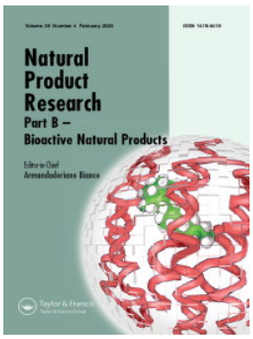

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Stilbenes, phenanthrenes and antiproliferative activity of *Cattleya intermedia*

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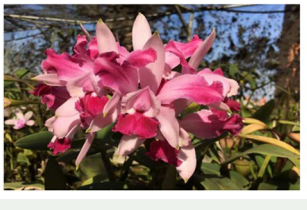
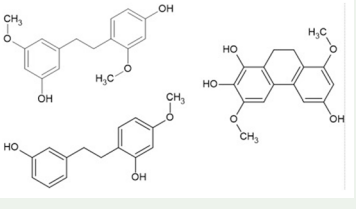
ABSTRACT

The phytochemical study of *Cattleya intermedia* (Orchidaceae) led to the isolation of two new stilbenoids and one new 9,10-dihydrophenanthrene, 4′,5-dihydroxy-2′,3-dimethoxy-dihydrostilbene (**1**), 3,6′-dihydroxy-4′-methoxy-dihydrostilbene (**2**) and 1,2,6-trihydroxy-3,8-dimethoxy-9,10-dihydrophenanthrene (**3**), named cattleymediol, cattleyol and phenanmediol, respectively, in addition to other five known compounds (**4–8**). The structural elucidations of the isolated compounds were carried out through the analyses of the one-dimensional ^{1}H,^{13}C and NOE NMR spectra, and the 2D HSQC, HMBC, COSY and NOESY spectra, besides high-resolution mass spectrometry. In addition to this, the crude extract and its main fractions were analysed by ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-QTOF-MS/MS), leading to the putative identification of several other compounds, including flavonoids and organic acids derivatives. Finally, the main fractions of the crude extract, and the pure compounds cattleymediol (**1**) and lusiantridine (**7**), had their antiproliferative activities evaluated against human cancerous HeLa and non-cancerous VERO cells.





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1. Introduction

The Orchidaceae family is considered, among the Angiosperms, the largest and most evolved, with about 30,000 species that can be found from the Arctic to the tropics (Hossain 2011; Rodrigues 2011). Orchids are distributed in 650 genera, including the genus *Cattleya* Lindl., with about 114 species. They occur in the neotropics, and are distributed in South America, from Colombia to Uruguay and northern Argentina. This genus has high commercial value due to the exuberant flowers, with different colours and shades, and due to its capacity to generate thousands of hydribs through genetic recombination with species of other closely related orchid genera (Moraes et al. 2009; Van Der Berg 2014; Carneiro et al. 2017).

In the study carried out by Baskin and Bliss (1969), sugars, glucose, sucrose and fructose were isolated from the species *Cattleya bowringiana*. Filho and Castro (2019), through the investigation of the species *Cattleya walkeriana*, identified the presence, in the leaves of the plant, of organic acids, flavonoids, saponins, coumarin and tannins. Ishii (1979), isolated a novel phenolic compound characterised as 5′-hydroxy-eucomic acid and reported the presence of dopamine in the species *Cattleya trianaei*. In our research group, studies were carried out with species belonging to the genus *Cattleya* and subtribe Laeliinae, to which *C. intermedia* belongs. Belloto et al. (2017), reported seven compounds from the orchid *Laelia marginata*, one of them being the unprecedented natural product crispoic acid, a derivative of eucomic acid. From the orchid *Cattleya tigrina*, Ferreira et al. (2021) characterised thirteen compounds, namely stilbenoids, phenanthrenoids, steroids and phenolic acids, including a phenanthrenequinone, which showed antiproliferative activity against cancerous cell line.

In this work, the first phytochemical study of the orchid *Cattleya intermedia* Grah. was carried out. This species is endemic to the South and Southeast regions of Brazil, in the coastal areas of the Atlantic rain forest, and it is considered to be at risk of extinction in the natural environment (Buzzato et al. 2010; Endres Junior et al. 2015).

