Essential Oil from Bark of Aniba parviflora (Meisn.) Mez (Lauraceae) Reduces HepG2 Cell Proliferation and Inhibits Tumor Development in a Xenograft Model

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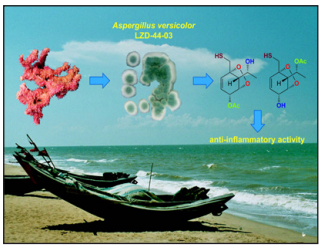

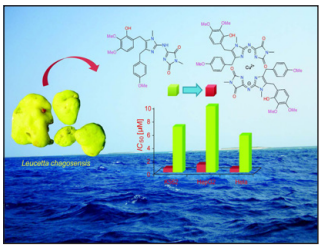

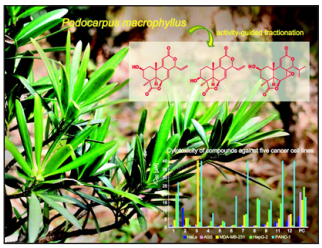

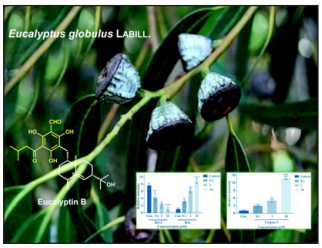

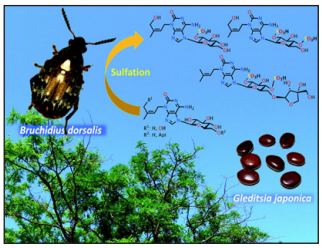

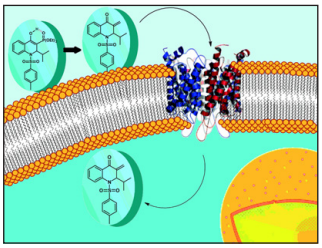

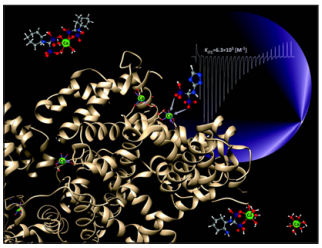

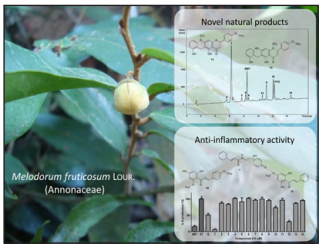

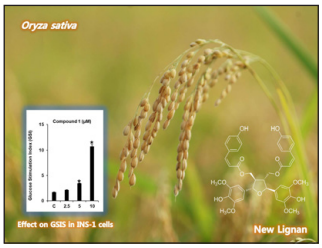

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FULL PAPER

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Essential Oil from Bark of *Aniba parviflora* (Meisn.) Mez (Lauraceae) Reduces HepG2 Cell Proliferation and Inhibits Tumor Development in a Xenograft Model

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Aniba parviflora (Meisn.) Mez (Lauraceae) is an aromatic plant of the Amazon rainforest, which has a tremendous commercial value in the perfumery industry; it is popularly used as flavoring sachets and aromatic baths. In Brazilian folk medicine, *A. parviflora* is used to treat victims of snakebites. Herein, we analyzed the chemical composition of *A. parviflora* bark essential oil (EO) and its effect on the growth of human hepatocellular carcinoma HepG2 cells *in vitro* and *in vivo*. EO was obtained by hydrodistillation and characterized by GC-MS and GC-FID. The main constituents of EO were linalool (16.3 ± 3.15), α-humulene (14.5 ± 2.41 %), δ-cadinene (10.2 ± 1.09 %), α-copaene (9.51 ± 1.12 %) and germacrene B (7.58 ± 2.15 %). Initially, EO's cytotoxic effect was evaluated against five cancer cell lines (HepG2, MCF-7, HCT116, HL-60 and B16-F10) and one non-cancerous one (MRC-5), using the Alamar blue method after 72 h of treatment. The calculated IC_{50} values were 9.05, 22.04, > 50, 15.36, 17.57, and 30.46 μg/mL, respectively. The best selectivity was for HepG2 cells with a selective index of 3.4. DNA Fragmentation and cell cycle distribution were quantified in HepG2 cells by flow cytometry after a treatment period of 24 and 48 h. The effect of EO on tumor development *in vivo* was evaluated in a xenograft model using C.B-17 SCID mice engrafted with HepG2 cells. *In vivo* tumor growth inhibition of HepG2 xenograft at the doses of 40 and 80 mg/kg were 12.1 and 62.4 %, respectively.

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Keywords: *Aniba parviflora*, antitumor agents, cytotoxicity, HepG2, Lauraceae.

Introduction

Aniba parviflora (Meisn.) Mez (synonym Aniba fragrans Ducke) (Lauraceae), popularly known as 'macacaporanga' or 'louro rosa', is an aromatic plant of 15 to 30 meters found in the Amazon rainforest of South American countries, such as Brazil, Guyana, Suriname, Peru, Colombia and Venezuela.[1] This plant has great commercial value in perfumery industry due to the presence of about 35% of linalool in its essential oil.[2] Because they are very similar morphologically, *A. parviflora* and *A. rosaeodora* are confused by small farmers and oil producers, whose the latter has a linalool content between 78% and 93%.[3,4] In addition, the branches and wood of *A. parviflora* are dried and powdered for use as flavoring sachets.[5] Its leaves are also used to prepare 'banhos de cheiro' (aromatic baths), common during the annual São João festival (a June Brazilian folk festival).[6]

In the Brazilian Amazon, *A. parviflora* barks are used in folk medicine in the preparation of teas by decoction for the treatment of snakebite victims.[7] Interestingly, the anti-snakebite effect of the aqueous extract of the bark of *A. parviflora* was reported, while it was able to inhibit the hemorrhagic activity induced by *Bothrops jararaca* venom.[7] The ethanolic extract of the leaves showed an antiparasitic effect against *Plasmodium falciparum*, *Trypanosoma cruzi* and *Leishmania amazonensis*.[8] In addition, the essential oils of *A. parviflora* from leaves and/or branches have antioxidant,[9] antidepressant,[10] antibacterial,[9,11,12] anesthetic[13] and cytotoxic[9] activities. Moreover, 6-aryl-4-methoxy-2-pyrones, 6-*trans*-styryl-4-methoxy-2-pyrones, 6-aryl-2-pyrones, 6-styryl-2-pyrones, 6-(4′-hydroxy-*trans*-styryl)-2-pyrone and 6-(3′,4′-dihydroxy-*trans*-styryl-2-pyrone were isolated from trunk wood of *A. parviflora*.[14,15] Tetrahydroyangonin and dihydromethysticin were also identified in ethanolic branch extract of *A. parviflora*.[8]

In this present work, we analyzed the chemical composition of *A. parviflora* bark essential oil (EO) and its effect on the growth of human hepatocellular carcinoma HepG2 cells in the culture and development of tumors in a xenograft model. Interestingly, this EO inhibited the development of HepG2 cells in both *in vitro* and *in vivo*.

