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Short communication

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

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ABSTRACT

Conobea scoparioides (Cham. & Schltdl.) Benth. (syn. *Sphaerotheca scoparioides* Cham. & Schldtl.) (Plantaginaceae), popularly known as "pataqueira", "vassourinha-do-brejo" and/or "hierba-de-sapo", is a popular medicinal plant used to treat leishmaniasis, pain and beriberi. In addition, inhibition of cell adhesion, antioxidant, cytotoxic and leishmanicidal activities of compounds or fractions of *C. scoparioides* have been reported. In the present work, chemical constituents and in vitro and in vivo anti-liver cancer potential of essential oil (EO) from leaves of *C. scoparioides* were investigated using human hepatocellular carcinoma HepG2 cells as a cell model. EO was obtained by hydrodistillation using a Clevenger-type apparatus and characterized by GC–MS and GC–FID. The in vitro cytotoxic effect was evaluated on three human cancer cell lines (MCF-7, HepG2 and HCT116) and one human non-cancerous cell line (MRC-5) using the Alamar blue assay. Phosphatidylserine externalization and cell cycle distribution were quantified in HepG2 cells by flow cytometry after 48 h incubation. The effectiveness of EO in anti-liver cancer model was studied with HepG2 cells grafted on C.B. 17 SCID mice. The main constituents of EO were thymol methyl ether (62 %), thymol (16 %) and α-phellandrene (14 %). EO displayed an in vitro cytotoxic effect against all human cancer cell lines and caused externalization of phosphatidylserine and DNA fragmentation in HepG2 cells, suggesting induction of apoptotic-like cell death. In vivo tumor mass inhibition of 36.7 and 55.8 % was observed for treatment with EO at doses of 40 and 80 mg/kg, respectively. These results indicate in vitro and in vivo anti-liver cancer potential of EO from leaves of *C. scoparioides*.

1. Introduction

Liver cancer has high incidence and lethality worldwide. For 2018, the GLOBOCAN database intimated a death rate of 0.93 for liver cancer (841,080 new cases for 781,631 deaths), indicating a poor prognosis [1]. Hepatocellular carcinoma (HCC) is responsible for about 75 % of primary liver cancer cases, and is diagnosed mainly at a late stage when surgery or transplantation is not an option. Sorafenib, a tyrosine kinase

Abbreviations: 5-FU, 5-fluorouracil; ANOVA, analysis of variance; ATCC, American Type Culture Collection; SBCAL, Brazilian Association of Laboratory Animal Science; CTL, control group; DOX, doxorubicin; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EO, essential oil; GC-FID, gas chromatography with flame ionization detection; GC–MS, gas chromatography coupled to mass spectrometry; HCC, hepatocellular carcinoma; IC_{50}, half of the maximum inhibitory concentration; INPA, National Research Institute of the Amazon; PI, propidium iodide; RI, retention indexes; SCID, severe combined immunodeficient; SPF, specific pathogen-free

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inhibitor, is a standard systemic therapy for advanced HCC, but extends survival by only 3 months [2–4]. Consequently, new approaches of the effective treatment of liver cancer are urgently needed.

Conobea scoparioides (Cham. & Schltdl.) Benth. (syn. *Sphaerotheca scoparioides* Cham. & Schldtl.) (Plantaginaceae family), popularly known as "pataqueira", "vassourinha-do-brejo" and/or “hierba-de-sapo”, is a tropical tree found in humid areas of rivers and streams of South America [5,6]. In relation to ethnopharmacological uses, aerial parts of *C. scoparioides* are used to treat leishmaniasis and as an anticonceptive agent in Western Colombia [6,7]. In Brazilian Amazon, the entire plant is used to prepare aromatic baths at the June folk festivals (annual São João festival), as well as to treat beriberi (a nutritional disease caused by vitamin B1 deficiency) [5].

Some pharmacological activities of this plant have been reported, including inhibition of cell adhesion, antioxidant, cytotoxic and leishmanicidal activities [6,8,9]. Fractionation of stem and leaves of *C. scoparioides* led to some cucurbitacins with cell adhesion inhibiting activity ($IC_{50} < 2$ μM) in JY (human lymphoblastoid cells)/HeLa (human cervical adenocarcinoma) cell adhesion assay [8]. The essential oil (from fresh and air-dried plant material) and methanol extract (from air-dried material) of *C. scoparioides* showed antioxidant capacity in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ($EC_{50} > 60$ μg/mL) assay and cytotoxicity in brine shrimp bioassay (*Artemia salina*), in which essential oil samples were more potent than methanol extract [9]. Thymol and thymol methyl ether were found as main constituents of these essential oil samples [5,9]. Moreover, the methylene chloride extract from the leaves of *C. scoparioides* showed potent leishmanicidal activity (IC_{50} = 1.3 μg/mL) in intracellular amastigotes of *Leishmania (Viannia) panamensis* and presented cytotoxicity to human myeloid leukemia U-937 cells [5]. Although the cytotoxic potential in vitro has been reported for this plant, its antitumor effect in vivo has not been investigated. In the present work, we studied the chemical constituents and in vitro and in vivo anti-liver cancer potential of essential oil (EO) from leaves of *C. scoparioides*.

2. Methods

2.1. Plant material

Fresh leaves from the species *C. scoparioides* were purchased in May 2014 at the Ver-o-Peso market located in the municipality of Belém, Pará, Brazil (1°27’08.7” S, 48°30’13.6” W). The plant was validated by the botanist Mike Hopkins, comparing the material with exsiccates (#347, #141715 and #188340) previously deposited at the herbarium of the National Research Institute of the Amazon (INPA).

2.2. Essential oil extraction and chemical analysis

The collected material was extracted directly by hydrodistillation in a Clevenger-type apparatus. For this stage, 500 g of fresh and crushed material was extracted for a period of 4 h in 1200 mL of ultrapure water (18.2 MΩ). Once obtained, the oil was separated from the aqueous layer by extraction with CH_2Cl_2 and then, dried over anhydrous Na_2SO_4 to remove any trace of water. At the end, the oil was filtered through a nylon membrane (pore size 0.22 μm) and the resulting EO weighed in flasks and stored at −4 °C until chemical analysis.