2. Results and discussion

2.1. Isolation and structural characterisation of the new natural products

The compound **1** (18 mg) was isolated as a wax from the chloroform and *n*-hexane fractions of the crude extract, being soluble in the organic solvents chloroform and methanol. Through the ^{1}H NMR spectrum, in the aromatic region, the signals of the hydrogens H-2, H-4 and H-6 were observed, as a triplet with *meta* coupling at 6.33 ppm ($J = 2$ Hz; 1H; H-6), and a doublet with *meta* couping at 6.28 ppm ($J = 2$ Hz; 2H; H-2 and 3). In the other aromatic ring, the hydrogens H-3′, H-5′ and H-6′ were observed as a signal with *ortho* coupling at 6.85 ppm ($J = 8$ Hz; 1H; H-6′), an *ortho-meta* double doublet at 6.70 ppm ($J = 8$ Hz and 2 Hz; 1H; H-5′) and, finally, a doublet with *meta* coupling at 6.64 ppm ($J = 2$ Hz; 1H; H-3′). The COSY spectrum confirmed the correlation between the hydrogens H-2, H-4 and H-6, and in the other aromatic ring between the hydrogens H-3′, H-5′ and H-6′. In ^{13}C NMR spectrum, the signals at 55.50 and 56.10 ppm stand out, characteristic of carbons linked to oxygen, which are correlated to the methoxy hydrogens at 3.75 and 3.84 ppm, respectively, as shown by the HSQC data.

The final structure of the compound was arrived at with the help of HMBC data, highlighting the correlations between the hydrogens H-2 and H-6 with the carbon signal at 38.50 ppm, of the hydrogens H-3′ and H-6′ with the carbon signal at 146.50 ppm (C-OCH_3), the H-3′ and H-5′ with carbon signal at 143.85 ppm (C-OH), H-2 and H-4 with carbon signal at 160.99 ppm (C-OCH_3), and the correlation between H-4 and H-6 with carbon signal at 156.86 ppm (C-OH). In addition to the NMR spectra, the sample was also subjected to high resolution mass spectrometry analyses in negative and positive modes, and it was possible to confirm the molecular formula of the compound, $C_{16}H_{18}O_4$ (*m/z* 274.1169) by means of the peaks at *m/z* 273.1135 [(M-H); error (ppm): 4.85] and *m/z* 275.1287 [(M + H); error (ppm): 3.29].

The compound **2** (5.2 mg) was isolated as a wax from the ethyl acetate fraction of the methanolic crude extract, being soluble in the organic solvents chloroform and methanol. Through the ^{1}H NMR spectrum, the signals of the hydrogens H-2, H-4, H-5 and H-6, were observed in the aromatic region, as a triplet with *meta* coupling at 6.66 ppm (2H; $J = 2$ Hz; H-2 and H-4), a multiplet at 6.60 ppm (1H; H-6), a triplet with *meta* coupling at 7.06 ppm (1H; $J = 2$ Hz; H-5). The other aromatic ring hydrogens H-2′, H-3′ and H-5′ were observed as a multiplet at 6.22 ppm (2H; H-2′ and H-3′), and a triplet with *meta* coupling at 6.18 ppm (1H; $J = 2$ Hz; H-5′). Through the COSY spectrum, the correlation between the hydrogens H-4, H-5 and H-6 was confirmed. In the ^{13}C NMR spectrum, the signal at 54.27 ppm stands out, characteristic of carbon bonded to oxygen, which is correlated to the hydrogen simplet signal at 3.69 ppm, integrating for 3 hydrogens, as shown by the HSQC data. The final structure of the compound was arrived at with the help of HMBC data, highlighting the correlations between the hydrogens H-2 and H-6 with the carbon signal at 37.65 ppm (CH_2), the hydrogen H-3′ with the carbon at 37.98 ppm (CH_2), the hydrogens H-a and H-b with the carbons at 143.47 ppm (C-1) and 144.24 ppm (C-1′), and finally, a correlation of the hydrogen H-5 with the carbons signals at 143.47 and 157.11 ppm (C-3). Through the NOE 1D spectrum, irradiation on the hydrogen signal at 3.69 ppm (OCH_3) caused signal increments in the signals of hydrogens at 6.22 and 6.18 ppm, adding further evidence for the proposed position of the methoxy group. High resolution mass spectrometry analysis was performed, and it was possible to confirm the molecular formula of the compound **2**, $C_{15}H_{16}O_3$ (*m/z* 244.1105) by means of the peak at *m/z* 245.1186 [(M + H); error (ppm): 1.22].