Results and Discussion

Chemical Analysis of Aniba Parviflora *Bark Essential Oil*

The oil recovery was 2.21 ± 0.47% (*w/w*), in which sesquiterpenes were prevalent (> 70%) (*Table S1*). This sample displayed 26 compounds that were satisfactorily identified, comprising more than 99% of the EO composition. The oxygenated aliphatic monoterpene linalool (16.3 ± 3.15%) was the main compound of this EO, as already observed for leaves and branches of *A. parviflora*,[12] as well as in other species of *Aniba*.[16] Other main compounds were α-humulene (14.5 ± 2.41%), δ-cadinene (10.2 ± 1.09%), α-copaene (9.51 ± 1.12%) and germacrene B (7.58 ± 2.15%).

As mentioned above, EO chemistry of this species was the subject of a previous investigation in search of antioxidant and cytotoxic compounds,[9] in which linalool was observed in the same range as this work. Briefly, the EO from the leaves presented β-phellandrene (15.1%), linalool (14.1%) and γ-eudesmol (12.9%) as main constituents, whereas the EO from the branches presented γ-eudesmol (16.8%), β-caryophyllene (15.7%), linalool (12.4%), β-phellandrene (6.7%) and bicyclogermacrene (6.0%) as the main ones. In this study, leaves and branches of *A. parviflora* were collected in Belém (Pará state, Brazil) during the dry season.[9] Curiously, eudesmol isomers were not observed in this work. Moreover, linalool was also observed in the same range in a study carried out with *A. parviflora* collected Santarém (Pará state, Brazil) during the rainy season (22.8% for leaves and 11.90% for branches).[12] This chemical variation may be attributed to soil nutrients, age and development stage of the plant, presence of predators, abiotic factors, *etc.*

Aniba Parviflora *Bark Essential Oil Induces Cytotoxicity to Cancer Cells in Culture*

The evaluation of cytotoxicity was carried out by Alamar blue method on five cancer cells (HepG2, MCF-7, HCT116, HL-60 and B16-F10) and one non-cancerous cells (MRC-5) after 72 h of treatment with EO in a concentration-response curve. The half-maximum inhibitory concentration (IC_{50}) values found are displayed in *Table 1*. The OE showed IC_{50} values of 9.05 μg/mL for HepG2, 22.04 μg/mL for MCF-7, > 50 μg/mL for HCT116, 15.36 μg/mL for HL-60 and

Table 1. IC_{50} values of the cytotoxicity of *Aniba parviflora* bark essential oil (EO).

Cells	Histological type	IC_{50}[a] DOX[b]	5-FU[b]	EO
Cancer cells				
HepG2	human hepatocellular carcinoma	0.03 0.01–0.19	0.46 0.22–0.65	9.05 1.70–18.02
MCF-7	human breast adenocarcinoma	0.28 0.20–0.39	1.74 0.71–2.22	22.04 18.45–26.33
HCT116	human colon carcinoma	0.13 0.08–0.21	0.63 0.35–1.14	>50
HL-60	human promyelocytic leukemia	0.04 0.02–0.08	1.91 1.32–2.58	15.36 9.91–23.80
B16-F10	mouse melanoma	0.20 0.17–0.23	0.72 0.45–0.98	17.57 15.69–19.67
Non-cancerous cells				
MRC-5	human lung fibroblast	2.09 1.51–2.89	5.81 4.79–8.23	30.46 24.42–37.99

[a] Data are presented as IC_{50} values with 95% confidence interval obtained from three independent experiments carried out in duplicate, as measured by Alamar blue method after 72 h of treatment. [b] Doxorubicin (DOX) and 5-fluorouracil (5-FU) were used as positive controls.

17.57 μg/mL for B16-F10. In non-cancerous cells, EO displayed an IC_{50} value of 30.46 μg/mL. The best selectivity was found for HepG2 cells, while the EO presented SI (= selectivity index) of 3.4. *Table 2* shows the calculated SI. Doxorubicin was used as a positive control and presented IC_{50} values ranging from 0.03 to 0.28 μg/mL for HepG2 and MCF-7, respectively, and an IC_{50} value of 2.09 μg/mL for MRC-5 cells. 5-Fluorouracil was also used as a positive control and exhibited IC_{50} values ranging from 0.46 to 1.91 μg/mL for HepG2 and HL-60, respectively, and an IC_{50} value of 5.81 μg/mL for MRC-5 cells.

Table 2. Selectivity indices of *Aniba parviflora* bark essential oil (EO).[a]

Cancer cells[b]	Non-cancerous cells[c]		
	MRC-5		
	DOX[d]	5-FU[d]	EO
HepG2	68.7	12.6	3.4
MCF-7	7.4	3.3	1.4
HCT116	15.9	9.2	N.d.[e]
HL-60	51.5	3.0	2.0
B16-F10	10.3	8.1	1.7

[a] Data are presented as selectivity index (SI) calculated using the following formula: SI = IC_{50}[non-cancerous cells]/IC_{50}[cancer cells]. [b] Cancer cells: HepG2 (human hepatocellular carcinoma); MCF-7 (human breast adenocarcinoma); HCT116 (human colon carcinoma); and HL-60 (human promyelocytic leukemia); B16-F10 (mouse melanoma). [c] Non-cancerous cells: MRC-5 (human lung fibroblast). [d] Doxorubicin (DOX) and 5-fluorouracil (5-FU) were used as positive controls. [e] N.d: not determined.

In our screening program for the discovery and development of cytotoxic drugs for cancer therapy, extracts/essential oils with IC_{50} values < 30 μg/mL, in cytotoxic assays using cancer cells, are considered promising.[17–21] Therefore, we tested the EO of *A. parviflora* bark in a range of concentration of 0.4 to 50 μg/mL and we found an IC_{50} value of 9.05 μg/mL for HepG2 cells and an SI of 3.4, suggesting it as a potential phytotherapeutic with action against liver cancer. Corroborating these data, Silva *et al.*[9] previously reported the cytotoxicity of *A. parviflora* EO obtained from leaves (IC_{50} = 67.9 μg/mL) and branches (IC_{50} = 102.2 μg/mL) against MCF-7 cells. The cytotoxicity of EO of *A. parviflora* bark was evaluated for the first time at this communication.

