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In vitro and in vivo inhibition of HCT116 cells by essential oils from bark and leaves of *Virola surinamensis* (Rol. ex Rottb.) Warb. (Myristicaceae)

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ABSTRACT

Ethnopharmacological relevance: Virola surinamensis (Rol. ex Rottb.) Warb. (Myristicaceae), popularly known in Brazil as "mucuíba", "ucuúba", "ucuúba-branca" or "ucuúba do igapó", is a medicinal plant used to treat a variety of diseases, including infections, inflammatory processes and cancer.

Aim of the study: In the present work, we investigated the chemical constituents and the in vitro and in vivo inhibition of human colon carcinoma HCT116 cells by essential oils obtained from the bark (EOB) and leaves (EOL) of *V. surinamensis*.

Materials and methods: EOB and EOL were obtained by hydrodistillation and analyzed via gas chromatography with flame ionization detection and gas chromatography coupled to mass spectrometry. *In vitro* cytotoxic activity was determined in cultured cancer cells HCT116, HepG2, HL-60, B16–F10 and MCF-7 and in a non-cancerous cell line MRC-5 by the Alamar blue assay after 72 h of treatment. Annexin V/propidium iodide staining, mitochondrial transmembrane potential and cell cycle distribution were evaluated by flow cytometry in HCT116 cells treated with essential oils after 24 and 48 h of treatment. The cells were also stained with May-Grunwald-Giemsa to analyze cell morphology. In vivo antitumor activity was evaluated in C.B-17 SCID mice with HCT116 cells.

Results: The main constituents in EOB were aristolene (28.0 ± 3.1%), α-gurjunene (15.1 ± 2.4%), valencene (14.1 ± 1.9%), germacrene D (7.5 ± 0.9%), δ-guaiene (6.8 ± 1.0%) and β-elemene (5.4 ± 0.6%). On the other hand, EOL displayed α-farnesene (14.5 ± 1.5%), β-elemene (9.6 ± 2.3%), bicyclogermacrene (8.1 ± 2.0%), germacrene D (7.4 ± 0.7%) and α-cubebene (5.6 ± 1.1%) as main constituents. EOB showed IC_{50} values for cancer cells ranging from 9.41 to 29.52 μg/mL for HCT116 and B16–F10, while EOL showed IC_{50} values for cancer cells ranging from 7.07 to 26.70 μg/mL for HepG2 and HCT116, respectively. The IC_{50} value for a non-cancerous MRC-5 cell was 34.7 and 38.93 μg/mL for EOB and EOL, respectively. Both oils induced apoptotic-like cell death in HCT116 cells, as observed by the morphological characteristics of apoptosis, externalization of phosphatidylserine, mitochondrial depolarization and fragmentation of internucleosomal DNA. At a dose of 40 mg/kg, tumor mass inhibition rates were 57.9 and 44.8% in animals treated with EOB and EOL, respectively.

Conclusions: These data indicate *V. surinamensis* as possible herbal medicine in the treatment of colon cancer.

Abbreviations: 5-FU, 5-fluorouracil; ANOVA, analysis of variance; ATCC, american type culture collection; DMSO, dimethyl sulfoxide; DOX, doxorubicin; EOB, essential oil from bark; EOL, essential oil from leaves; GC-FID, gas chromatography with flame ionization detection; GC-MS, gas chromatography coupled to mass spectrometry; IC_{50}, half-maximum inhibitory concentration; INPA, National Institute of Research of the Amazon; NC, negative control; PI, propidium iodide; RI, retention indices; SCID, severe combined immunodeficient; SI, selectivity index; TBE, trypan blue exclusion.

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1. Introduction

Virola surinamensis (Rol. ex Rottb.) Warb. (Myristicaceae), popularly known in Brazil as "mucuíba", "ucuúba", "ucuúba-branca" or "ucuúba do igapó", is a woody tree that grows in swampy areas (igapós) and/or close to the riverbanks. It can reach up to 40 m in height and is widely distributed in the Brazilian Amazon. Commercial exploration of this species was carried out on a large scale for its valuable wood, which transformed the plant into a threat of extinction (Galuppo and Carvalho, 2001; Rodrigues, 1980).

Regarding the ethnopharmacological uses of *V. surinamensis*, its leaf tea is used to treat colic, dyspepsia, inflammation, fever and liver problems, and bark resin is used to treat erysipelas and other inflammatory processes (Schultes and Holmsted, 1971; Correa, 1984; Lorenzi and Matos, 2002; Di Stasi and Hiruma-Lima, 2002). Latex is used externally mixed with water to treat venereal diseases. Stem bark and its sap are used for the treatment of cancer, inflammation, infections, gastritis and ulcers (Di Stasi and Hiruma-Lima, 2002; Costa et al., 2008). The decoction of bark and fruits is topically used to treat furunculosis and intestinal infections (Sarquis et al., 2019), while indigenous people in the Amazon inhale the vapor of the leaves to treat malaria (Lopes et al., 1999).

This variety of popular medicinal uses for *V. surinamensis* has led some previous ethnopharmacological studies with this medicinal plant, in which some pharmacological properties have been reported for its extracts/essential oils or secondary metabolites, including anti-schistosomal (Barata et al., 1978), tryponosomicidal (Lopes et al., 1998), antimalarial (Lopes et al., 1999), antileishmanial (Barata et al., 2000; Veiga et al., 2017), antifungal (Costa et al., 2008), antibacterial (Costa et al., 2008), antiulcerogenic (Hiruma-Lima et al., 2009), anti-nociceptive (Carvalho et al., 2010) and anti-inflammatory activities (Carvalho et al., 2010).

Regarding its anticancer property, *V. surinamensis* sap extract was active in a cytotoxic assay using brine shrimp (Beloz, 1992); however, studies based on cancer cells have not been conducted. In the present work, we investigated the chemical constituents and the in vitro and in vivo inhibition of human colon carcinoma HCT116 cells by essential oils from bark (EOB) and leaves (EOL) of *V. surinamensis*.

2. Material and methods

2.1. Plant material

Bark and leaves of *V. surinamensis* were collected in December 2014 in an area flooded in the green preserved area of a private farm owned by H.H.F. Koolen located in the municipality of Rio Preto da Eva, state of Amazonas, Brazil (2° 43′ 11.7″ S e 59° 31′ 08.2″ W). The confirmation of the plant's authenticity was performed at the Herbarium of the National Institute of Research of the Amazon (INPA) compared to a previously deposited voucher (#244270), since the material collected was infertile. This work was registered (SISGEN: AED7F3A) and carried out under Brazilian laws to access genetic resources.

2.2. Essential oil extraction

After collection, the fresh plant tissues were crushed with aid of a metal blender (Colombo, Itajobi, Brazil) and directly subjected to a hydrodistillation with a Clevenger type apparatus (Diogolab, Poá, Brazil). For this purpose, 1.2 kg of crushed barks and 900 g of leaves were extracted for a period of 4 h in 4 L of ultrapure water (18.2 MΩ) each at 100 °C. Then, the obtained essentials oils were extracted three times with chloroform, dried over anhydrous Na_2SO_4 and filtered through a nylon membrane (pore size 0.22 μm, Whatman, Maidstone, UK). Samples were maintained at −80 °C prior chemical analysis.

2.3. Chemical analysis

The identifications of the constituents of each essential oils were performed by gas chromatography coupled to mass spectrometry (GC-MS) with a GCMS/QP2010 Plus device (Shimadzu, Kyoto, Japan) equipped with a capillary column Rtx-5 MS (30 m × 0.25 mm x 0.25, Restek). Helium at a flow rate of 1.02 mL/min was the carrier, and injections of 1 μL were performed with 1.0 mg/mL stock solutions in chloroform with a partition ratio of 1:50 to EOB and EOL. The column temperature program was 55–285 °C with gradual increases of 3 °C/min. The injector and ion source temperatures were 215 °C and 265 °C, respectively. The identifications were based on comparisons of the spectra obtained with those stored in the library of the 8th edition of Wiley (similarities < 90% were discontinued). Confirmations were performed by calculating the retention indices (RI) according to the equation of Van den Dool and Kratz (Van Den Dool and Kratz, 1963) compared to a homologous series composed of linear hydrocarbons ranging from *n*-C_7 to *n*-C_{30} (Sigma Aldrich, St. Louis, MO, United States).

A semi-quantitative analysis was performed to obtain the relative amount of each component of EOB and EOL. For this, gas chromatography with flame ionization detection (GC-FID) was applied. A system consisting of GC2010 equipment (Shimadzu) equipped with an Rtx-5 capillary column was used under the same conditions as the GC-MS analysis. Relative quantities (%) were calculated in relation to the total chromatogram area of three independent replications.