The compound **3** (1.5 mg) was isolated as an amorphous solid from the *n*-hexane fraction of the methanol crude extract. Through the ^{1}H NMR spectrum, the signals of the hydrogens H-4, H-6 and H-8 were observed in the aromatic region, as a singlet in 6.93 ppm (1H; s; H-4), a doublet with *meta* coupling at 6.86 ppm ($J = 1.5$ Hz; H-6) and a doublet with *meta* coupling at 6.72 ppm ($J = 1.5$ Hz; H-8). In the ^{13}C NMR spectrum, the signals at 56.52 and 57.32 ppm stand out, characteristic of carbons linked to oxygen, which are correlated to the hydrogens 3.87 and 3.95, respectively, as shown by the HSQC spectrum. These signals were attributed to the methoxy groups. With the help of the HMBC correlations map, it was possible to define that each methoxy substituent group is present in a different aromatic ring, through the correlations between carbon signal at 147.07 ppm (C-3) with the hydrogen signal at 6.93 ppm (H-4), and carbon signal at 161.02 ppm (C-7) with hydrogen signal at 6.86 ppm (H-6). It is also noted that the hydrogen signal at 6.86 ppm also correlates with the carbon

signal at 155.45 ppm (C-5), which indicates that this hydrogen is between two substituent groups. Through the COSY spectrum, the correlation between the hydrogens H-6 and H-8 was confirmed. High resolution mass spectrometry analysis was performed, and it was possible to confirm the molecular formula of the compound **3**, $C_{16}H_{16}O_5$ (*m/z* 288.1003) by means of the peak at *m/z* 287.0932 [(M-H); error (ppm): 2.79].

In addition to the new natural products, the compounds 3,3′-dihydroxy-5-methoxy-dihydrostilbene (**4**, batatasin III, 17.3 mg, Agustina et al. 2018), 3,4′-dihydroxy-3′,5-dimethoxy-dihydrostilbene (**5**, gigantol, 23.3 mg, Woo et al. 2014), 2,5-dihydroxy-7-methoxy-9,10-dihydrophenanthrene (**6,** lusianthridine, 53.6 mg, Xu et al. 2019), 2,7-dihydroxy-4-methoxy-dihydrophenanthrene (**7**, coelonin, 6.5 mg, Xu et al. 2019) and phloretic acid (**8**; 70.9 mg, Almeida et al. 2014), were also isolated and identified from *C. intermedia* in this work (Figure 1).

2.2. Analyses of compounds present in the crude extract fractions by UHPLC-HR-MS-MS

The crude extract (EBCI), the *n*-hexane and chloroform fractions of the crude extract (CLCI, HXCI) were analysed using the technique of ultra-high efficiency liquid chromatography coupled to high resolution mass spectrometry (UHPLC-QTOF-MS-MS). These analyses led to the putative identification of the presence of 15 compounds, whose identifications were proposed according to comparisons of the acquired data with databases, such as HMDB, KEGG, NIST and PUBCHEM, in addition to comparisons with the literature (Xu et al. 2019). Among identified compounds, there are some previously isolated and identified by the classic techniques of purification and

