

Antitumor Properties of the Essential Oil From the Leaves of *Duguetia gardneriana*

Authors

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Key words

- ◉ *Duguetia gardneriana*
- ◉ Annonaceae
- ◉ essential oil
- ◉ cytotoxic
- ◉ antitumor

received March 9, 2015
revised April 26, 2015
accepted April 28, 2015

Bibliography

DOI <http://dx.doi.org/10.1055/s-0035-1546130>
Published online June 30, 2015
Planta Med 2015; 81: 798–803
© Georg Thieme Verlag KG
Stuttgart · New York ·
ISSN 0032-0943

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Abstract

Duguetia gardneriana, popularly known in the Brazilian northeast as “jaquinha”, is a species belonging to the family Annonaceae. The aim of this work was to assess the chemical composition and antitumor properties of the essential oil from the leaves of *D. gardneriana* in experimental models. The chemical composition of the essential oil was analyzed via gas chromatography-flame ionization detector and gas chromatography-mass spectrometry. *In vitro* cytotoxic activity was determined in cultured tumor cells, and *in vivo* antitumor activity was assessed in B16-F10-bearing mice. The identified compounds were β -bisabolene (80.99%), elemicin (8.04%), germacrene D

(4.15%), and cyperene (2.82%). The essential oil exhibited a cytotoxic effect, with IC₅₀ values of 16.89, 19.16, 13.08, and 19.33 μ g/mL being obtained for B16-F10, HepG2, HL-60, and K562 cell lines, respectively. On the other hand, β -bisabolene was inactive in all of the tested tumor cell lines (showing IC₅₀ values greater than 25 μ g/mL). The *in vivo* analysis revealed tumor growth inhibition rates of 5.37–37.52% at doses of 40 and 80 mg/kg/day, respectively. Herein, the essential oil from the leaves of *D. gardneriana* presented β -bisabolene as the major constituent and showed cytotoxic and antitumor potential.

Supporting information available online at <http://www.thieme-connect.de/products>

Introduction

Duguetia gardneriana Mart., popularly known as “jaquinha”, is a species belonging to the family Annonaceae that has been found in the northeast Brazilian states of Sergipe, Pernambuco, and Paraíba [1]. Plants from the genus *Duguetia* have been used in folk medicine to treat several diseases. Moreover, some species have been reported to be a source of cytotoxic agents, such as *Duguetia hadrantha* R.E. Fr. [2], *Duguetia glabriuscula* R.E. Fr. [3], *Duguetia odorata* J.F. Macbr. [4], *Duguetia furfuracea* Saff. [5], and *Duguetia lanceolata* A.St.-Hil. [6].

There are currently only two available scientific research reports on *D. gardneriana*. In the first, Almeida et al. [7] described the isolation of three alkaloids (discretamine, tetrahydrojathorrhizine, and tetrahydropalmatine) and a cinnamate derivative (methyl 3,5-dihydroxy-4-ethoxycinnamate) from the ethanol extract of the stem bark. The second reported the chemical composition and antimicrobial activity of essential oil from the leaves obtained in the state of Paraíba, Brazil,

which presented germacrene D, viridiflorene, β -pinene, α -pinene, and β -caryophyllene as the major constituents and showed antimicrobial activity against *Staphylococcus aureus* and *Candida guilliermondii* [8]. The aim of this work was to assess the chemical composition and antitumor properties of the essential oil from the leaves of *D. gardneriana*, obtained in the northeast Brazilian state of Sergipe, in experimental models.

Results and Discussion

In relation to its dry weight, the leaves of *D. gardneriana* resulted in 0.13 \pm 0.04% essential oil (light yellow). As shown in **Table 1**, four compounds were identified. The essential oil from the leaves of *D. gardneriana* is composed predominantly of sesquiterpene compounds. The identified compounds were β -bisabolene (80.99%), elemicin (8.04%), germacrene D (4.15%), and cyperene (2.82%). The presence of β -bisabolene in *D. gardneriana* as a major compound is reported here for the first time; however, this compound has

Compounds		RI ^a	RI ^b	Leaves oil %
1	Cyperene	1396	1398	2.82 ± 0.46
2	Germacrene D	1479	1484	4.15 ± 0.24
3	β -Bisabolene	1507	1505	80.99 ± 0.35
4	Elemicin	1547	1555	8.04 ± 0.01
Sesquiterpene identified				96.00 ± 0.45
Total identified				96.00 ± 0.45

Table 1 Chemical composition of the essential oil from the leaves of *D. gardneriana*.