Interestingly, EO extracted from rosewood *A. rosaeodora*, also rich in linalool, showed cytotoxicity in the A431 cell line of human epidermoid carcinoma, in immortalized HaCaT cells, in normal keratinocytes HEK001 transformed by HPV16 E6/E7, and in primary

normal human keratinocytes NHEK.[22] Linalool has also been reported as a cytotoxic agent, which showed an IC_{50} value of 2.59 and 11.02 μM in human histiocytic lymphoma U937 cells and human cervical adenocarcinoma HeLa cells, respectively.[23]

Cell morphology was analyzed by flow cytometry in EO-treated HepG2 cells after 24 and 48 h. Interestingly, EO induced a reduction in cell volume, as observed by the drop in forward light scatter after 48 h of treatment (*Figure S1*), morphological changes characteristic of apoptotic cell death. No alteration was observed after 24 h of treatment (data not shown).

The content of intracellular DNA was measured by flow cytometry in EO-treated HepG2 cells after 24 and 48 h of treatment to quantify the fragmentation of internucleosomal DNA and the distribution of the cell cycle. All DNA sub-diploid in size (sub-G_1) was considered internucleosomal DNA fragmentation. HepG2 cells treated with EO significantly increased the fragmentation of internucleosomal DNA after 48 h of treatment ($P < 0.05$; *Figure 1*). The EO increased DNA fragmentation to 7.9%, 8.3%, and 19.1%, at concentrations of 5, 10, and 20 μg/mL, respectively, against 7.0% found in the control group after 48 h of treatment, indicating induction of cell death. No alteration was observed after 24 h of treatment (data not shown). Doxorubicin, at 0.5 μg/mL, also caused DNA fragmentation.

A. rosaeodora EO caused caspase-dependent cell death in precancerous and cancerous skin cells, along with the production of reactive oxygen species, induced depolarization of the mitochondrial membrane and externalization of phosphatidylserine.[22] It also caused cell cycle arrest in U937 cells in the G_0/G_1 phase and HeLa cells in the G_2/M phase, followed by apoptotic cell death.[23] Likewise, linalool induced the apoptosis of HCT116 cells, inducing cancer-specific oxidative stress.[24]

Aniba Parviflora *Bark Essential Oil Inhibits the Development of HepG2 Cells in a Xenograft Model*

Using C.B-17 severe combined immunodeficient (SCID) mice grafted with HepG2 cells, the EO was evaluated for its ability to inhibit tumor growth in vivo at doses of 40 and 80 mg/kg (i.p. daily for 21 consecutive days; *Figure 2*). In animals treated with EO, the mean tumor weights were 0.40 ± 0.06 g and 0.17 ± 0.04 g at the lowest and the highest doses, respectively. An average of 0.45 ± 0.05 g was observed in the control group. *In vivo* tumor inhibition rates were 12.1–62.4%. 5-Fluorouracil (10 mg/kg) was used as a positive control and exhibited a tumor inhibition rate of 48.4%.

Morphological aspects of xenograft tumors were analyzed by bright field microscopy and hematoxylin and eosin chromogens. The observed patterns were consistent with an encapsulated carcinoma, organized in nodules. Highly malignant hyperchromatic pleomorphic cells and aberrant mitosis were observed in all groups (*Figure 3*). Necrosis and vascular congestion were more frequent in the OE 40 mg/kg and 5-fluorouracil groups, although the OE 40 mg/kg presents smaller nodules compared to the other groups.

The *in vivo* antitumor of linalool has been previously evaluated in immunocompromised mouse model with xenograft human colon carcinoma. Linalool was administered in doses of 100 or 200 μg/kg orally every three days in SCID mice with HCT116 xenografts. At the highest dose, linalool reduced the average tumor weight by 55% when compared with mice in the control group, and no significant weight loss or organ damage was observed.[24] Herein, we demonstrated for the first time that *A. parviflora* bark EO inhibits tumor growth of HepG2 xenograft by 12.1 and 62.4% at the doses of 40 and 80 mg/kg, respectively.

Regarding toxicological aspects, 100% survival was observed in all groups. No significant alteration in body weight or in the relative weight of the organs in any experimental groups was found ($P > 0.05$; *Figure S2*). In addition, no significant alteration was also observed in the hematological parameters from animals treated with EO ($P > 0.05$) (data not shown).

Histopathological analyzes of the liver, kidneys, lungs and heart were performed for all experimental groups (data not shown). Although two animals in the EO groups and in all animals in the 5-fluorouracil group showed focal areas of coagulation necrosis, the hepatic and portal architecture were preserved in most livers. Some alterations include, hydropic degeneration, vascular congestion, focal inflammatory infiltrate, predominantly of mononuclear cells, in portal region, ranging from mild to severe. In the kidneys, the tissue architecture was maintained in negative control, 5-fluorouracil and EO 40 mg/kg groups. Coagulation necrosis of the renal tubules (distal and proximal) was observed in animals treated with EO 80 mg/kg. In the kidneys of all experimental groups, vascular congestion and thickening of the basal membrane of the renal glomerulus was observed, with a decrease in the urinary space, ranging from mild to severe. The architecture of the pulmonary parenchyma was partially maintained and thickening of the alveolar

