PHYTOCHEMICAL STUDY OF ENDEMIC COSTA RICAN ANNONACEAE SPECIES *Annona pittieri* AND *Cymbopetalum costaricense*

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ABSTRACT

Phytochemical profile of the Central American rainforest endemic Annonaceae species, *Annona pittieri* and *Cymbonopetallum costaricense*, were studied in search of novel bioactive compounds. The acetogenin squamocin (**1**) isolated from *A. pittieri* showed cytotoxic activity against human acute lymphocytic leukaemia with low activity against healthy blood cells. In addition, other eight compounds were isolated from *A. pittieri*, including a novel 2-azaanthraquinone alkaloid, 4-methoxybenzo[*g*]isoquinoline-5,10-dione (**2**). Furthermore, from *C. costaricense* three compounds were isolated including a novel 1-azaanthraquinone alkaloid, 6-hydroxy-9-methoxy-cleistopholine (**3**).

Keywords: Alkaloids, Annonaceae, *Annona pittieri*, *Cymbopetalum costaricense*, squamocin.

INTRODUCTION

Natural products are an important source of structural scaffolds for drug discovery, with particular relevance in the development of chemotherapeutics[1]. In particular, alkaloids and acetogenins from Annonacea species have been widely studied as potential cytotoxic compounds[2,3].

The Annonaceae species, *Annona pittieri* Donn. Sm. and *Cymbopetalum costaricense* Donn. Sm. are native plants of the Costa Rican and Panamanian rainforest[4,5] that are still understudied in terms of their phytochemical profile. The use of *C. costaricense* as a medicinal plant in the treatment of snake bites by Ngäbe people of Panama and Costa Rica was reported previously[6], while there are no records of medicinal uses for *A. pittieri*. Although cyanogenic compounds were previously reported in both species[7], there are no preceding studies on their phytochemical composition. Thus, in this work, *A. pittieri* and *C. costaricense* were studied as a source of new bioactive compounds.

EXPERIMENTAL

Plant material

Leaves, bark and wood of *Annona pittieri* were collected in La Cruz, Guanacaste, Costa Rica. Leaves, bark and wood of *Cymbopetalum costaricense* were collected in Sarapiquí, Heredia, Costa Rica. Both specimens were identified by the botanist Luis Poveda and stored in the Juvenal Valerio herbarium of the National University of Costa Rica.

Extraction

The plant material was dried and grounded. Separately, leaves (550 g) and wood (4250 g) of *A. pittieri* were extracted with a mixture of methyl *tert*-butyl ether (MTBE) and methanol (MeOH) (9:1) by maceration during 48 h. Then, the remaining plant material was alkalized with NH_3 (1%) and the extraction with MTBE-MeOH (9:1) was repeated. Finally, the remaining plant material was extracted once more with MTBE-MeOH (7:3). Leaves and wood (300 g) of *C. costaricense* were extracted with the same procedure mentioned above.

Compound isolation

Each crude extract was purified by open column chromatography using silica gel (70-230 mesh, Merck®) and a gradient system of solvents of hexane, MTBE and MeOH mixtures. Pure compounds were isolated from fractions using flash chromatography with silica gel (60 mesh, Merck®) and thin-layer chromatography (TLC) with silica gel (60 F_{254}, Merck®).

Structural elucidation

Structures of the isolated compound were elucidated by nuclear magnetic resonance spectroscopy (NMR). The NMR spectra were recorded in deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD) on a Bruker® Ascend® 600 MHz and a Varian® Mercury® 400 MHz spectrometers. The structure **1** was confirmed by mass spectrometry (MS) on a high-resolution atmospheric pressure chemical ionization (HRAPCI) with an Orbitrap® mass spectrometer (Thermo Fisher®), while the structure **2** on electrospray ionization (ESI) with Quadrupole Time-of-flight tandem (QTOF) mass spectrometer (Waters®), and the structure **3** on a direct infusion electrospray ionization (DIESI) with Triple Quadrupole Linear Ion Traps (QTRAP) mass spectrometer (SCIEX®).