Data are expressed as mean ± SD of three analyses. RI (retention indices): ^a calculated on an RTX[®]-5MS column according to Van Den Dool and Kratz [19], based on a homologous series of normal alkanes; ^b according to ADAMS [20]

Cell lines	IC ₅₀ (μg/mL) ^a		
	Essential oil	β -Bisabolene	5-FU ^b
Tumor cells ^c			
B16-F10	16.89 13.96–20.43	> 25	0.68 0.21–1.45
HepG2	19.16 16.06–22.85	> 25	0.04 0.01–1.22
HL-60	13.08 7.937–21.55	> 25	0.15 0.01–1.86
K562	19.33 11.06–33.80	> 25	0.29 0.21–0.38
Non-tumor cells ^d			
PBMC	46.58 36.43–59.57	> 25	14.00 9.83–23.31

Table 2 *In vitro* cytotoxic evaluation of the essential oil from the leaves of *D. gardneriana* and of its major constituent, β -bisabolene.

^a Data are presented as IC₅₀ values, in μg/mL, and their 95% confidence interval obtained by nonlinear regression from three independent experiments performed in duplicate, measured by Alamar blue assay after 72 h incubation; ^b 5-Fluorouracil (5-FU) was used as the positive control; ^c Tumor cells: B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), HL-60 (human promyelocytic leukemia), and K562 (human chronic myelocytic leukemia); ^d Non-tumor cell: PBMC (human peripheral blood mononuclear cells activated with concanavalin A – human lymphoblast)

been detected as a minor constituent in the essential oil of *Uvaria pierrei* Finet & Gagnep. and *Uvaria dac* Pierre ex Finet & Gagnep., two species belonging to the family Annonaceae [9]. The results presented in this work are very different from those of Almeida et al. [8]. In addition, other compounds are described for the first time in the genus *Duguetia*, such as elemicin and cyperene. These variations in the reported composition of the major constituents of *D. gardneriana* and in the contents of all of its components may be related to soil and climate conditions, water stress, the site of collection, nutrition, and other abiotic factors.

Since this major component presented a good separation and resolution by thin-layer chromatography (TLC), it was submitted to preparative TLC from the oil product and confirmed as β -bisabolene via MS and based on the subsequent NMR 1D and 2D spectra. TLC is an efficient, reproducible, and cheap technique, and, although β -bisabolene is a volatile compound, it was isolated in a sufficient amount for all tests. The ¹H and ¹³C NMR data for this compound are presented in Supporting Information and are described for the first time here.

The chemical constituents of essential oils from plants belonging to the genus *Duguetia* include spathulenol and muurolol in the fine stems and germacrene D, cyperene, and muurolol in the stem bark of *Duguetia flagellaris* [10]. In the essential oil from the leaves of *D. glabriuscula*, the presence of aromadendrane sesquiterpenoids was observed [11]. On the other hand, in the essential oil from the bark of *D. lanceolata*, β -elemene, caryophyllene oxide, β -selinene, β -eudesmol, and humulene epoxide II were observed as major compounds [6].

The *in vitro* cytotoxic effects of the essential oil and its major constituent, β -bisabolene (● Fig. 1), were assessed in four tumor cell lines using the Alamar blue assay after 72 h of incubation (● Table 2). The essential oil exhibited a cytotoxic effect on all of the tested tumor cell lines, with half-maximal inhibitory concentration (IC₅₀) values of 16.89, 19.16, 13.08, and 19.33 μg/mL being obtained in B16-F10, HepG2, HL-60, and K562 cells, respectively. β -Bisabolene was inactive in all of the tested tumor cell lines (showing IC₅₀ values greater than 25 μg/mL). In our preclinical anticancer drug screening program, extracts/oils presenting IC₅₀ values below 30 μg/mL and pure compounds presenting IC₅₀ values below 4 μg/mL in the tumor cell line assays are considered promising [12–18]. Therefore, the essential oil obtained from *D. gardneriana* presented promising results, and these effects were associated with a mixture of its constituents. For 5-fluorouracil (5-FU), which was used as the positive control, IC₅₀ values of 0.04 to 0.68 μg/mL were observed in the HepG2 and B16-F10 cell lines, respectively.