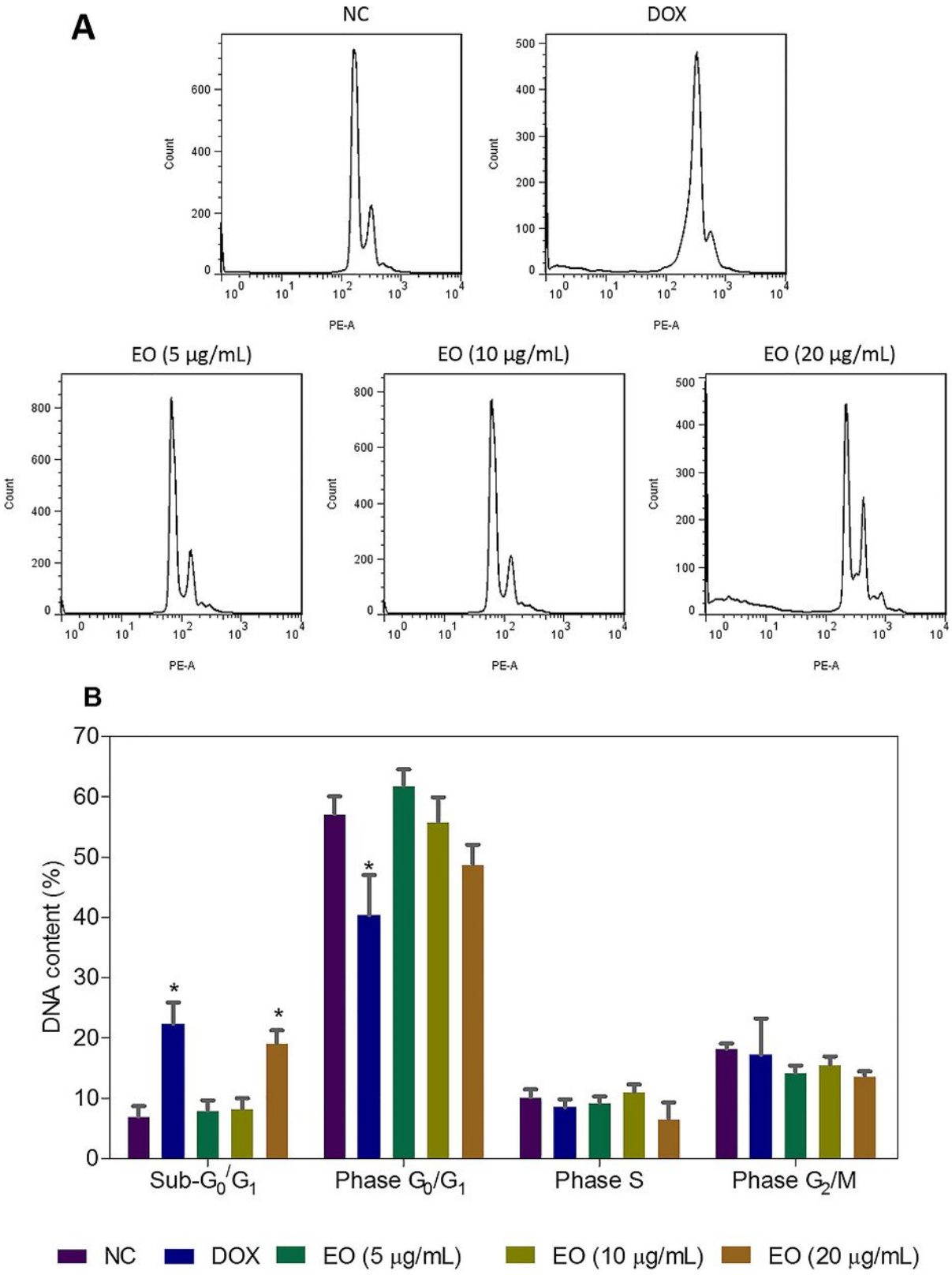

Figure 1. Effect of *Aniba parviflora* bark essential oil (EO) on the cell cycle distribution of HepG2 cells after 48 h of treatment. Data are presented as mean ± S.E.M. of three independent experiments carried out in duplicate. Ten thousands of events were recorded per sample and cell debris was omitted from analyze. Negative control (NC) was treated with the vehicle (0.5 % DMSO). Doxorubicin (DOX, 0.5 µg/mL) was used as a positive control. * $P < 0.05$ compared with the negative control by ANOVA, followed by Bonferroni's multiple comparison test.

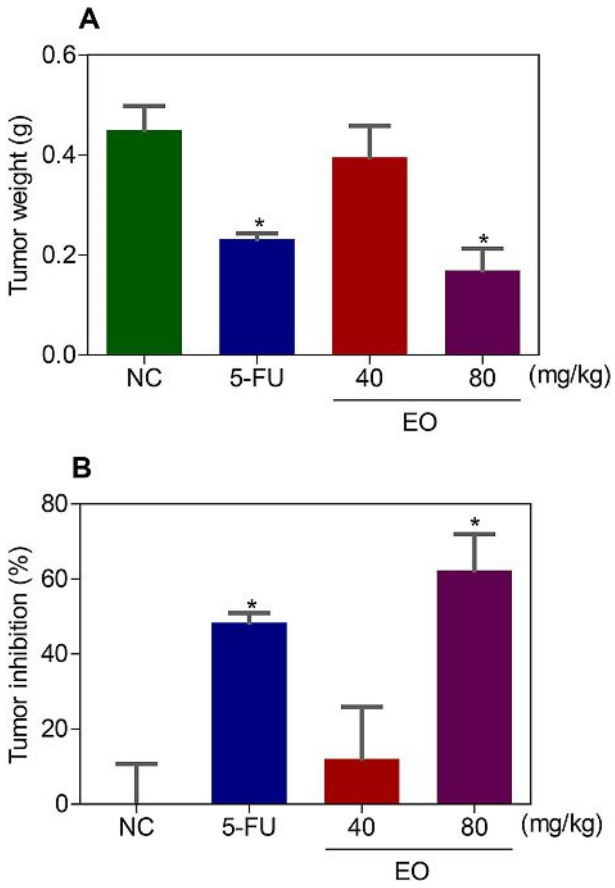

Figure 2. *In vivo* anti-tumor effect of *Aniba parviflora* bark essential oil (EO) in C.B-17 SCID mice with HepG2 cell xenografts. A) Tumor weight [g] after treatment. B) Tumor inhibition [%] after treatment. Negative control (NC) was treated with the vehicle (5% DMSO). 5-Fluorouracil (5-FU, 10 mg/kg) was used as a positive control. Data are presented as mean ± S.E.M. of 10 animals. * $P < 0.05$ compared to the negative control by ANOVA, followed by Bonferroni's multiple comparison test.

septum was observed, with a decrease in air space, varying from mild to moderate, in all experimental groups. Inflammatory infiltrate, predominantly of mononuclear cells, edema, vascular congestion and hemorrhage were observed in the lungs of all groups, ranging from mild to severe. No histological changes were observed in the hearts of all experimental groups.

Conclusion

In summary, *A. parviflora* bark EO shows linalool, α-humulene, δ-cadinene, α-copaene and germacrene B as the main constituents. Interestingly, this EO is capable to suppress the growth of HepG2 cells in culture (in vitro studies), as well as, inhibit the development of tumor in a xenograft model (in vivo study). Overall, these data indicate *A. parviflora* bark EO as a potential phytotherapeutic in cancer therapy.

