Research Article

Evaluation of anti-onchocercal activity of pseudopalmatine, a quaternary protoberberine alkaloid of *Enantia chlorantha* (Syn. *Annickia chlorantha*).

Kennedy D. Nyongbela[1*], Moses Samje[2], Angelbert F. Awantu[3], Tiku E. Tiku[2], Fidelis Cho-Ngwa[2]

[1]Pharmacochemistry Research Laboratory, Department of Chemistry, Faculty of Science, University of Buea. P.O. Box 63 Buea, South West Region Cameroon.

[2]ANDI Centre of Excellence on Onchocerciasis Research, Faculty of Science, University of Buea, P. O. Box 63 Buea, South West Region Cameroon.

[3]Department of Chemistry, Faculty of Science, University of Bamenda, P. O. Box 39 Bambili, North West Region, Cameroon.

*Corresponding author. knyongbela@gmail.com

ABSTRACT

As part of our efforts towards identifying and subsequently developing lead compounds from medicinal plants of Cameroon to combat neglected tropical diseases, we embarked to phytochemically investigate *Enantia chlorantha* (Syn. *Annickia chlorantha*). The rationale for choosing this plant is its numerous uses in folk medicine in Cameroon and other parts of Africa. In Cameroon the quaternary protoberberine alkaloids, columbamine (**2**), palmatine (**3**) and jatrorrizine (**4**) have been isolated from the stem back and combined to produce a phytomedicine (HEPAZOR®) used in the treatment of viral hepatitis. An alkaloidal extraction of the methanolic extract of the stem bark of the plant was carried-out and this afforded yellow amorphous solids whose structure was obtained using routine nuclear magnetic resonance spectroscopy (NMR), high pressure liquid chromatography coupled to an electrospray mass spectrometer (HPLC-ESI-MS) and comparison with literature. The compound was identified as pseudopalmatine (**1**), a quaternary protoberberine alkaloid. Preliminary screening of the compound on both adult and juvenile worms of *Onchocerca ochengi*, a close relative of *Onchocerca volvulus*, the parasite responsible for human onchocerciasis (river blindness), showed that compound **(1)** was inactive at a concentration of 500 µg/mL on the adult worms, but inhibited microfilariae motility completely at this same concentration and by 50 % at 250 µg/mL and was thus considered active. While this work to the best of our knowledge constitutes the first report on the anti-onchocercal activity of quaternary protoberberine alkaloids in general and pseudopalmatine (**1**) in particular isolated from *E. Chlorantha*, it has however opened a window for further investigation of the anti-onchoceral activity of this class of compounds.

Key words: anti-onchocercal, pseudopalmatine, alkaloid, *Enantia chlorantha*

RÉSUMÉ

Dans le cadre de nos efforts pour identifier et ensuite developer des substances naturelles ayant une activite antiparasitaires et antihelminthiques des plantes médicinales du Cameroun, nous nous sommes attelés à étudier *Enantia chlorantha* (Syn.*Annickia chlorantha*), une plante médicinale utiliser généralement au Cameroun pour le traitement des divers maladies parmi lesquels le palludisme, la rougeole et l'hépatite. Au Cameroun, les alcaloïdes tels que la columbamine (**2**), palmatine (**3**) et jatrorrhizine (**4**) isolé des ecorces de cette plante, sont combiner pour produire une phytomédicament (HEPAZOR®) appliquée dans le traitement du l'hépatite virale. L'extrait au méthanol des ecorces de cette plante à été sujet a une extraction des alcaloïdes. Une composé s'est cristallisée sous forme de poudre jaune et à été séparer et purifier. La structure de ce composé a été identifiée à celle du pseudopalmatine (**1**), un alcaloïde protoberberine quaternaire, sur la base des techniques spectroscopiques. La pseudopalmatine (**1**) a été soumis au teste sur les verres des macros et microfilaires de *l'Onchorcerca ochengi*, une proche relation de l'*Onchocerca volvulus*, la parasite responsable de la maladie d'onchocercose humaine. Pseudoplamatine (**1**) na pas inhiber la motilité des macrofilaires à une concentration de 500 µg/mL et est considéré inactif , mais par contre, à inhiber la motilité des microfilaires complètement à cette concentration et 50 % à 250 µg/mL et est considéré actif. Etant donner que ce travail constitue le premier rapport sur l'activité anti-onchocercose des alcaloïdes protoberberine quaternaire en générale et la pseudopalmatine (**1**) en particulier, ces résultats cependant ont ouvrir une fenêtre pour la continuation de recherche sur activité anti-onchocercose de cette classe des composés.