The cytotoxicity of the essential oil was also evaluated against non-tumor cells (PBMCs). The selectivity index (SI) was determined as IC₅₀[PBMC]/IC₅₀[HL-60]. The essential oil showed an SI of 3.6, while 5-FU showed an SI of 93.3.

In the genus *Duguetia*, the alkaloids hadranthine B, imbiline-1, and sampangine from the stem bark of *D. hadrantha* have been shown to induce cytotoxic effects in different tumor cell lines [2]. Matos et al. [3] reported the cytotoxic effects of seven constituents isolated from *D. glabriuscula* in Hep2 human larynx carcinoma cells, in which β -sitosterol exhibited an IC₅₀ value below 5 μg/mL. Oliveroline isolated from *D. odorata* inhibits the G₂

DNA damage checkpoint in MCF-7 mp53 cells [4]. Duguetine and duguetine β -N-oxide isolated from the subterranean stem bark of *D. furfuracea* present potent cytotoxic effects in different tumor cell lines [5]. Moreover, the essential oil from the bark of *D. lanceolata* shows a cytotoxic effect in brine shrimp [6]. Additionally, we have been continually investigating the antitumor potencial of essential oils from species belonging to the family Annonaceae. Among these, some essential oils from plants from the genera *Xylopi*a and *Guatteria* have been shown to exhibit significant *in vitro* and *in vivo* anticancer activity, without substantial systemic toxicity [12, 14–17]. In this context, the *in vitro* cytotoxic effect, as well as, *in vivo* antitumor activity of the essential oil from the leaves of *D. gardneriana* were assessed for the first time in this study.

In vivo antitumor activity was evaluated using C57BL/6 mice subcutaneously inoculated with B16-F10 melanoma cells and treated once a day for nine consecutive days with the essential oil via the intraperitoneal route (● Fig. 2). On the 16th day, the average tumor weight in the control mice was 3.87 ± 0.20 g. In the presence of the essential oil, administered at 40 and 80 mg/kg/day, the average tumor weights were 3.66 ± 0.38 and 2.42 ± 0.39 g, respectively. The observed tumor growth inhibition rates were 5.37–37.52%. The administration of 5-FU (25 mg/kg/day), as the positive control, reduced tumor weight by 43.11%. In addition, tumor sections removed from all groups were subjected to histopathological analyses and showed intense pleomorphism, lumpy chromatin, abundant intracellular melanin pigment, and atypical mitoses.

Microscopic and macroscopic analyses of the organs and analyses of biochemical and hematological parameters were performed in the essential oil-treated mice to evaluate systemic toxicity. No significant changes in body or organ (liver, kidney, spleen, lung, and heart) weight were observed in the essential oil-treated groups ($p > 0.05$, Table 2S, Supporting Information). Microscopic analyses of the liver and kidneys revealed hydropic degeneration and vascular congestion in all groups. Atelectasis, focal hemorrhage, and vascular congestion were observed in the lungs of animals treated with the essential oil and 5-FU. In the spleen, moderate hyperplasia of the white pulp was observed in the essential oil-treated mice. All of these acutely injured cells returned to a homeostatic state when the stimulus ended. Microscopic analyses of the heart did not reveal alterations in any group. Moreover, no significant changes in peripheral blood biochemistry (alkaline phosphatase, gamma-glutamyl transpeptidase, alanine aminotransferase, albumin, globulin, total protein, blood urea nitrogen, creatinine, uric acid, phosphorus, calcium, amylase, cholesterol, and glucose) or hematological (total erythrocytes and leukocytes, and the proportions of neutrophils, lymphocytes, monocytes, eosinophils, and basophils) parameters were observed in the essential oil-treated groups ($p > 0.05$, Tables 3S and 4S, Supporting Information).

In conclusion, the essential oil from the leaves of *D. gardneriana* presents β -bisabolene as the major constituent and shows cytotoxic and antitumor potential.

Materials and Methods



Cells

The tumor cells lines B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukemia), and HL-60 (human promyelocytic leukemia) were do-

nated by the A. C. Camargo Hospital, São Paulo, SP, Brazil. The cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco-BRL) medium supplemented with 10% fetal bovine serum (Cultilab), 2 mM L-glutamine (Vetec Química Fina), and 50 μ g/mL gentamycin (Novafarma). Adherent cells were harvested via treatment with a 0.25% trypsin EDTA solution (Gibco-BRL). All cell lines were cultured in cell culture flasks at 37 °C in 5% CO₂ and subcultured every three to four days to maintain exponential growth. All experiments were conducted with cells in the exponential growth phase. All cell lines were tested for mycoplasma using the Lookout[®] Mycoplasma qPCR detection kit (Sigma-Aldrich), and all cells were shown to be free from contamination.