Experimental Section

Plant Material

Barks of *A. parviflora* were collected from a sample previously identified at the Experimental Farm of Curauá-PEMATEC, municipality of Santarém, state of Pará, Brazil (2°33′45.68″ S and 54°37′00.37″ W) in March of 2014 during the Amazonian winter. A voucher was deposited at the Herbarium of the National Institute for Amazonian Research (#254.490). This work was registered (SISGEN: A7 C3 A27) and carried out under Brazilian law to access genetic resources with exclusively scientific interests.

Essential Oil Extraction and Chemical Analysis

Plant material of *A. parviflora* was extracted through hydrodistillation with a Clevenger-type apparatus. For this, the plant material was dried out in a stove at 40 °C during 24 h. After dried, the material was manually crushed and powdered in a glass blender. For the extraction, a portion of 500 g of the bark powder was extracted for a period of 4 h in 2 L of ultrapure water (18.2 MΩ). Finally, EO obtained was extracted three times with HPLC-grade chloroform, dried with anhydrous Na_2SO_4 and filtered through a nylon membrane (pore size 0.22 μm).

The chemical composition of the EO of *A. parviflora* bark was accessed by gas chromatography coupled to mass spectrometry (GC-MS) analysis with an equipment model *GC-MS/QP2010 Plus* (*Shimadzu*) equipped with a capillary column *Rtx-5MS* (30 m × 0.25 mm × 0.25 μm, *Restek*). Helium in a flow of 1 mL/min was the vehicle and injections of 1 μL were carried out with stock solutions of 0.8 mg/mL in chloroform with a 1 : 50 split ratio. The column temperature program was 55 to 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 obtained by calculating the retention indices (*RI*) according to the equation of Van den Dool and Kratz[25]

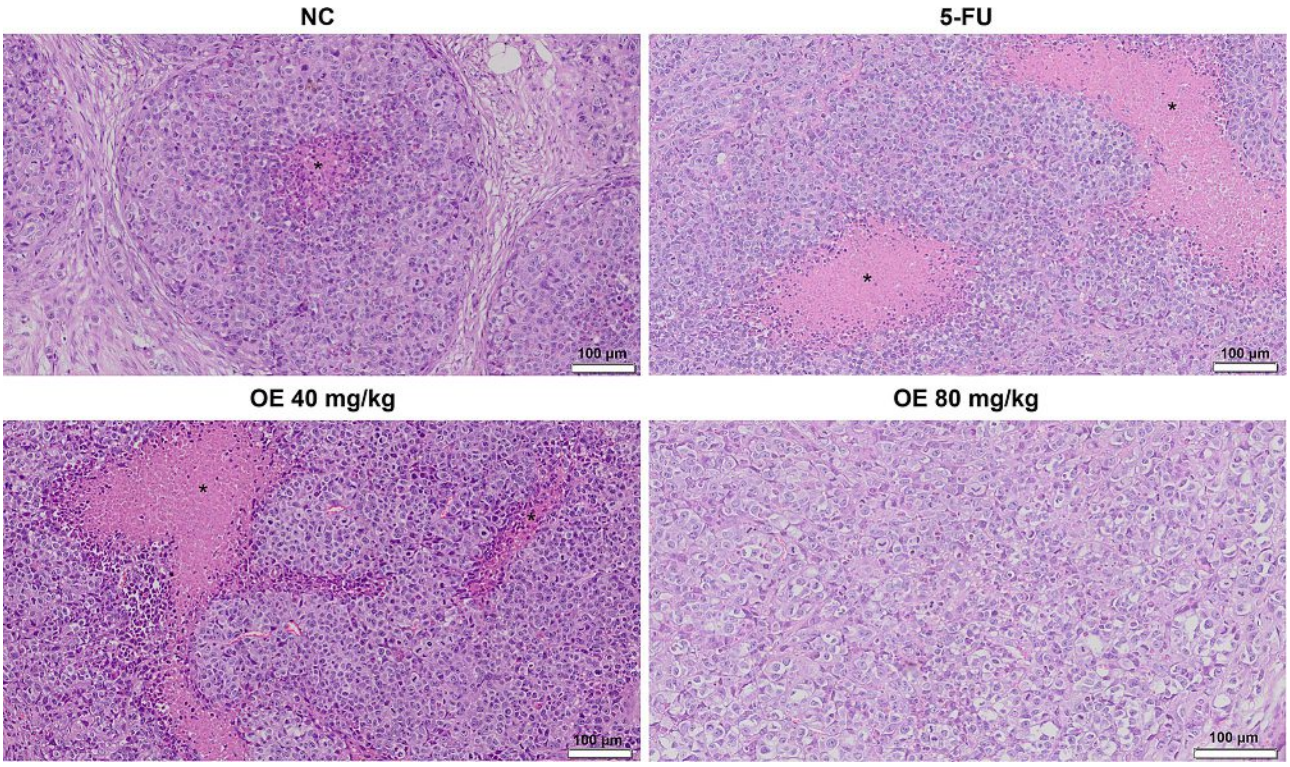

Figure 3. Representative photomicrographs of HepG2 tumors treated with *Aniba parviflora* bark essential oil (EO). Negative control (NC) was treated with the vehicle (5% DMSO). 5-Fluorouracil (5-FU, 10 mg/kg) was used as a positive control. Histological sections were stained with hematoxylin-eosin and analyzed using optical microscopy under 100×magnification (Bar = 100 µm). Asterisks represent areas of tissue necrosis.

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 carried out to obtain the relative quantity of each EO component. For this, gas chromatography with flame ionization detection (GC-FID) was applied. A system consisting of *GC2010* equipment (*Shimadzu*) equipped with a *Rtx-5* capillary column was used under the same conditions as the GC-MS analysis. Relative amounts [%] were calculated in relation to the total chromatogram area of three independent repetitions.

In Vitro *Study*

Cytotoxicity Assay. HL-60 (human promyelocytic leukemia), HepG2 (human hepatocellular carcinoma), HCT116 (human colon carcinoma), MCF-7 (human breast adenocarcinoma), B16-F10 (mouse melanoma) and MRC-5 (human pulmonary fibroblasts) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured following the instructions in the ATCC cell culture guide and were mycoplasma free as tested by mycoplasma staining kit (*Sigma–Aldrich*). Trypan blue exclusion assay was performed to measure the number of viable cells.