Mots Clé: anti-onchocercose, pseudopalmatine, alcaloïde, *EnantiachIorantha*

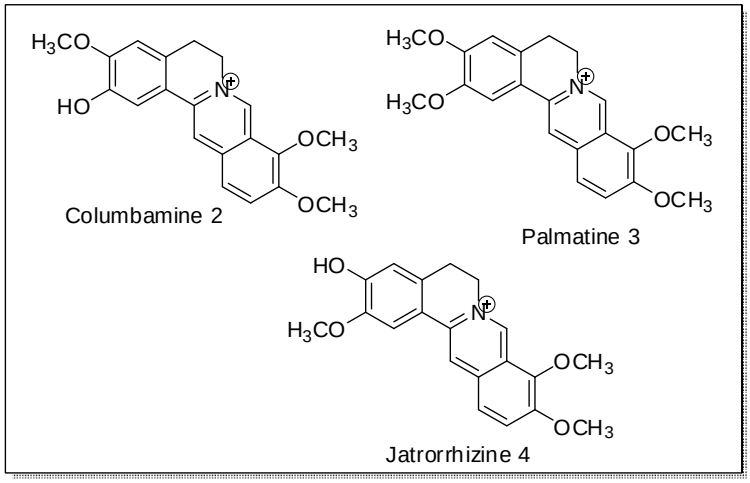
Received: 23/06/2018
Accepted: 27/12/2018
DOI: https://dx.doi.org/10.4314/jcas.v14i3.1

Introduction

While human onchocerciasis (river blindness) is not only a neglected tropical disease (NTD), it remains the world's second most leading infectious cause of blindness, with over 37 million patients and a risk population of over 120 million Samje et al. (2014). Ivermectin (Mectizan®, Merck), the currently available drug of choice for the treatment of river blindness, is limited only on the juvenile form of the parasite (microfilariae), and there are now reports of its suboptimal effect on these worms Cupp et al. (2011). This situation has led to an increasing interest by researchers to search for alternative treatments to combat this disease that continue to affect the population and retard socio-economic development in the affected countries.

Alkaloids are a group of natural products with diverse structures and possess very important biological activities(Roberts and Wink,1998). Although their role in plants has not yet been fully established, they are generally thought to confer protective mechanisms against herbivorous animals and possible parasites. Isoquinoline alkaloids are among the most abundant group of alkaloids in nature and constitute the basic skeleton of protoberberine alkaloids and many others. The protoberberine skeleton falls within the quaternary protoberberine alkaloids (QPA) that represent approximately 25% of all alkaloids isolated from natural sources Grycovš et al. (2007).

