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

**Abstract**

*Duguetia gardneriana* is a species belonging to the family Annonaceae, which has 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 haadrantha* R.E. Fr. [2], *Duguetia glabriscusula* 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, tetrahydrojathrorrizine, 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 Paraiba, 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.

**Introduction**

In relation to its dry weight, the leaves of *D. gardneriana* resulted in 0.13 ± 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 essential oil exhibited a cytotoxic effect, with IC₅₀ values of 16.89, 19.16, 13.08, and 19.33 µg/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 µg/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

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Germacrene D</td>
<td>80.99%</td>
</tr>
<tr>
<td>Elemicin</td>
<td>8.04%</td>
</tr>
<tr>
<td>Bisabolene</td>
<td>80.99%</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>4.15%</td>
</tr>
<tr>
<td>Cyperene</td>
<td>2.82%</td>
</tr>
</tbody>
</table>

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**References**

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 flagellartis* [10]. In the essential oil from the leaves of *D. glabriuscula*, the presence of aromadendrene sesquiterpenoids was observed [11]. On the other hand, in the essential oil from the bark of *D. lanceolata*, β-elemene, carpyphyllyl 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 0.68 to 0.69 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-sisoterol exhibited an IC50 value below 5 µg/mL. Oliveroline isolated from *D. odorata* inhibits the G2

### Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R1</th>
<th>R2</th>
<th>Leaves oil %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyperene</td>
<td>1396</td>
<td>1398</td>
</tr>
<tr>
<td>2</td>
<td>Germacrene D</td>
<td>1479</td>
<td>1484</td>
</tr>
<tr>
<td>3</td>
<td>β-Bisabolene</td>
<td>1507</td>
<td>1505</td>
</tr>
<tr>
<td>4</td>
<td>Elemicin</td>
<td>1547</td>
<td>1555</td>
</tr>
</tbody>
</table>

Sesquiterpene identified: 96.00 ± 0.45
Total identified: 96.00 ± 0.45

Data are expressed as mean ± SD of three analyses. R1 (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].

### Table 2

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Essential oil</th>
<th>IC50 (µg/mL)a</th>
<th>β-Bisabolene</th>
<th>5-FUb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16-F10</td>
<td>16.89</td>
<td>13.96–20.43</td>
<td>&gt; 25</td>
<td>0.68</td>
</tr>
<tr>
<td>HepG2</td>
<td>19.16</td>
<td>16.06–22.85</td>
<td>&gt; 25</td>
<td>0.04</td>
</tr>
<tr>
<td>HL-60</td>
<td>13.08</td>
<td>7.937–21.55</td>
<td>&gt; 25</td>
<td>0.15</td>
</tr>
<tr>
<td>K562</td>
<td>19.33</td>
<td>11.06–33.80</td>
<td>&gt; 25</td>
<td>0.29</td>
</tr>
<tr>
<td>Non-tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>46.58</td>
<td>36.43–59.57</td>
<td>&gt; 25</td>
<td>14.00</td>
</tr>
</tbody>
</table>

IC50 values are presented 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; a Tumor cells: B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), HL-60 (human promyelocytic leukemia), and K562 (human chronic myelocytic leukemia).
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 potential of essential oils from species belonging to the family Annonaceae. Among these, some essential oils from plants from the genera *Xylopia* and *Guatteria* have been shown to exhibit significant in vitro and in vivo antitumor 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 3.67 ± 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 30.2% and 41.3%. The administration of 5-FU (25 mg/kg/day), as the positive control, reduced tumor weight by 43.11%. In addition, the observed tumor growth inhibition rates were 30.2% and 41.3%.

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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, the observed tumor growth inhibition rates were 30.2% and 41.3%.

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-
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 Cleve-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 × 0.25 mm × 0.25 µm 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 µL, with a split ratio of 1:50. Retention indices were generated using a standard solution of *n*-alkanes (C₅-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 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. [α]D²⁰⁻64.2 (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 × 10⁵ cells/mL for adherent cells or 0.3 × 10⁶ cells/mL for cells suspended in 100 µL of medium). After 24 h, the essential oil (0.39–50.0 µg/mL) and its major constituent, β-bisabolene (0.19–25.0 µg/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 µL 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 was also measured at higher and lower wavelengths. The absorbance of the medium alone was sub-

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**Fig. 1** Chemical structure of β-bisabolene.

**Fig. 2** In vivo antitumor effect of the essential oil from the leaves of *D. gardneriana*. Mice were injected with 816-F10 mouse melanoma cells (2.0 × 10⁶ 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 ± SEM from nine animals per group. *P < 0.05 compared with the 5% DMSO group via ANOVA followed by the Student-Newman-Keuls test.
tracted from the absorbance of the medium plus alamar blue at the higher wavelength. This value was referred to as A0HW. 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 AOHW. A correction factor, R0, was calculated from AOHW and AOLW, where R0 = AOHW/AOHW. The percent of reduction in alamar blue was expressed as follows: % reduced = A0HW − (AHW × R0) / 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 × 10^4 cells per 500 µL) 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 µm were stained with hematoxylin and eosin, and a pathologist performed analyses under light microscopy.

Statistical analysis
Data are presented as means ± SEM/SD or IC50 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 ^13C) and 2D (COSY, HSQC and HMBC) NMR, GC-MS, GC-FID, and MS data, and systemic toxicological parameters (body and organ weight, peripheral blood biochemistry, and hematologic parameters) are available as Supporting Information.

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

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