The identification of EO constituents was performed by gas chromatography coupled to mass spectrometry (GC–MS) with a GCMS/QP2010 Plus (Shimadzu) equipped with a capillary column Rtx-5 MS (30 m x0.25 mm x 0.25, Restek). Helium was the carrier gas at a flow rate of 1.02 mL/min. Injections of 1 μL were performed with 1.5 mg/mL EO solutions in *n*-hexane using a 1:50 rate. The column temperature program was 60–280 °C with gradual increases of 3 °C/min. The injector and ion source temperatures were 220 °C and 260 °C, respectively. The preliminary identifications of the constituents were performed based on the comparison of the experimental spectra with those stored in the library of 8th edition of Wiley (similarities > 90 %). Confirmations were performed by calculating the retention indices (RI), according to Van den Dool and Kratz equation [10], in comparison with a homologous series of linear hydrocarbons (C_7-C_{30}).

The semiquantitative analysis was performed with gas chromatography with flame ionization detection (GC-FID), model GC2010 (Shimadzu) equipped with capillary column Rtx-5. The same thermal conditions of the GC–MS analysis were used to guarantee reproducibility. Relative amounts (%) were calculated in relation to the total chromatogram area.

Table 1
Chemical composition of essential oil from leaves of *Conobea scoparioides*.

Peak Number	Compounds	Retention Time (min)	R.I. theo.[a]	R.I. exp.	Proportion Area (%)[b]
1	*α*-thujene	5.52	924	924	0.46 ± 0.03
2	3-octanone	6.94	979	979	1.25 ± 0.12
3	*α*-phellandrene	7.60	1002	1000	13.10 ± 0.94
4	*m*-cymene	8.33	1023	1023	5.74 ± 0.45
5	Unknown	8.46	–	–	0.41 ± 0.01
6	Unknown	9.22	–	–	0.74 ± 0.04
7	(*Z*)-*β*-ocimene	9.51	1032	1031	0.49 ± 0.01
8	linalool	11.30	1095	1094	0.39 ± 0.01
9	Unknown	15.40	–	–	0.37 ± 0.01
10	sabinol	16.10	1139	1139	0.46 ± 0.02
11	thymol methyl ether	17.70	1232	1232	59.70 ± 3.86
12	thymol	20.60	1290	1290	14.90 ± 0.98
13	(*E*)-*α*-bergamotene	30.50	1432	1431	1.80 ± 0.27
$\Sigma_{\text{total detected peaks}}$					99.80
$\Sigma_{\text{monoterpenes}}$					95.10
$\Sigma_{\text{sesquiterpenes}}$					1.80
Identification coverage					98.20

[a] Retention indices calculated with the Van den Dool and Kratz equation [7].
[b] Area proportional to total area of the chromatogram after eliminate of peaks resulting from contamination and/or bleeding from the column.

2.3. Alamar blue assay

To assess the cytotoxicity of EO, three human cancer cell lines, MCF-7 (breast adenocarcinoma), HepG2 (hepatocellular carcinoma) and HCT116 (colon carcinoma), and a human non-cancerous cell line, MRC-5 (pulmonary fibroblast), were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured as recommended by ATCC animal cell culture guide. All cell lines were tested for mycoplasma using a mycoplasma staining kit (Sigma-Aldrich) and were free from contamination.

The quantification of cell viability was performed by Alamar blue assay, as previously described [11–13]. Briefly, the cells were seeded in 96-well plates and incubated for 72 h. The EO was tested on a concentration-response curve ranging from 0.4–50 μg/mL, obtained by serial dilution (eight different concentrations) of a 10 mg/mL stock dissolved in dimethyl sulfoxide (DMSO, Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil). Doxorubicin (doxorubicin hydrochloride, purity ≥ 95 %, Laboratory IMA S.A.I.C., Buenos Aires, Argentina) (in eight different concentrations ranging from 0.04–5 μg/mL) and 5-fluorouracil (in eight different concentrations ranging from 0.2–25 μg/mL) (Sigma-Aldrich) were used as positive controls. At the end of the treatment, 20 μL stock solution (0.312 mg/mL) of resazurin (Sigma-Aldrich Co., Saint Louis, MO, USA) was added to each well. Absorbances at 570 and 600 nm were measured using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

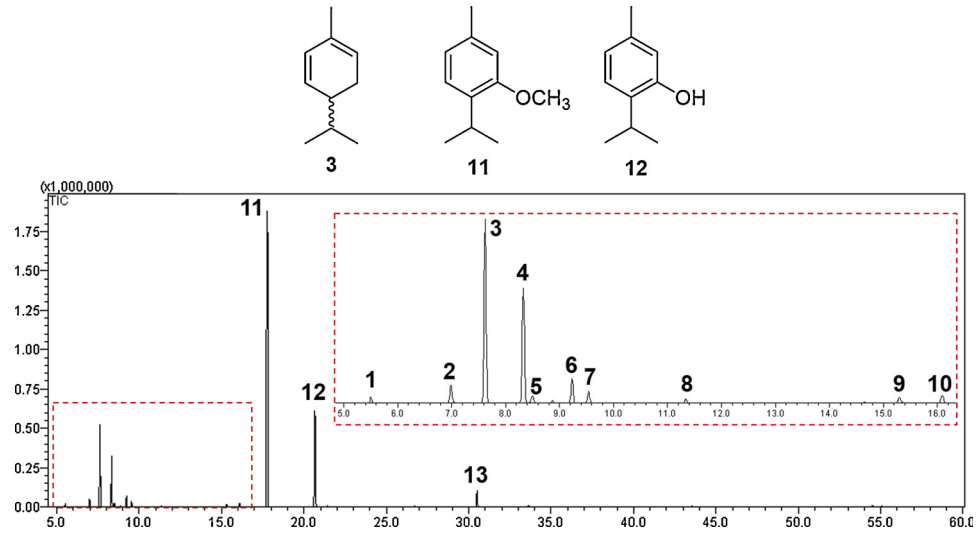

Fig. 1. Total ion chromatogram of essential oil from leaves of *Conobea scoparioides*.