2.4. In vitro studies

2.4.1. Cell lines culture and cell viability assay

The cell lines HCT116 (human colon carcinoma), MCF-7 (human breast adenocarcinoma), HL-60 (human promyelocytic leukemia), HepG2 (human hepatocellular carcinoma), B16–F10 (mouse melanoma) and MRC-5 (human pulmonary fibroblast) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured following the ATCC animal cell culture guide and were free of mycoplasma as tested using a mycoplasma staining kit (Sigma-Aldrich). Cells were cultured in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (Life, Carlsbad, CA, USA), 2 mM L-glutamine (Vetec Química Fina, Duque de Caxias, RJ, Brazil) and 50 μg/mL gentamycin (Life). All cell lines were cultured in flasks at 37 °C in 5% CO_2 and were subcultured every 3–4 days to maintain exponential growth. Adherent cells were collected by treatment with 0.25% trypsin-EDTA solution (Gibco-BRL). All cell lines were used in passages below 80th.

The trypan blue exclusion (TBE) test was used to quantify the number of viable cells before each experiment. In TBE assay, 90 μL was removed from the cell suspension, and 10 μL of trypan blue (0.4%) was added. Cell counting was performed by Neubauer chamber using a light microscope.

To assess the cytotoxicity of EOB and EOL, cell viability was measured by the Alamar blue assay, as previously described (Ahmed et al., 1994; Santos et al., 2017; Silva et al., 2018). In summary, the cells were seeded (30,000/well for suspension cells or 7000/well for adherent cells) in 96-well plates (Cat# 655180, Greiner Bio-One, Monroe, NC, USA) and EOB and EOL (ranging from 0.4 to 50 μg/mL at total volume of 200 μL/well), dissolved in dimethyl sulfoxide (DMSO, Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil), were added to each well and incubated for 72 h. 5-Fluorouracil (5-FU, ranging from 0.2 to 25 μg/mL) (purity > 99%; Sigma Chemical Co.) and doxorubicin (DOX, ranging from 0.04 to 5 μg/mL) (purity ≥ 95%, doxorubicin hydrochloride, Laboratory IMA S.A.I.C., Buenos Aires, Argentina) were used as positive controls. Alamar blue reagent was added to each well (20 μL of a stock solution of resazurin [Sigma-Aldrich Co.] at concentration of 0.312 mg/mL) 4 h before the end of the treatment. Absorbances at 570 nm and 600 nm were measured using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The values of the

half-maximum inhibitory concentration (IC_{50}) and their respective 95% confidence intervals were obtained by non-linear regression. Selectivity indices (SI) were calculated by the formula: SI = IC_{50} (non-cancerous cells)/IC_{50} (cancer cells).

2.4.2. Cell morphology analysis

The morphological changes of the cells stained with May-Grunwald-Giemsa were performed by optical microscopy (Olympus BX41), using Image-Pro software (Media Cybernetics).

2.4.3. Flow cytometry assays

The fragmentation of internucleosomal DNA and the distribution of the cell cycle were carried out as reported by Nicoletti et al. (1991). In summary, the cells were stained with propidium iodide (PI) using a permeabilization solution, containing 2 μg/mL propidium iodide, 0.1% sodium citrate, 0.1% triton X-100 and 100 μg/mL RNAse (all from Sigma-Aldrich Co). The cells were incubated in the dark for 15 min at room temperature, and cell fluorescence was quantified by flow cytometry.

Cell death was quantified using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) and the analysis was performed according to the manufacturer's instructions. Cell fluorescence was measured by flow cytometry, and cells were classified as viable (annexin V-FITC/PI double-negative cells), early apoptotic (annexin V-FITC-positive and PI-negative cells), late apoptotic (annexin V-FITC/PI double-positive cells), or necrotic stages (annexin V-FITC-negative and PI-positive cells).

Mitochondrial transmembrane potential was assessed by rhodamine 123 assay, as reported by Sureda et al. (1997). In summary, the cells were incubated with rhodamine 123 (5 μg/mL, Sigma-Aldrich Co.) for 15 min. After wash with saline solution, the cells were incubated in saline for 30 min and cell fluorescence was quantified by flow cytometry.

For all flow cytometry analysis, 10,000 events per sample were recorded using a BD LSRFortessa cytometer, BD FACSDiva Software (BD Biosciences, San Jose, CA, EUA) and Flowjo Software 10 (Flowjo LCC, Ashland, OR, USA). Cell debris was omitted from the analyzes.

2.5. In vivo studies

2.5.1. Human colon carcinoma xenograft model

The human colon carcinoma xenograft model was carried out as previously described (Baliza et al., 2019; Silva et al., 2019). A total of 40 C.B-17 severe combined immunodeficient (SCID) mice (females, 25–30 g) were obtained and kept at the animal facilities of the Gonçalo Moniz Institute-FIOCRUZ (Salvador, Bahia, Brazil). All animals were housed in cages with free access to food and water and were subjected to a 12:12 h light-dark cycle (lights on at 6:00 a.m.). The animal ethics committee of the Gonçalo Moniz Institute-FIOCRUZ approved the experimental protocol used (number #06/2015).

HCT116 cells (10^7 cells/500 μL/subcutaneous) were inoculated in the left frontal axils of the mice. The animals were randomly divided into four groups at the beginning of the experiment, as described: G1, animals treated with a vehicle (5% DMSO, $n = 10$); G2, animals treated with 5-FU (10 mg/kg, $n = 10$); G3, animals treated with EOB (40 mg/kg, $n = 10$); and G4, animals treated with EOL (40 mg/kg, $n = 10$). The dose (40 mg/kg) of EOB and EOL was selected based on our previous works with essential oils (Lima et al., 2018, 2020; Nogueira et al., 2020). All animals were treated intraperitoneally for 15 consecutive days beginning 1-day after tumor inoculation. One day after the end of the treatment, the animals were anesthetized (50 mg/kg of thiopental) and samples of peripheral blood were collected from the brachial artery. The mice were euthanized by anesthetic overdose (100 mg/kg of thiopental) and the tumors were excised and weighed using an analytic balance.

Table 1
Chemical composition of the essential oil from bark (EOB) and leaves (EOL) of *Virola surinamensis*.

Compounds	Molecular Weight	RI[a]	EOB composition (%)[b]	EOL composition (%)[b]
α-thujene	136	923	0.33 ± 0.03	–
artemisia triene	136	925	1.05 ± 0.22	–
α-pinene	136	933	–	1.28 ± 0.31
β-pinene	136	964	–	0.60 ± 0.10
myrcene	136	991	–	0.11 ± 0.02
limonene	136	1031	0.34 ± 0.05	1.08 ± 0.25
terpinolene	136	1086	0.59 ± 0.08	0.88 ± 0.11
α-cubebene	204	1345	4.47 ± 0.52	5.69 ± 0.67
β-elemene	204	1375	5.42 ± 0.55	9.61 ± 1.02
α-copaene	204	1376	2.30 ± 0.45	5.02 ± 0.77
methyl eugenol	178	1401	–	0.48 ± 0.02
α-gurjunene	204	1407	15.0 ± 3.17	–
aristolene	204	1416	28.4 ± 5.03	–
β-caryophyllene	204	1418	1.94 ± 0.32	1.70 ± 0.23
β-cedrene	204	1418	–	0.65 ± 0.09
γ-elemene	204	1430	–	2.72 ± 0.40
trans α-bergamotene	204	1435	–	0.35 ± 0.01
α-guaiene	204	1439	5.18 ± 0.87	–
α-humulene	204	1452	–	0.95 ± 0.08
alloaromandredene	204	1461	–	1.41 ± 0.12
γ-muurolene	204	1477	–	1.20 ± 0.16
α-muurolene	204	1480	–	0.56 ± 0.10
germacrene D	204	1480	7.58 ± 2.15	7.44 ± 1.80
α-amorphene	204	1485	–	1.70 ± 0.88
valencene	204	1491	14.1 ± 4.87	–
bicyclogermacrene	204	1494	–	8.10 ± 2.42
ledene	204	1496	–	1.16 ± 0.10
α-muurolene	204	1499	–	1.90 ± 0.25
α-farnensene	204	1508	–	14.5 ± 3.24
δ-guaiene	204	1509	6.86 ± 2.40	1.80 ± 0.30
γ-cadinene	204	1512	–	0.30 ± 0.04
elemicin	208	1540	–	4.80 ± 1.13
elemol	222	1547	–	0.44 ± 0.08
germacrene B	204	1560	–	0.94 ± 0.08
spathulenol	220	1575	2.61 ± 0.78	3.05 ± 0.49
caryophyllene oxide	222	1581	0.80 ± 0.45	2.57 ± 0.50
viridiflorol	220	1590	–	1.88 ± 0.52
Δ-cadinol	222	1636	–	2.33 ± 0.84
α-cadinol	222	1653	–	2.72 ± 0.66
cembrene A	276	1942	1.70 ± 0.94	–
$\Sigma_{hydrocarbon\ monoterpenes}$			*2.31*	*3.95*
$\Sigma_{hydrocarbon\ sesquiterpenes}$			*91.25*	*67.70*
$\Sigma_{oxygenated\ sesquiterpenes}$			*3.41*	*12.99*
$\Sigma_{hydrocarbon\ diterpenes}$			*1.70*	-
$\Sigma_{phenylpropanoids}$			-	*5.28*
$\Sigma_{total\ identified}$			*98.67*	89.92

[a] Experimental retention indices according to equation of the Van den Dool & Kratz.
[b] Relative percentage.

