



Original Article

 Isocryptolepine, an indoloquinoline alkaloid from *Cryptolepis sanguinolenta* promotes LDL uptake in HepG2 cells

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ABSTRACT

About 31 percent of deaths worldwide result from atherosclerotic cardiovascular disease. Hyperlipidemia remains the major risk factor for this disease and therefore, it is necessary to identify antihyperlipidemic compounds for drug development. The crude ethanolic extract of *Cryptolepis sanguinolenta* (Lindl.) Schltr., Apocynaceae, has demonstrated antihyperlipidemic properties. However, the chemical constituents responsible for this action are unknown. Hence, to identify chemical constituent(s) of *C. sanguinolenta* with anti-hyperlipidemic effect, five indoloquinoline alkaloids were isolated and evaluated in 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate labeled low density lipoprotein uptake assay using HepG2 cells. The minor alkaloid, isocryptolepine, showed strong activity in promoting low lipid lipoprotein uptake by 1.85-fold. Isocryptolepine may, therefore, serve as a lead compound for future studies in the development of novel antihyperlipidemic drugs.

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Introduction

Atherosclerotic cardiovascular disease (ASCVD) is responsible for 31 percent of all deaths worldwide (Benjamin et al., 2017). Hyperlipidemia remains the major risk factor in the development of ASCVD and thus, reduction in low-density lipoprotein-cholesterol (LDL-C) is considered a suitable intervention. Indeed, a number of studies have shown that reduction in LDL-C improves cardiovascular outcomes of ASCVD (LaRosa et al., 2005).

Cryptolepis sanguinolenta (Lindl.) Schltr., Apocynaceae, is widely distributed throughout the west coast of Africa (Iwu, 1993). Decoctions of the dried root are used in Ghanaian traditional medicine to treat various forms of fevers, rheumatism, and diabetes (Boye and Ampofo, 1990; Wright et al., 1996; Paulo and Houghton, 2002). The major class of compounds associated with *C. sanguinolenta* are indoloquinoline alkaloids which are considered unique to this West African plant (Collins, 2012). As a result, a number of studies have focused on these compounds. Many of the biological activities exhibited by this plant have been attributed to its major alkaloid, cryptolepine, **1**, and its isomers; and they include antihyperglycemic,

antibacterial, antifungal, antiprotozoal, antitumoral, anti-inflammatory, hypotensive, antithrombotic and vasodilation as reviewed by Lavrado et al. (2010). To the best of our knowledge, there is no report on the antihyperlipidemic activity of indoloquinoline alkaloids.

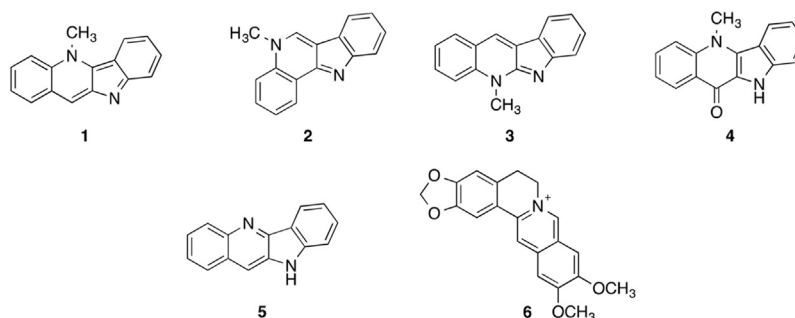
Ajayi et al. (2012) in a previous study demonstrated a significant reduction in total cholesterol, triacylglyceride, and LDL-C in Wistar albino rats after administration of 50 mg/kg, 150 mg/kg, and 250 mg/kg body weight of ethanol extract of *C. sanguinolenta* for 21 days. Compound **1**, being the major alkaloid, was suggested to be responsible for these effects. However, this suggestion has not been confirmed.

Therefore, since reduction in plasma LDL-C levels has been shown to reduce the risk of ASCVD (Catapano et al., 2016), it is rational to identify the LDL lowering agent(s) present in *C. sanguinolenta* which may serve as promising lead compound(s) for the development of an antihyperlipidemic drug(s).

Consequently, as part of our search for natural products with antihyperlipidemic activity, five indoloquinoline alkaloids; **1**, isocryptolepine (**2**) (Pousset et al., 1995), neocryptolepine (**3**) (Sharaf et al., 1996), cryptolepinone (**4**) (Cooper et al., 1996) and quinoline (**5**) (Dwuma-Badu et al., 1978) were isolated from *C. sanguinolenta* as described herein. These compounds were tested for their LDL lowering effect in lipid (DiI-LDL) uptake assay using HepG2 cells for the first time.