Cellular assays

Cytotoxicity of squamocin isolated from *A. pittieri* was tested against three tumour cell lines: CCRF-CEM (human T-cell acute lymphoblastic leukaemia), CEM-ADR5000 (human T-cell acute lymphoblastic leukaemia resistant to doxorubicin) and MIA-PaCa-2 (human pancreatic carcinoma), as well as against peripheral blood mononuclear cells (PBMC) from healthy human subjects; according to the method published by Calderón *et al*[8]. PBMCs were isolated from human buffy coats obtained from the Freiburg University Clinic, Freiburg, Germany (ethical permission number from the ethics commission, University of Freiburg: 356/13; 2013). Briefly, the cells were maintained at 37 °C under 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/mL streptomycin, and 100 U/mL penicillin. The cells were seeded in 96-well plates (1.2×10^4 cells/well for MIA-PaCa-2 cells, 4×10^4 cells/well for leukemic cells, and 2×10^5 PBMCs/well in 150 µL complete medium). Squamocin was dissolved in DMSO, and the cells were incubated for 24 h with various concentrations of squamocin or the positive control, respectively. Camptothecin and doxorubicin were used as the positive controls, and DMSO 0.1% was the solvent control. The viability of the tumour cells was quantified using an MTT assay. The IC_{50} values were obtained by nonlinear regression using the GraphPad® Prism® 5. The data are expressed as means ± SD.

Squamocin (1): White wax. ^{1}H NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 2.24 (2H, d, *J*=7.8, H-3), 1.52 (2H, m, H-4), 1.23 (m, H-5, 6, 7, 8, 9, 10, 11, 12, 30, 31), 1.41 (m, H-13, 27, 29), 1.36 (m, H-14), 3.38 (1H, m, H-15), 3.82 (1H, m, H-16), 1.56 (3H, m, H_a-17, 18, 21), 1.95 (3H, m, H_b-17, 18, 21), 3.90 (1H, m, H-19), 3.82 (1H, m, H-20), 1.80 (1H, d, *J*=6, H_a-22), 1.89 (1H, d, *J*=6, H_b-22), 3.94 (1H, m, H-23), 3.88 (1H, m, H-24), 1.35 (m, H-25), 1-37 (m, H_a-26), 1.65 (1H, d, *J*=9.6,

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H_b-26), 3.57 (1H, m, H-28), 1.23 (m, H-32, 33), 0.86 (3H, t, *J*=6.6, H-34), 6.98 (1H, d, *J*=1.2, H-35), 4.98 (1H, dq, *J*=1.2, 6.6, H-36), 1.39 (3H, d, *J*=6.6, H-37); ^{13}C NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 174.7 (C-1), 134.7 (C-2), 25.0 (C-3), 27.2 (C-4), 29.0-29,6 (C-5, 6, 7, 8, 9, 10, 11, 12, 30, 31), 25.5 (C-13), 32.2 (C-14), 74.4 (C-15), 83.5 (C-16), 28.7 (C-17, 18, 21), 82.7 (C-19), 82.3 (C-20), 24.6 (C-22), 83.0 (C-23), 71.4 (C-24), 33.0 (C-25), 21.8 (C-26), 37.3 (C-27), 71.8 (C-28), 37.1 (C-29), 31.7 (C-32), 22.4 (C-33), 13.8 (C-34), 149.5 (C-35), 77.6 (C-36), 19.0 (C-37). HRAPCI (negative mode) m/z 621.4736 $[M - H]^-$ (calcd for $C_{37}H_{65}O_7^-$, 621.4730). TLC Rf 0.42 ($CHCl_3$-MeOH; 95:5).

5-Methoxyflavone: Pale yellow needles. ^{1}H NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 6.75 (1H, s, H-3), 6.82 (1H, d, *J*=8.4, H-6), 7.58 (1H, dd, *J*=8.4, H-7), 7.14 (1H, d, *J*=8.4, H-8), 7.90 (2H, m, H-2', 6'), 7.51 (2H, m, H-3', 5'), 7.50 (1H, s, H-4'), 3.99 (3H, s, H-5-OCH_3); ^{13}C NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 161.3 (C-2), 109.2 (C-3), 178.6 (C-4), 159.8 (C-5), 106.5 (C-6), 133.9 (C-7), 110.3 (C-8), 158.4 (C-9), 114.7 (C-10), 131.5 (C-1'), 126.2 (C-2', 6'), 129.1 (C-3', 5'), 131.5 (C-4'), 56.6 (C-5-OCH_3). TLC Rf 0.81 (C_6H_6-CH_2Cl_2-MTBE; 1:1:1).