2.5.2. Toxicological analysis

Regarding the toxicological evaluation, all mice were also observed for signs of abnormality throughout the study. Initial and final body weight of the animals was also measured. Hematological analyzes were carried out using an Advia 60 hematology system (Bayer, Leverkusen, Germany). Some organs that are usually targets of toxicity (livers, kidneys, lungs and hearts) were collected, weighed and examined for signs of thick lesion formation, color change and/or hemorrhage. After macroscopic examination, the tumors, livers, kidneys, lungs and hearts were fixed in 4% buffered formalin and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin (and Periodic acid-Schiff for livers and kidneys slides), and a pathologist performed the analysis under an optical microscope.

Table 2
The IC_{50} values of the cytotoxicity of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis*.

Cells	IC_{50} and 95%CI in μg/mL (μM)			
	DOX	5-FU	EOB	EOL
Cancer cells				
HCT116	0.13 (0.24)	0.61 (4.69)	9.41	26.70
	0.08–0.21	0.33–1.10	5.63–15.75	12.92–36.93
HepG2	0.03 (0.06)	0.43 (3.31)	16.93	7.07
	0.01–0.19	0.21–0.57	8.69–32.99	1.55–32.22
HL-60	0.04 (0.07)	1.93 (14.84)	20.64	22.76
	0.02–0.08	1.32–2.38	16.00–26.63	17.77–29.15
B16–F10	0.20 (0.37)	0.68 (5.23)	29.52	18.80
	0.17–0.23	0.33–0.92	21.59–40.37	11.46–30.83
MCF-7	0.28 (0.52)	1.69 (12.99)	15.88	21.39
	0.20–0.39	0.72–2.34	11.89–21.20	8.91–51.36
Non-cancerous cells				
MRC-5	3.32 (6.11)	6.74 (51.82)	34.07	38.93
	1.82–6.03	5.22–9.04	29.54–39.30	34.04–44.51

The data are presented as IC50 values, in μg/mL (μM), with their respective 95% confidence interval (95% CI) obtained by nonlinear regression of three independent experiments performed in duplicate, as measured by the Alamar blue assay after 72 h of treatment. Cancer cells: HCT116 (human colon carcinoma); HepG2 (human hepatocellular carcinoma); HL-60 (human promyelocytic leukemia); B16–F10 (mouse melanoma); and MCF-7 (human breast adenocarcinoma). Non-cancerous cells: MRC-5 (human pulmonary fibroblast). Doxorubicin (DOX) and 5-fluorouracil (5-FU) were used as positive controls.

2.6. Statistical analysis

Data were presented as mean ± S.E.M. or as IC_{50} values with 95% confidence intervals obtained by nonlinear regressions. The differences between the experimental groups were compared by analysis of variance (ANOVA) followed by the Bonferroni's multiple comparison test ($p < 0.05$). All statistical analyzes were performed using the GraphPad Prism (Intuitive Software for Science; San Diego, CA, USA).

3. Results

3.1. Chemical analysis of essential oils from bark and leaves of Virola surinamensis

Oil recoveries were 1.21 ± 0.10% (w/w) for EOB and 1.78 ± 0.17% for EOL, in which a composition dominated by sesquiterpenes (>90%) was observed for both samples (Table 1). EOL exhibited a more complex composition (39 compounds), while EOB exhibited almost half of EOL compounds (19 compounds). Cyclic hydrocarbon sesquiterpenes dominated the EOB composition, highlighting aristolene (28.4 ± 5.03%), α-gurjunene (15.0 ± 3.17%), valencene (14.1 ± 4.87%), germacrene D (7.58 ± 2.15%), δ-guaiene (6.86 ± 2.40%) and β-elemene (5.42 ± 0.55%). On the other hand, EOL presented as a main constituent a sesquiterpene aliphatic hydrocarbon, α-farnesene (14.5 ± 3.24%). Moreover, β-elemene (9.61 ± 1.02%), bicyclogermacrene (8.10 ± 2.42%), germacrene D (7.44 ± 1.80%) and α-cubebene (5.69 ± 0.67%) were other main constituents.

3.2. In vitro cytotoxicity induced by essential oils from bark and leaves of Virola surinamensis

The cytotoxicity of essential oils from the bark and leaves of *Virola surinamensis* was evaluated in cell lines of different histological types of cancer, using the alamar blue assay after 72 h of treatment. Table 2 shows the IC_{50} values obtained. EOB showed IC_{50} values for cancer cells ranging from 9.41 to 29.52 μg/mL for HCT116 and B16–F10, while EOL showed IC_{50} values for cancer cells ranging from 7.07 to 26.70 μg/mL for HepG2 and HCT116, respectively. Cytotoxicity was also evaluated in the non-cancerous fibroblast cell line MRC-5, since fibroblast cells are among the targets of toxicity for chemotherapeutic agents. The IC_{50} value for a non-cancerous MRC-5 cell was 34.7 and 38.93 μg/mL for EOB and EOL, respectively. DOX and 5-FU were used as positive controls and were also cytotoxic to all tested cell lines. DOX exhibited IC_{50} values ranging from 0.03 to 0.28 μg/mL for HepG2 and MCF-7, respectively, exhibiting an IC_{50} value of 3.32 μg/mL for MRC-5 cells. 5-FU showed IC_{50} values ranging from 0.61 to 1.93 μg/mL for HCT116 and HL-60, respectively, and an IC_{50} value of 6.74 μg/mL for MRC-5 cells. Table 3 shows the calculated SI.

As HCT116 was among the EOB and EOL sensitive cell lines, the effect of EOB and EOL on cell viability of HCT116 cells was confirmed by the TBE test in HCT116 cells treated at concentrations of 5, 10 and 20 μg/mL after 24 and 48 h of treatment (Fig. 1). After 48 h, EOB reduced the number of viable cells by 36.3, 44.5 and 58.3%, and EOL reduced by 43.4, 58.7 and 78.6% at concentrations of 5, 10 and 20 μg/mL,

Table 3
Selectivity index of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis*.

Drug	Selectivity index per cell line				
	HCT116	HepG2	HL-60	B16–F10	MCF-7
DOX	25.5	110.7	83.0	16.6	11.9
5-FU	11.5	15.7	3.5	9.9	4.0
EOB	3.6	2.0	1.7	1.2	2.2
EOL	1.5	5.5	1.7	2.1	1.8

The data are presented the selectivity index (SI) calculated using the following formula: SI = IC_{50}[non-cancerous cells]/IC_{50}[cancer cells]. Doxorubicin (DOX) and 5-fluorouracil (5-FU) were used as positive controls.

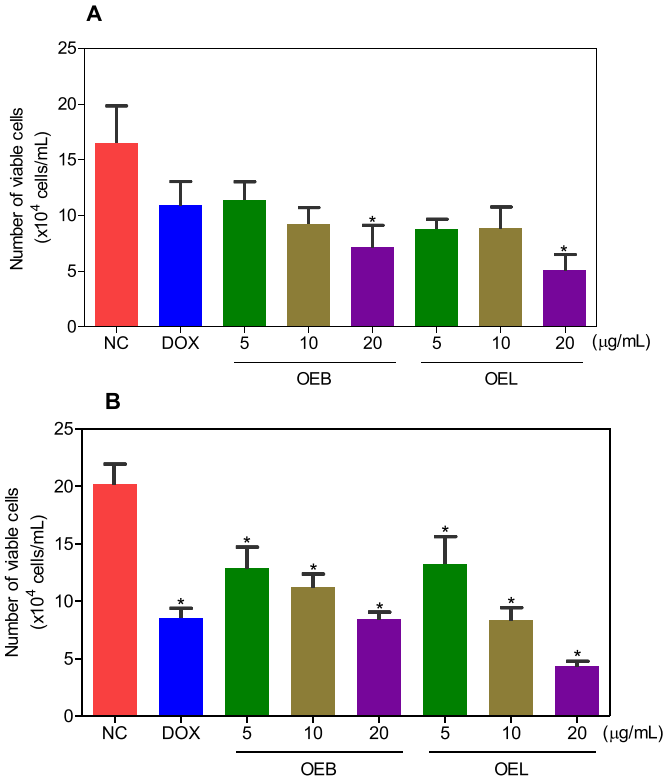

Fig. 1. Effect of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* on the viability of HCT116 cells, as measured by the trypan blue dye exclusion test after 24 (**A**) and 48 (**B**) h of treatment. The negative control (NC) was treated with a vehicle (0.2% DMSO) used to dilute EOB and EOL, and doxorubicin (DOX, 0.5 μg/mL) was used as a positive control. Data are presented as mean ± S.E.M. of three independent experiments carried out in duplicate. *$p < 0.05$ compared to the negative control by ANOVA, followed by the Bonferroni's multiple comparison test.