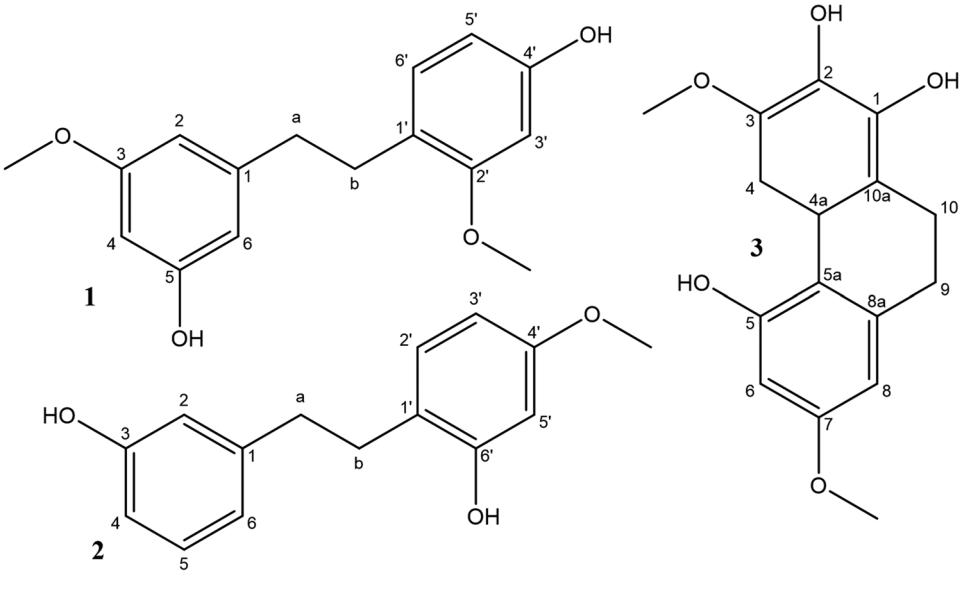

Figure 1. New natural products 4′,5-dihydroxy-2′,3-dimethoxy-dihydrostilbene (1, cattleymediol), 3,6′-dihydroxy-4′-methoxy-dihydrostilbene (2, cattleyol), 1,2,5-trihydroxy-3,7-dimethoxy-9,10-dihydrophenanthrene (3, phenanmediol), isolated from *C. intermedia*.

structural identification by NMR, as the phloretic acid (**8**), coelonin (**7**), the new natural stilbenoid 4′,5-hydroxy-2′,3-methoxy-dihydrostilbene (**1**) and batatasin III (**4**). It is important to highlight that this technique also allowed the putative identification of flavonoids isovitexin-2′′-*O*-beta-*D*-glucoside and acacetin. The results are shown in Table 1.

2.3. Antiproliferative activity assays

Biological cytotoxicity tests against HeLa tumour cells and VERO non-tumour cells were performed with crude extract (EBCI), *n*-hexane (HXCI), chloroform (CLCI), ethyl acetate (AECI) and methanolic (MECI) fractions, and with the isolated compounds **1** and **7**. The CLCI fraction showed the best performance against the HeLa cell line ($CC_{50\%}$ = 64.03 ± 5.48 µg/mL), however, the cytotoxicity was higher in VERO cells ($CC_{50\%}$ = 60.92 ± 16.86). Compound **7** showed very similar results for both cell lines ($CC_{50\%}$ = HeLa 55.15 ± 20.06; VERO 55.15 ± 22.98 µg/mL). The new natural product **1** was poorly active against both cell lines ($CC_{50\%}$ = HeLa 294.29 ± 3.03; Vero 138.13 ± 5.75 µg/mL). For all samples, the Selectivity Index (SI) was calculated, which according to Suffness and Pezzuto (1991), a compound will be selective to tumour cells if its SI was greater than or equal to 2.0. In this work, none of the samples tested showed significant results according to this parameter. Results are shown in Table 2.