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Materials and methods

General experimental procedures

NMR spectra were obtained on a Bruker DXR-500 spectrometer operating at 400 MHz for ^1H NMR and 126 MHz for ^{13}C NMR. HRESIMS were measured using Agilent Technologies G6224A TOF spectrometer. Precoated silica gel plates, GF254 (Yantai, PR China) were used for thin-layer chromatography (TLC). Sephadex LH-20 (20–80 μm ; Amersham Pharmacia Biotech AB); Silica gel H (200–300 mesh, Qingdao Marine Chemical Ltd), NH silica gel (100–200 mesh; Fuji Silysia Chemical Ltd., Japan) and Toyopearl HW40F gel were used for column chromatography. Preparative HPLC was carried out on Agilent 1100 series.

Plant material

Dried roots of *Cryptolepis sanguinolenta* (Lindl.) Schltr., Apocynaceae, were collected in Mampong in the Eastern Region of Ghana, at the arboretum of the Centre for Plant Medicine Research in June 2015. It was identified by Mr. Blagooee, and a voucher specimen SIMMLJXCS1 was deposited at the herbarium of Shanghai Institute of Materia Medica, Shanghai, PR China.

Extraction and isolation

The air-dried, powdered roots of *C. sanguinolenta* (8 kg) was extracted with ethanol at room temperature for 7 days. After removal of organic solvent, the sample was suspended in H_2O and sequentially extracted with petroleum ether (PET) and ethyl acetate (EtOAc). The EtOAc extract (42.78 g) was subjected to silica gel column chromatography eluting with PET/acetone (Ace) (6:1) to afford fifteen fractions; 1–15. Fraction 14 was then chromatographed on Toyopearl HW40F gel column with CH_3OH (50–100%) to get five sub-fractions, 14A–14E. Sub-fraction 14C was further ran on NH Silica gel, eluting with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (35:1), and subsequently purified on Sephadex LH-20 using CH_3OH to afforded **1** (1.76 g). Fraction 13 was chromatographed on NH Silica gel column with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (25:1) to obtain sub-fractions 13A and 13B. Sub-fraction 13B was then subjected to silica gel TLC with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{NH}_3$ (10:1:1%) as mobile phase to obtain sub-fractions 13B1–13B3. Sub-fraction 13B3 was further chromatographed on Sephadex LH-20 gel column with CH_3OH to get three sub-fractions 13B31–13B33. Successive column chromatography of 13B31 on Toyopearl HW40F gel column with CH_3OH (70%) and NH Silica gel column with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (30:1) yielded **2** (75 mg). Sub-fraction 13B32 was ran on NH Silica gel column with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (30:1) to obtain **3** (34 mg). Fraction 3 was chromatographed on Sephadex LH-20 gel column using $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1) to afforded **4** (721 mg). Fraction 2 was also chromatographed on Sephadex LH-20 gel column with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1) to obtain sub-fractions 2A–2B. Sub-fraction 2B was further ran on silica gel column using $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ (30:1–EtOAc) to get seven

sub-fractions 2B1–2B7. Sub-fraction 2B2 was then chromatographed on Sephadex LH-20 gel column using $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1) to obtain **5** (1.4 g). The structures of the isolated compounds were determined by comparing their spectroscopic data with literature values (Dwuma-Badu et al., 1978; Cooper et al., 1996; Tousek et al., 2008) (see supplementary material for spectroscopic data).

LDL uptake

Cell culture

HepG2 cells (ATCC HB-8065) were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Gibco Invitrogen China Limited, Shanghai, China). Incubation of cells was carried out under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37 °C.

Isolation and DiI-LDL preparation

With approval of the local Ethics Committee (No. 2017015), and after informed consent, human plasma was obtained from Shanghai Xuhui Central Hospital, China. The procedures conformed the Helsinki Declaration. Human LDL and lipoprotein-deficient serum (LPDS) were separated from the pooled plasma of normal cholesterolemic volunteers by ultracentrifugation and were extensively dialyzed against dialysis buffer and PBS. The LDL was labeled with the fluorescent probe-DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Biotium, California, USA) as previously described, with some modifications. Briefly, DiI in DMSO (15 mg/ml) was added to the LDL/LPDS mixture (v/v, 1:2) to a final concentration of 300 mg DiI/mg LDL protein, and was incubated overnight at 37 °C. The DiI-labeled LDL was isolated by ultracentrifugation, dialyzed against a dialysis buffer, sterilized using a 0.45 μm filter (Millipore, Massachusetts, USA) and stored at 4 °C.

