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Abstract

Croton linearis is a shrub that grows in Caribbean regions, which is rich in metabolites such as alkaloids. The main aim of this study was to evaluate the antiplasmodial effect of alkaloids from this species. Three isoquinoline alkaloids, i.e. reticuline, laudanidine and 8,14-dihydrosalutaridine, were isolated from the leaves of *C. linearis* by flash chromatography and semi-preparative HPLC-DAD-MS. Their structures were elucidated by spectroscopic techniques. Antiplasmodial activity against the chloroquine-resistant strain *Plasmodium falciparum* K1 and cytotoxicity against MRC-5 cells (human fetal lung fibroblast cells) were assessed *in vitro*. Reticuline, laudanidine and 8,14-dihydrosalutaridine showed moderate antiplasmodial activity with IC_{50} values of 46.8 ± 0.6, 17.7 ± 0.6 and 16.0 ± 0.5 µM, respectively, but no cytotoxicity was observed in a concentration up to 64.0 µM. This is the first report on the antiplasmodial activity of laudanidine and 8,14-dihydrosalutaridine.

Keywords: *Croton linearis*, isoquinoline alkaloids, antiplasmodial activity, MRC5

1. Introduction

Among the neglected tropical diseases, malaria is one the most occurring protozoal infections caused by *Plasmodium* species and transmitted by female *Anopheles* mosquitoes (Varo et al., 2020). The World Health Organization reported 1.5 billion malaria cases and 7.6 million malaria deaths globally in the period 2000-2019. Most of the cases (82%) and deaths (94%) occurred in the African Region followed by South-East Asia (cases 10% and deaths 3%) (WHO, 2020).

Nowadays, this disease still is a serious public health concern in many countries due to the rapid emergence and spread of parasite strains resistant against available drugs (Bordignon et al., 2017). Therefore, the scientific global community joins efforts to accomplish the discovery of new antimalarial drugs. Natural products, more in particular medicinal plants, are a valuable source of bioactive compounds. A recent review conducted by Tajuddeen and Van Heerden (2019) reported a total of 1524 natural compounds assayed against at least one strain of *Plasmodium* between 2010-2017.

Different classes of compounds from plants display promising antiplasmodial activity, including alkaloids, terpenes, steroids and flavonoids (Nogueira and Lopes, 2011). Several classes of alkaloids, including terpenoidal, indole, bisindole, quinolone and isoquinoline alkaloids, are well-recognized to possess antimalarial activity (Uzor, 2020).

Croton linearis Jacq. (Euphorbiaceae) is an evergreen shrub that grows on the Southern coast of the Eastern region of Cuba and in other Caribbean regions. It is used by Jamaican peasants as a hair wash, and in Browne's day it is still used in baths for the treatment of fever and colds, while alternatively a tea made with the plant may also be consumed (Asprey and Thornton, 1955). Also in Cuba it is used to treat fever (Roig, 1974).

Some species of the genus *Croton* produce alkaloids, specifically isoquinoline type alkaloids. Recently, our research group reported the isolation of six alkaloids (laudanidine, laudanosine, reticuline, corydine, glaucine and cularine) and one flavonoid glycoside, isorhamnetin-3-*O*-(6″-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside from *Croton linearis* Jacq. leaves. The alkaloid reticuline showed activity against *Leishmania infantum* (IC_{50} 148.0 ± 1.2 µM), and the flavonoid was found to be active against *Trypanosoma cruzi* (IC_{50} 35.6 ± 2.3 µM) (Garcia et al., 2019). In addition, activity against promastigote and amastigote forms of *L. amazonensis* of essential oil was observed with IC_{50} values of 20.0 ± 4.9 µg/mL and 13.8 ± 4.3 µg/mL, respectively (Garcia et al., 2018). In a continuation of our ongoing investigation of the antiprotozoal potential of the metabolites from leaves of *C. linearis*, the aim of the current study was to evaluate the antiplasmodial activity against *Plasmodium falciparum* K1 (chloroquine-resistant) of alkaloids from this species using the lactate dehydrogenase assay.

