Antitumor Effect of the Essential Oil from Leaves of *Guatteria pogonopus* (Annonaceae)

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Guatteria pogonopus Martius, a plant belonging to the Annonaceae family, is found in the remaining Brazilian Atlantic Forest. In this study, the chemical composition and antitumor effects of the essential oil isolated from leaves of *G. pogonopus* was investigated. The chemical composition of the oil was determined by GC-FID and GC/MS analyses. The *in vitro* cytotoxicity was evaluated against three different tumor cell lines (OVCAR-8, NCI-H358M, and PC-3M), and the *in vivo* antitumor activity was tested in mice bearing sarcoma 180 tumor. A total of 29 compounds was identified and quantified in the oil. The major compounds were γ-patchoulene (13.55%), (*E*)-caryophyllene (11.36%), β-pinene (10.37%), germacrene D (6.72%), bicyclogermacrene (5.97%), α-pinene (5.33%), and germacrene B (4.69%). The essential oil, but neither (*E*)-caryophyllene nor β-pinene, displayed *in vitro* cytotoxicity against all three tumor cell lines tested. The obtained average IC_{50} values ranged from 3.8 to 20.8 μg/ml. The lowest and highest values were obtained against the NCI-H358M and the OVCAR-8 cell lines, respectively. The *in vivo* tumor-growth-inhibition rates in the tumor-bearing mice treated with essential oil (50 and 100 mg/kg/d) were 25.3 and 42.6%, respectively. Hence, the essential oil showed significant *in vitro* and *in vivo* antitumor activity.

Introduction. – The genus *Guatteria* (Annonaceae) comprises *ca.* 300 species and is distributed from southeastern Mexico to southern Brazil [1][2]. Numerous biological properties have been reported for the plants belonging to this genus, including antioxidant [3], antimicrobial [4], antimalarial [5], antileishmanial [6], insecticide [7], and cytotoxic effects [8]. In particular, cytotoxic activity has been found for *Guatteria hispida* [8], *G. blepharophylla* [8], *G. boliviana* [9], and *G. friesiana* [10].

G. pogonopus Martius is a tree (4–10-m-tall) characterized by very large leaves, which often have a rounded base. It was reported to grow in the Brazilian states of Bahia, Espírito Santo, and Minas Gerais [11]. In addition, in this work, we were able to find it in the Brazilian state of Sergipe. Several beneficial biological activities had been reported for *Guatteria* species [3–10], but up to now, no chemical or pharmacological scientific research was published for the species *G. pogonopus.*

Hence, in this study, the chemical composition of the essential oil from leaves of *G. pogonopus* was characterized by GC-FID and GC/MS analyses, and the *in vitro* and *in vivo* antitumor effects of this oil were investigated.

Results and Discussion. – *Chemical Composition.* Hydrodistillation of *G. pogonopus* leaves gave a crude, red essential oil with a yield of 0.28 ± 0.00% (*v*/*w*, based on the dry weight of the plant material). As shown in *Table 1*, 29 compounds were identified by GC-FID and GC/MS analyses. The major compounds were γ-patchoulene (13.55%), (*E*)-caryophyllene (11.36%), β-pinene (10.37%), germacrene D (6.72%), bicyclogermacrene (5.97%), α-pinene (5.33%), and germacrene B (4.69%). The presence of some of these major compounds, along with spathulenol (3.57%), was also detected in other essential oils from *Guatteria* species [4][7][12][13], indicating that *G. pogonopus* is a typical member of the Annonaceae family. However, recent chemical

Table 1. *Chemical Composition of the Essential Oil Isolated from Leaves of* Guatteria pogonopus