Table 2
In vitro cytotoxicity of essential oil (EO) from leaves of *Conobea scoparioides*.

Cells	Histological type	IC_{50} (μg/mL)		
		EO	DOX	5-FU
Cancer cells				
MCF-7	Human breast adenocarcinoma	45.52 31.61 – 65.57	0.22 0.15 - 0.32	9.25 7.47 – 13.84
HepG2	Human hepatocellular carcinoma	41.86 33.27 – 59.84	0.04 0.02 - 0.20	3.93 1.74 – 6.63
HCT116	Human colon carcinoma	13.50 5.14 – 35.47	0.08 0.05 - 0.14	0.55 0.31– 1.08
Non-cancer cells				
MRC-5	Human pulmonary fibroblast	> 50	2.09 1.51 - 2.89	3.74 1.27 – 8.56

Data are presented as IC_{50} values in μg/mL with their respective 95 % confidence interval obtained by nonlinear regression from three independent experiments performed in duplicate, evaluated by Alamar blue assay after 72 h incubation. Doxorubicin (DOX) and 5-fluorouracil (5-FU) were used as positive controls.

2.4. Flow cytometry assays

To quantify cell death, the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) was used and the analysis was performed according to the manufacturer's instructions. Cell fluorescence and light scattering features were determined by flow cytometry.

DNA fragmentation and cell cycle distribution were determined using 2 μg/mL propidium iodide (PI) in cells permeabilized with 0.1 % triton X-100, 0.1 % sodium citrate and 100 μg/mL RNAse (all from Sigma-Aldrich Co.), as previously described [14], and cell fluorescence was assessed by flow cytometry.

For all analyzes by flow cytometry, 10,000 events were recorded per sample with a BD LSRFortessa cytometer, analyzed with BD FACSDiva Software (BD Biosciences) and Flowjo Software 10 (Flowjo LCC, Ashland, OR, USA, and cellular debris were omitted from the analysis.

2.5. Human liver cancer xenograft model

To assess the anti-liver cancer potential of EO, HepG2 cells were grafted into C.B-*17* severe combined immunodeficient (SCID) mice as previously described [15]. Fifty specific pathogen-free (SPF) C.B-*17* SCID mice (females, 25–30 g) were obtained and kept in the animal facilities of FIOCRUZ-Bahia (Salvador, Bahia, Brazil). The animals were housed in cages with free access to food and water. All animals were submitted to a 12: 12 light-dark cycle (lights on at 6 am). The animals were treated according to ethical principles for animal experimentation of SBCAL (Brazilian Association of Laboratory Animal Science), Brazil. The Animal Ethics Committee of FIOCRUZ-Bahia (Salvador, Bahia, Brazil) approved the experimental protocol (number 06/2015).

HepG2 cells (10^7 cells/500 μL) were implanted subcutaneously in the left frontal axils of the mice. The animals were randomly divided into four groups: group 1, animals that received a vehicle (5% DMSO) (n = 20); group 2, animals that received the positive control 5-fluorouracil (10 mg/kg, n = 10); group 3, animals that received EO at 40 mg/kg (n = 10); and group 4, animals that received EO at 80 mg/kg (n = 10). One day after tumor implantation, the animals were treated intraperitoneally once a day for 21 consecutive days. These doses were selected based on previous works, using EO in mouse tumor models [15,16]. One day after treatment, the animals were anesthetized (thiopental, 50 mg/kg) and samples of peripheral blood were collected from the brachial artery. Then, the animals were euthanized by anesthetic overdose (thiopental, 100 mg/kg) and the tumors were excised and immediately weighed using an analytical balance with 4-digit precision. Inhibition ratio (%) was calculated by formula: inhibition ratio (%) = [(A–B)/A] x 100, where A is average tumor weight of negative control, and B is tumor weight of treated group.

The animals were weighed on the first and last day of the experiment to analyze possible toxicological effects. The animals were observed for signs of abnormality throughout the study. Hematological analysis was performed using the Advia 60 hematology system (Bayer, Leverkusen, Germany). Livers, kidneys, lungs and hearts were removed, weighed and examined for signs of formation of a thick lesion, color change and/or hemorrhage. After fixation in 4% formaldehyde, the histological analysis of tumors, livers, kidneys, hearts and lungs was performed under optical microscopy, using Hematoxylin/Eosin staining (and Periodic Acid-Schiff staining for liver), by an experienced pathologist.

2.6. Statistical analysis

Data were presented as mean ± S.E.M. or as values of half of the maximum inhibitory concentration (IC_{50}) with their 95 % confidence intervals obtained by nonlinear regressions. All in vitro experiments were performed in duplicate and repeated at least three times to