As part of our efforts to discover and develop novel lead compounds from medicinal plants of Cameroon to combat neglected tropical diseases, we embark to phytochemically screen *Enantia chlorantha* (Syn. *Annickia chlorantha*), a medicinal plant of Cameroon whose bark is used in folk medicine for the treatment of malaria and jaundice (Ajanohoun et al.,1996), HEPAZOR®, a phytomedicine developed by the Laboratoire Santé Nature (PHYTORICA) in Douala, Cameroun, comprising the quaternary protoberberine alakaloids columbamine (**2**), 15%, palmatine (**3**), 65% and jatrorrhizine (**4**), 20%, (Labothera, (2005) is used for the treatment of viral hepatitis. The stem back of this plant was extracted in methanol and later subjected to an alkaloidal extraction procedure (scheme **1**). We report herein the isolation and identification of pseudopalmatine (**1**), a diisoquinoline quaternary protoberberine alkaloid and its preliminary screening *in vitro* against adult and juvenile worms of *Onchocerca ochengi*, a close relative of *Onchocerca volvulus,* the parasite responsible for human onchocerciasis (river blindness). While this is the first report on the anti-onchocercal screening of quatenary protoberberine alkaloids in general and pseudopalmatine (**1**) in particular, it has nonetheless opened a window for further investigation of anti-onchocercal activity of these class of compounds.



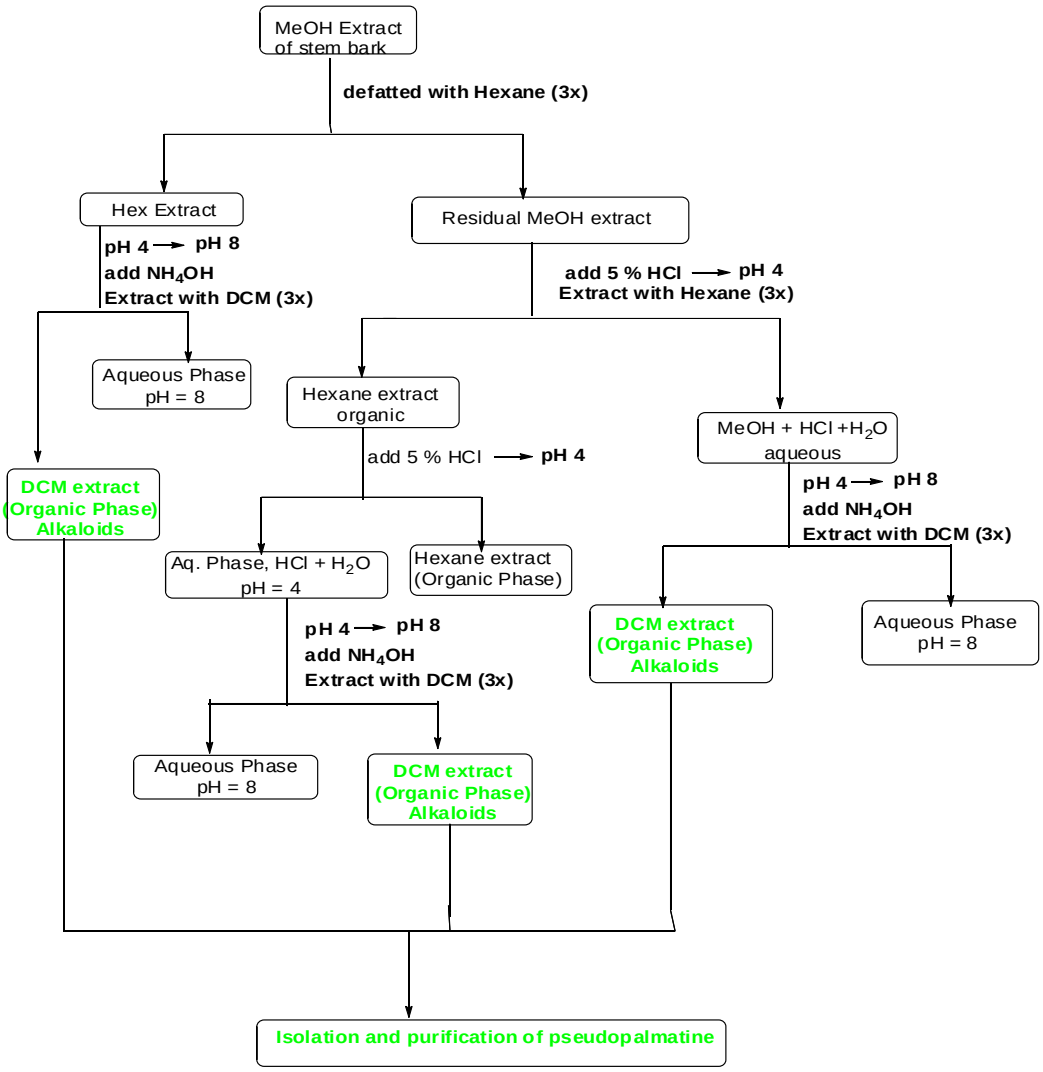
Materials and Methods.