Compound name and class	RI_{exp} [a])	RI_{lit} [b])	Content [%] [c])
(*E*)-Hex-3-enol	841	844	1.85 ± 0.41
(*Z*)-Hex-2-enol	854	859	0.77 ± 0.20
α-Pinene	930	932	5.33 ± 1.10
β-Pinene	975	974	10.37 ± 1.71
o-Cymene	1023	1022	0.58 ± 0.10
Sylvestrene	1028	1025	2.52 ± 0.26
β-Phellandrene	1029	1025	1.03 ± 0.11
(*E*)-β-Ocimene	1046	1044	2.90 ± 0.30
Linalool	1099	1095	0.40 ± 0.03
δ-Elemene	1339	1335	0.55 ± 0.01
α-Ylangene	1373	1373	0.54 ± 0.04
α-Copaene	1379	1374	0.58 ± 0.03
β-Elemene	1391	1389	0.89 ± 0.03
(*E*)-Caryophyllene	1421	1417	11.36 ± 0.50
γ-Elemene	1430	1434	3.55 ± 0.03
cis-Muurola-3,5-diene	1450	1448	0.35 ± 0.02
Spirolepechinene	1454	1449	1.90 ± 0.10
α-Humulene	1457	1452	0.66 ± 0.03
Germacrene D	1483	1484	6.72 ± 0.15
γ-Amorphene	1495	1495	0.73 ± 0.04
Bicyclogermacrene	1497	1500	5.97 ± 0.05
γ-Patchoulene	1507	1502	13.55 ± 0.41
δ-Cadinene	1519	1522	0.98 ± 0.03
Germacrene B	1561	1559	4.69 ± 0.20
Spathulenol	1578	1577	3.57 ± 0.27
Globulol	1588	1590	0.76 ± 1.31
Viridiflorol	1591	1592	1.66 ± 1.44
Rosifoliol	1595	1600	1.07 ± 0.45
Alloaromadendrene epoxide	1634	1639	0.36 ± 0.06
Alcohols			2.62
Monoterpenes			23.13
Sesquiterpenes			60.44
Total identified			86.19

[a]) RI_{exp}: Retention index determined on a *Rtx®-5MS* column rel. to the t_R of a series of *n*-alkanes, according to *Van Den Dool* and *Kratz* [13]. [b]) RI_{lit}: Retention index according to *Adams* [14]. [c]) Contents are expressed as mean ± SD ($n = 3$).

investigations have demonstrated significant variations in the chemical composition of the essential oils from species belonging to this genus.

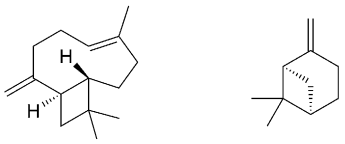
Maia et al. [12] analyzed the chemical composition of four Amazon *Guatteria* species (*G. juruensis*, *G. microcalyx*, *G. poeppigiana*, and *G. blepharophylla*) and observed variations in their chemical composition. Indeed, the main compounds found in the leaf oil of *G. juruensis* were spathulenol (77.5%) and α-pinene (4.5%). The leaf oil of *G. microcalyx* was dominated by caryophyllene oxide (44.2%), α-pinene (11.9%), and β-pinene (6.3%). The major constituents identified in the leaf oil of *G. poeppigiana* were spathulenol (53.0%), kushinol (10.9%), and humulene epoxide II (5.7%), whereas those found in the leaf oil of *G. blepharophylla* were caryophyllene oxide (51.0%), humulene epoxide II (6.8%), and (*E*)-14-hydroxy-9-epicaryophyllene (4.1%).

Aciole et al. [7] also analyzed the chemical composition of three Amazon *Guatteria* species (*G. blepharophylla*, *G. friesiana*, and *G. hispida*) and found significant differences in their chemical composition. Caryophyllene oxide (70.0%) predominated in the essential oil from the leaves of *G. blepharophylla*, while α-, β-, and γ-eudesmol (15.1, 52.0, and 24.0%, resp.) were the main compounds in the essential oil from the leaves of *G. friesiana*. The major constituents identified in the leaf oil of *G. hispida* were α- and β-pinene (31.0 and 36.0%, resp.) and (*E*)-caryophyllene (21.0%). These results are in agreement with those reported by *Costa et al.* [4].