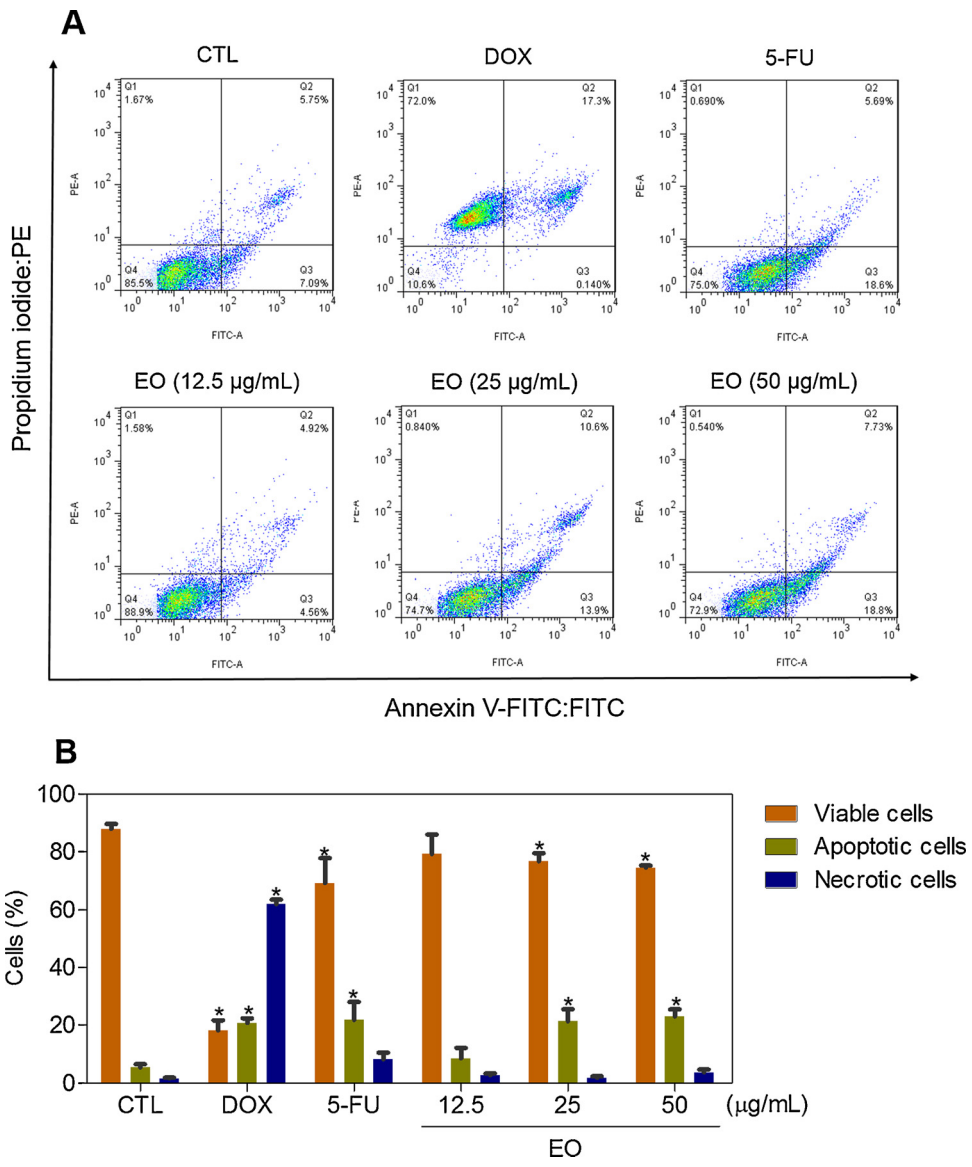

Fig. 2. Effect of essential oil (EO) from leaves of *Conobea scoparioides* in induction of cell death in HepG2 cells after 48 h incubation quantified by flow cytometry using annexin V-FITC/PI staining. (A) Representative flow cytometric dot plots. (B) Quantification of viable (annexin V-FITC/PI double negative cells), apoptotic (early apoptosis [annexin V-FITC positive, but PI negative cells] plus late apoptosis [annexin V-FITC/PI double positive cells]) and necrotic cells (PI positive, but annexin V-FITC negative cells). Doxorubicin (DOX, 1 µg/mL) and 5-fluorouracil (5-FU, 5 µg/mL) were used as positive controls, and negative control group (CTL) was treated with vehicle (0.5 % DMSO) used to dissolve and dilute EO. Ten thousand events were evaluated per experiment, and cellular debris was omitted from analysis. Data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. * $P < 0.05$ compared with negative control by ANOVA, followed by Bonferroni's Multiple Comparison Test.

analyze biological variation. The difference between the experimental groups was compared through the analysis of variance (ANOVA) followed by the Bonferroni's Multiple Comparison Test ($P < 0.05$). All statistical analyses were performed using the GraphPad Prism 5 (Intuitive Software for Science; San Diego, CA, USA).

3. Results

3.1. Chemical components of essential oil from leaves of Conobea scoparioides

A total oil recovery from leaves of *C. scoparioides* was 1.07 % ± 0.11 (w/w) and its chemical composition is shown in Table 1. Thirteen compounds were detected, of which 10 (representing 98.2 % of EO) were identified based on their retention indices and mass spectral-fragments (Fig. 1). Terpenoids were 96.9 % of the composition of EO sample, in which monoterpenes were dominant (95.1 %), followed by two sesquiterpene identified, (*E*)-α- bergamotene (1.80 %) and a ketone hydrocarbon named 3-octanone (1.25 %). The main constituents of EO were thymol methyl ether (59.7 %), thymol (14.9) and α-phellandrene (13.1 %).

3.2. Essential oil from leaves of Conobea scoparioides displays in vitro cytotoxic effect against human cancer cells

The in vitro cytotoxic effect of EO from leaves of *C. scoparioides* was evaluated in three human cancer cell lines (MCF-7, HepG2 and HCT116) and in a human non-cancerous cell line (MRC-5) by alamar blue assay after 72 h incubation. Table 2 shows the IC_{50} values obtained through concentration-response curves. EO displayed in vitro cytotoxicity against all human cancer cell lines tested. The IC_{50} values found for EO were 45.52 µg/mL for MCF-7 cells, 41.86 µg/mL for HepG2 cells, 13.50 µg/mL for HCT116 cells and > 50 µg/mL for MRC-5 cells. For doxorubicin, used as positive control, IC_{50} values observed ranged from 0.03 to 0.22 µg/mL for HepG2 and MCF-7 cancer cells, respectively, and was 2.09 µg/mL for non-cancerous cells MRC-5. 5-Fluorouracil, another drug used as a positive control, had IC_{50} values ranging from 0.55 to 9.25 µg/mL for HCT116 and MCF-7 cancer cells, respectively, and was 3.74 µg/mL for non-cancerous cells MRC-5.