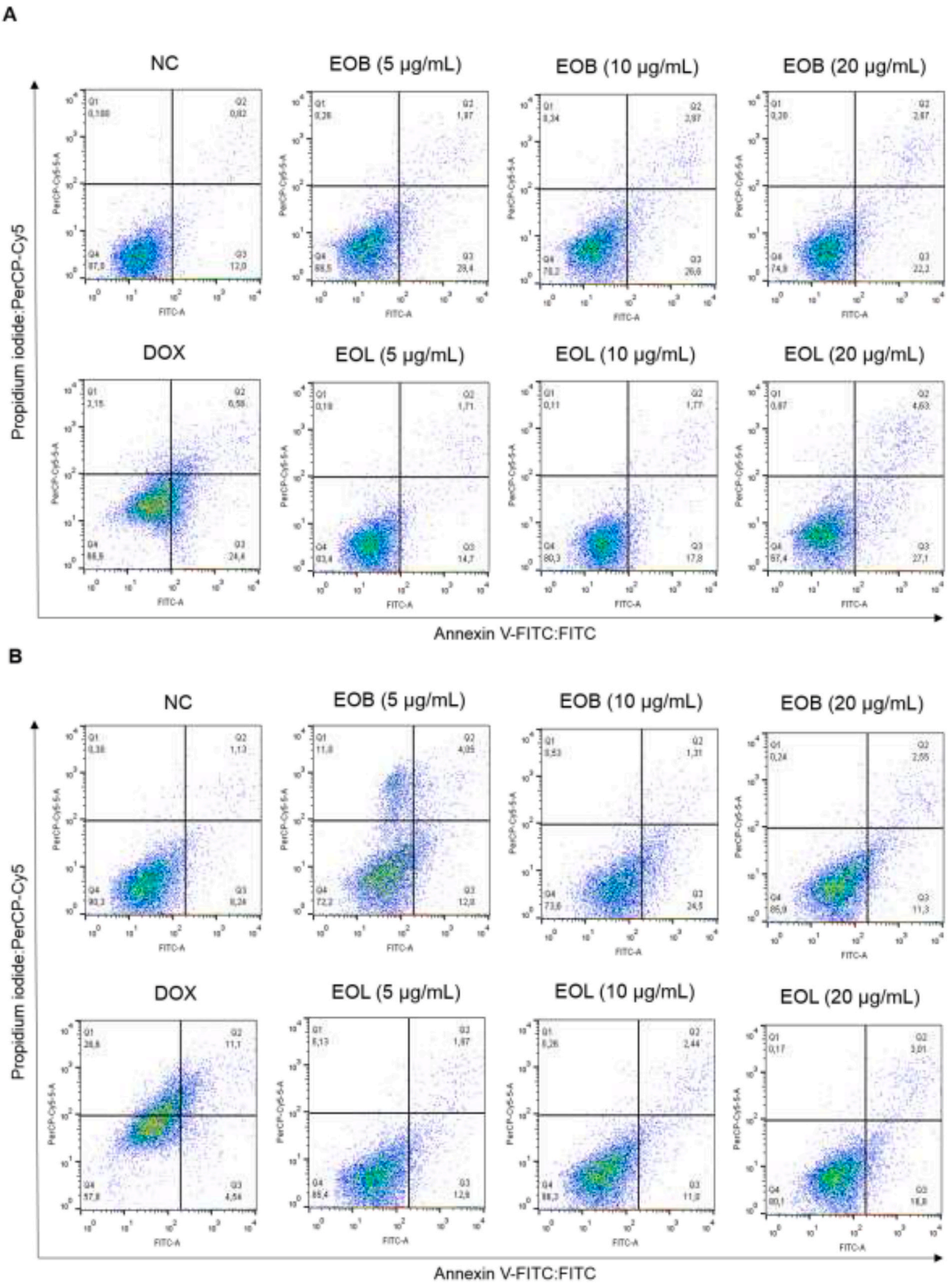

Fig. 2. Representative flow cytometric dotplots of the cell death induction of HCT116 cells treated with essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis*, as measured by flow cytometry using annexin V-FITC/PI staining after 24 (**A**) and 48 (**B**) h of treatment. The negative control (NC) was treated with a vehicle (0.2% DMSO) used to dilute EOB and EOL, and doxorubicin (DOX, 0.5 µg/mL) was used as a positive control.

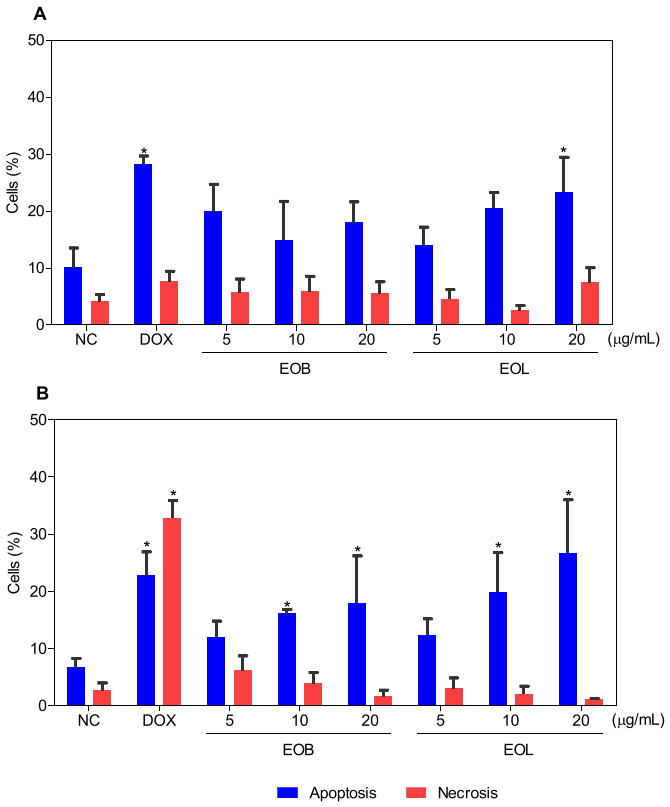

Fig. 3. Effect of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* on the induction of cell death in HCT116 cells, measured by flow cytometry, using annexin V-FITC/PI double staining after 24 (**A**) and 48 (**B**) h of treatment. The cells were classified into viable, apoptosis (early apoptosis + late apoptosis) and necrosis stages. The negative control (NC) was treated with a vehicle (0.2% DMSO) used to dilute EOB and EOL, and doxorubicin (DOX, 0.5 μg/mL) was used as a positive control. Ten thousand events were evaluated per experiment and cell debris was omitted from the analysis. Data are presented as mean ± S.E.M. of three independent experiments carried out in duplicate. *$p <$ 0.05 compared to the negative control by ANOVA, followed by the Bonferroni's multiple comparison test.

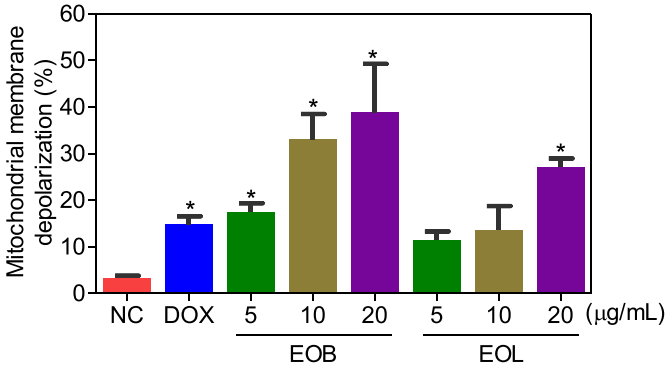

Fig. 4. Effect of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* on the potential of mitochondrial membrane of HCT116 cells, as determined by flow cytometry using rhodamine 123 staining after 24 h of treatment. The negative control (NC) was treated with a vehicle (0.2% DMSO) used to dilute EOB and EOL, and doxorubicin (DOX, 0.5 μg/mL) was used as a positive control. Ten thousand events were evaluated per experiment and cell debris was omitted from the analysis. Data are presented as mean ± S.E.M. of three independent experiments carried out in duplicate. *$p < 0.05$ compared to the negative control by ANOVA, followed by the Bonferroni's multiple comparison test.

respectively. DOX, at 0.5 μg/mL, reduced the number of viable cells by 57.5% after 48 h.