Plant Collection

The stem bark of *E. Chlorantha* was collected at Likombe village, around mount Cameroon, South West Region in June 2016. It was chopped, air-dried and ground to powder to give 1.5 kg of dry powder. This powder was macerated in methanol (3 Litres) for 2 days after which the solvent was filtered and evaporated on a rotary evaporator. This maceration process was repeated twice to yield 300 g of combined methanolic extract.

Alkaloid Extraction.

250 g of the methanolic extract was subjected to an alkaloid extraction procedure as described by Bruneton J. (1993) and shown below (Scheme **1**).



Scheme 1: The alkaloid extraction of the methanolic extract of the stem bark of *E. chlorantha*

Scheme 1: The alkaloid extraction of the methanolic extract of the stem bark of *E. chlorantha*

Extraction and screen for *O.ochengi* microfilariae

The microfilaria of *O. ochengi* were extracted by the method of Titanjiet al. (1990), with some slight modifications. Cattle skin got from the slaughterhouse was thoroughly cleaned and sterilized as described below. The skin was then firmly attached onto a sterilized flat wooden board using autoclaved thumbnails. The outer surface was carefully shaved with a sterile razor blade, and then rinsed twice with distilled water. A clean dry adsorbent cloth was used to remove excess moisture from the skin. The entire skin was covered with 70% ethanol and allowed to evaporate in a laminar flow hood. This sterilization process was done twice. Once the alcohol had completely evaporated from the skin, skin snips were obtained from different locations of the skin. These sleeves were carefully scrapped and the snips submerged in 15 ml of complete culture medium. The assemblage was incubated at room temperature for 2 hours to allow for emergence of microfilaria. The highly motile microfilaria that emerged were concentrated by centrifugation at 400 x g for 10 minutes. The supernatant was decanted, and the pelleted mfs were re-suspended in fresh complete culture medium (RPMI-1640 supplemented with 25 mM HEPES, 2 g/L sodium bicarbonate, 20 mM L-glutamine, 10% new born calf serum [SIGMA, USA], 200 µg/mL penicillin, 200µg/ mlstreptomycin and 2.5 µg/mL amphotericin B). The highly motile microfilaria were quantified using an inverted microscope (Euromex, Holland). One hundred microlitres of culture medium containing microfilaria were distributed into 96 well culture plate containing LLC-MK2 cell layers to obtain an average of 12-15 mfs per well. The cultures were carried out at 37°C under and atmosphere of 5% CO_2 in humidified air(Thermo Electron, Germany) and lasted for five days after adding drugs. In the primary screen, the pure compound was tested in duplicate wells at single concentrations 500 µg/mL. In the secondary screen, the active molecule was testedper drug concentration and at serial dilutions of 8 concentrations starting from 500 µg/mL 7.8 µg/ mL in order to determine the $IC_{50.}$ Ivermectin at 10 µg/ml serve as positive control whilethe negative control wells received only the drug diluents (≤ 2% DMSO in RPMI). Viability of microfilaria was assessed by reading their mean motility scores every 24 hours using an inverted microscope for 120 hours on a scale of 0 (immotile), through 0.25 (only tail or head shaking occasionally), through 0.5 (whole body motile, but sluggish) to 1 (vigorous motility).