3.3. Essential oil from leaves of Conobea scoparioides causes cell death in HepG2 cells

To confirm the cytotoxicity of the EO from leaves of *C. scoparioides*, cell death and cell cycle distribution were quantified by flow cytometry

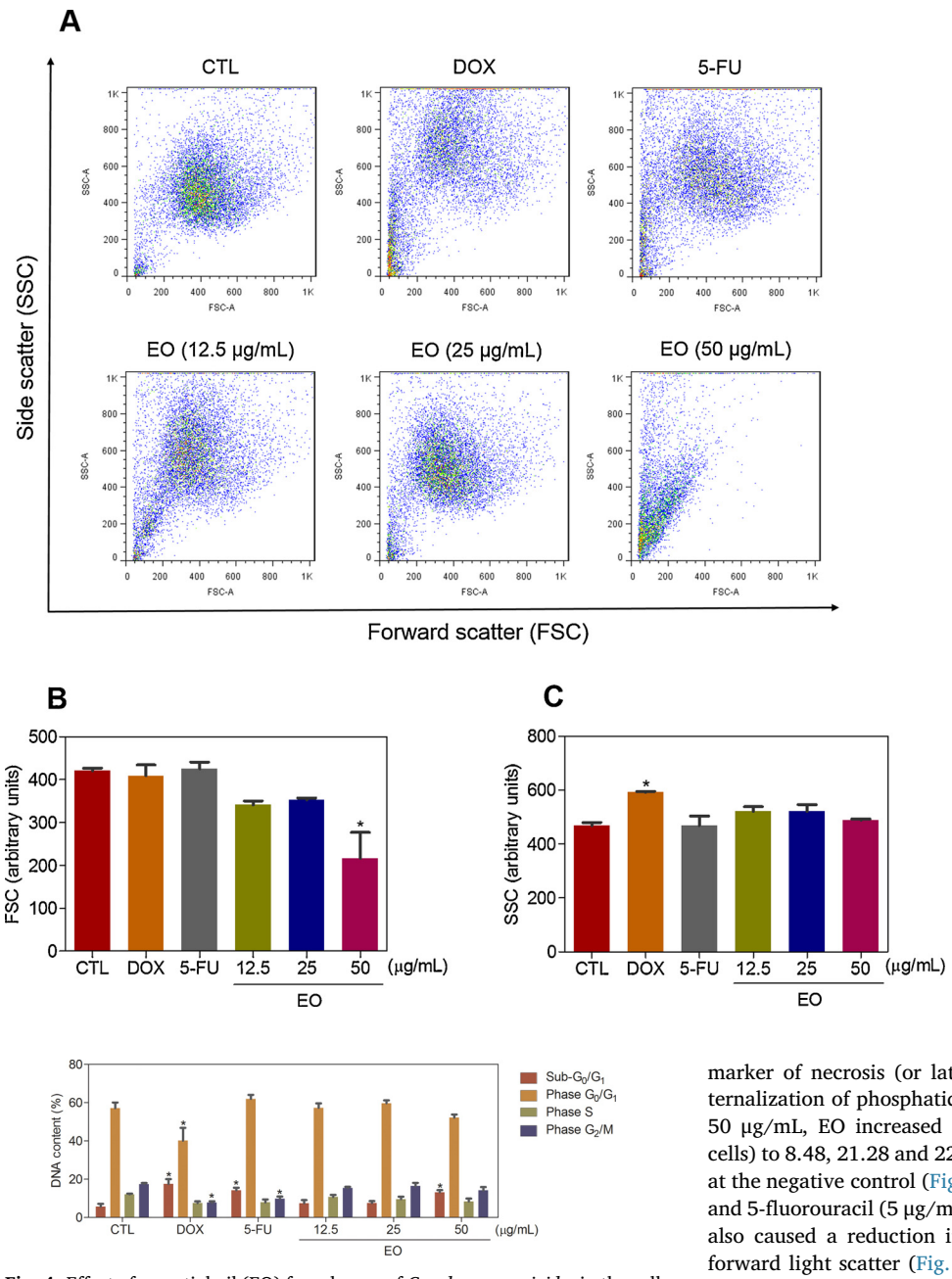

Fig. 3. Effect of essential oil (EO) from leaves of *Conobea scoparioides* in HepG2 cell morphology, as determined by light-scattering features detected by flow cytometry after 48 h incubation. Doxorubicin (DOX, 1 µg/mL) and 5-fluorouracil (5-FU, 5 µg/mL) were used as positive controls, and negative control (CTL) was treated with vehicle (0.5 % DMSO) used to dissolve and dilute EO. Ten thousand events were evaluated per experiment, and cellular debris was omitted from analysis. Data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. * $P < 0.05$ compared with negative control by ANOVA, followed by Bonferroni's Multiple Comparison Test.

Fig. 4. Effect of essential oil (EO) from leaves of *Conobea scoparioides* in the cell cycle distribution of HepG2 cells after 48 h incubation. Doxorubicin (DOX, 1 µg/mL) and 5-fluorouracil (5-FU, 5 µg/mL) were used as positive controls, and negative control group (CTL) was treated with vehicle (0.5 % DMSO) used to dissolve and diluting EO. Ten thousand events were evaluated per experiment, and cellular debris was omitted from analysis. Data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. * $P < 0.05$ compared with negative control by ANOVA, followed by Bonferroni's Multiple Comparison Test.

in HepG2 cells after 48 h incubation. Annexin V-FITC and PI double fluorescence staining was used to quantify the percentage of viable (annexin V-FITC/PI double-negative cells), early apoptosis (annexin V-FITC-positive and PI-negative cells), late apoptosis (annexin V-FITC/PI double-positive cells) and necrosis (annexin V-FITC-negative and PI-positive cells) cells. In this assay, annexin V-FITC stains in green fluorescence cells with externalization of phosphatidylserine and PI in red fluorescence cells with loss of cell membrane integrity. Therefore, the externalization of phosphatidylserine is used as a marker of apoptotic cell death, and the loss of cell membrane integrity is used as a marker of necrosis (or late apoptosis, when found together with externalization of phosphatidylserine). At concentrations of 12.5, 25 and 50 µg/mL, EO increased the apoptotic cells (early + late apoptotic cells) to 8.48, 21.28 and 22.96 %, respectively, against 5.25 % observed at the negative control (Fig. 2). Incubation with doxorubicin (1 µg/mL) and 5-fluorouracil (5 µg/mL) also increased cell death. Additionally, EO also caused a reduction in cell volume, as observed by the drop in forward light scatter (Fig. 3), morphological changes characteristic of apoptotic cell death.