Cell death was also quantified by flow cytometry using annexin V-FITC and PI double staining in HCT116 cells after 24 and 48 h of treatment (Figs. 2 and 3). Treatment with EOB and EOL increased the percentage of apoptosis cells after 48 h of treatment ($P < 0.05$). After 48 h of treatment, EOB led 70.4% of apoptosis (early apoptosis + late apoptosis), while EOL caused 80.2% at a concentration of 20 μg/mL. DOX also increased the percentage of apoptotic cells ($P < 0.05$). The mitochondrial transmembrane potential was assessed in HCT116 cells treated with EOB and EOL by flow cytometry using staining with rhodamine 123. Both oils also caused loss of the mitochondrial transmembrane potential (Fig. 4). In addition, the cell morphology of HCT116 cells stained with May-Grunwald-Giemsa was also analyzed under an optical microscope. Both oils led to cell shrinkage, chromatin condensation and DNA fragmentation (Fig. 5).

The fragmentation of internucleosomal DNA and the cell cycle distribution in HCT116 cells treated with EOB and EOL were measured by DNA content using flow cytometry after 24 and 48 h of treatment, as shown in Figs. 6 and 7. All DNA sub-diploid in size (sub-G_0/G_1) was considered fragmented. Treatment with EOB and EOL induced a significant increase in cells with DNA fragmentation ($P < 0.05$). DOX also significantly caused fragmentation of internucleosomal DNA ($P < 0.05$). At the concentrations of 5, 10, and 20 μg/mL, EOB increased DNA fragmentation to 8.8%, 8.4%, and 19.5%, while EOL increased DNA fragmentation to 8.7%, 16.7%, and 47.8%, against 7.0% observed in the control group after 48 h of treatment, respectively. The phases of the cell cycle G_0/G_1, S, and G_2/M were reduced proportionally. DOX, at 0.5 μg/mL, caused cell cycle arrest in the G_2/M phase.

3.4. *In vivo* antitumor caused by essential oils from bark and leaves of *Virola surinamensis*

The in vivo antitumor activity of essential oils from bark and leaves of *Virola surinamensis* was investigated in a human colon carcinoma xenograft model using C.B-17 SCID mice with HCT116 cell xenografts (Fig. 8). The animals were treated with a dose of 40 mg/kg intraperitoneally, once a day for 15 consecutive days. Both oils reduced the development of the HCT116 tumor in vivo. In EOB-treated animals, mean tumor weight was 0.27 ± 0.05 g, while in EOL-treated animals, mean tumor weight was 0.35 ± 0.05 g versus 0.65 ± 0.06 g observed in the control group. The rates of tumor inhibition in vivo were 57.9% for EOB and 44.8% for EOL. 5-FU was used as a positive control and showed a tumor inhibition rate in vivo of 56.1%. Through H&E, the histological changes of xenograft tumors were analyzed in all experimental groups (EOB and EOL), compared with negative control and 5-FU groups (Fig. 9). All groups exhibited a solid epithelial tumor with clear intratumor heterogeneity, aberrant mitosis and cellular pleomorphism. Areas of necrosis were found common to all groups, being more evident in the tumors of animals treated with 5-FU and EOL. An increase in secretory cells and the infiltration of inflammatory cells in the tumor and peripheral tissue were observed in EOL group.

Toxicological parameters were also observed in animals treated with EOB and EOL. There was no significant change in body weight or in the relative weight of organs in any of the groups evaluated ($P > 0.05$) (Table 4). Hematological parameters were analyzed. The data reveal that there were no statistically significant changes in the hematological parameters evaluated in the peripheral blood samples from animals treated with EOB and EOL ($P > 0.05$) (Table 5).

Morphological analyzes of the liver, kidneys, lungs and hearts in all groups were performed (Fig. 10). In the kidneys, tissue architecture was preserved in all experimental groups, but morphological changes were observed, such as vascular congestion and thickening of the basal membrane of the renal glomerulus, with decreased urinary space in all kidneys, ranging from mild to severe. In livers, acinar architecture and centrilobular veins were also preserved in the EOB and EOL groups and

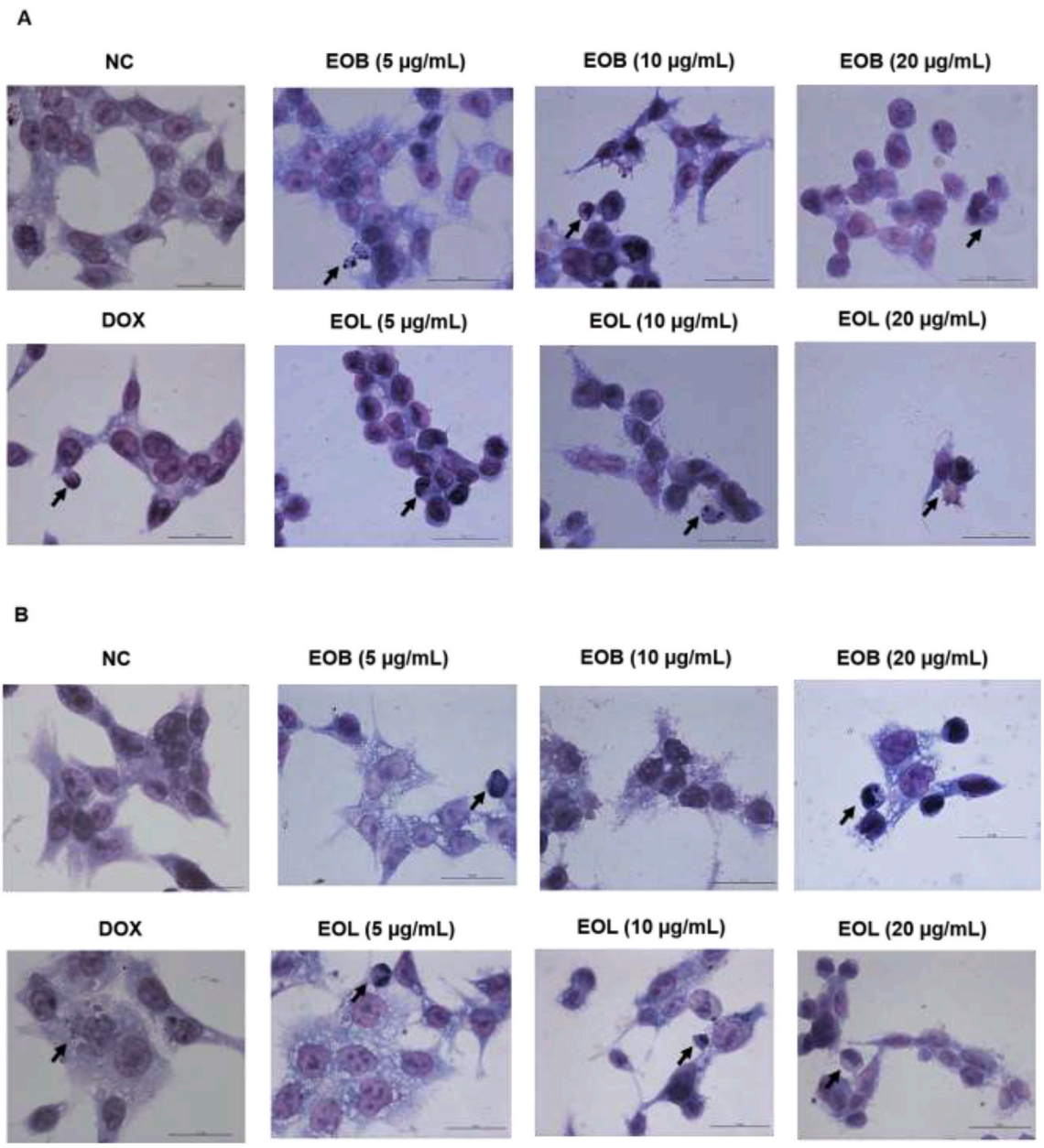

Fig. 5. Effect of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* on HCT116 cell morphology after 24 (**A**) and 48 (**B**) h of treatment. The cells were stained with May-Grunwald-Giemsa and examined by optical microscopy (bar = 50 µm). The negative control (NC) was treated with a vehicle (0.2% DMSO) used to dilute EOB and EOL, and doxorubicin (DOX, 0.5 µg/mL) was used as a positive control. Arrows indicate cell shrinkage, cells with nuclear condensation or DNA fragmentation.

partially preserved in the negative and positive control groups, DMSO and 5-FU respectively. Focal areas of inflammation and congestion were observed in all experimental groups. Other findings, such as hydropic degeneration and coagulation necrosis, were found in the DMSO and 5-FU groups, ranging from mild to moderate. In the lungs, the architecture of the parenchyma was partially maintained in all groups, while a thickening of the alveolar septum was observed, resulting in a decrease in air space, ranging from focal to generalized areas in the analyzed tissues. Histopathological analyses of the lungs revealed significant inflammation, predominantly acute, with edema, pulmonary parenchyma cell hyperplasia and vascular congestion. Focal hemorrhage and hemosiderin pigments were observed in the lungs of some animals in this study. It is important to note that tumor embolism were observed in the lungs of two animals in the negative control group (DMSO 5%). The hearts of the animals in this study showed no significant morphological changes.