Heparinized blood (from healthy, 20- to 35-year-old, nonsmoking donors who had not taken any drugs for at least 15 days prior to sampling) was collected, and peripheral blood mononuclear cells (PBMCs) were isolated via a standard protocol using a Ficoll density gradient in GE Ficoll-Paque Plus. The PBMCs were washed and resuspended at a concentration of 0.3×10^6 cells/mL in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, and 50 μ g/mL gentamycin at 37 °C with 5% CO₂. Concanavalin A (ConA; Sigma Chemical Co.) was used as a mitogen to trigger cell division in T-lymphocytes. ConA (10 μ g/mL) was added at the beginning of culture, and the cells were treated with the test drugs after 24 h. The Research Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (number 031 019/2013). All participants signed a written informed consent form to participate in the study.

Cell viability was examined using the Trypan blue exclusion assay for all experiments. Over 90% of the cells were viable at the beginning of culture.

Animals

A total of 36 specific pathogen-free C57BL/6 mice (males, 25–30 g) were obtained and maintained at the animal facilities of the Gonçalo Moniz Research Center-FIOCRUZ (Salvador, Bahia, Brazil). Animals were housed in cages with free access to food and water. All animals were kept under a 12:12-h light-dark cycle (lights on at 6:00 a.m.). The animals were treated according to the ethical principles for animal experimentation of the SBCAL (Brazilian Association of Laboratory Animal Science), Brazil. The Animal Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (number 01/2013).

General experimental procedures

MS spectra were acquired on a Shimadzu QP2010 GC/MS system equipped with an AOC-20i auto-injector, and 1D and 2D NMR data were recorded at 303 K in CDCl₃ on a Bruker AVANCE III 600 NMR spectrometer operating at 14.1 Tesla, observing ¹H and ¹³C at 600 and 150 MHz, respectively. All of the observed ¹H and ¹³C NMR chemical shifts (δ) are presented in ppm in relation to the TMS signal at 0.00 ppm as an internal reference and the coupling constant (*J*) in Hz. For analytical (0.25 mm) and preparative (1.00 mm) TLC, silica gel 60 F₂₅₄ was used. Compounds were visualized via exposure under UV_{254/365} light and spraying the p-anisaldehyde reagent, followed by heating on a hot plate.

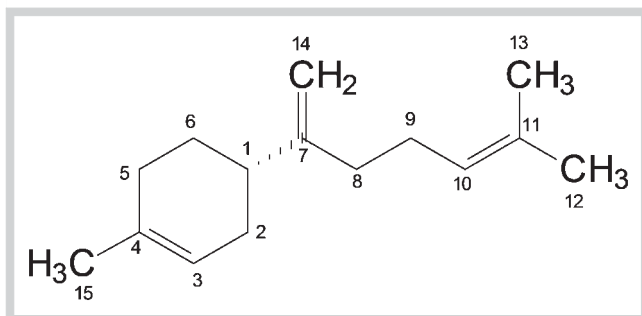


Fig. 1 Chemical structure of β -bisabolene.

Botanical material

Leaves of *D. gardneriana* were collected in November 2013, in the Itabaiana Mountain National Park, between the cities of Itabaiana and Areia Branca, Sergipe State, Brazil (coordinates: S 10°45' 17.1" and W 37°20' 32.6"). The identity of the plant was confirmed by Dr. Ana Paula do Nascimento Prata of the Department of Biology of Federal University of Sergipe (UFS), Brazil, and a voucher specimen (32822) was deposited in the Herbarium of UFS (ASE/UFS). The authors have authorization from the Chico Mendes Institute for Biodiversity Conservation from the Brazilian Ministry of the Environment for plant collection (number 25637-1). This work was performed according to special authorization for access to genetic resources in Brazil # 010344/2014-4, issued by CNPq/MCTI.

Hydrodistillation of the essential oil

Essential oil from the dried leaves (for 24 h) of *D. gardneriana* (200 g) was obtained via hydrodistillation for 3 h using a Clevenger-type apparatus. The essential oil was dried over anhydrous sodium sulfate, and the percentage content was calculated on the basis of the dry weight of plant material. The oil was then stored in a freezer until analysis. Hydrodistillation was performed in duplicate.