Palazzo et al. [13] analyzed the chemical composition of three *Guatteria* species from Costa Rica and also observed differences in their chemical composition. The essential oil from leaves of *G. costaricensis* was constituted mainly of α- and β-pinene (36.3 and 48.2%, resp.) as well as (*E*)-caryophyllene (5.4%). The leaf oil of *G. diospyroides* was composed principally of germacrene D (46.4%), (*Z*)-β-ocimene (17.4%), (*E*)-β-ocimene (12.0%), and (*E*)-caryophyllene (10.3%). Germacrene D predominated in the leaf oil of *G. oliviformis* (73.3%), but α- and β-pinene (3.4 and 4.4%, resp.) and bicyclogermacrene (4.5%) were also detected in considerable amounts.

These significant variations in the major oil constituents as well as the varying contents of all oil components of the various *Guatteria* species might be explained by the different climate conditions of these regions. Nevertheless, the presence at high concentrations of α- and β-pinene, (*E*)-caryophyllene, germacrene D, and bicyclogermacrene appears to be a common characteristic of the essential oils of *Guatteria* species.

In vitro *Cytotoxicity.* The *in vitro* cytotoxicity of the essential oil isolated from the leaves of *G. pogonopus* and its components (*E*)-caryophyllene and β-pinene (*Fig. 1*) was evaluated against three different human tumor cell lines, *i.e.*, OVCAR-8, NCI-H358M, and PC-3M, using the MTT assay. *Table 2* summarizes the IC_{50} values for the cytotoxic activity. The essential oil, but neither (*E*)-caryophyllene nor β-pinene, showed *in vitro* cytotoxicity against all tested tumor cell lines. The obtained average IC_{50} values ranged from 3.8 to 20.8 μg/ml. The lowest and highest values were obtained against the NCI-H358M and the OVCAR-8 cell lines, respectively. The positive control doxorubicin showed IC_{50} values ranging from 0.9 (NCI-H358M cells) to 1.6 μg/ml (PC-3M cells). Concerning our screening program for cytotoxic activity, essential oils and pure compounds with IC_{50} values below 30 and 1 μg/ml, respectively, are considered



(*E*)-Caryophyllene

β-Pinene

Fig. 1. *Chemical structures of (*E*)-caryophyllene and β-pinene*

Table 2. In vitro *Cytotoxic Activity of the Leaf Essential Oil of* Guatteria pogonopus

	IC_{50} [μg/ml][a])		
	OVCAR-8	NCI-H358M	PC-3M
Essential oil	20.8 (16.1–26.9)	3.8 (2.6–5.5)	17.0 (12.3–23.4)
β-Pinene	>5	>5	>5
(*E*)-Caryophyllene	>5	>5	>5
Doxorubicin[b])	1.2 (0.9–1.6)	0.9 (0.6–1.3)	1.6 (1.1–2.4)

[a]) The IC_{50} values were obtained by nonlinear regression from two independent experiments, performed in duplicate and measured with the MTT assay after 72 h of incubation of the human tumor cell lines OVCAR-8 (ovarian adenocarcinoma), NCI-H358M (bronchoalveolar lung carcinoma), and PC-3M (metastatic prostate carcinoma) with the oil or the test compounds; values are means with 95% confidence limits in parentheses. [b]) Doxorubicin was used as positive control.

promising [16–19]. Therefore, the *G. pogonopus* essential oil was considered to possess potent cytotoxic activity. On the other hand, (*E*)-caryophyllene and *β*-pinene were regarded as compounds without cytotoxic potential ($IC_{50}>5$ μg/ml). The cytotoxic activity of (*E*)-caryophyllene and *β*-pinene have previously been tested, and they showed IC_{50} values higher than 25 μg/ml and of *ca.* 24 μg/ml, respectively [8]. In another study, (*E*)-caryophyllene showed cytotoxic activity against a renal adenocarcinoma (ACHN) and an amelanotic melanoma (C32) cell line with IC_{50} values of *ca.* 20 μg/ml [20]. Probably, the potent cytotoxic activity of the *G. pogonopus* essential oil tested might be attributed to additive and/or synergic effects of its main and minor constituents.