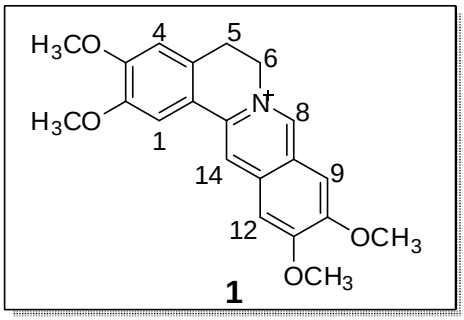
Extraction and screen for *O. ochengi* adult worms

Extraction of *O. ochengi* adult worms was done by the method of Cho-Ngwa*et al*., (2010) with slight modification. Fresh pieces of umbilical cattle skin containing palpable nodules were washed with tap water and soap and then rinsed thoroughly with distilled water. The skin was placed on a clean wooden board whose surface had been covered with 70% ethanol. The nodules containing pale orange-yellow *O. ochengi* adult worm masses were extracted using a razor blade into 2 mL of CCM distributed into a 12 well plate. The compound was tested in quadruplicate wells at a single concentration of 500 µg/mL. Auranofin (10mM) served as the positive control. All cultures were terminated on day 5 post addition of compound. Biochemical evaluation of adult worm viability was doneusing the MTT/formazan colorimetric assay. The worms were placed in 500 µL/well of 0.5 mg/mL MTT(Sigma, USA) in serum free media and then incubated under the culture conditions for 30 minutes. Adult worm viability was taken as mean percent inhibition of formazan formation relative to negative control.

Results

Isolation and identification

The alkaloid extraction method afforded 50 mg of a yellow amorphous powder which precipitated from the mixture and were purified by washing several times with acetone. It showed a positive test for alkaloid by giving an orange coloured spot on TLC plate when sprayed with Dragendorf solution. High pressure liquid chromatography coupled to an Electrospray ionization mass spectroscopy in Atmospheric Pressure Chemical Impact (HPLC-ESI+ APCI) in the positive mode (Figure 1), gave a molecular ion at m/z 352.0 corresponding to a compound with molecular formula $C_{21}H_{22}N^{+}O_4$. Its ^{1}H NMR spectrum recorded in DMSO (Figure 2), showed a signal at δ 3.30 (2H, t, 5-H_2) and δ 5.21 (2H, t, 6-H_2) corresponding to the methylene protons at H-5 and H-6 respectively. Four methoxy protons were registered at δ 3.99 (C_3-OCH_3), δ 4.10 (C_2 and C_{10}-OCH_3) and δ 4.25 (C_{11}-OCH_3). Finally five aromatic proton singlets were recorded at . δ6.84 (H-1, s), δ7.40 (H-4, s), δ7.83 (H-9, s and H-12, s), δ8.35 (H-14, s) and δ 10.34 (H-8, s). The above NMR data was consistent with published data for the compound by Yusupov et al., (1993) and Grycovš et al.,(2007) (Table 1). The compound was identified as the diisoquinoline quaternary protoberberine alkaloid, pseudopalmatine (**1**).



1

Table 1: Comparison of Proton NMR data of compound 1 with reference (Chemical shifts in ppm)

Proton Number	Compound 1	Ref. Grycovà et al.,(2007)	Ref. Yusupov et al.,(1993)
H-1	6.84	6.72	6.85
H-4	7.40	7.45	7.48
H-5 (2 Protons)	3.30	3.15	3.25
H-6 (2 Protons)	5.21	5.00	4.97
H-8	10.34	10.34	9.75
H-9	7.83	7.67	7.90
H-12	7.83	7.45	7.90
H-14	8.35	8.40	8.65
OCH_3-2	4.10	4.09	4.03
OCH_3-3	3.99	3.98	3.99
OCH_3-10	4.10	4.06	4.03
OCH_3-11	4.25	4.13	4.14