Gas chromatography-flame ionization detector and gas chromatography-mass spectrometry analysis

GC-FID and GC-MS analyses were performed on a Shimadzu GC-2010 Plus GCMS-QP2010 Ultra GC-FID equipped with a Shimadzu AOC-20i auto-injector. Separation of the compounds was achieved in an RTX®-5MS fused capillary chromatography column (30 m \times 0.25 mm \times 0.25 μ m film thickness) coated with 5%-diphenyl-95%-dimethylpolysiloxane. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The column temperature program was 40 °C/4 min, followed by an increase at a rate of 4 °C/min to 240 °C, then an increase at 10 °C/min to 280 °C and holding 280 °C/2 min. The injector and detector temperatures were 250 °C and 280 °C, respectively. The injection volume of the essential oil in dichloromethane (10.0 mg/mL) was 1.0 μ L, with a split ratio of 1 : 50. Retention indices were generated using a standard solution of *n*-alkanes (C₈–C₁₈). Peak areas and retention times were measured with an electronic integrator. The relative amounts of individual compounds were computed from the obtained GC peak areas without an FID response factor correction. MS were collected at 70 eV, with a scan interval of 0.5 s and fragments of 40–550 Da. The components of the essential oil were identified by comparing the retention times of the GC peaks with standard compounds run under identical conditions and by comparison of

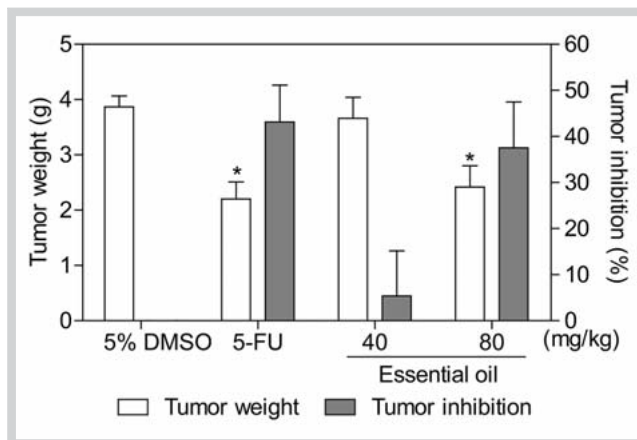


Fig. 2 *In vivo* antitumor effect of the essential oil from the leaves of *D. gardneriana*. Mice were injected with B16-F10 mouse melanoma cells (2.0×10^6 cells/animal, s. c.). The animals were then treated via intraperitoneal administration for nine consecutive days, starting one day after tumor implantation. As the positive control, 5-fluorouracil (5-FU, 25 mg/kg) was used. The negative control was treated with the vehicle used for diluting the tested substance (5% DMSO). Data are presented as the mean \pm SEM from nine animals per group. * $P < 0.05$ compared with the 5% DMSO group via ANOVA followed by the Student-Newman-Keuls test.

the obtained retention indices [19] and MS [20] with those available in the literature in addition to comparison of the MS with those stored in the NIST and Wiley libraries. The analyses of the essential oil were performed in duplicate.

β -Bisabolene isolation

A portion of the essential oil (200.0 mg) was also subjected to preparative TLC, eluted with a mixture of *n*-hexane-ethyl acetate (95 : 05, v/v), producing 136.5 mg of the β -bisabolene compound (GC-FID, 95.0%).

β -bisabolene: Colorless oil. $[\alpha]_D^{25} -64.2$ (c 0.7 g/100 mL, CHCl₃); ¹H and ¹³C NMR data, see Supporting Information; MS (EI, 70 eV): *m/z* (%) = 204 (M⁺, 4), 161 (8), 121 (9), 119 (18), 109 (18), 107 (19), 94 (24), 93 (57), 92 (13), 91 (26), 81 (10), 79 (34), 77 (21), 69 (100), 68 (15), 67 (47), 65 (10), 55 (21), 53 (21), 43 (10), 41 (90).