The cytotoxic activity of some essential oils from *Guatteria* species have been previously investigated by us. The antitumor effects of *G. friesiana* essential oil have been studied using both *in vitro* and *in vivo* models, and these effects seem to be assigned to its main components *α*-, *β*-, and *γ*-eudesmol [10]. The essential oils isolated from *G. blepharophylla* and *G. hispida* have also shown potent cytotoxic activity. In contrast to the *G. friesiana* oil, but similar to the *G. pogonopus* oil, the association of the activity of main and/or minor constituents seems to be responsible for their cytotoxic activity [8].

In vivo *Antitumor Activity.* For the study of the *in vivo* antitumor activity of *G. pogonopus* leaf essential oil, mice were subcutaneously transplanted with sarcoma 180 cells and treated with oil, by the intraperitoneal route, once a day for seven consecutive days. The effects of the essential oil on mice transplanted with sarcoma 180 tumor are presented in *Fig. 2*. On the eighth day, the average tumor weight of the control mice was 1.91 ± 0.14 g. In the presence of the essential oil (50 and 100 mg/kg/d), the average

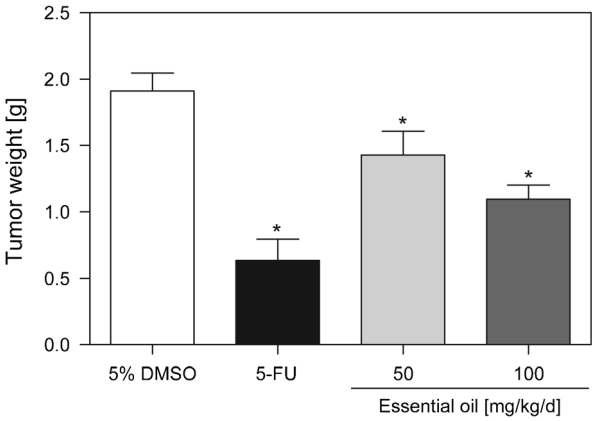


Fig. 2. In vivo *antitumor effect of the leaf essential oil of* Guatteria pogonopus. Mice were injected with sarcoma 180 tumor cells (2.0×10^6 cells/animal, *s.c.*) and treated by intraperitoneal administration of essential oil (50 and 100 mg/kg/d) or the positive control 5-fluorouracil (5-FU, 25 mg/kg/d) for seven consecutive days, starting one day after tumor implantation. The negative control group was treated with the vehicle used for the dilution of the tested substances (5% DMSO). Data are presented as mean ± SEM of 8–12 animals. Significant differences compared to control group (ANOVA followed by *Student–Newman–Keuls* test): $p < 0.05$ (*).

tumor weights were 1.43 ± 0.18 and 1.10 ± 0.11 g, respectively. Hence, the tumor-growth-inhibition rates were 25.3 and 42.6%. The tumor inhibition was significant for both doses compared to the control group ($p < 0.05$). At a dose of 25 mg/kg/d, the positive control 5-fluorouracil (5-FU) reduced the tumor weight by 66.8% within the same time period.

Some systemic toxicological parameters were also examined in the essential oil-treated mice. The treatment with essential oil did not significantly affect the body mass, the macroscopic structure of the organs (liver, kidney, and spleen), and the blood leukocyte counts ($p > 0.05$, data not shown). However, anal ulcers were observed at the end of the treatment in mice receiving essential oil at the dose of 100 mg/kg/d. In contrast, the positive control 5-FU reduced the body weight of mice as well as the spleen weight, and it induced a decrease in the total leukocytes ($p < 0.05$, data not shown).

In conclusion, the essential oil isolated from leaves of *G. pogonopus* presented as major constituents γ-patchoulene, (E)-caryophyllene, β-pinene, germacrene D, bicyclogermacrene, α-pinene, and germacrene B and showed significant *in vitro* and *in vivo* antitumor activity.