4. Discussion

In this study, we demonstrated for the first time that essential oils from bark and leaves of *V. surinamensis* inhibit the proliferation of HCT116 cells in vitro and in vivo models. The main constituents in EOB were aristolene, α-gurjunene, valencene, germacrene D, δ-guaiene and β-elemene, while EOL presented α-farnesene, β-elemene, bicyclogermacrene, germacrene D and α-cubebene. Interestingly, previous work describing the circadian and seasonal variations of the leaves of *V. surinamensis* showed that monoterpenes dominanted sesquiterpenes during the dry season in the Amazon (June to November), while the opposite occurs during the rainy season (December to April) (Lopes et al., 1997). Our records reinforce those findings, since the collection took place in the rainy season.

In our cytotoxic screening program, extracts/essential oils with IC_{50} values below 30 µg/mL in assay based on cancer cells are considered

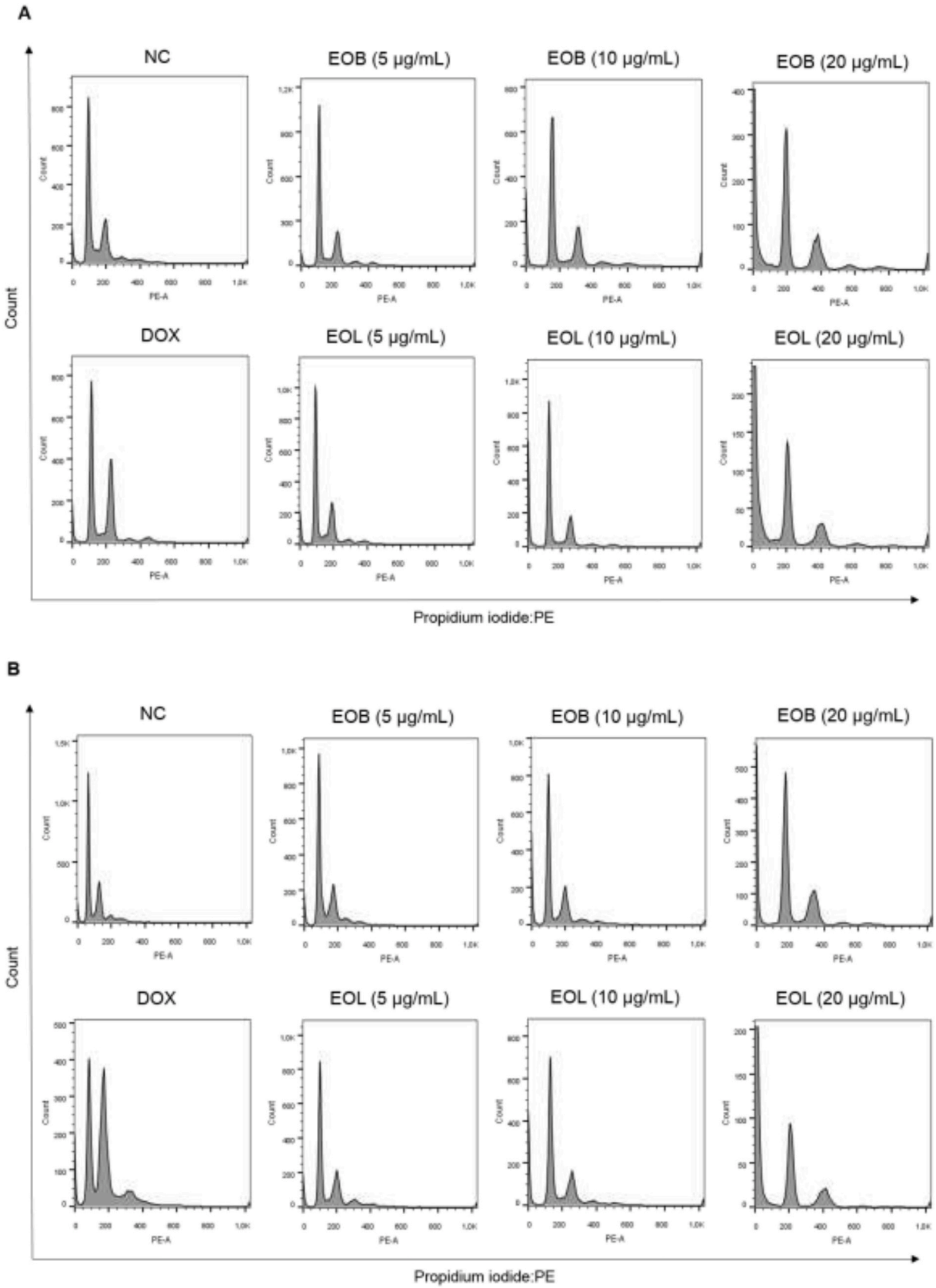

Fig. 6. Representative flow cytometric histograms of the cell cycle distribution of HCT116 cells treated with essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* after 24 (**A**) and 48 (**B**) h of treatment. The negative control (NC) was treated with a vehicle (0.2% DMSO) used to dilute EOB and EOL, and doxorubicin (DOX, 0.5 µg/mL) was used as a positive control.

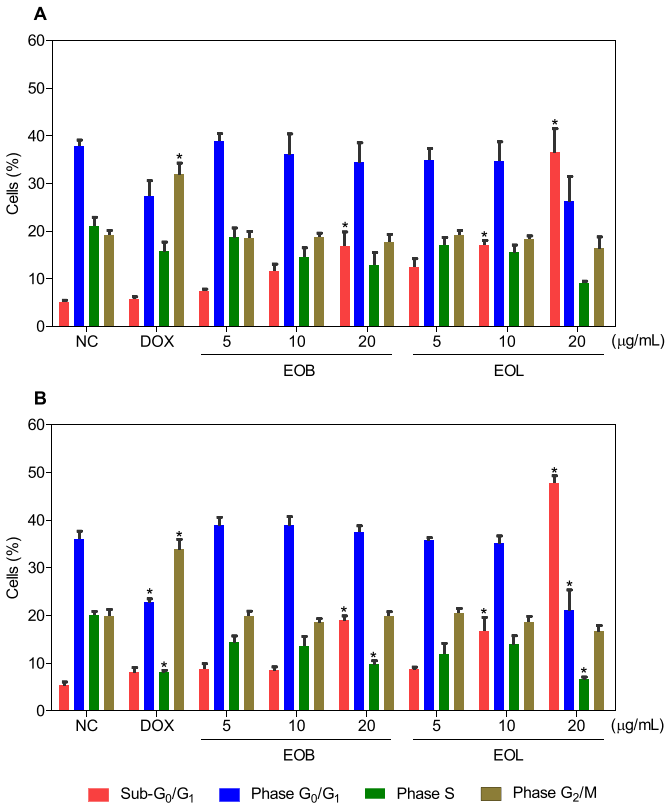

Fig. 7. Effect of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* on the cell cycle distribution of HCT116 cells after 24 (**A**) and 48 (**B**) h of treatment. The negative control (NC) was treated with a vehicle (0.2% DMSO) used to dilute EOB and EOL, and doxorubicin (DOX, 0.5 μg/mL) was used as a positive control. Data are presented as mean ± S.E.M. of three independent experiments carried out in duplicate. Ten thousand events were evaluated per experiment and cell debris was omitted from the analysis. *$P <$ 0.05 compared to the negative control by ANOVA, followed by the Bonferroni's multiple comparison test.

