switzerland is also gratefully acknowledged for this collaboration.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Fabian I. Eze and Patience O. Osadebe designed the study; Fabian I. Eze did the laboratory works and prepared the manuscript. Xavier Siwe-Noundou assisted in the laboratory work, manuscript editing and spectral analysis. Michelle Isaacs did bioassays, Srinivas Patnala proof-read and edited the manuscript and made useful suggestions. Patience Osadebe and Rui M Krause supervised the work and provided the mentorship, scientific and material support. All authors have read and approved the manuscript for publication.

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