Alamar blue method was used to assess the cell viability, as previously described.[26–28] Briefly, cells were plated on 96-well plates (3×10^4 cells/well for suspension cells and 7×10^3 cells/well for adherent cells). EO (concentration range from 0.4 to 50 µg/mL), dissolved in dimethyl sulfoxide (DMSO, *Vetec Química Fina Ltda*, Duque de Caxias, RJ, Brazil), was added to each well and incubated for 72 h. After treatment time, 20 µL of resazurin (0.312 mg/mL, *Sigma–Aldrich*) was added to each well. Absorbances at 570 and 600 nm were quantified by a *SpectraMax 190* Microplate Reader (*Molecular Devices*, Sunnyvale, CA, USA). Doxorubicin (concentration range from 0.04 to 5 µg/mL; purity ≥ 95%, *Laboratory IMA S.A.I.C.*, Buenos Aires, Argentina) and 5-fluorouracil (concentration range from 0.2 to 25 µg/mL) (purity > 99%; *Sigma–Aldrich*) were used as positive controls.

Flow Cytometry Assays. Cell morphology was analyzed by flow cytometry thought light scattering features. Fragmentation of internucleosomal DNA and distribution of the cell cycle were evaluated using propidium iodide (PI).[29] For this, the cells were stained with PI using a permeabilization solution (2 μg/mL PI, 0.1% *triton X-100*, 0.1% sodium citrate and 100 μg/mL RNAse, all from *Sigma–Aldrich*). The cells were incubated in the dark for 15 min and cell fluorescence was acquired by flow cytometry. Ten thousands of events were recorded per sample using a *BD LSRFortessa* cytometer using BD FACSDiva Software (*BD Biosciences*, San Jose, CA, EUA) and analyzed by Flowjo Software 10 (*Flowjo LCC*, Ashland, OR, USA). Cell debris was omitted from analyze.

In Vivo Study

Human Hepatocellular Carcinoma Xenograft Model. Human hepatocellular carcinoma xenograft model was carried out as previously reported.[30–32] Fifty C.B-17 SCID mice (females, 25–30 g) obtained at the animal facilities of the Gonçalo Moniz Institute-FIOCRUZ (Salvador, Bahia, Brazil) were used. All mice were housed in cages with free access to food and water, and under a 12:12 h light-dark cycle (lights on at 6:00 a.m.). The experimental protocol (number #06/2015) was approved by the animal ethics committee of Gonçalo Moniz Institute-FIOCRUZ.

Animals were grafted subcutaneously with HepG2 cells (10^7 cells per 500 μL), in the left armpit and randomly divided into four groups: (G1) vehicle-treated mice (5% DMSO, $n=20$); (G2) 5-FU-treated mice (10 mg/kg, $n=10$); (G3) EO-treated mice (40 mg/kg, $n=10$); and (G4) EO-treated mice (80 mg/kg, $n=10$). These doses were based on previous studies with EO.[33–37] Starting one day after tumor implantation, the animals were treated intraperitoneally for 21 consecutive days. All animals were also observed for signs of abnormality during treatment. On the 22nd day, all mice were anesthetized, using 50 mg/kg thiopental, and peripheral blood samples were collected by brachial artery. Hematological analyzes were carried out using an *Advia 60* hematology system (*Bayer*, Leverkusen, Germany). After that, all mice were euthanized by anesthetic overdose, using 100 mg/kg thiopental, and the tumors, lungs kidneys, livers and hearts were collected, weighed and examined for color change, signs of thick lesion formation and/or hemorrhage. Tumors, lungs kidneys, livers and hearts were also fixed in 4% buffered formalin and embedded in paraffin. The tissue sections stained with hematoxylin/eosin (and periodic acid–Schiff for livers and kidneys) were examined by a pathologist using an optical microscope under 100× magnification (Bar = 100 μm).

Statistical Analysis

Data were shown as mean ± S.E.M. or as IC_{50} and their 95% confidence intervals obtained by non-linear regression. Analysis of variance (ANOVA) followed by the Bonferroni's multiple comparison test ($P<0.05$) was used to assess the differences between the experimental groups and control group. All statistical analyzes were carried out by GraphPad Prism (*Intuitive Software for Science*, San Diego, CA, USA).

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Author Contribution Statement

Conceived and designed the experiments: F. P. O., H. H. F. K. and D. P. B. Collected the plant material and performed the chemical experiments: E. J. S. P. L., S. D. J., P. M. A., E. S. L., J. F. C. G., G. A. B., E. V. C., F. M. A. S. and H. H. F. K. Performed the *in vitro* and *in vivo* experiments: F. P. O., A. C. B. C. R., T. A. A., M. L. N., V. R. S. and L. S. S. Performed the histological analysis: R. B. D. and C. A. G. R. Analyzed the data: F. P. O., R. B.D., M. B. P. S., C. A. G. R., H. H. F. K. and D. P. B. Contributed reagents/materials/analysis tools: M. B. P. S., C. A.G. R., E. S. L., H. H. F. K. and D. P. B. Wrote the paper: H. H. F. K. and D. P. B.

References

[1] J. E. L. S. Ribeiro, M. J. G. Hopkins, A. Vicentini, C. A. Sothers, M. A. S. Costa, J. M. Brito, M. A. D. Souza, L. H. P. Martins,

L. G. Lohmann, P. A. C. L. Assunção, E. C. Pereira, C. F. Silva, M. R. Mesquita, L. C. Procópio,'Flora da Reserva Ducke: Guia de identificação das plantas vasculares de uma floresta de terra firme na Amazônia Central', 1a ed., Manaus, INPA/DFID, 1999.

[2] C. A. Marques, 'Importância econômica da família Lauraceae Lindl', *Floresta Ambienta* **2001**, *8*, 195–206.

[3] J. G. S. Maia, R. H. V. Mourão, 'Amazon Rosewood (*Aniba rosaeodora* Ducke) Oils', in 'Essential Oils in Food Preservation, Flavor and Safety', Ed. V. R. Preedy, Academic Press, 2016, pp. 193–201.

[4] R. S. Galaverna, P. T. B. Sampaio, L. E. S. Barata, M. N. Eberlin, C. H. V. Fidelis, 'Differentiation of two morphologically similar Amazonian *Aniba* species by mass spectrometry leaf fingerprinting', *Anal. Methods* **2015**, *7*, 1984–1990.

[5] M. P. Correa, 'Dicionário das plantas úteis do Brasil e das exóticas cultivadas', 5a ed., Rio de Janeiro, IBDF, 1974.