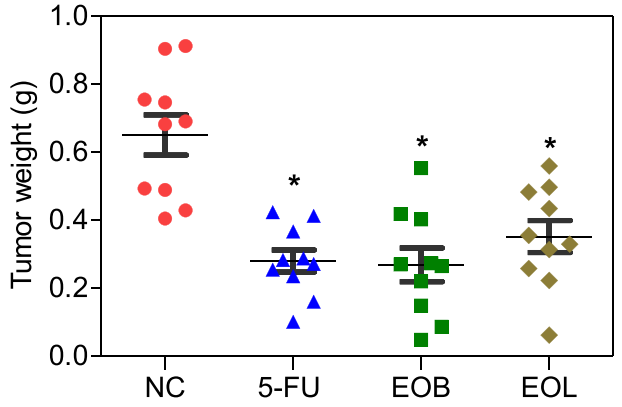

Fig. 8. In vivo antitumor effect of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* in C.B-17 SCID mice with HCT116 cell xenografts. The negative control (NC) was treated with a vehicle (5% DMSO) used to dilute EOB (40 mg/kg) and EOL (40 mg/kg), and 5-fluorouracil (5-FU, 10 mg/kg) was used as a positive control. Beginning 1 day after tumor implantation, the animals were treated intraperitoneally for 15 consecutive days. Data are presented as mean ± S.E.M. of 10 animals. *$p < 0.05$ compared to the negative control by ANOVA, followed by the Bonferroni's multiple comparison test.

promising (Suffness and Pezzuto, 1990; Ribeiro et al., 2012; Ferraz et al., 2013; Rodrigues et al., 2015, 2019; Silva et al., 2016). Both oils showed IC_{50} values below 30 μg/mL and were selected for future studies. There are no reports of cytotoxicity of another essential oil obtained from *Virola* species; however, (Z)-3,5,4′-trimethoxystilbene, a polyphenol found in the *Virola cuspidate* and *Virola elongata*, is cytotoxic to Caco-2 (human colon adenocarcinoma, IC_{50} = 0.25 μM), SW-480 (human colon adenocarcinoma, IC_{50} = 0.23 μM), SW-620 (metastatic cells derived from SW-480, IC_{50} = 0.20 μM), KB (human head and neck cancer, IC_{50} = 0.08 μM) and TK-6 cells (human lymphoma, IC_{50} = 0.10 μM). It also inhibited the polymerisation of tubulin and caused the interruption of the cell cycle in the G_2/M phase of Caco-2 cells (Chabert et al., 2006).

Similar to our cytotoxic potential found in the essential oils of *V. surinamensis,* some essential oils from plants of the Amazon rainforest biodiversity have also been reported as potent cytotoxic agents with IC_{50} values below 30 μg/mL in cancer cells. These include *Guatteria friesiana* (W. A. Rodrigues) Erkens & Maas (Annonaceae) (Britto et al., 2012), *Guatteria blepharophylla* Mart. (Annonaceae) (Ferraz et al., 2014), *Guatteria hispida* (R.E. Fr.) Erkens & Maas (Annonaceae) (Ferraz et al., 2014), *Croton matourensis* Aubl. (Euphorbiaceae) (Lima et al., 2018), *Eugenia uniflora* L. (Myrtaceae) (Figueiredo et al., 2019), *Iryanthera polyneura* Ducke (Myristicaceae) (Martins et al., 2019), *Cyperus articulatus* L. (Cyperaceae) (Nogueira et al., 2020), *Conobea scoparioides* (Cham. & Schltdl.) Benth. (Plantaginaceae) (Lima et al., 2020) and *Guatteria megalophylla* Diels (Annonaceae) (Costa et al., 2020).

Here, we also demonstrated that the essential oils of *V. surinamensis* induce apoptotic-like cell death in HCT116 cells, as observed by the morphological characteristics of apoptosis, externalization of phosphatidylserine and fragmentation of internucleosomal DNA. The amount of EOB and EOL was a limitation to evaluate them in additional in vitro and in vivo protocols. Ferraz et al. (2014) found cell morphology related to apoptotic cell death, increased fragmentation of internucleosomal DNA and activation of caspase-3 in HepG2 cells treated with *G. blepharophylla* and *G. hispida* leaf essential oils. *C. matourensis* leaf essential oil caused cell death in HepG2 cells associated with the externalization of phosphatidylserine and DNA fragmentation without loss of cell membrane integrity (Lima et al., 2018). Future investigations should be directed to EOB and EOL to better understand their mechanism of action.

Cytotoxic studies with the main constituents of EOB and EOL are scarce, and only β-elemene has been reported as cytotoxic agent. β-Elemene is a sesquiterpene found in the traditional Chinese medicine plant *Curcuma longa* L. (Zingiberaceae), which is used to treat different types of cancer (Zhai et al., 2019). Liu et al. (2017) reported that β-elemene induces oxidative stress and apoptosis of non-small-cell lung cancer cells by PERK/IRE1alpha/ATF6 pathway. It also inhibits proliferation of glioblastoma cells through p38 MAPK Activation (Yao et al., 2008). β-Elemene also causes G_2/M phase arrest and led to apoptosis in liver cancer cells by augment of Fas and FasL (Dai et al., 2013). Since β-Elemene represents only part of EOB and EOL, the mixture of the main and minor constituents of these essential oils must be responsible for their cytotoxic potential.

The essential oils of *V. surinamensis* also inhibited the development of HCT116 cells in the xenograft model. At a dose of 40 mg/kg, tumor mass inhibition rates were 57.9 and 44.8% in animal treated with EOB and EOL, respectively. The antitumor effect of *G. friesiana* leaf essential oil was previously studied in mice with sarcoma 180 and showed tumor growth inhibition rates of 43.4–54.2% and 6.6–42.8%, when administrated intraperitoneally (50 and 100 mg/kg) and orally (100 and 200 mg/kg), respectively (Britto et al., 2012). The essential oil of *G. megalophylla* leaf EO (50 and 100 mg/kg) was also evaluated in C. B-17 SCID mice with HL-60 cell xenografts and exhibited tumor mass inhibition rates of 16.6–48.8% (Costa et al., 2020).

Regarding the bioavailability of essential oil constituents, Michiels et al. (2008) demonstrated an almost total absorption in the stomach and in the proximal small intestine of carvacrol, thymol, eugenol and

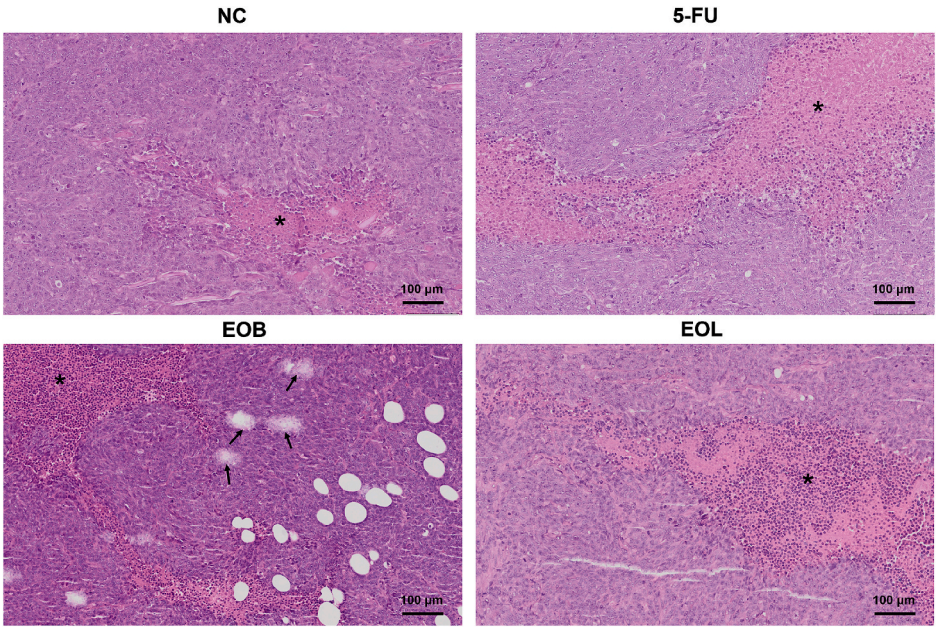

Fig. 9. Representative histological analysis of tumors stained with hematoxylin and eosin and analyzed by optical microscopy. Asterisks indicate areas of necrosis with infiltration of inflammatory cells and arrows indicate the presence of mucous material. The negative control (NC) was treated with a vehicle (5% DMSO) used to dilute EOB (40 mg/kg) and EOL (40 mg/kg), and 5-fluorouracil (5-FU, 10 mg/kg) was used as a positive control.

Table 4
Effect of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* on the body and on the relative weight of the organs of C.B-17 SCID mice with HCT116 cell xenografts.

