

Chemical Constituents and Anticancer Effects of the Essential Oil from Leaves of *Xylopi* *laevigata*

Authors

Jullyana de S. S. Quintans¹, Bruno M. Soares², Rosana P. C. Ferraz¹, Allan C. A. Oliveira¹, Thanany B. da Silva³, Leociley R. A. Menezes³, Marília F. C. Sampaio³, Ana Paula do N. Prata⁴, Manoel O. Moraes², Claudia Pessoa², Angelo R. Antoniolli¹, Emmanoel V. Costa³, Daniel P. Bezerra¹

Affiliations

The affiliations are listed at the end of the article

Key words

- *Xylopi* *laevigata*
- Annonaceae
- essential oil
- chemical composition
- antitumor

Abstract

Xylopi *laevigata*, popularly known as “meiú” and “pindaíba”, is a medicinal plant used in the folk medicine of the Brazilian Northeast for several purposes. The chemical constituents of the essential oil from leaves of *X. laevigata*, collected from wild plants growing at three different sites of the remaining Atlantic forest in Sergipe State (Brazilian Northeast), were analyzed by GC/FID and GC/MS. The effect of the essential oil samples was assessed on tumor cells in culture, as well on tumor growth *in vivo*. All samples of the essential oil were dominated by sesquiterpene constituents. A total of 44 compounds were identified and quantified. Although some small differences were observed in the chemical composition, the presence of γ -muurolene (0.60–17.99%), δ -cadinene (1.15–13.45%), germacrene B (3.22–7.31%), α -copaene (3.33–5.98%), germacrene D (9.09–

60.44%), bicyclogermacrene (7.00–14.63%), and (*E*)-caryophyllene (5.43–7.98%) were verified as major constituents in all samples of the essential oil. In the *in vitro* cytotoxic study, the essential oil displayed cytotoxicity to all tumor cell lines tested, with the different samples displaying a similar profile; however, they were not hemolytic or genotoxic. In the *in vivo* antitumor study, tumor growth inhibition rates were 37.3–42.5%. The treatment with the essential oil did not significantly affect body weight, macroscopy of the organs, or blood leukocyte counts. In conclusion, the essential oil from the leaves of *X. laevigata* is chemically characterized by the presence of γ -muurolene, δ -cadinene, germacrene B, α -copaene, germacrene D, bicyclogermacrene, and (*E*)-caryophyllene as major constituents and possesses significant *in vitro* and *in vivo* anticancer potential.

received August 30, 2012
revised October 15, 2012
accepted Dec. 2, 2012

Bibliography

DOI <http://dx.doi.org/10.1055/s-0032-1328091>
Published online January 10, 2013
Planta Med 2013; 79: 123–130
© Georg Thieme Verlag KG
Stuttgart · New York ·
ISSN 0032-0943

Correspondence

Prof. Dr. Daniel P. Bezerra
Department of Physiology
Federal University of Sergipe
Av. Marechal Rondon, Jardim
Rosa Elze
49100–000, São Cristóvão
Sergipe
Brazil
Phone: + 55 79 21 05 66 44
danielpbezerra@gmail.com

Introduction

The *Xylopi* species (family Annonaceae) are aromatic plants that have both nutritional and medicinal uses. Several studies have reported cytotoxic activity for plants belonging to this genus, such as *Xylopi* *aethiopic* (Dunal) A. Rich. [1], *Xylopi* *aromatic* (Lam.) Mart. in Mart. [2], *Xylopi* *langsdorffiana* St.-Hil. & Tul. [3], and *Xylopi* *sericea* A. St.-Hil. [4].

Xylopi *laevigata* (Mart.) R. E. Fries is a medicinal plant found in the tropical American forest. In Brazil, it is popularly known as “meiú” and “pindaíba” and can be found in the States of Piauí, Paraíba, Sergipe, Rio de Janeiro, and São Paulo [5]. Although the principles of the popular use of this plant are almost unknown, by interview of the local population, we found that a *decoction* of its leaves and flowers is used in the folk medicine of the Brazilian Northeast for several purposes, in-

cluding painful disorders, heart disease, and inflammatory conditions. In addition, some *Xylopi* species have been used in the folk medicine for treating tumors. Nonetheless, almost no scientific research was made of this plant.