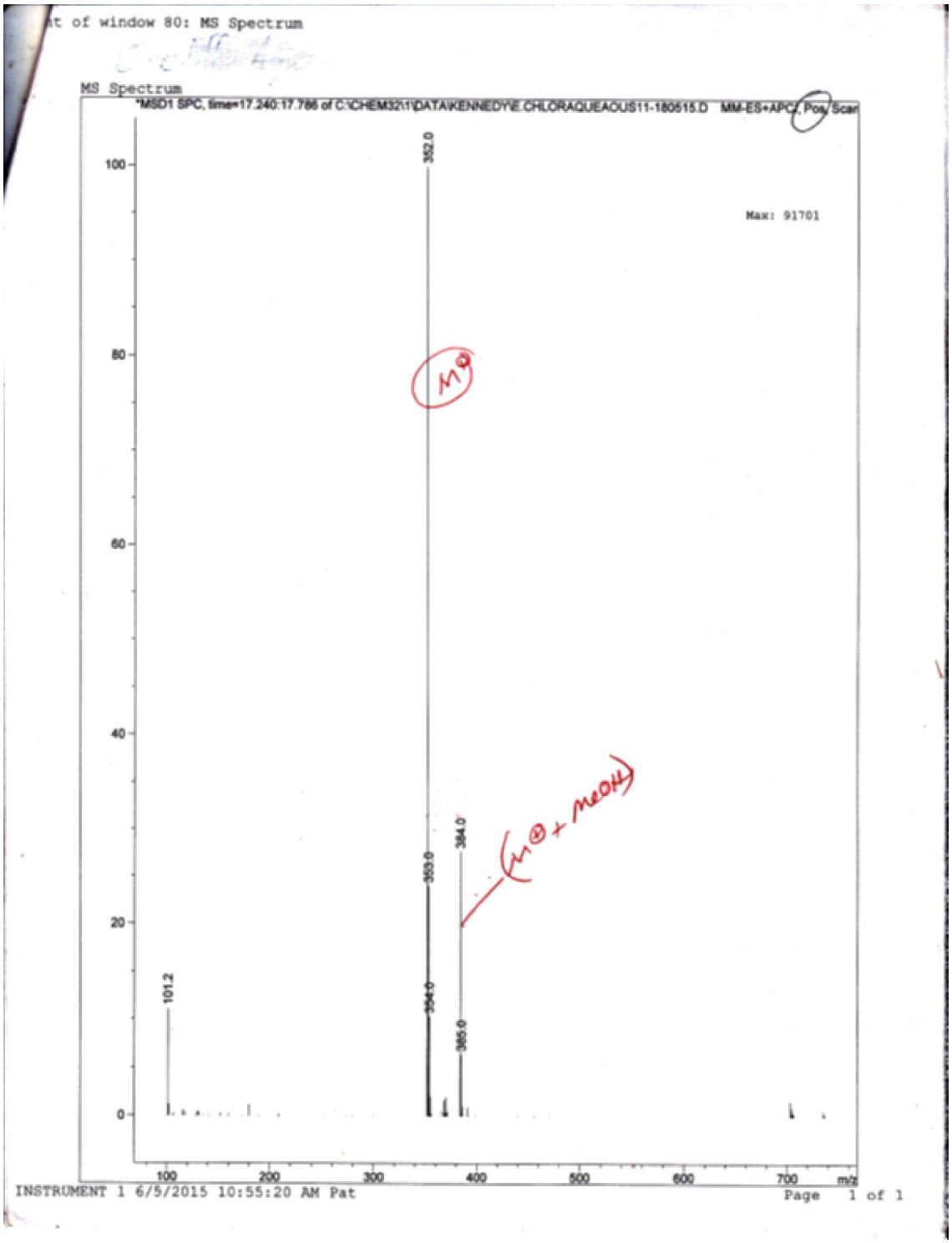


Figure 1: Electrospray Ionization Mass Spectrum in the positive mode of Pseudopalmatine