In vitro cytotoxic activity assay

Cell growth was quantified using the alamar blue assay as previously described [21]. Cells were seeded into 96-well plates for all experiments (0.7×10^5 cells/mL for adherent cells or 0.3×10^6 cells/mL for cells suspended in 100 μ L of medium). After 24 h, the essential oil (0.39–50.0 μ g/mL) and its major constituent, β -bisabolene (0.19–25.0 μ g/mL), dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), were added to each well and incubated for 72 h. As a positive control, 5-FU (purity > 99%; Sigma-Aldrich) was used. The negative controls received the vehicle employed for diluting the tested compound (0.5% DMSO). Four hours (for cell lines) or 24 hours (for PBMCs) before the end of incubation, 20 μ L of a stock solution (0.312 mg/mL) of alamar blue (resazurin, Sigma-Aldrich Co.) was added to each well. Absorbance was measured using a SpectraMax 190 multiplate reader, and the drug effect was quantified as the percentage of the control absorbance at 570 nm and 600 nm. The absorbance of alamar blue in the culture medium was measured at higher and lower wavelengths. The absorbance of the medium alone was sub-

tracted from the absorbance of the medium plus alamar blue at the higher wavelength. This value was referred to as AO_{HW} . The absorbance of the medium alone was subtracted from the absorbance of the medium plus alamar blue at the lower wavelength. This value was referred to as AO_{LW} . A correction factor, R_0 , was calculated from AO_{HW} and AO_{LW} , where $R_0 = AO_{LW}/AO_{HW}$. The percent of reduction in alamar blue was expressed as follows: % reduced = $A_{LW} - (A_{HW} \times R_0) \times 100$.

In vivo antitumor activity assay

The *in vivo* antitumor effect was evaluated in C57BL/6 mice inoculated with B16-F10 melanoma cells as previously described [18]. Tumor cells (2×10^6 cells per 500 μ L) were implanted subcutaneously into the left hind groin of the mice. The essential oil was dissolved in 5% DMSO and given to the mice intraperitoneally once a day for nine consecutive days. Mice were divided into four groups at the beginning of the experiment: Group 1 (negative control, $n = 9$): animals treated via injection of the vehicle (5% DMSO); Group 2 (positive control, $n = 9$): animals treated via injection of 5-FU (25 mg/kg); Group 3: animals treated via injection of the essential oil (40 mg/kg, $n = 9$); and Group 4: animals treated via injection of the essential oil (80 mg/kg, $n = 9$). The treatments were initiated one day after tumor injection. On day 16, peripheral blood samples from the mice were collected from the retro-orbital plexus under phenobarbital (Aventis Pharma Ltda) for biochemical and hematological analyses, as described below. The animals were euthanized through cervical dislocation, and the tumors were excised and weighed. Drug effects are expressed as the percent of inhibition in relation to the control.

Systemic toxicological evaluation

Systemic toxicological effects were investigated as previously described [18]. The mice were weighed at the beginning and end of the experiment. The animals were observed for signs of abnormalities throughout the study. Their livers, kidneys, spleens, lungs, and hearts were removed, weighed, and examined for any signs of gross lesions or color changes and hemorrhage. Biochemical analyses of serum samples were performed using a Vet-16 rotor and quantified using an Analyst bench-top clinical chemistry system (Hemagen Diagnostics, Inc.). Hematological analyses were performed using light microscopy. Following a gross macroscopic examination, representative tissue sections of the tumors, livers, kidneys, spleens, lungs, and hearts were fixed in 4% formalin buffer and embedded in paraffin. Tissue sections with a thickness of 4 μ m were stained with hematoxylin and eosin, and a pathologist performed analyses under light microscopy.

Statistical analysis

Data are presented as means \pm SEM/SD or IC_{50} values and their 95% confidence intervals (CI 95%), obtained via nonlinear regression. Differences between experimental groups were compared using ANOVA (analysis of variance) followed by the Student-Newman-Keuls test ($p < 0.05$). All statistical analyses were performed using the GraphPad program (Intuitive Software for Science).

Supporting information

Spectrometric data, including 1D (1H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR, GC-MS, GC-FID, and MS data, and systemic toxicological parameters (body and organ weight, periph-

eral blood biochemistry, and hematologic parameters) are available as Supporting Information.

Acknowledgements

This work was financially supported by the Brazilian agencies CNPq, CAPES, FAPESB and FAPITEC/SE. The authors are grateful to Professor Dr. Andersson Barison for 1D and 2D NMR spectra and to Marlene da Silva Cerqueira for technical assistance and the histotechnology platform of FIOCRUZ-Bahia for performing histological techniques. The English was edited by American Journal Experts (key# 6F4C-C714-BC72-8A16-991B).

Conflict of Interest

The authors have declared that there is no conflict of interest.

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