A phytochemical and biological study of the stem from *X. laevigata* was performed by Silva et al. [6]. The *ent*-kaurane diterpenoids, *ent*-kaur-16-en-19-oic acid, 4-*epi*-kaurenic acid, *ent*-16 β -hydroxy-17-acetoxy-kauran-19-al, *ent*-3 β -hydroxy-kaur-16-en-19-oic acid, and *ent*-16 β ,17-dihydroxy-kauran-19-oic acid, as well as spathulenol and a mixture of β -sitosterol, stigmaterol, and campesterol, were isolated from the hexane extract from *X. laevigata* stem. Potent larvicidal activity against *Aedes aegypti* larvae and antifungal activity against *Candida glabrata* and *Candida dubliniensis* were found for *ent*-3 β -hydroxy-kaur-16-en-19-oic acid. However, chemical analysis or investigation of the biological properties

of the essential oil from leaves of *X. laevigata* has not been previously performed.

The chemical composition of the essential oil from leaves of *X. laevigata*, collected from wild plants growing at three different sites of the remaining Atlantic forest in Sergipe State (Brazilian Northeast), was analyzed by GC/FID and GC/MS. In addition, the effect of the essential oil samples was assessed on tumor cells in culture as well as on tumor growth *in vivo*.

Material and Methods



Reagents

5-Fluorouracil (5-FU, purity >99%), doxorubicin (purity >98%), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), ficoll-hypaque, phytohemagglutinin, and resazurin were purchased from Sigma Chemical Co. Triton X-100 (purity >98%) was purchased from Vetec. RPMI 1640 medium, fetal bovine serum, penicillin, and streptomycin were purchased from Cultilab. Carbon dioxide (CO₂) was purchased from White Martins. All other reagents were of analytical grade.

Cells

The cytotoxicity of the essential oil was tested against HL-60 (promyelocytic leukemia), OVACAR-8 (ovarian carcinoma), SF-295 (glioblastoma), and HCT-116 (colon carcinoma) human cancer cell lines, all obtained from the National Cancer Institute, Bethesda, MD, USA. 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 °C with a 5% CO₂ atmosphere.

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

In order to get healthy human peripheral blood mononuclear cells (PBMC), heparinized blood (from healthy, nonsmoking donors who had not taken any drug at least 15 days prior to sampling) was collected, and the PBMC were isolated by a standard method of density-gradient centrifugation over ficoll-hypaque. The PBMC were washed and resuspended. Cells were grown under the same conditions as above plus the addition of phytohemagglutinin (4%). The Human Research Ethics Committee approved this experimental protocol.

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. 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.). Animals were treated according to the ethical principles for animal experimentation of SBCAL (the Brazilian association of laboratory animal science), Brazil. The Animal Studies Committee from the Federal University of Sergipe approved the experimental protocol (number 08/2012).

Plant material

X. laevigata leaves were collected from wild plants growing at three different sites of the remaining Atlantic forest in Sergipe State (Brazilian Northeast). Sample A (voucher number 15440) was collected in April 2010 at Itabaiana Mountain National Park in the Municipality of Itabaiana (coordinates: S 10°44' 53" W 37°20' 21"). Sample B (voucher number 15443) was collected in

March 2010 at "Mata do Crasto" in the Municipality of Santa Luzia do Itanhý (coordinates: S 11°22' 54" W 37°25' 15"). And sample C (voucher number 17359) was collected in July 2010 near the campus of the Federal University of Sergipe in the Municipality of São Cristóvão (coordinates: S 10°55' 08" W 37°06' 13"). All specimens were obtained from flowering plants. The species was identified by Dr. Ana Paula do Nascimento Prata, a plant taxonomist from the Department of Biology at the Federal University of Sergipe. The voucher botanic specimens were deposited at the Herbarium of the Federal University of Sergipe.