Experimental Part

Plant Material. The *Guatteria pogonopus* leaves were collected in February 2012 in the Itabaiana Mountain National Park, Municipality of Itabaiana, Sergipe, Brazil (coordinates: 10°45′16.8″ S, 37°20′32.7″ W). The leaves were obtained from a flowering and fructifying plant. The plant material was identified by Dr. *Ana Paula do Nascimento Prata*, a plant taxonomist from the Department of Biology, Federal University of Sergipe, Brazil, and a voucher specimen (No. 22793) has been deposited with the Herbarium of the Federal University of Sergipe.

Chemical Compounds and Reagents. 5-Fluorouracil (5-FU, purity >99%), doxorubicin (purity >98%), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from *Sigma Chemical Co.* (St Louis, MO, USA). *RPMI 1640* Medium, fetal bovine serum, penicillin, and streptomycin were obtained from *Cultilab* (Campinas, SP, Brazil), and CO_2 was purchased from *White Martins* (Rio de Janeiro, Brazil). The compounds (*E*)-caryophyllene (purity ≥86 %) and β-pinene (purity ≥97 %) were obtained from *Aldrich Chemical Company*, Milwaukee, Wisconsin, USA. All other reagents were of analytical grade.

Cells. The cytotoxicity assay was performed using OVCAR-8 (ovarian adenocarcinoma), NCI-H358M (*bronchoalveolar lung carcinoma*), and PC-3M (metastatic prostate carcinoma) human tumor cell lines, all obtained from the National Cancer Institute, Bethesda, MD, USA. The cells were grown in *RPMI-1640* medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin and incubated at 37° in a 5% CO_2 atmosphere.

The sarcoma 180 tumor cells, which had been maintained in the peritoneal cavity of *Swiss* mice, were obtained from the Laboratory of Experimental Oncology, Federal University of Ceará.

Animals. A total of 40 Swiss mice (males, 25–30 g) obtained from the central animal house of the Federal University of Sergipe, Brazil, were used. The animals were housed in cages with free access to food and water and kept under a standard light-dark cycle of 12 h (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*), and the experimental protocol (No. 08/2012) was approved by the Animal Studies Committee of the Federal University of Sergipe.

Hydrodistillation of the Volatile Constituents. Portions of *G. pogonopus* leaves (3 × 200 g) were dried in a stove with circulating air at 40° for 72 h and submitted to hydrodistillation for 4 h using a *Clevenger*-type apparatus (*Amitel*, São Paulo, Brazil) [21]. The essential oil was dried (anh. Na_2SO_4), and its yield in % (*v*/*w*) was calculated on the basis of the dry weight of the plant material. The essential oil was stored in a freezer until analyses. The hydrodistillation was performed in triplicate.

GC-FID and GC/MS Analyses. The GC-FID and GC/MS analyses were performed with a *GC-2010 Plus* and a *GCMS-QP2010 Ultra* (*Shimadzu Corporation*, Kyoto, Japan) apparatus, resp., equipped with an *AOC-20i* (*Shimadzu*) autosampler and an *Rtx®-5MS* (*Restek*) fused-silica cap. column (5% diphenyl/95% dimethylpolysiloxane; 30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temp. was programmed isothermal at 40° for 1.5 min, then rising from 40 to 230° at 4°/min, and finally kept isothermal at 230° for 5 min (total analysis time, 54 min); carrier gas, He (99.999%; 1.2 ml/min); split ratio, 1:10; injection volume, 0.5 μl of the essential oils in AcOEt (5.0 mg/ml).

The MS and FID data were simultaneously acquired employing a detector splitting system with a split-flow ratio of 4:1 (MS/FID). Restrictor tubes (capillary columns) of 0.62 m × 0.15 mm i.d. and 0.74 m × 0.22 mm i.d. were used to connect the splitter to the MS and the FID detector, resp. The injector and ion-source temp. were 250 and 200°, resp. MS Spectra were taken at 70 eV with a scan interval of 0.3 s over the mass range 40–350 Da. The FID temp. was set to 250°, and the gas supplies for the FID were H_2, air, and He at flow rates of 30, 300, and 30 ml/min, resp.