The cell cycle distribution was quantified by analyzing the DNA content using PI staining in permeabilized cells (Fig. 4). All DNA that was sub-diploid in size (sub-G_1) was considered fragmented. We observed a significant increase in DNA fragmentation in HepG2 cells treated with EO at concentration of 50 µg/mL (13.16 % versus 5.68 % observed in the negative control). Doxorubicin (1 µg/mL) and 5-fluorouracil (5 µg/mL) increased DNA fragmentation to 17.47 % and 14.23 %, respectively.

3.4. Essential oil from leaves of *C. scoparioides* inhibits HepG2 cells development in xenograft model

Since the EO from leaves of *C. scoparioides* displayed in vitro cytotoxicity in liver cancer HepG2 cells, in vivo anti-liver cancer potential of this EO was investigated in the development of HepG2 cells in xenograft model. A significant reduction in the growth of HepG2 cell was observed in both groups treated with EO (40 and 80 mg/kg, i.p., once daily for 21 consecutive days). After the treatment period, mean tumor

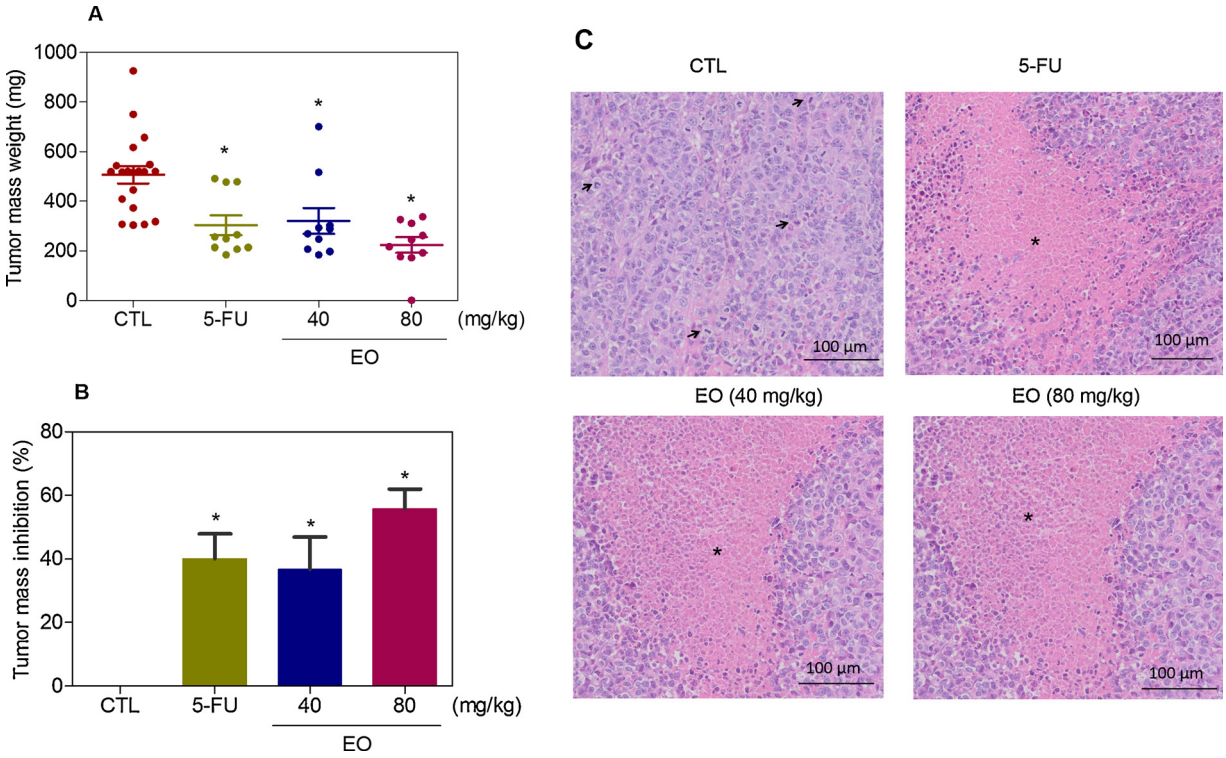

Fig. 5. In vivo anti-liver cancer effect of essential oil (EO) from leaves of *Conobea scoparioides* in C.B-17 SCID mice xenografted with HepG2 cells. (**A**) Tumor mass weight (g). (**B**) Tumor mass inhibition (%). (**C**) Representative histological analysis of tumors stained with hematoxylin and eosin and analyzed by light microscopy. Arrows indicate atypical mitosis and areas of necrosis were represented by asterisks. 5-Fluorouracil (5-FU, 10 mg/kg) was used as positive control, and negative control (CTL) received vehicle (5% DMSO) used to dissolve and dilute EO. Data are presented as mean ± S.E.M. of 10-20 animals. * $P < 0.05$ compared to negative control by ANOVA followed by Bonferroni's Multiple Comparison Test.

Table 3
Effect of essential oil (EO) from leaves of *Conobea scoparioides* on body and relative organ weight from C.B-17 SCID mice with HepG2 cell xenografts.