Essential oil isolation and chemical analysis

Oil isolation: The *X. laevigata* leaf specimen samples (200 g each) were dried separately in a stove with circulating air at 40 °C for 24 h and submitted to hydrodistillation for 3 h using a Clevenger-type apparatus (Amitel). The hydrodistillation was performed in triplicate. The essential oil was dried over anhydrous sodium sulphate and stored in a freezer until analysis. The essential oil yields (v/w), calculated from dry plant weight, were 1.18 ± 0.13% for sample A, 1.58 ± 0.12% for sample B, and 1.40 ± 0.14% for sample C.

GC/FID analysis: GC analyses were carried out using a Shimadzu GC-17A fitted with a flame ionization detector (FID) and an electronic integrator. Separation of the compounds was achieved employing a ZB-5MS fused capillary column (30 m × 0.25 mm × 0.25 µm film thickness) coated with 5%-phenyl-arylene-95%-methylpolysiloxane. Helium was the carrier gas at 1.2 mL/min flow rate. The column temperature program was: 50 °C/2 min, then a temperature increase of 4 °C/min to 200 °C, followed by another temperature increase of 15 °C/min to 300 °C, finishing with 300 °C/15 min. The injector and detector temperatures were 250 °C and 280 °C, respectively [7]. Samples (0.5 µL in CH₂Cl₂) were injected with a 1:100 split ratio. Retention indices were generated with a standard solution of *n*-alkanes (C₉–C₁₈). Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without FID response factor correction. **GC/MS analysis:** GC/MS analyses were performed on a Shimadzu QP5050A GC/MS system equipped with an AOC-20i auto-injector. A J&W Scientific DB-5MS (coated with 5%-phenyl-95%-methylpolysiloxane) fused capillary column (30 m × 0.25 mm × 0.25 µm film thickness) was used as the stationary phase. MS were taken at 70 eV with a scan interval of 0.5 s and fragments from 40–500 Da. All other conditions were similar to the GC analysis [7].

Identification of constituents

The essential oil components were identified by comparing the retention times of the GC peaks with standard compounds ran under identical conditions, by comparison of retention indices [8] and MS [9] with those in the literature, and by comparison of MS with those stored in the NIST and Wiley databases.

In vitro cytotoxic evaluation of the essential oil

Determination of the effect of the essential oil on cultured tumor and normal cells: Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye MTT to a purple formazan product, as described by Mossman [10]. For all experiments, 100 µL of a solution of cells (0.7 × 10⁵ cells/mL for adherent cells or 0.3 × 10⁶ cells/mL) were seeded in 96-well plates. After 24 h, the essential oil (0.39–50 µg/mL), dissolved in dimethyl sulfoxide (DMSO), was added to each well (using the HTS – highthroughput screening – Biomek 3000; Beckman Coulter, Inc.) and incubated

for 72 h. For this, a stock solution (10 mg/mL) was prepared using DMSO, and then this stock solution was diluted using medium. Doxorubicin was used as the positive control. Negative control was treated with the vehicle used for diluting the test substance (0.5% DMSO). At the end of incubation, the plates were centrifuged, and the medium was replaced by fresh medium (150 μ L) containing 0.5 mg/mL MTT. Three hours later, the formazan product was dissolved in 150 μ L of DMSO, and the absorbance was measured using a multiplate reader (DTX 880 Multimode Detector; Beckman Coulter, Inc.). The drug effect was quantified as the percentage of control absorbance of reduced dye at 595 nm.

PBMC cell growth was determined by the Alamar blue assay, as described by Ahmed et al. [11]. For all experiments, 100 μ L of a solution of cells (0.3×10^6 cells/mL) were seeded in 96-well plates. After 24 h, the essential oil (0.39–50 μ g/mL), dissolved in DMSO, was added to each well (using HTS, high-throughput screening, Biomek 3000, Beckman Coulter, Inc.) and incubated for 72 h. Doxorubicin was used as the positive control. Negative control was treated with the vehicle used for diluting the test substance (0.5% DMSO). Twenty-four hours before the end of incubation, 10 μ L of stock solution (0.312 mg/mL) of Alamar blue (Resazurin) was added to each well. The absorbance was measured using a multiplate reader (DTX 880 Multimode Detector; Beckman Coulter®), and the drug effect was quantified as the percentage of control absorbance at 570 nm and 595 nm.