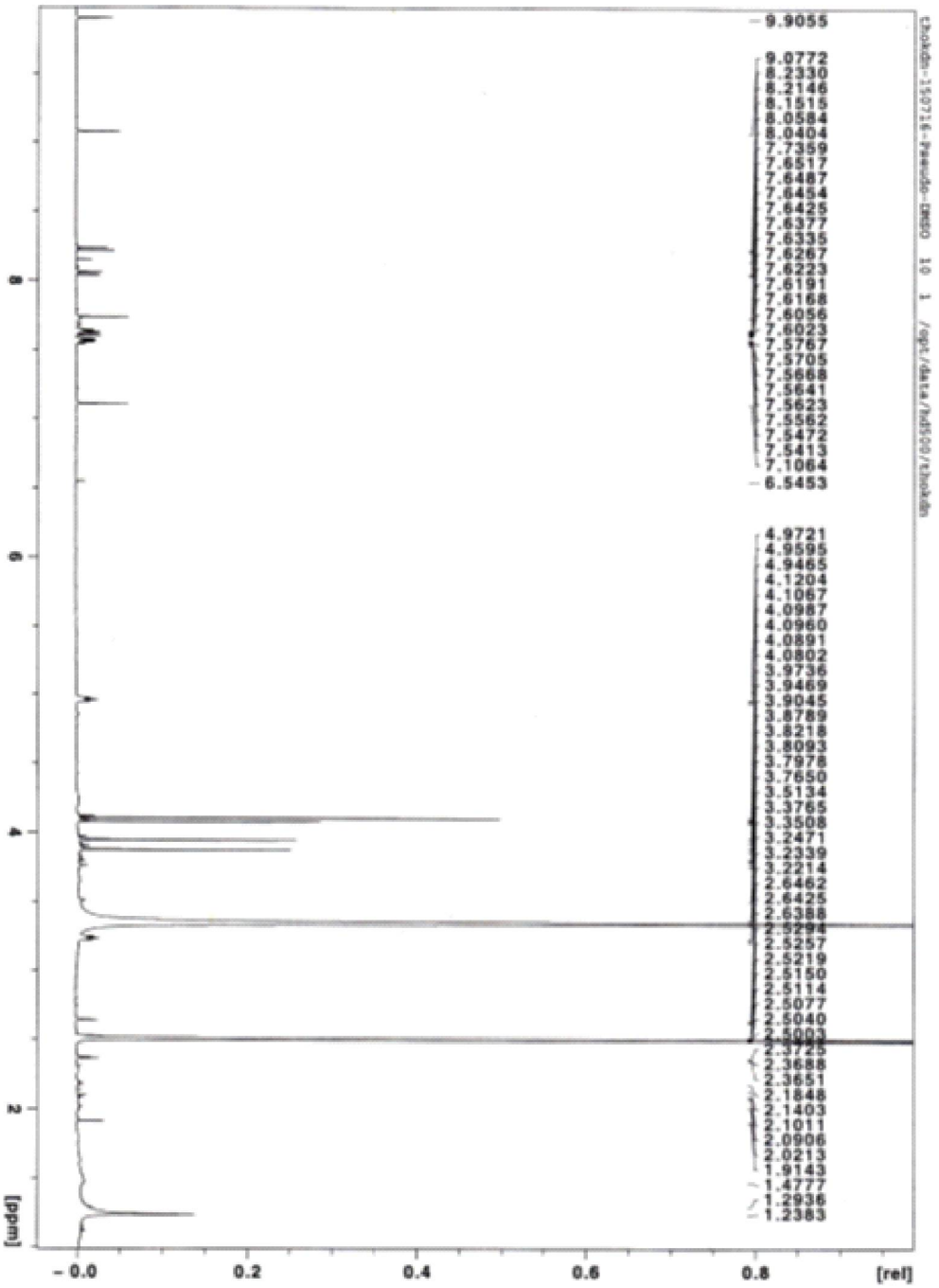


Figure 2: Proton NMR spectrum of Pseudopalmatine recorded in DMSO (500 MHz)

Anti-onchocercal screening

Pseudopalmatine (**1**) was inactive at the highest concentration (500 µg/mL) tested on the adult worms. The compound however showed 100 and 50 % inhibition of *O. ochengi* microfilaria with IC_{50} of 500 and 250 µg/mL respectively and was thus considered active (Table 1).