DiI-LDL uptake assay

The LDLR activity of the HepG2 cells was determined using DiI-LDL uptake assay as described by Stephan and Yurachek (1993) with minor modifications. Briefly, after different treatment, HepG2 cells grown in 24-well plates were incubated in DMEM and 20 $\mu\text{g}/\text{ml}$ DiI-LDL for 3 h at 37 °C in the dark. The cells were rinsed twice with PBS containing 0.4% albumin and washed three times with PBS after incubation. 500 μl of isopropanol was then added into each well followed by a 20 min incubation under constant shaking at room temperature. Afterwards, 200 μl aliquots were used for the analysis with a SpectraMax M2e Microplate Reader (Molecular Devices, 520–570 nm). Nagilactone B was used as the positive control (Gui et al., 2016).

Table 1
DiI-LDL uptake results for indoloquinoline alkaloids from *Cryptolepis sanguinolenta* and berberine in HepG2 cells.^a

Compounds (5 μM)	Fold of control (DMSO)
1	0.78 ± 0.02
2	1.85 ± 0.01
3	1.00 ± 0.03
4	0.96 ± 0.01
5	0.97 ± 0.01
6	1.38 ± 0.01
Nagilactone B	1.44 ± 0.01

^a Data are presented as the mean ± SEM of at least three independent experiments. $p < 0.05$, $p < 0.01$ vs the normal group.

Results and discussion

Currently, statins alone or in combination with fibrates are considered effective first line treatment for hyperlipidemia. However, they often result in unpleasant side effects (Mattar and Obeid, 2009). Hence, identifying compounds with antihyperlipidemic properties which may aid the development of novel drugs is required. Previous studies have demonstrated that plant secondary metabolites could serve as potential source of such compounds (Sahebkar et al., 2016; Wang et al., 2016). Therefore, given that the crude extract of *C. sanguinolenta* reduces total cholesterol, triacylglyceride, and LDL-C, we investigated the plant to identify the active antihyperlipidemic compound(s).

Five indoloquinoline alkaloids, **1–5**, were isolated from *C. sanguinolenta*. The compounds were identified by comparing their ¹H and ¹³C NMR as well as their HRESIMS data with literature values and the data agreed with those reported. The results from the DiI-LDL uptake assay as seen in Table 1, showed that the major alkaloid **1** was inactive (0.78 fold of the control) contrary previous suggestions (Ajayi et al., 2012). Also, compound **3** as well as compounds **4** and **5**; were inactive with 1.00, 0.96 and 0.97-fold respectively when compared with the control.

Interestingly, isocryptolepine (**2**), a regioisomers of **1** and a minor alkaloid found in *C. sanguinolenta* showed dramatic LDL uptake activity of 1.85-fold. The LDL uptake activity of **2** was better when compared with that of the widely studied antihyperlipidemic plant alkaloid, berberine (Sahebkar et al., 2016) by about 0.5 measuring units (fold of control). It was again better than the positive standard, nagilactone B, employed in this study. This is the first report of LDL uptake promoting activity of an indoloquinoline alkaloid, and a demonstration that isocryptolepine, **2**, may be the major LDL lowering constituent of *C. sanguinolenta* as opposed to the major alkaloid, cryptolepine.

Again, the results also give an insight into the structure-activity relationship necessary for LDL uptake activity of indoloquinoline scaffolds. The N5-methyl is claimed to be important for the anticancer and anti-hyperglycemic activity of this molecular framework (Bierer et al., 1998; Lu et al., 2008). However, for LDL uptake activity, the skeletal connectivity of the core structure may be of primary importance. Thus, this work may serve as the basis for future SAR studies of indoloquinolines with regards to their LDL lowering activity.

From the results, we conclude that isocryptolepine (**2**) may serve as a lead compound in the development of novel antihyperlipidemic drugs and therefore warrants further research attention.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with

those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contribution

MOA, YW, and LXJ and contributed to the concept and experimental designs. MOA and WQW took part in the extraction and characterization of test compounds. HHL and YW conducted the DiI-LDL uptake assay. LXJ, YW, WS, and MOA interpreted the results. MOA prepared the manuscript and all authors contributed to its critical reading and approval for submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.08.008.

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