Determination of the effect of the essential oil on mouse erythrocytes: The hemolytic activity was performed using mouse erythrocyte, as described by Jimenez et al. [12]. The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM CaCl₂. The essential oil samples were tested at concentrations ranging from 31.25 to 500 μ g/mL. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed, and the hemoglobin released was measured spectrophotometrically as the absorbance at 540 nm.

Determination of the genotoxic effect of the essential oil by the alkaline comet assay: The alkaline (pH > 13) version of the comet assay (Single Cell Gel Electrophoresis) was performed as described by Singh et al. [13] with minor modifications [14]. Slides were prepared in duplicate, and 100 cells were screened per sample (50 cells from each duplicate slide) using a fluorescence microscope (Zeiss) equipped with a 515–560 nm excitation filter, a 590 nm barrier filter, and a 40X objective. Cells were scored visually into five classes according to tail length: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length 1–2 \times the diameter of the head; (4) class 3: with a tail longer than 2 \times the diameter of the head; (5) class 4: comets with no heads. A value of damage index (DI) was assigned to each comet according to its class, using the formula: $DI = (0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)$, where n = number of cells in each class analyzed. The damage index ranged from 0 (completely undamaged: 100 cells \times 0) to 400 (with maximum damage: 100 cells \times 4). DI is based on migration length and on the amount of DNA in the tail, and it is considered a sensitive DNA measure [15].

In vivo antitumor evaluation of the essential oil

Determination of the effect of the essential oil on the growth of solid tumor in vivo: The *in vivo* antitumor effect was evaluated using sarcoma 180 ascites tumor cells following protocols previously described [16–18]. Ten-day-old sarcoma 180 ascites tumor cells (2×10^6 cells per 500 μ L) 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 7 consecutive days. At the beginning of the experiment, the mice were divided into four groups, as follows: Group 1: animals treated by i.p. injection of vehicle 5% DMSO ($n = 12$); Group 2: animals treated by i.p. injection of 5-FU (25 mg/kg/day) ($n = 10$); Group 3: animals treated by i.p. injection of the essential oil (50 mg/kg/day) ($n = 8$); Group 4: animals treated by i.p. injection of the essential oil (100 mg/kg/day) ($n = 10$). The treatments were started one day after tumor injection. The dosages were determined based on previous articles [18,19]. On day 8, the animals were sacrificed by cervical dislocation, and the tumors were excised and weighed. The drug effects were expressed as the percent inhibition of control.

Systemic toxicological evaluation: Body weight loss, organ weight alteration, and hematological analyses were determined at the end of the above experiment, as previously described [17,18]. 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. In hematological analysis, total leukocyte counts were determined by standard manual procedures using light microscopy.

Statistical analysis

Data were presented as mean \pm SEM (or SD) or IC₅₀ values, and their 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 ($p < 0.05$). All statistical analyses were performed using the GraphPad program (Intuitive Software for Science).

Results



Initially, chemical compositions of three essential oil samples were analyzed by GC/FID and GC/MS. Hydrodistillation of the *X. laevigata* leaf specimens yielded essential oil superior to 1% in relation to the dry weight of the plant material. The best yield was observed for sample B with $1.58 \pm 0.12\%$. All samples of the essential oil were dominated by sesquiterpene constituents with 95.35% in sample A, 98.60% in sample B, and 91.18% in sample C. **Fig. 1** presents a representative chromatogram from GC-MS analysis of the essential oil samples. As shown in **Table 1**, it was possible to identify 44 compounds: 37 in sample A, 19 in sample B, and 28 in sample C. The major compounds identified in the essential oil of sample A were γ -muurolene (17.99%), δ -cadinene (13.45%), germacrene D (9.09%), bicyclogermacrene (7.00%), α -copaene (5.98%), (*E*)-caryophyllene (5.43%), γ -cadinene (4.52%), aromadendrene (3.99%), γ -amorphene (3.92%), and germacrene B (3.22%), while germacrene D (60.44%), bicyclogermacrene (11.65%), (*E*)-caryophyllene (6.37%), germacrene B (3.67%), γ -cadinene (3.38%), and α -copaene (3.33%) were the main constituents in the essential oil of sample B. In sample C, the following predominated: germacrene D (43.62%), bicyclogermacrene (14.63%), (*E*)-caryophyllene (7.98%), germacrene B (7.31%), δ -elemene (4.50%), and α -copaene (3.56%). Although some small differences were observed in the chemical composition of the essential oil samples from *X. laevigata* specimens, the presence of γ -muurolene (0.60–17.99%), δ -cadinene (1.15–13.45%), germacrene B (3.22–7.31%), α -copaene (3.33–5.98%), germacrene D