2. Materials and methods

2.1. Plant Material

The leaves of *C. linearis* were harvested in the Siboney-Juticí Ecological Reserve, Santiago de Cuba, Cuba in September 2018. The plant was authenticated and a voucher specimen was deposited at the Herbarium of the Eastern Center of Ecosystems and Biodiversity BSC “Jorge Sierra Calzado” under number 21659. Leaves were air-dried for 20 days, shaded from direct sunlight, and were ground in a blade mill (MRC Model KM 700/Germany).

2.2. Extraction and isolation of alkaloids

A dried ethanolic extract (150 g) was obtained according to the extraction procedure previously described (Garcia et al., 2019). This extract was subjected to acid-base liquid-liquid partition

with solvents of different polarity, yielding a total of six main fractions. The dichloromethane fraction (6.27 g) was selected for further purification, since TLC analysis indicated the presence of alkaloids in this fraction (detection with iodoplatinate reagent). Next, 3 g of this fraction was subjected to flash chromatography under similar conditions to the ones reported before (Garcia et al., 2019). A detailed description of the conditions used during flash chromatography can be found as Supplementary Data. All the subfractions were joined according to their TLC profile resulting in 12 subfractions (**CL-1** to **CL-12**). TLC analysis performed on all subfractions indicated the presence of alkaloids in **CL-4** to **CL-11**.

Based on HPLC-DAD profiling, subfractions **CL-6** (35 mg) and **CL-9** (70 mg), were selected for isolation of alkaloids by semi-preparative HPLC-DAD-MS under similar conditions as reported before (Garcia et al., 2019). A detailed description of these conditions can be found as Supplementary Data. Fraction **CL-6** yielded 4 subfractions: A6-1 (4.7 mg), A6-2 (8.9 mg), A6-3 (5.4 mg) and A6-4 (2.5 mg). Fraction **CL-9** yielded 9 subfractions: A9-1 (10.2 mg), A9-2 (21.6 mg), A9-3 (2.3 mg), A9-4 (12.3 mg), A9-5 (3.0 mg), A9-6 (2.2 mg), A9-7 (7.7 mg), A9-8 (1.6 mg) and A9-9 (2.4 mg).

The isolated compounds were characterized by means of ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR spectroscopy. In addition, DEPT-135, DEPT-90 and 2D NMR (COSY, HSQC and HMBC) experiments were carried out. NMR spectra were recorded on a Bruker DRX-400 instrument (Rheinstetten, Germany), equipped with either a 3 mm broadband inverse (BBI) probe or a 5 mm dual ^{1}H/^{13}C probe, using standard Bruker pulse sequences. NMR data processing was performed with MestReNova software version 6.1.0-6224. Methanol-d_4 (99.8% D), chloroform-*d* (99.8% D) and DMSO-d6 (99.9 % D) were used as solvents and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Based on the MS and NMR data, subfraction A6-2 was identified as reticuline (**1**), A6-3 as laudanidine (**2**) and A9-2 as 8,14-dihydrosalutaridine (**3**) (Fig. 1). The rest of the subfractions were impure.

*Reticuline (**1**):* yellow powder (8.9 mg). ESI-MS (positive mode): *m/z* 330 $[M+H]^+$ ($C_{19}H_{23}NO_4$). The NMR spectra and data can be consulted in the Supplementary Data file. This alkaloid was previously reported by our research group (Garcia et al., 2019).

*Laudanidine (**2**):* yellow powder (5.4 mg). ESI-MS (positive mode): *m/z* 344 $[M+H]^+$ ($C_{20}H_{25}NO_4$). The NMR spectra and data can be consulted in the Supplementary Data file. This alkaloid was previously reported by our research group (Garcia et al., 2019).