3. Experimental

3.1. Equipment

The 1D (^{1}H,^{13}C, NOE) and 2D (COSY, NOESY, HMBC and HSQC) nuclear magnetic resonance spectra were obtained using a Bruker Avance III HD spectrometer, operating at 300 MHz for ^{1}H, using the reference standard tetramethylsilane (0.0 ppm) or typical solvent signals. For the preparation of the analysed samples, the deuterated solvents $CDCl_3$, CD_3OD, $(CD_3)_2SO$ from Sigma-Aldrich or Cambridge Isotope Laboratories, Inc. (Tewksbury, MA) were used. Chromatography columns and thin layer chromatography plates were made with a stationary phase of silica gel 60 or Sephadex LH 20, and the organic solvents hexane, chloroform, dichloromethane, ethyl acetate and methanol were of analytical grade, and distilled before using. For QTOF-HR-MS-MS analyses, samples were analysed by direct infusion using an Impact II Q-tof mass spectrometer (Bruker Daltonics Corporation, Bremen, Germany). The mass spectrometer was equipped with an electrospray ionisation (ESI) source, and was operated in negative ionisation mode, with capillary voltage set to 4.50 kV, source temperature of 200 °C. The desolvation gas flow was 8 L min^{-1} and the nebulisation gas pressure was 4 bar. Data were collected between the *m/z* range from 50 to 700, with an acquisition rate of 5 Hz, with the precursor ion selected for fragmentation (auto MS/MS).

3.2. Plant material

The orchid *Cattleya intermedia* Grah. was kindly donated by the commercial grower Mr. Vanderlei F. Silva (‘Orquidário Progresso’, Iguatemi District, Maringá, Paraná, Brazil)

Table 1. Compounds identified from EBCI, CLCI and HXCI by UHPLC-QTOF-HR-MS-MS, in negative mode.

Compound	Sample	MF	Theoretical mass [M-H] (*m/z*)	Experimental molecular mass [M-H] (*m/z*)	Error (ppm)	RT (min)	Main fragments	Database or reference
Succinic acid	EBCI	$C_4H_6O_4$	117.0182	117.0181	−0.85	3.48	73; 99; 100	HMDB0000254
Malic acid	EBCI	$C_4H_6O_5$	133.0132	133.0132	0	2.44	71; 72; 89; 115	KEGG-C00149
Phloretic acid	EBCI	$C_9H_{10}O_3$	165.0546	165.0544	−1.21	5.93	93;119	KEGG-C01744
2-isopropilmalic acid	EBCI	$C_7H_{12}O_5$	175.0601	175.0597	−2.28	5.88	85; 115; 119; 157	NIST 1052052
Mannitol	HXCI	$C_6H_{14}O_6$	181.0706	181.0706	0	1.34	71; 89; 101; 119;163	PUBCHEM-6251
2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid	EBCI	$C_{11}H_{12}O_6$	239.0550	239.0548	−0.84	5.68	87; 107; 149; 177; 179	KEGG-C05817
	HXCI			239.0548	-0.84	5.69		
Coelonin	EBCI	$C_{15}H_{14}O_3$	241.0859	241.0849	−4.15	6.36	199; 226; 277	KEGG-C10257
Batatasin III	HXCI	$C_{15}H_{16}O_3$	243.1016	243.1011	−2.06	6.42	151; 198; 226; 243	PUBCHEM-10466989
2′,3-dimethoxy-4′,5-dihydroxy-dihydrostilbene (**1**)	HXCI	$C_{16}H_{18}O_4$	273.1121	273.1114	−2.56	6.39	93; 121; 151; 226; 243; 258	New natural product
Linoleic acid	CLCI	$C_{18}H_{32}O_2$	279.2319	279.2302	−6.09	13.41	112; 279; 280;	KEGG-C01595
Acacetin	EBCI	$C_{16}H_{12}O_5$	283,0601	283.0589	−4.24	7.15	267; 268; 269	Silmen et al. (2017)
	CLCI			283.0586	−4.59	7.17		
Gitogenin	CLCI	$C_{27}H_{44}O_4$	431.3156	431.3131	−5.56	12.27	117; 145; 431	KEGG-C08899
Proscilaridin	CLCI	$C_{30}H_{42}O_8$	530.2874	530.2842	−6.03	12.34	175; 295; 471	PUBCHEM-5284613
Isovitexin-2′′-*O*-beta-D-glucoside	HXCI	$C_{27}H_{30}O_{15}$	593.1501	593.1489	−2.38	5.48	89; 101; 33; 383; 413	KEGG-C04199

Table 2. Cytotoxicity against HeLa and VERO cells of the crude extract main fractions and pure compound **7**.