Parameters	CTL	5-FU	EO	
Dose (mg/kg)	–	10	40	80
Survival	20/20	10/10	10/10	10/10
Initial body weight (g)	21.26 ± 0.53	19.06 ± 0.27	23.05 ± 0.54	20.45 ± 0.69
Final body weight (g)	22.03 ± 0.48	20.56 ± 0.60	23.55 ± 0.58	20.86 ± 0.64
Liver (g/100 g body weight)	4.76 ± 0.17	4.80 ± 0.24	4.76 ± 0.28	5.14 ± 0.16
Kidneys (g/100 g body weight)	1.52 ± 0.05	1.55 ± 0.06	1.38 ± 0.07	1.49 ± 0.08
Heart (g/100 g body weight)	0.56 ± 0.05	0.55 ± 0.02	0.48 ± 0.03	0.55 ± 0.02
Lung (g/100 g body weight)	0.77 ± 0.03	0.75 ± 0.05	0.70 ± 0.04	0.83 ± 0.05

Beginning 1 day after tumor implantation, the animals were treated through intraperitoneal route for 21 consecutive days. 5-Fluorouracil (5-FU, 10 mg/kg) was used as positive control, and negative control group (CTL) received vehicle (5% DMSO) used to dissolve and dilute EO. Data are presented as mean ± S.E.M. of 10–20 animals.

mass weight were 320.6 ± 51.7 mg and 223.9 ± 31.5 mg in the groups treated with EO at lowest and highest doses, respectively, while 506.8 ± 34.8 mg was found in group treated with the vehicle (5% DMSO) (Fig. 5A). This represents an inhibition of the tumor mass of 36.7 and 55.8 %, respectively ($P < 0.05$) (Fig. 5B). 5-Fluorouracil (10 mg/kg, i.p. for the same treatment period) was used as positive control and inhibited the tumor mass weight by 40.1 %. Histopathological examination of the tumors was also performed on all slides stained with hematoxylin and eosin (Fig. 5C).

All groups were classified as undifferentiated carcinomas and exhibited highly proliferative tumors with hyperchromatic and pleomorphic cells (anisokaryosis and anisocytosis). Several bizarre mitotic figures were observed, especially in the vehicle (5% DMSO) group. Necrotic areas were more frequent in the 5-fluorouracil- and EO-treated groups. Hemorrhage and areas of calcification were observed mainly in EO-treated groups.

Although animals treated with EO (80 mg/kg) and 5-fluorouracil showed a slight reduction in the number of leukocytes (that means possible immunosuppression), neither the weight of the body and organs (liver, kidney, lung and heart) (Table 3) nor hematological parameters (Table 4) presented statistically significant changes in EO-treated groups ($P > 0.05$).

Moreover, morphological analyses of the lungs, liver, kidneys and heart were performed for all groups (Table 5). In the lungs, parenchyma architecture was partially maintained in all experimental groups, and a thickening of the alveolar septum with decreased air space was observed in all groups, ranging from mild to moderate. In addition, significant inflammation, predominantly by mononuclear cells, edema, congestion and hemorrhage, was frequently observed in all groups, ranging from mild to severe. In the livers, hepatic architecture and portal space were partially preserved in all experimental groups. Histopathological changes included congestion and hydropic degeneration, as well as, focal areas of mixed inflammation and coagulation necrosis were found in all groups, ranging from mild to moderate. In the kidneys, tissue architecture was maintained; however, some histopathological changes were observed in all experimental groups, such as moderate vascular congestion and thickening of the basal membrane of the renal glomerulus with decreased urinary space, ranging from mild to moderate. The heart showed no changes in any group.

Table 4
Effect of essential oil (EO) from leaves of *Conobea scoparioides* on hematological parameters of peripheral blood from C.B-17 SCID mice with HepG2 cell xenografts.

Parameters	CTL	5-FU	EO	
Dose (mg/kg)	–	10	40	80
Erythrocytes (10^6/mm^3)	5.62 ± 1.08	7.23 ± 0.87	4.82 ± 1.08	7.86 ± 0.96
Hemoglobin (g/dL)	21.21 ± 4.83	17.74 ± 3.06	26.65 ± 0.76	16.43 ± 2.29
MCV (fL)	43.78 ± 0.43	45.00 ± 3.00	42.25 ± 0.25	45.50 ± 0.50
Platelets (10^3/mm^3)	407.9 ± 108.9	222.1 ± 41.6	577.0 ± 161.0	325.8 ± 96.19
Leukocytes (10^3/mm^3)	4.39 ± 0.76	1.98 ± 0.38	6.42 ± 0.11	2.65 ± 0.35
Differential leukocytes (%)				
Granulocytes	24.12	28.40	29.63	29.00
Lymphocytes	41.51	46.08	40.01	38.39
Monocytes	33.58	25.52	30.34	32.61

Beginning 1 day after tumor implantation, the animals were treated through intraperitoneal route for 21 consecutive days. 5-Fluorouracil (5-FU, 10 mg/kg) was used as positive control, and negative control group (CTL) received vehicle (5% DMSO) used to dissolve and dilute EO. Data are presented as mean ± S.E.M. of 7–14 animals. MCV: Mean Corpuscular Volume.

Table 5
Effect of essential oil (EO) from leaves of *Conobea scoparioides* on histopathological analysis of lungs, liver, kidneys and heart from C.B-17 SCID mice with HepG2 cell xenografts.

Organ	Parameters	CTL	5-FU	EO	
				40	80
Lung					
	Parenchyma architecture	partially maintained	partially maintained	partially maintained	partially maintained
	Thickening of the alveolar septum with decreased air space	mild to moderate	mild to moderate	mild to moderate	mild to moderate
	Inflammation	mild to severe	mild to severe	mild to severe	mild to severe
Liver					
	Liver architecture and portal space	partially preserved	partially preserved	partially preserved	partially preserved
	Congestion and hydropic degeneration	mild to moderate	mild to moderate	mild to moderate	mild to moderate
	Inflammation	mild to moderate	mild to moderate	mild to moderate	mild to moderate
	Coagulation necrosis	mild to moderate	mild to moderate	mild to moderate	mild to moderate
Kidneys					
	Tissue architecture	maintained	maintained	maintained	maintained
	Vascular congestion	mild to moderate	mild to moderate	mild to moderate	mild to moderate
	Thickening of the basal membrane of the renal glomerulus	mild to moderate	mild to moderate	mild to moderate	mild to moderate
Heart	–	No alteration observed	No alteration observed	No alteration observed	No alteration observed

Beginning 1 day after tumor implantation, the animals were treated through intraperitoneal route for 21 consecutive days. 5-Fluorouracil (5-FU, 10 mg/kg) was used as positive control, and negative control group (CTL) received vehicle (5% DMSO) used to dissolve and dilute EO. Histological analysis was performed under optical microscopy using Hematoxylin/Eosin and Periodic Acid-Schiff (liver) staining.