5,2'-Dimethoxyflavone: Pale yellow needles. ^{1}H NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 7.09 (1H, s, H-3), 6.82 (1H, d, *J*=7.8, H-6), 7.56 (1H, dd, *J*=7.8, H-7), 7.11 (1H, d, *J*=7.8, H-8), 7.04 (1H, d, *J*=8.4, H-3'), 7.47 (1H, dd, *J*=8.4, 7.2, H-4'), 7.11 (1H, m, H-5'), 7.90 (1H, d, *J*=7.2, H-6'), 4.01 (3H, s, H-5-OCH_3), 3.94 (3H, s, H-2'-OCH_3); ^{13}C NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 158.7 (C-2), 114.3 (C-3), 179.3 (C-4), 159.8 (C-5), 106.2 (C-6), 133.7 (C-7), 110.3 (C-8), 158.6 (C-9), 114.3 (C-10), 120.5 (C-1'), 158.2 (C-2'), 111.8 (C-3'), 132.4 (C-4'), 120.8 (C-5'), 129.2 (C-6'), 56.6 (C-5-OCH_3), 55.8 (C-2'-OCH_3). TLC Rf 0.71 (C_6H_6-CH_2Cl_2-MTBE; 1:1:1).

4-Methoxybenzo[*g*]isoquinoline-5,10-dione (2): Yellow solid. ^{1}H NMR and ^{13}C NMR data, see Table 1. ESIQTOF (positive mode) m/z 240.066 $[M + H]^+$ (calcd for $C_{14}H_{10}NO_3^+$, 240.0661). TLC Rf 0.44 ($CHCl_3$-MeOH; 6:4).

Liriodenine: Yellow solid. ^{1}H NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 7.15 (1H, d, *J*=4.8, H-3), 7.75 (1H, dd, *J*=4.8, H-4), 8.73 (1H, dd, *J*=4.8, H-5), 8.45 (1H, dd, *J*=3, 7.8, H-8), 7.51 (1H, ddd, *J*=3, 7.8, 7.5, H-9) 7.70 (1H, ddd, *J*=3, 7.8, 7.5, H-10), 8.58 (1H, dd, *J*=3, 7.8, H-11), 6.32 (2H, d, *J*=3, H-1-OCH_2O-2); ^{13}C NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 148.4 (C-1), 152.1 (C-2), 103.3 (C-3), 136.1 (C-3a), 123.6 (C-3b), 124.7 (C-4), 144.4 (C-5), 144.9 (C-6a), 182.6 (C-7), 131.0 (C-7a), 128.6 (C-8), 128.7 (C-9), 134.2 (C-10), 127.5 (C-11), 133.0 (C-11a), 107.9 (C-11b), 102.7 (C-1-OCH_2O-2). TLC Rf 0.71 ($CHCl_3$- *i*PrOH; 95:5).

Tamgermanetin: Yellow solid. ^{1}H NMR (MeOD, 600 MHz, *J*/Hz): δ 7.11 (1H, d, *J*=1.8, H-2), 6.79 (1H, d, *J*=8.4, H-5), 7.02 (1H, dd, *J*=1.8, 8.4, H-6), 7.42 (1H, d, *J*=15.6, H-7), 6.40 (1H, d, *J*=15.6, H-8), 7.05 (2H, d, *J*=8.4, H-2', 6'), 6.71 (2H, d, *J*=8.4, H-3', 5'), 2.75 (2H, t, *J*=7.2, H-7'), 3.46 (2H, t, *J*=7.2, H-8'), 3.87 (3H, s, H-4-OCH_3); ^{13}C NMR (MeOD, 600 MHz, *J*/Hz): δ 128.3 (C-1), 111.5 (C-2), 149.8 (C-3), 149.3 (C-4), 116.3 (C-5), 123.2 (C-6), 142.0 (C-7), 118.7 (C-8), 169.2 (9), 130.7 (C-1'), 130.7 (C-2', 6'), 116.3 (C-3', 5'), 156.9 (C-4'), 35.8 (C-7'), 42.5 (C-8'), 56.4 (C-4-OCH_3). TLC Rf 0.38 ($CHCl_3$- *i*PrOH; 9:1).