Sample	HeLa $CC_{50\%}$ µg/mL		VERO $CC_{50\%}$ µg/mL		SI
HXCI	80.01	± 5.06	108.64	± 13.32	1.36
CLCI	64.03	± 5.48	60.92	± 16.86	0.95
AECI	83.7	± 0.14	71.91	± 1.17	0.86
MECI	95.79	± 2.07	–	–	–
Compound **7**	55.15	± 20.06	55.10	± 22.98	1.00
Compound **1**	294.29	± 3.03	138.13	± 5.74	0.47

and identified by Prof. Dr. Maria Auxiliadora Milaneze-Gutierre, from the Department of Biology at State University of Maringá. An exsiccate was deposited in the Herbarium of the State University of Maringá (HUEM), Brazil, under code 36312. The work was registered in Sisgen.

3.3. Isolation of compounds

The plant organs (roots, rhizomes, pseudobulbs and leaves) were washed in water, crushed and dried at room temperature, resulting in 319.00 g of dry material. For the crude extract preparation (EBCI), exhaustive extraction with methanol was used, in which the dried and crushed plant material was immersed in 2 L of methanol, until solvent saturation, at room temperature. After saturation, the solution was filtered and evaporated under reduced pressure. The process was repeated 15 times, obtaining the EBCI (102.00 g). Then, the EBCI was fractionated through direct contact with the organic solvents *n*-hexane, chloroform, ethyl acetate and methanol. This process was repeated until no traces of sample were observed in the supernatant, thus resulting in the *n*-hexanic (HXCI; 6.65 g), chloroform (CLCI; 7.07 g), ethyl acetate (AECI; 3.00 g) and methanolic (MECI; 15.32 g) fractions. Part of the HXCI fraction (4.65 g) was subjected to separation by column chromatography on silica gel 60 and eluted with the solvents *n*-hexane, chloroform, ethyl acetate and methanol, in gradient polarity. The more polar fractions resulting from this initial separation were purified by column chromatography with Sephadex LH 20, eluted isocratically with methanol or methanol/chloroform 80:20, which resulted in the isolation of compounds **1**, **3** and **5**.

Part of the CLCI fraction (6.43 g) was subjected to separation by column chromatography on silica gel 60 and eluted with the solvents *n*-hexane, chloroform, ethyl acetate and methanol. The samples obtained from this separation were further purified on silica gel 60 columns and recrystallised with methanol/chloroform and chloroform/hexane resulting, thus, in the isolation of compounds **1**, **4**, **6** and **7**.

Part of the AECI fraction (1.58 g) was separated and further purified by column chromatography on Sephadex LH 20 eluted isocratically with methanol or methanol/chloroform 80:20. This procedure, in addition to recrystallisations with chloroform/methanol, led to the isolation the compounds **2** and **8**. All isolated compounds were structurally characterised upon 1D ^{1}H, ^{13}C and NOE NMR analyses, bidimensional COSY, HMBC, HSQC and NOESY NMR analyses, besides high resolution mass spectrometry. The spectra of the new natural products 1, **2** and **3** are shown in the Supplementary Material file.

Compound **1**, cattleymediol (4',5-dihydroxy-2',3-methoxy-dihydrostilbene). ^{1}H NMR (300 MHz; $CDCl_3$; ppm): 6.85 (1H; d; J = 7.99 Hz; H-6'), 6.70 (1H; dd; J = 8.0 and 1.9 Hz; H-5'), 6.64 (1H; d; J = 1.84 Hz; H-3'), 6.33 (1H; t; J = 1.99 Hz; H-6), 6.28 (2H; t; J = 2.1 Hz; H-2 and 4), 3.84 (3H, s; OCH_3), 3.75 (3H, s; OCH_3), 2.81 (4H; m; H- a and b). ^{13}C NMR (75 MHz; $CDCl_3$; ppm): 160.99 (C-3), 156.86 (C-5), 146.49 (C-2'), 144.75 (C-1), 143.85 (C-4'), 133.93 (C-1'), 121.20 (C-5'), 114.45 (C-6'), 111.42 (C-3'), 108.33 (C-2), 106.99 (C-6), 99.26 (C-4), 56.10 (OCH_3), 55.50 (OCH_3), 38.50 (C-a), 37.44 (C-b).