Table 1: Activity of Pseudopalmatine (**1**) on microfilaria of *O. ochengi* at 120th hour post addition of compound

Concentration (µg/mL)	% inhibition of *O. ochengi* microfilaria motility
500	100
250	50
125	25
62.5	0
31.25	0
15.625	0
7.8	0

Discussions

Although previous works, Yusupov et al., (1993) and Grycovš et al., (2007) have reported the isolation of pseudopalmatine (**1**) from other plant species, this work to the best of our knowledge, is the first report on the isolation of this compound from *Enantia chlorantha* (Syn: *Annickia chlorantha*) and its subsequent screening for anti-onchocercal activity. Even though the compound(**1**) showed no activity on the macrofilaria (adult worms), activity was however seen on the juvenile form of the parasite. These results have therefore opened a window into the anti-onchocerca screening of quaternary protoberberine alkaloids. We equally think that following structure modification of compound **(1)** and the synthesis of other compounds from this parent molecule we can be able to enhance the activity to desirable levels.

Conclusion

While this work constitutes the first report on the anti-onchocercal activity of quaternary protoberberine alkaloids in general and pseudopalmatine in particular isolated from the stem bark of *E. Chlorantha* (Syn: *Annickia chlorantha*), it has opened a window for further investigation of the anti-onchoceral activity of this class of compounds

Acknowledgements.

This work was done using resources and facilities provided by the University of Buea and the WHO ANDI Centre of Excellence for Onchocerciasis Research to the authors. We are grateful to Mr. Elias Ndive of the Limbe Botanical Garden for the collection and identification of the plant.

Conflict of Interest: None

References

Ajanohoun E, Aboubakar N, Dramane K, Ebot ME, Ekpere JA, Enow-Orock EG. (1996). Contribution to ethanobotanical and floristic studies in Cameroon. Cameroon: CSTR/OUA.

Bruneton J. (1993). Pharmacognosie: Phytochimie Plantes Médicinales: Alkaloids. 2e Ed. TEC.DOC. Paris, pp 633-638.

Cho-Ngwa F, Abongwa M, Ngemenya NM, Nyongbela KD: Selective activity of extracts of *Margaritaria discoidea* and *Homalium africanum* on Onchocercaochengi. BMC Complement Altern Med 2010, 10:62.

Cupp EW, Sauerbrey M, Richards F. (2011). Elimination of human onchocerciasis: history of progress and current feasibility using ivermectin (Mectizan®) monotherapy. Acta Trop. 120: S100-S108.

Grycovš L,Dostál J, Marek R. (2007). Quaternary protoberberine alkaloids. Phytochemistry 68: 150 – 175.

Roberts MF, Wink M. (1998). Biochemistry, ecology, and medicinal applications. In Roberts MF and Wink M, (eds.) Alkaloids, Plenum Press, New York, London, pp 1-7.

Samje M., Metuge JA, Mbah JA, Nguesson B, Cho-Ngwa F. (2014). In vitro anti-*Onchocerca ochengia* ctivities of extracts and chromatographic fractions

of *Craterispermum laurinum*and *Morinda Lucida*. BMC Complement. Altern. Med. *14*: 325. https://doi.org/10.1186/1472-6882-14-325

Titanji VP, Evehe MS, Ayafor JF, Kimbu SF. (1990). Novel *Onchocerca volvulus* filaricides from *Carapa procera, Polyalthia suaveolens* and *Pachypodanthium staudtii*. Acta Leiden. 59 (1-2): 377-82.

http//www.labothera.weekly.com/hepazor-antiviral-heacutepatique.html

Yusupov MM, Karimov A, Levkovich MG, Abdullaev ND, Shakirov R. (1993). Chem. Nat. Comp. 29: 43