4. Discussion

Herein, chemical composition and in vitro and in vivo anti-liver cancer potential of EO of *C. scoparioides* were described for the first time. As mentioned above, EO displayed in vitro cytotoxicity against all human cancer cell lines tested and thymol methyl ether, thymol and α-phellandrene were the main chemical constituents found. Although it has some quantitative variations, the composition recorded in this work corroborates previous findings on the chemistry of *C. scoparioides* [5,9]. These variations may be related to water stress, place of collection, nutrition, soil and climate conditions, and other abiotic factors.

As previously mentioned, the cytotoxicity of the essential oil from fresh and air-dried whole plant material of *C. scoparioides* was previously evaluated in the brine shrimp assay (*Artemia salina*), where the oil from fresh sample presented LC_{50} of 7.8 μg/mL and the oil from dried one presented LC_{50} of 7.5 μg/mL [9]; however, no study has been evaluated using cancer cell lines. In the present study, EO showed cytotoxicity to cancer cells MCF-7 (IC_{50} = 45.52 μg/mL), HepG2 (IC_{50} = 41.86 μg/mL) and HCT116 (IC_{50} = 13.50 μg/mL). The cytotoxic effect has also been observed previously in methanol extract (LC_{50} = 77.6 μg/mL in brine shrimp assay) [9] and in methylene chloride extract (IC_{50} = 63.4 μg/mL in U-937 cells) [5] of *C. scoparioides*. Here, more potent cytotoxicity was observed for *C. scoparioides* EO using liver, colon and breast cancer cell lines.

Biochemical and morphological aspects observed during apoptotic cell death include phosphatidylserine exposure, activation of caspases, DNA fragmentation, reduction of cell volume and membrane blebbing [17]. In the present work, we observed that EO from leaves of *C. scoparioides* caused both externalization of phosphatidylserine and DNA fragmentation along with reduction of cell volume in HepG2 cells, suggesting induction of apoptotic-like cell death. There are no reports of cytotoxicity of another EO obtained from *Conobea* species. However, some of the main chemical constituents found in EO from leaves of *C. scoparioides* have also been found in different EOs with cytotoxic potential, including EO from aerial parts of *Oliveria decumbens* and its main constituent, thymol, caused oxidative stress, mitochondrial membrane potential loss, caspase-3 activation, S-phase cell cycle arrest, DNA damage in human breast adenocarcinoma MDA-MB-231 cells, indicating induction of apoptotic cell death [18]. Treatment with EO from leaves of *Lippia gracilis,* which presents thymol as one of its main constituents, caused G_1 arrest in HepG2 cells followed by induction of DNA fragmentation and caspase-3 activation, suggesting induction of apoptotic cell death [19]. EO from aerial parts of *Monarda citriodora* and its major constituent thymol, induced apoptosis in HL-60 cells along with inhibition of downstream and upstream signaling of PI3K/AKT/mTOR pathway [20]. These data indicate that the cytotoxic effect of EO from leaves of *C. scoparioides* may be associated with a mixture of its main constituents.

The in vivo anti-liver cancer potential of EO from leaves of *C. scoparioides* was demonstrated in the development of HepG2 cells in xenograft model. Inhibition of tumor mass of 36.7 and 55.8 % was observed in EO-treated mice at doses of 40 and 80 mg/kg, respectively. 5-

Fluorouracil (10 mg/kg), a clinically useful chemotherapeutic drug used in this study as a positive control, showed an inhibition rate of 40.1 %. Interestingly, EO from leaves of *Annona vepretorum* that has α-phellandrene as one of its main constituents, inhibited the growth of B16-F10 cells in vivo by 34.5 % at 50 mg/kg, and was improved to 62.7 % when complexed with β-cyclodextrin in a microencapsulation [21]. The EO from aerial parts of *Lippia microphylla*, which has thymol as its main constituent, reduced the growth of mouse sarcoma 180 tumor cells in mice by 38 % and 60 % at doses of 50 and 100 mg/kg, respectively [22]. These data corroborating that antitumor potential of EO from leaves of *C. scoparioides* are probably due a mixture of its main constituents that include thymol and α-phellandrene.

In addition, we observed that there were no changes in body weight (or organs) or in the hematological analysis of peripheral blood in mice treated with OE. Regarding histopathological analysis, the changes observed, including hydropic change, vascular congestion and focal areas of inflammation, are acute cellular responses to non-lethal agents and, generally, when the aggression ends, the damaged cells can return to the state of homeostasis. Similar toxicological profile was reported for EO from leaves of *Croton matourensis* [15] and EO from leaves of *Guatteria megalophylla* [23].

These results indicate in vitro and in vivo anti-liver cancer potential of EO from leaves of *C. scoparioides,* suggesting that it may be a new herbal medicine candidate. Additional investigations should be directed to this new drug candidate to identify secondary metabolites with cytotoxic potential, as well as studies on the mechanism of action and toxicological profile to accelerate the development of *C. scoparioides* as a source of antineoplastic drugs. This exploitation of the anti-liver cancer potential of EO from leaves of *C. scoparioides* can also contribute to the preservation of biodiversity, an inexhaustible source of drugs, with a great diversity of pharmacological actions with innovative mechanisms of action.

Authors' contributions

Conceived and designed the experiments: HHFK and DPB. Performed the experiments: EJSPL, SSF, MLN, VRS, LSS, GMAD, RBD and CBSS. Analyzed the data: EJSPL, SSF, MLN, CAGR, MAVS, MBPS, EVC, FMAS, HHFK and DPB. Contributed the reagents/materials/analysis tools: CAGR, MAVS, MBPS, EVC, FMAS, HHFK and DPB. Wrote the paper: HHFK and DPB. All authors read and approved the final manuscript for submission.

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Declaration of Competing Interest

None.

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