Compound **2**, cattleyol (3,6'-dihydroxy-4'-methoxy-dihydrostilbene). ^{1}H (300 MHz; CD_3OD; ppm): 6.18 (1H; t; H-5'), 6.22 (2H; m; H-2' and H-3'), 6.60 (1H; m; H-6'), 6.66 (2H; m; H-2 and H-4), 7.06 (1H; t; J = 7.76 Hz; H-5), 3.69 (3H, s; OCH_3), 2.76 (4H; m; H- a and b). ^{13}C NMR (75 MHz; CD_3OD; ppm): 161.10 (C-6'), 158.17 (C-4'), 157.11 (C-3), 144.24 (C-1'), 143.47 (C-1), 129.06 (C-5), 119.66 (C-2), 115.19 (C-6), 112.55 (C-4), 107.99 (C-3'), 105.31 (C-2'), 98.63 (C-5'), 54.27 (OCH_3), 37.98 (C-b), 37.65 (C-a).

Compound **3**, phenanmediol (1,2,5-trihydroxy-3,7-dimethoxy-9,10-dihydrophenanthrene). ^{1}H NMR (300 MHz; $CDCl_3$; ppm): 6.72 (1H; d; J = 1.42 Hz; H-8), 6.86 (1H; d; J = 1.47 Hz; H-6), 6.93 (1H; s; H-4), 3.87 (3H; s; OCH_3), 3.95 (3H; s; OCH_3), 3.25 (4H, m; 2 CH_2). ^{13}C NMR (75 MHz; $CDCl_3$; ppm): 161.02 (C-7), 155.45 (C-5), 147.74 (C-2), 147.07 (C-3), 140.05 (C-1), 129.54 (C-10a), 117.82 (C-5a), 112.20 (C-4a), 108.72 (C-8), 96.04 (C-6), 94.90 (C-4), 57.32 (OCH_3), 56.52 (OCH_3), 25.99 (CH_2), 22.35 (CH_2).

3.4. Evaluation of cytotoxicity against HeLa and VERO cells

When at least 80% confluence was verified in the culture bottles, the cells were suspended using trypsin (Gibco, Grand Island, NY) and the cell concentration was adjusted to 2×10^5 cells mL^{-1} in RPMI 1640 medium (Roswell Park Memorial Institute, Gibco, Grand Island, NY) to 10% SFB (SFB, Gibco, Grand Island, NY) and then added to the wells of a microplate. After 24 h, samples EBCI, HXCI, CLCI, AECI and MECI were added to the microplate in serially diluted concentrations (1/2) (100, 50, 25, 12.5 and 6.25 µg mL^{-1}) and pure compounds **1** and **7** (2000, 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 3.90 µg mL^{-1}) in triplicate and incubated for a further 24 h at 37 °C under 5% CO_2. After this period, the compounds were removed and the wells were washed with a potassium phosphate buffer (PBS, 0.1 M, pH 6.8). Then, 50 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diph enyltetrazolium (MTT) were added to wells containing pure compounds **1** and **7**, diluted in DMEM (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich, St. Louis, MO), or 20 µL of the Neutral Red dye solution (0.5 mg mL^{-1}, INLAB, Diadema, SP, Brazil), diluted in DMEM, were added in the wells containing the samples crude extract and fractions. The plate was incubated again for 3 h. After this period, for the MTT wells the formazan crystals were solubilised with dimethylsulphoxide (DMSO – Synth, São Paulo, SP, Brazil), while the wells containing the Neutral Red dye were treated with ethanol-acetic acid solution (1% v/v acetic acid in 50% solution of ethanol in water). The spectrophotometric analyses were performed in a spectrophotometer (SpectraMax Plus 384, Molecular Devices, San José, CA), at 540 nm Repetto et al. (2008). The negative and positive controls were performed under the same conditions; however, the negative control did not contain cells ISO Document 10993-5 (2009).

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Disclosure statement

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