Parameters	NC	5-FU	OEB	OEL
Dose (mg/kg)	–	10	40	40
Survival	10/10	10/10	10/10	10/10
Initial body weight (g)	23.40 ± 0.31	23,20 ± 0.93	21.85 ± 0.50	23.40 ± 0.61
Final body weight (g)	19.83 ± 0.53	17.03 ± 0.99	19.70 ± 0.55	19.09 ± 0.36
Liver (g/100 g body weight)	4.26 ± 0.14	4.60 ± 0.26	4.70 ± 0.19	4.85 ± 0.23
Kidney (g/100 g body weight)	1.42 ± 0.04	1.41 ± 0.12	1.50 ± 0.08	1.51 ± 0.07
Heart (g/100 g body weight)	0.51 ± 0.02	0.58 ± 0.05	0.55 ± 0.03	0.54 ± 0.02
Lung (g/100 g body weight)	0.74 ± 0.02	0.81 ± 0.97	0.74 ± 0.06	0.77 ± 0.03

Data are presented as mean ± S.E.M. of 10 animals. The negative control (NC) was treated with a vehicle (5% DMSO) used to dilute EOB and EOL, and 5-fluorouracil (5-FU, 10 mg/kg) was used as a positive control.

Table 5
Effect of essential oils from bark (EOB) and leaves (EOL) of *Virola surinamensis* on hematological parameters of peripheral blood samples from C.B-17 SCID mice with HCT116 cell xenografts.

Parameters	NC	5-FU	OEB	OEL
Dose (mg/kg)	–	10	40	40
Erythrocytes (10^6/mm^3)	7.44 ± 0.79	6.88 ± 0.83	8.94 ± 0.39	8.46 ± 0.51
Hemoglobin (g/dL)	13.20 ± 1.04	9.37 ± 1.17	12.2 ± 0.55	11.4 ± 0.63
Hematocrit (%)	41.04 ± 3.40	29.43 ± 3.55	37.54 ± 1.62	36.29 ± 2.17
MCV (fL)	45.43 ± 2.10	42.71 ± 0.29	42.29 ± 0.18	43.00 ± 0.38
Platelets (10^3/mm^3)	1072.0 ± 137.0	916.9 ± 110.0	1486.0 ± 78.18	1297.0 ± 90.99
Leukocytes (10^3/mm^3)	8.90 ± 1.65	7.69 ± 1.72	9.63 ± 0.86	9.0 ± 0.79
Differential leukocytes (%)				
Granulocytes	23.06 ± 2.41	21.35 ± 3.19	17.73 ± 1.85	26.73 ± 2.42
Lymphocytes	60.13 ± 2.65	63.20 ± 3.33	65.4 ± 2.5	54.5 ± 2.7
Monocytes	16.81 ± 0.55	15.45 ± 0.37	16.86 ± 0.79	17.31 ± 0.70

Data are presented as mean ± S.E.M. of 7 animals. The negative control (NC) was treated with a vehicle (5% DMSO) used to dilute EOB and EOL, and 5-fluorouracil (5-FU, 10 mg/kg) was used as a positive control.

trans-cinnamaldehyde after a single dose mixed with the feed (13.0, 13.2, 12.5 and 12.7 mg/kg of body weight, respectively), administered orally to piglets. In another study with eugenol, oral administrations of 40 mg/kg eugenol for 5 days in male Sprague-Dawley rats had a long biological half-life, indicating accumulation of this compound after repeated administration (Guénette et al., 2007). Thymol was not detected in plasma or urine; however, the metabolites thymol sulfate and thymol glucuronide were found in the urine after oral

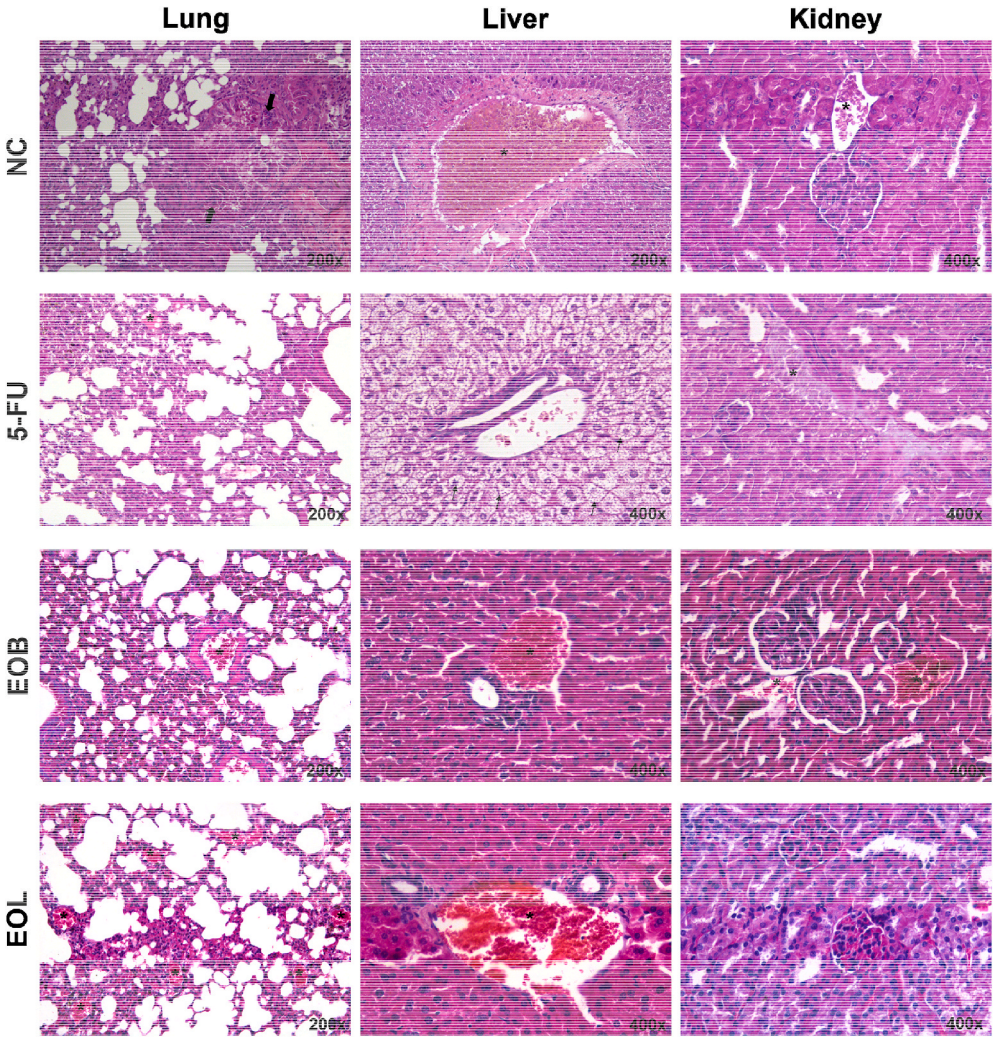

Fig. 10. Representative histological analysis of the lungs, livers and stained with hematoxylin and eosin and analyzed by optical microscopy. The negative control (NC) was treated with a vehicle (5% DMSO) used to dilute EOB (40 mg/kg) and EOL (40 mg/kg), and 5-fluorouracil (5-FU, 10 mg/kg) was used as a positive control. Asterisks indicate the areas of vascular congestion; thick arrows represent tumor emboli in the lung and thin arrows indicate microgoticular steatosis in the liver.

administration to 12 healthy volunteers at a dose equivalent to 1.08 mg (Kohlert et al., 2002). In addition to these studies by oral administration, the intraperitoneal route, used in this work with EOB and EOL, has greater absorption than the oral route. Although there are no investigations available for the main constituents of EOB and EOL, due to their chemical structure and similar physicochemical characteristics, these compounds may also have adequate bioavailability.

In summary, the essential oils from bark and leaves of *V. surinamensis* have chemical constituents dominated by sesquiterpenes. Both oils also induce cell death in HCT116 cells and inhibit tumor growth in xenograft model, indicating promising antitumor activity in colon cancer cells.

Author contributions

Conceived and designed the experiments: TAA, RBD, HHFK and DPB. Collected the plant material and performed the chemical experiments: EJSPL, EVC, FMAS and HHFK. Performed the in vitro and in vivo experiments: TAA, RGAC, VRS and LSS. Performed the histological analysis: RBD and CAGR. Analyzed the data: TAA, MBPS, RBD, CAGR, HHFK and DPB. Contributed reagents/materials/analysis tools: MBPS, CAGR, HHFK and DPB. Wrote the paper: DPB.

Declaration of competing interest

The authors have declared that there are no conflicts of interest.

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