[6] R. M. Rodrigues, 'A flora da Amazônia', Belem, CEJUP, vol. 4, 1989.

[7] V. M. Moura, N. C. Guimarães, L. T. Batista, L. A. Freitas-de-Sousa, J. S. Martins, M. C. S. Souza, P. D. O. Almeida, W. M. Monteiro, R. B. de Oliveira, M. C. Dos-Santos, R. H. V. Mourão, 'Assessment of the anti-snakebite properties of extracts of *Aniba fragrans* Ducke (Lauraceae) used in folk medicine as complementary treatment in cases of envenomation by *Bothrops atrox*', *J. Ethnopharmacol.* **2018**, *213*, 350–358.

[8] Y. C. Silva, E. M. S. Silva, N. S. Fernandes, N. L. Lopes, P. P. Orlandi, C. V. Nakamura, E. V. Costa, V. F. Veiga-Júnior, 'Antimicrobial substances from Amazonian *Aniba* (Lauraceae) species', *Nat. Prod. Res.* **2021**, in press, DOI: 10.1080/14786419.2019.1603225.

[9] J. K. Silva, G. S. Maia, N. S. Dosoky, W. N. Setzer, 'Antioxidant, Antimicrobial, and Cytotoxic Properties of *Aniba parviflora* Essential Oils from the Amazon', *Nat. Prod. Commun.* **2016**, *11*, 1025–1028.

[10] E. R. Q. Santos, C. S. F. Maia, E. A. Fontes-Junior, A. S. Melo, B. G. Pinheiro, J. G. S. Maia, 'Linalool-rich essential oils from the Amazon display antidepressant-type effect in rodents', *J. Ethnopharmacol.* **2018**, *212*, 43–49.

[11] S. L. F. Sarrazin, R. B. Oliveira, J. G. S. Maia, R. H. V. Mourão, 'Antibacterial Activity of the Rosewood (*Aniba rosaeodora* and *A. parviflora*) Linalool-rich Oils from the Amazon', *Eur. J. Med. Plants*, **2016**, *12*, EJMP.22901.

[12] L. T. Batista, S. L. F. Sarrazin, V. M. De Moura, I. G. C. Dos Santos, S. Duvoisin Jr., P. M. Albuquerque, 'Chemical composition, antimicrobial and antioxidant activity of the essential oil of *Aniba parviflora* (Meisn) Mez.', *Rev. Fitos* **2019**, *13*, 181–191.

[13] B. Baldisserotto, L. E. S. Barata, A. S. Silva, W. F. F. Lobato, L. L. Silva, C. Toni, L. V. F. Silva, 'Anesthesia of tambaqui *Colossoma macropomum* (Characiformes: Serrasalmidae) with the essential oils of *Aniba rosaeodora* and *Aniba parviflora* and their major compound, linalool', *Neotrop. Ichthyol.* **2018**, *16*, e170128.

[14] C. M. A. M. Rezende, M. V. Bülow, O. R. Gottlieb, S. L. V. Pinho, A. I. Rocha, 'The 2-pyrones of *Aniba* species', *Phytochemistry* **1971**, *10*, 3167–3172.

[15] A. M. Bittencourt, O. R. Gottlieb, W. B. Mors, M. T. Magalhães, S. Mageswaran, W. D. Ollis, I. O. Sutherland, 'The natural occurrence of 6-styryl-2-pyrones and their synthesis', *Tetrahedron* **1971**, *27*, 1043–1048.

[16] D. P. Souza, R. B. Q. Pimentel, A. S. Santos, P. M. Albuquerque, A. V. Fernandes, S. Duvoisin Jr., J. T. A. Oliveira, M. V. Ramos, B. Rathinasabapathi, J. F. C. Gonçalves, 'Fungicidal properties and insights on the mechanisms of the action of volatile oils from Amazonian Aniba trees', *Ind. Crops Prod.* **2020**, *143*, 111914.

[17] M. Suffness, J. M. Pezzuto, 'Assays related to cancer drug discovery', in: 'Methods in plant biochemistry: assays for bioactivity', Ed. K. Hostettmann, Academic Press, London, 1991, pp. 71–133.

[18] S. S. Ribeiro, A. M. Jesus, C. S. Anjos, T. B. Silva, A. D. C. Santos, J. R. Jesus, M. S. Andrade, T. S. Sampaio, W. F. Gomes, P. B. Alves, A. A. Carvalho, C. Pessoa, M. O. Moraes, M. L. B. Pinheiro, A. P. N. Prata, A. F. Blank, R. Silva-Mann, V. R. S. Moraes, E. V. Costa, P. C. L. Nogueira, D. P. Bezerra, 'Evaluation of the Cytotoxic Activity of Some Brazilian Medicinal Plants', *Planta Med.* **2012**, *78*, 1601–1606.

[19] T. B. C. Silva, C. O. D'Sousa Costa, A. F. C. Galvão, L. M. Bomfim, A. C. B. Rodrigues, M. C. S. Mota, A. A. Dantas, T. R. Santos, M. B. P. Soares, D. P. Bezerra, 'Cytotoxic potential of selected medicinal plants in northeast Brazil', *BMC Complementary Altern. Med.* **2016**, *16*, 199.

[20] A. C. B. C. Rodrigues, F. P. Oliveira, R. B. Dias, C. B. S. Sales, C. A. G. Rocha, M. B. P. Soares, E. V. Costa, F. M. A. Silva, W. C. Rocha, H. H. F. Koolen, D. P. Bezerra, 'In vitro and in vivo anti-leukemia activity of the stem bark of *Salacia impressifolia* (Miers) A. C. Smith (Celastraceae)', *J. Ethnopharmacol.* **2019**, *231*, 516–524.

[21] R. G. A. Costa, T. A. Anunciação, M. S. Araujo, C. A. Souza, R. B. Dias, C. B. S. Sales, C. A. G. Rocha, M. B. P. Soares, F. M. A. Silva, H. H. F. Koolen, E. V. Costa, D. P. Bezerra, 'In vitro and in vivo growth inhibition of human acute promyelocytic leukemia HL-60 cells by *Guatteria megalophylla* Diels (Annonaceae) leaf essential oil', *Biomed. Pharmacother.* **2020**, *122*, 109713.

[22] J. Sœur, L. Marrot, P. Perez, I. Iraqui, G. Kienda, M. Dardalhon, J.-R. Meunier, D. Averbeck, M.-E. Huang, 'Selective cytotoxicity of *Aniba rosaeodora* essential oil towards epidermoid cancer cells through induction of apoptosis', *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **2011**, *718*, 24–32.