(+)-Catechin: Brown solid. ^{1}H NMR (MeOD, 600 MHz, *J*/Hz): δ 4.56 (1H, d, *J*=7.8, H-2), 3.97 (1H, ddd, *J*=5.4, 8.4, 7.8, H-3), 2.50 (1H, dd, *J*=16.2, 8.4, H_a-4), 2.84 (1H, dd, *J*=16.2, 8.4, H_b-4), 5.93 (1H, s, H-6), 5.85 (1H, s, H-8), 6.83 (1H, d, *J*=1.8, H-2'), 6.76 (1H, d, *J*=8.4, H-5'), 6.71 (1H, d, *J*=1.8, 8.4, H-6'); ^{13}C NMR (MeOD, 600 MHz, *J*/Hz): δ 82.7 (C-2), 68.7 (C-3), 28.4 (C-4), 100.8 (C-4a), 157.4 (C-5), 96.3 (C-6), 157.6 (C-7), 95.5 (C-8), 156.8 (C-8a), 132.1 (C-1'), 115.2 (C-2'), 146.1 (C-3'), 146.2 (C-4'), 116.1 (C-5'), 120.0 (C-6'). TLC Rf 0.18 ($CHCl_3$- *i*PrOH; 9:1).

(±)-Marmesin: Brown solid. ^{1}H NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 6.22 (1H, d, *J*=9.3, H-3), 7.59 (1H, d, *J*=9.3, H-4), 7.22 (1H, s, H-5), 6.74 (1H, s, H-8), 4.74 (1H, t, *J*=8.4, H-2'), 3.21 (2H, m, H-3'), 1.24 (3H, s, H-4'-CH_{3a}), 1.37 (3H, s, H-4'-CH_{3b}); ^{13}C NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 161.9 (C-2), 112.5 (C-3), 143.8 (C-4), 112.5 (C-4a), 123.6 (C-5), 125.2 (C-6), 163.3 (C-7), 98.1 (C-8), 155.8 (C-8a), 91.2 (C-2'), 29.6 (C-3'), 71.8 (C-4'), 24.4 (C_a-4'-CH_3), 26.3 (C_b-4'-CH_3). TLC Rf 0.38 (C_6H_6-CH_2Cl_2-MTBE; 4:4:2).

Methyl *ent*-16α,17-dihydroxy-kauran-19-oate: Yellow solid. ^{1}H NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 0.77 (1H, m, H_{ax}-1), 1.81 (1H, m, H_{eq}-1), 1.42 (1H, m, H_{ax}-2), 1.82 (1H, m, H_{eq}-2), 0.99 (1H, m, H_{ax}-3), 2.16 (1H, m, H_{eq}-3), 1.02 (1H, dd, *J*=1.8, 12, H-5), 1.73 (1H, m, H_{ax}-6), 1.83 (1H, m, H_{eq}-6), 1.43 (1H, m, H_{ax}-7), 1.63 (1H, m, H_{eq}-7), 0.98 (1H, m, H-9), 1.50 (1H, m, H_{ax}-11), 1.59 (1H, m, H_{eq}-11), 1.49 (1H, m, H_{ax}-12), 1.57 (1H, m, H_{eq}-12), 2.03 (1H, m, H-13), 1.60 (1H, m, H_{ax}-14), 1.92 (1H, m, H_{eq}-14), 1.43 (1H, m, H_a-15), 1.56 (1H, m, H_b-15), 3.66 (1H, d, *J*=11.1, H_a-17), 3.76 (1H, d, *J*=11.1, H_b-17), 1.16 (3H, s, H-18), 0.82 (3H, s, H-20), 3.64 (3H, s, H-19-OCH_3); ^{13}C NMR ($CDCl_3$, 600 MHz, *J*/Hz): δ 40.6 (C-1), 18.9 (C-2), 38.0 (C-3), 43.7 (C-4), 56.9 (C-5), 21.9 (C-6), 41.9 (C-7), 44.6 (C-8), 55.7 (C-9), 39.4 (C-10), 18.3 (C-11), 26.0 (C-12), 45.2. (C-13), 37.1 (C-14), 53.1 (C-15), 82.1 (C-16), 66.4 (C-17), 28.6 (C-18), 178.8 (C-19), 15.1 (C-20), 51.2 (C-19-OCH_3). TLC Rf 0.27 (C_6H_6-CH_2Cl_2-MTBE; 4:4:2).