*8,14-Dihydrosalutaridine (**3**):* yellow powder (21.6 mg). ^{1}H NMR (DMSO-d6): δ 1.77 (td, *J*= 12.3, 3.7 Hz, 1H, H-15); 1.92 (d, *J*= 12.0 Hz, 1H, H-15); 2.23-2.06 (m, 1H, H-16); 2.32-2.23 (m, 2H, H8); 2.36 (s, 3H, N-CH_3); 2.46 (d, *J*= 13.1 Hz, 1H, H-14); 2.59 (d, *J*= 9.8 Hz, 1H, H-16); 2.71 (dd, *J*= 18.7, 5.5 Hz, 1H, H-10); 2.94 (s, 1H, H-10); 2.99 (s, 1H, H-9); 3.54 (s, 3H, 6-OCH_3); 3.74 (s, 3H, 3-OCH_3); 6.62 (d, *J*= 8.3 Hz, 1H, H-1); 6.74 (s, 1H, H-5); 6.80 (d, *J*= 8.3 Hz, 1H, H-2). ^{13}C NMR (DMSO-d6): δ 22.95 (C-10), 35.67 (C-15), 36.63 (C-13), 38.85 (C-8), 41.64 (N-CH3), 42.52 (C-14), 46.78 (C-16), 54.17 (6-OCH3), 55.38 (C-9), 55.73 (3-OCH3), 110.12 (C-2), 118.11 (C-1), 124.60 (C-12), 126.92 (C-5), 128.40 (C-11), 144.04 (C-4), 146.14 (C-3), 147.58 (C-6), 192.26 (C-7). The NMR spectra can be consulted in the Supplementary Data file. ESI-MS (positive mode): *m/z* 330 $[M+H]^+$ ($C_{19}H_{23}NO_4$). These data were in agreement with those reported for 8,14-dihydrosalutaridine isolated from *C. linearis* by Haynes et al. (1967).

2.3. Biological assays

2.3.1. Antiplasmodial assay

The antiplasmodial activity was tested against *Plasmodium falciparum* K1 (chloroquine-resistant). The strain was supplied by the culture collection of the Laboratory for Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Belgium. The parasite was maintained in continuous log phase growth in RPMI-1640 medium supplemented with 2% penicillin/streptomycin solution, 0.37 mM hypoxanthine, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 25 mM $NaHCO_3$, and 10% O^+ human serum together with 4% human O^+ erythrocytes. The antiplasmodial activity was assessed using the lactate dehydrogenase assay (Cos et al., 2006; Mesia et al., 2010). The test compounds were dissolved in DMSO and serially diluted (2- or 4-fold) in demineralized water to ensure a final in-test DMSO concentration below 1%. The final concentrations tested ranged from 0.125 to 64 µM. Dilutions of compounds and malaria parasite inoculum with 1% parasitaemia and 2% haematocrit were incubated. After haemolysis, Malstat® reagent, phenazine ethosulfate and NBT (Nitro Blue Tetrazolium Grade III) were added. The plates were kept in the dark for 2 h and change in colour was measured spectrophotometrically at 655 nm. The results were expressed as percentage reduction in parasitemia (percentage of parasitized erythrocytes) compared to control wells. IC_{50} values were calculated from drug-concentration response curves. Chloroquine diphosphate was used as antiplasmodial reference drug. Statistical comparison of the obtained IC_{50} values was performed by Anova with Tukey HSD post hoc testing, using the STATGRAPHICS Centurion XV.II software (Windows, V 15.2.14, 2007). p-values < 0.05 were considered significantly different.

2.3.2. Cytotoxicity assay

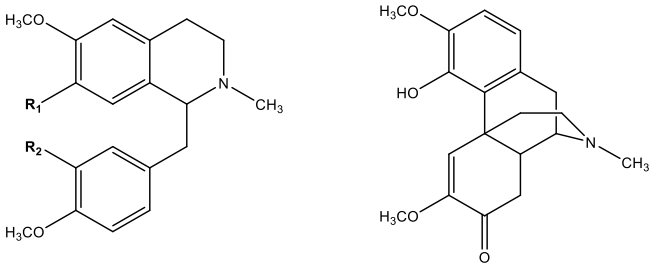
MRC-5_{SV2} (human fetal lung fibroblasts) cells were purchased from ATCC (American Type Culture Collection). Cells were cultured in MEM+ Earl's salts-medium, supplemented with L-

glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5% inactivated fetal calf serum. The *in vitro* cytotoxic assay was performed as reported previously (Cos et al., 2006; Garcia et al., 2019). Compounds were tested in concentrations ranging from 0.125 to 64 µM. The results were expressed as percent reduction in cell growth/viability compared to control wells and IC_{50} was determined. Tamoxifen was used as reference drug.

3. Results and discussion

3.1. Isolation of Alkaloids

Isolation was carried out by liquid-liquid partition and chromatographic methods (flash chromatography and semi-preparative HPLC-DAD-MS) and three alkaloids were obtained. Based on their NMR and ESI-MS data and by comparison with literature, their structures (Fig. 1) were established as: reticuline (**1**), laudanidine (**2**), and 8,14-dihydrosalutaridine (**3**).



Compound	R_1	R_2
1	-OH	-OH
2	$-OCH_3$	-OH

Compound 3

Fig. 1. Alkaloids with antiplasmodial activity isolated from leaves of *C. linearis.*

3.2. Antiplasmodial activity and cytotoxicity

The three isoquinoline alkaloids **1**, **2** and **3** demonstrated antiplasmodial activity with IC_{50} values of 46.8 ± 0.6, 17.7 ± 0.6 and 16.0 ± 0.6 µM (n=2, see **Table 1**), respectively, against *P. falciparum* K1. This study is the first report of antiplasmodial activity of the alkaloids laudanidine (**2**) and 8,14-dihydrosalutaridine (**3**). In contrast, this activity has already been well-

described for reticuline (**1**) (Zahari et al., 2014; Fadaeinasab et al., 2015). The *in vitro* cytotoxic activity of the compounds was also assessed to determine their selectivity. None of the tested compounds showed cytotoxic effects on MRC-5_{SV2} up to a concentration of 64.0 µM.

Table 1

In vitro antiplasmodial activity and cytotoxicity of alkaloids from *C. linearis* leaves.

Compound	**Cytotoxicity** (CC_{50} µM)	**Antiplasmodial activity** (IC_{50} µM)	**Selectivity Index** (CC_{50}/IC_{50})
1	>64.0	46.8 ± 0.6*	>1.4
2	>64.0	17.7 ± 0.6	>3.6
3	>64.0	16.0 ± 0.6	>4.0

Reference compounds: Tamoxifen (MRC-5) IC_{50} 0.12 µM; Chloroquine (*Plasmodium falciparum* K1) IC_{50} 0.12 µM

* Significant difference (α= 0.05)

In literature various *Croton* species are reported to be used in folk medicine as a treatment for malaria and, several studies with *in vitro* assays of various extracts and compounds were published (Moremi et al., 2021). An extract from *C. pendulilorus* showed good activity against three strains of *Plasmodium falciparum* (chloroquine-sensitive (FCB), chloroquine-resistant (W2), and field isolate (CAM06)) with IC_{50} values of 5.37 ± 0.18, 14.03 ± 17.04 and 14.66 ± 2.02 µg/mL respectively (Laryea and Borquaye, 2019). The methanolic stem bark extract of *C. zambesicus* from Cameroon was active *in vitro* against *P. falciparum* W2 , with an IC_{50} of 5.69 µg/mL (Boyom et al., 2009).