The content of each constituent was estimated by FID peak-area normalization (%). The analyses of the essential oil were performed in triplicate.

Identification of the Oil Constituents. The essential oil components were identified by comparison of *i*) their retention times (t_R) with those of standard compounds analyzed under identical conditions, *ii*) their retention indices (*RI*s, determined on a *Rtx®-5MS* column rel. to the t_R of a series of *n*-alkanes, according to *Van Den Dool* and *Kratz* [14]) with those published in the literature [14], and *iii*) their mass spectra with those listed in the *NIST* and *Wiley* mass spectral libraries and those published in the literature [15].

In vitro *Cytotoxicity Assay.* The tumor cell growth was determined by the ability of living cells to reduce the yellow dye MTT to a purple formazan product, as described by *Mosmann* [22]. For all experiments, cells were seeded in 96-well plates in 100 μl of medium (0.7×10^5 cells/ml for adherent cells and 0.3×10^6 cells/ml for suspended cells). After 24 h, the essential oil or compounds to be tested (0.78–50 μg/ml for the oil and 0.078–5 μg/ml for isolated compounds) were dissolved in pure DMSO and added to each well, using the high-throughput screening (HTS) system *Biomek 3000* (*Beckman Coulter Inc.*, Fullerton, CA, USA). Then, the cells were incubated for 72 h. Doxorubicin was used as the positive

control. At the end of incubation, the plates were centrifuged and the medium was replaced by 150 µl fresh medium containing 0.5 mg/ml MTT. After 3 h, the formazan product was dissolved in 150 µl DMSO and the absorbance was measured using a multiplate reader (*DTX 880 Multimode Detector*, *Beckman Coulter Inc.*, Fullerton, CA, USA). The effects of the oil and test compounds were expressed as percentage of the absorbance of reduced dye at 595 nm of the control.

In vivo *Antitumor-Activity Assay.* The *in vivo* antitumor effect was evaluated using sarcoma 180 ascites tumor cells and following protocols previously described [10][16][23][24]. Ten-day old sarcoma 180 ascites tumor cells (2×10^6 cells per 500 µl) were implanted subcutaneously into the left hind groin of mice. The essential oil was dissolved in 5% DMSO and given to mice intraperitoneally once a day for seven consecutive days. At the beginning of the experiment, the mice were divided into four groups of 8–12 animals as follows: *Group 1*, animals treated by injection of vehicle (5% DMSO; $n = 12$); *Group 2*, animals treated by injection of 5-FU (25 mg/kg/d; $n = 10$); *Group 3*, animals treated by injection of the essential oil (50 mg/kg/d; $n = 10$); *Group 4*, animals treated by injection of the essential oil (100 mg/kg/d; $n = 8$). The treatments were started one day after tumor injection. The dosages were determined based on previous studies. On day eight, the animals were sacrificed by cervical dislocation, and the tumors were excised and weighed. The effects of the oil and test compounds were expressed as percent inhibition of tumor growth compared to the control (*Group 1*).

Systemic-Toxicity Evaluation. Body mass loss, organ weight alterations, and changes in the leukocyte counts were determined at the end of the *in vivo* antitumor-activity assay as previously described [10][16][24]. Peripheral blood samples of the mice were collected from the retro-orbital plexus under light ether anesthesia, and the animals were sacrificed by cervical dislocation. After sacrifice, the liver, kidney, and spleens were removed and weighed. For the hematological analysis, total leukocyte counts were determined by standard manual procedures using light microscopy.

Statistical Analysis. Data are presented as mean ± SEM (or SD) or as IC_{50} values with 95% confidence intervals (CI 95%) obtained by nonlinear regression. The differences between experimental groups were compared by 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*, San Diego, CA, USA).

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