4-Methyl-2(1H)-quinolinone: Yellow solid. ^{1}H NMR ($CDCl_3$, 400 MHz, *J*/Hz): δ 6.69 (1H, d, *J*=1.2, H-3), 8.24 (1H, dd, *J*=1.2, 7.6, H-5), 7.8 (1H, ddd, *J*=1.2, 7.6, 7.6, H-6), 7.87 (1H, ddd, *J*=1.2, 7.6, 7.6, H-7), 8.19 (1H, dd, *J*=1.2, 7.6, H-8), 2.71 (4H, d, *J*=1.2, H-4-CH_3), 9.70 (H-NH); ^{13}C NMR ($CDCl_3$, 400 MHz, *J*/Hz): δ 161.0 (C-2), 128.0 (C-3), 152.8 (C-4), 116.5 (C-4a), 128.2 (C-5), 134.2 (C-6), 136.3 (C-7), 127.1 (C-8), 143.4 (C-8a), 22.6 (C-4-CH_3). TLC Rf 0.76 ($CHCl_3$- *i*PrOH; 9:1).

6,7-Dimethoxy-1-methyl-2(1H)-quinolinone: Orange amorphous solid. ^{1}H NMR ($CDCl_3$, 400 MHz, *J*/Hz): δ 6.40 (1H, d, *J*=7.2, H-3), 6.99 (1H, d, *J*=7.2, H-4), 7.80 (1H, s, H-5), 6.85 (1H, s, H-8), 3.59 (3H, s, H-NCH_3), 4.00 (3H, s, H-6-OCH_3), 3.97 (3H, s, H-7-OCH_3); ^{13}C NMR ($CDCl_3$, 400 MHz, *J*/Hz): δ 162.6 (C-2), 105.8 (C-3), 131.5 (C-4), 120.5 (C-4a), 107.8 (C-5), 149.8 (C-6), 153.8 (C-7), 106.2 (C-8), 133.0 (C-8a), 37.0 (C-NCH_3), 56.0 (C-6-OCH_3), 56.2 (C-7-OCH_3). TLC Rf 0.65 ($CHCl_3$- *i*PrOH; 9:1).

6-Hydroxy-9-methoxy-cleistopholine (3): Purple needles. ^{1}H NMR and ^{13}C NMR data, see Table 2. ESIQTRAP (positive mode) m/z 269.21 $[M]^+$. TLC Rf 0.47 ($CHCl_3$- *i*PrOH; 9:1).

RESULTS AND DISCUSSION

In this work, it is reported the phytochemical profile of two native Annonaceae species from Costa Rican rainforest. Furthermore, the cytotoxic activity of an acetogenin is described, and two novel alkaloids are reported.

The acetogenin squamocin (**1**)[9]; the flavonoids 5-methoxyflavone[10], 5,2'-dimethoxyflavone[11], and catechin[12]; the aporphine alkaloid liriodenine[13]; the tyramine tamgermanetin[14]; the coumarin marmesin[15]; and *ent*-kaurane diterpene methyl *ent*-16α,17-dihydroxy-kauran-19-oate[16] were isolated from *Annona pittieri* and identified by NMR.

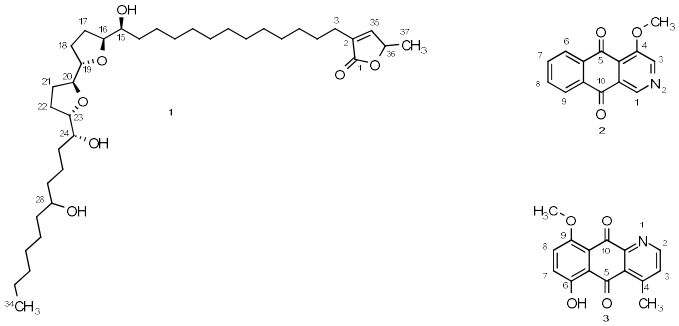

Figure 1. Compounds isolated from *Annona pittieri* (**1** and **2**) and *Cymbopetalum costaricense* (**3**).