Various alkaloid types showed a good activity against different strains of *Plasmodium* spp (Nogueira and Lopes, 2011; Tajuddeen and Van Heerden, 2019; Hassan and Dangani, 2019). In our current study two benzylisoquinoline alkaloids and one morphinandienone alkaloid were isolated. For both classes antiplasmodial effects have already been reported (Uzor, 2020). Nasrullah et al. (2013) described the isolation of four alkaloids from the stem bark of *Cryptocarya nigra*. The benzylisoquinoline alkaloid (+)-*N*-methylisococlaurine showed strong

antiplasmodial activity (IC_{50} 5.40 µM) against a chloroquine-resistant strain of *P. falciparum* (K1 strain). Seven isoquinoline alkaloids isolated from the bark of *Actinodaphne macrophylla* showed potent antiplasmodial activity against *P. falciparum* 3D7 (IC_{50} values ranging from 0.05 to 3.11 µM). More recently, two benzyltetrahydroisoquinoline alkaloids, laudanine and laudanosine, from *C. gratissimus* fruits displayed similar antiplasmodial activities with IC_{50} values of 78.6 ± 0.4 and 76.1 ± 21.6 µM respectively (Mahmoud et al., 2020).

For the alkaloid reticuline an IC_{50} value of 1.18 µM was reported (Fadaeinasab et al., 2015). In another study conducted by Zahari et al., (2014) reticuline (isolated from *Dehaasia longipedicellata*) exhibited an IC_{50} of <30.4 µM against the K1 strain of *P. falciparum,* using a histidine-rich protein II (HRPII) assay.

Furthermore, the morphinandienone alkaloids sebiferine and (-)-milonine isolated from this species, displayed potent to moderate activity against *P. falciparum* K1 with an IC_{50} value of 0.097 µM and 22.46 µM, respectively (Zahari et al., 2014). In addition, sebiferine isolated from the leaves of *Phoebe tavoyana* (Meissn.) Hook f. exhibited a potent inhibitory activity against a *P. falciparum* 3D7 clone, with an IC_{50} value of 8.09 µM (Omar et al., 2019).

In the current study, a significant increase in activity of compound **2** compared to compound **1** is observed. This behavior, could be related to the methylation of one of the phenolic groups of compound **1**, which increases lipophilicity and which may facilitate membrane cell transit, providing accumulation of compounds inside of the parasite and increased interaction with cellular targets. However, given the limited number of compounds tested, drawing such conclusions is highly preliminary. More compounds should be tested in order to determine whether this hypothesis is true.

In literature it is well-established that the isoquinoline moiety is important for antiprotozoal activity (Osorio et al., 2008). This moiety could contribute to DNA intercalation or minor groove binding. In addition, it could also bind with other targets such as functional proteins of the protozoa (Osorio et al., 2008; Khan and Kumar, 2015). However, a high variability of potencies is observed and only limited Structure-Activity Relationship (SAR) studies of these classes of compounds are available, which do not allow establishing rational structural modification strategies with the aim of improving compound potencies. Nevertheless, the current results indicate the potential of these alkaloids as lead compounds for the development of new antimalarial drugs. Moreover, these findings support the potential of this medicinal plant growing in the Eastern region of Cuba as antiprotozoal agent (Garcia, et al., 2018, 2019) and for mosquito control (Rodriguez et al., 2020). In addition, these results confirm that the biological activity of leaves of *C. linearis* could be related to alkaloids and other constituents, like flavonoids and/or the essential oil.

4. Conclusions

In conclusion, this study revealed that three alkaloids isolated from leaves of *C. linearis* showed antiplasmodial activity, without *in vitro* cytotoxic effects on human fetal lung fibroblasts cells. In addition, it is the first report of the antiplasmodial activity of laudanidine (**2**) and 8,14-dihydrosalutaridine (**3**). These findings are a valuable contribution in the ongoing search for alkaloids with antimalarial potential.

CRediT authorship contribution statement

Jesús García Díaz: Conceptualization, Methodology, Investigation, Writing - Original Draft, Visualization. **Emmy Tuenter:** Investigation, Writing - Review & Editing, Visualization. **Julio Cesar Escalona Arranz:** Writing - Review & Editing, Supervision. **Gabriel Llauradó Maury:**

Writing - Review & Editing. **Paul Cos:** Writing - Review & Editing, Supervision. **Luc Pieters:** Writing - Review & Editing, Supervision.

Conflict of interest

The authors declare no conflict of interest.

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