[23] M.-Y. Chang, D.-E. Shieh, C.-C. Chen, C.-S. Yeh, H.-P. Dong, 'Linalool Induces Cell Cycle Arrest and Apoptosis in Leukemia Cells and Cervical Cancer Cells through CDKIs', *Int. J. Mol. Sci.* **2015**, *16*, 28169–28179.

[24] K. Iwasaki, Y.-W. Zheng, S. Murata, H. Ito, K. Nakayama, T. Kurokawa, N. Sano, T. Nowatari, M. O. Villareal, Y. N. Nagano, H. Isoda, H. Matsui, N. Ohkohchi, 'Anticancer effect of linalool *via* cancer-specific hydroxyl radical generation in human colon cancer', *World J. Gastroenterol.* **2016**, *22*, 9765–9774.

[25] H. Van Den Dool, P. D. Kratz, 'A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography', *J. Chromatogr. A* **1963**, *11*, 463–471.

[26] S. A. Ahmed, R. M. Gogal Jr., J. E. Walsh, 'A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H]-

thymidine incorporation assay', *J. Immunol. Methods* **1994**, *170*, 211–224.
[27] L. S. Santos, V. R. Silva, L. R. A. Menezes, M. B. P. Soares, E. V. Costa, D. P. Bezerra, 'Xylopine Induces Oxidative Stress and Causes G_2/M Phase Arrest, Triggering Caspase-Mediated Apoptosis by p53-Independent Pathway in HCT116 Cells', *Oxid. Met.* **2017**, 7126872.
[28] V. R. Silva, R. S. Correa, L. S. Santos, M. B. P. Soares, A. A. Batista, D. P. Bezerra, 'A ruthenium-based 5-fluorouracil complex with enhanced cytotoxicity and apoptosis induction action in HCT116 cells', *Sci. Rep.* **2018**, *8*, 288.
[29] I. Nicoletti, G. Migliorati, M. C. Pagliacci, F. Grignani, C. Riccardi, 'A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry', *J. Immunol. Methods* **1991**, *139*, 271–279.
[30] E. J. S. P. Lima, R. G. Alves, G. M. A. D'Elia, T. A. D. Anunciação, V. R. Silva, L. S. Santos, M. B. P. Soares, N. M. D. Cardozo, E. V. Costa, F. M. A. Silva, H. H. F. Koolen, D. P. Bezerra, 'Antitumor Effect of the Essential Oil from the Leaves of *Croton matourensis* Aubl. (Euphorbiaceae)', *Molecules* **2018**, *23*, 2974.
[31] N. C. Carvalho, S. P. Neves, R. B. Dias, L. F. Valverde, C. B. S. Sales, C. A. G. Rocha, M. B. P. Soares, E. R. dos Santos, R. M. M. Oliveira, R. M. Carlos, P. C. L. Nogueira, D. P. Bezerra, 'A novel ruthenium complex with xanthoxylin induces S-phase arrest and causes ERK1/2-mediated apoptosis in HepG2 cells through a p53-independent pathway', *Cell Death Dis.* **2018**, *9*, 79.
[32] S. P. Neves, N. C. Carvalho, M. M. Silva, A. C. B. C. Rodrigues, L. M. Bomfim, R. B. Dias, C. B. S. Sales, C. A. G. Rocha, M. B. P. Soares, A. A. Batista, D. P. Bezerra, 'Ruthenium Complexes Containing Heterocyclic Thioamidates Trigger Caspase-Mediated Apoptosis Through MAPK Signaling in Human Hepatocellular Carcinoma Cells', *Front. Oncol.* **2019**, *9*, 562.
[33] R. P. C. Ferraz, D. S. Bomfim, N. C. Carvalho, M. B. P. Soares, T. B. Silva, W. J. Machado, A. P. N. Prata, E. V. Costa, V. R. S. Moraes, P. C. L. Nogueira, D. P. Bezerra, 'Cytotoxic effect of leaf essential oil of *Lippia gracilis* Schauer (Verbenaceae)', *Phytomedicine* **2013**, *20*, 615–621.
[34] A. C. B. C. Rodrigues, L. M. Bomfim, S. P. Neves, L. R. A. Menezes, R. B. Dias, M. B. P. Soares, A. P. N. Prata, C. A. G. Rocha, E. V. Costa, D. P. Bezerra, 'Antitumor Properties of the Essential Oil from the Leaves of *Duguetia gardneriana*', *Planta Med.* **2015**, *81*, 798–803.
[35] M. L. Nogueira, E. J. S. P. Lima, A. A. X. Adrião, S. S. Fontes, V. R. Silva, L. S. Santos, M. B. P. Soares, R. B. Dias, C. A. G. Rocha, E. V. Costa, F. M. A. Silva, M. A. Vannier-Santos, N. M. D. Cardozo, H. H. F. Koolen, D. P. Bezerra, '*Cyperus articulatus* L. (Cyperaceae) Rhizome Essential Oil Causes Cell Cycle Arrest in the G_2/M Phase, Cell Death in HepG2 Cells and Inhibits the Development of Tumors in a Xenograft Model', *Molecules* **2020**, *25*, 2687.
[36] E. J. S. P. Lima, S. S. Fontes, M. L. Nogueira, V. R. Silva, L. S. Santos, G. M. A. D'Elia, R. B. Dias, C. B. S. Sales, C. A. G. Rocha, M. A. Vannier-Santos, M. B. P. Soares, E. V. Costa, F. M. A. Silva, H. H. F. Koolen, D. P. Bezerra, 'Essential oil from leaves of *Conobea scoparioides* (Cham. & Schltdl.) Benth. (Plantaginaceae) causes cell death in HepG2 cells and inhibits tumor development in a xenograft model', *Biomed. Pharmacother.* **2020**, *129*, 110402.
[37] T. A. Anunciação, R. G. A. Costa, E. J. S. P. de Lima, V. R. Silva, L. S. Santos, M. B. P. Soares, R. B. Dias, C. A. G. Rocha, E. V. Costa, F. M. A. Silva, H. H. F. Koolen, D. P. Bezerra, 'In vitro and in vivo inhibition of HCT116 cells by essential oils from bark and leaves of *Virola surinamensis* (Rol. ex Rottb.) Warb. (Myristicaceae)', *J. Ethnopharmacol.* **2020**, *262*, 113166.

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