Acetogenins compounds have been widely studied as cytotoxic agents, which activity is explained through inhibition of mitochondrial complex I (NADH:ubiquinone oxidoreductase) of the respiratory chain and inhibition of the sodium-potassium ATPase[17]. Squamocin (**1**) isolated from *A. pittieri* leaves showed activity against pancreatic carcinoma and leukaemia cells (Figure 2), while its higher activity was against human T-cell acute lymphoblastic leukaemia resistant to doxorubicin (CEM-ADR500). Furthermore, the cytotoxic activity was lower against healthy blood cells (Figure 3), showing selective activity against cancer cells. Although cytotoxic activity in leukaemia cells was reported previously for squamocin[18]. This results demonstrated in particular, a higher activity against resistant cell lines. A similar cytotoxic profile was described for other acetogenins and cell lines[19,20].

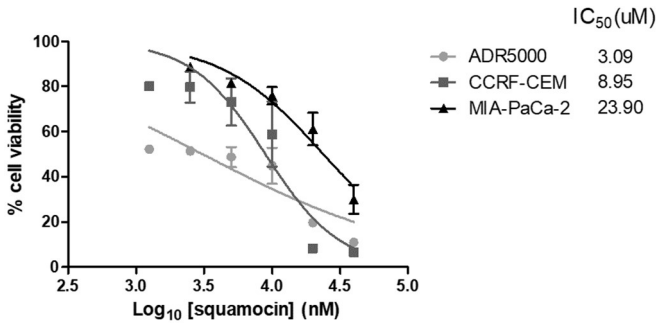

Figure 2. Cell viability of the 24 h treatment with squamocin isolated from *A. pittieri* in cancer cell lines. Results presented mean ± SD (n=3).

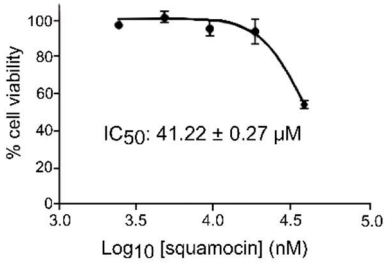

Figure 3. Cell viability of the 24 h treatment with squamocin isolated from *A. pittieri* PBMC cells. Results represent mean ± SD (n=3).

In addition, a novel 2-azaanthraquinone alkaloid, 4-methoxybenzo[*g*]isoquinoline-5,10-dione (**2**), was isolated from *A. pittieri* stem. The ESI-TOF MS of **2** in positive mode showed a molecular ion at *m/z* 240.066 $[M + H]^+$, suggesting a molecular formula of $C_{14}H_9NO_3$. The NMR data (Table 1) showed signals characteristics for aromatic protons at δ_H 8.29, 7.88 (2H) and 8.43, with a correlation between them in the H-H COSY spectrum. Additionally, the signals at δ_H 8.29 and 8.43 correlated with δ_C 181.9 and 180.9, respectively in the HMBC spectrum, suggesting a benzoquinone-like system. A signal at δ_H 9.18, which showed a correlation in H-H COSY with a signal at δ_H 7.63, is typical of alpha protons to nitrogen in the isoquinoline-like system, which agrees with a 2-azaanthracene system. Furthermore, a signal at δ_C 167.9 showed correlations with δ_H 7.63 and 4.09, which is characteristic of methoxyl groups, suggesting the structure **2**.

Table 1. ^{1}H RMN (600 MHz) y ^{13}C RMN (600 MHz) data for **2**. $CDCl_3$, δ [ppm] (J [Hz]).

Position	δ_H	δ_C
1	9.18 d (4.2)	155.2
3	7.63 d (4.2)	125.5
4	-	167.9
4a	-	142.5
5	-	181.9
5a	-	133.1
6	8.29 dd (1.8, 7.2)	127.7
7	7.88 ddd (1.8, 7.2, 6)	135.1
8	7.88 ddd (1.8, 7.2, 6)	135.2
9	8.43 dd (1.8, 7.2)	128.2
9a	-	132.7
10	-	180.9
10a	-	149.4
4-OCH_3	4.09 s	53.6

There are published reports of 1-azaanthraquinone alkaloid in others species of Annonaceae family, such as cleistopholine isolated from *Cleistopholis patens*[21] and 5-hydroxy-6-methoxycleistopholine isolated from *Porcelia macrocarpa*[22]. However, this is the first report of a 2-azaanthraquinone structure with a methoxyl group in the pyridine ring.

From *Cymbopetalum costaricense*, the quinolinone alkaloids, 4-methyl-2(1H)-quinolinone[23] and 6,7-dimethoxy-1-methyl-2(1H)-quinolinone[24], were isolated and identified by NMR. Despite these compounds are known structures, this is the first report of their biosynthetic origin.

In addition, a novel 1-azaanthraquinone alkaloid, 6-hydroxy-9-methoxy-cleistopholine (**3**), was isolated from *C. costaricense*. The ESI-QTRAP MS of **3** in positive mode showed a molecular ion at *m/z* 269.21 $[M]^+$, suggesting a molecular formula of $C_{15}H_{11}NO_4$, which agrees with NMR data. Moreover, signals at *m/z* 254.02 and *m/z* 226.07 suggested chemical transformations of demethylation and decarbonylation, respectively. The NMR data (Table 2) displayed characteristics signals of aromatic protons at δ_H 7.42 and 8.68, which correlate each other in the H-H COSY spectrum. Besides, the signal at δ_H 8.68 could be related to the alpha position to nitrogen in a pyridine ring, likewise discussed for **2**. Finally, the signal at δ_H 2.90, which correlate to δ_C 152.8 in the HMBC spectrum, suggesting a methyl group substituting the pyridine scaffold.

Table 2. ^{1}H RMN (400 MHz) and ^{13}C RMN (400 MHz) data for **3.** CD_3Cl, δ [ppm] (J [Hz]).

Position	δ_H	δ_C
2	8.68 d (4.8)	152.0
3	7.42 d (4.8)	131.1
4	-	152.8
4a	-	128.6
5	-	183.3
5b	-	159.2
6	-	169.9
7	7.60 d (8)	120.8
8	6.83 d (8)	111.8
9	-	158.4
9a	-	125.7
10	-	183.3
10a	-	148.2
4-CH_3	2.90 s	22.8
9-OCH_3	3.96 s	56.0

Two more aromatic proton signals at δ_H 6.83 and 7.60 suggest the presence of another aromatic system, where δ_H 7.60 correlated with δ_C 183.3, a typical carbonyl signal, in the HMBC spectrum. Moreover, protons at δ_H 6.83 and 7.60 correlated with δ_C 169.9 and 158.4, respectively, which are typical signals of phenoxyl group. Furthermore, the signal characteristic of phenoxy groups at δ_H 6.83, correlated in the HMBC spectrum with δ_C 158.4. All the HMBC correlations (Figure 3) are consistent with the structure **3**.

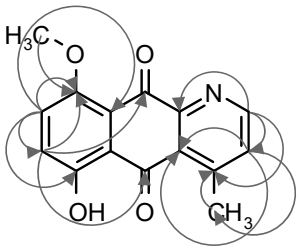

Figure 3. HMBC correlations for **3**.

The structure **3** is closely related to cleistopholine, an alkaloid mentioned above, isolated from Annonaceae species, such as *Cleistopholis patens*[21] and *Annona Cherimolia*[25]. Despite reports of hydroxyl and methoxyl derivatives of cleistopholine[26], this is the first report of the 6-hydroxy-9-methoxy-cleistopholine structure.

CONCLUSION

Annonaceae family is a well-known source of bioactive compounds. In this work, a cytotoxic acetogenin and two novel alkaloids from *Annona pittieri* and *Cymbopetalum costaricense* were described, supporting the importance of native species from the tropical rainforest as a source of bioactive compounds. In particular, *Cymbopetalum costaricense* proved to be a source of alkaloids with novel structures.

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