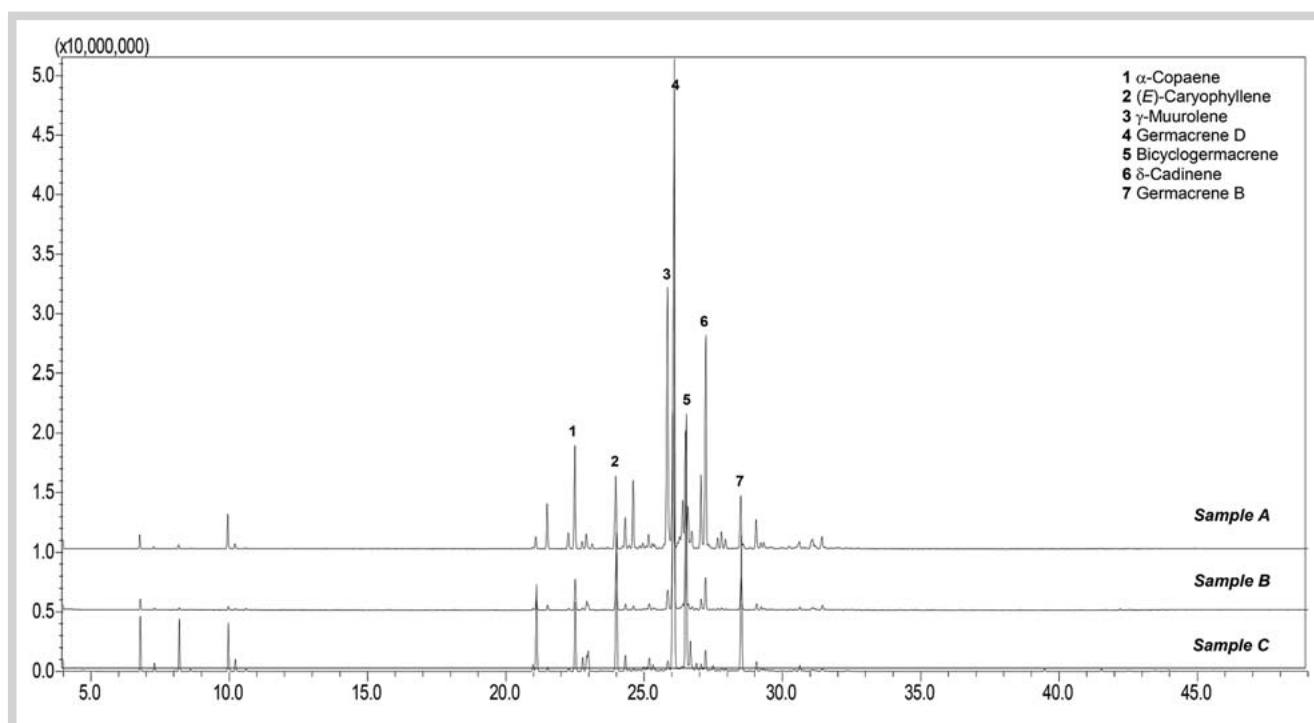


Fig. 1 Total ion current chromatogram from GC-MS analysis of the essential oil samples from leaves of *Xylopia laevigata*. Only the major compounds are presented in the GC-MS chromatogram. Sample A was collected at Itabaiana

Mountain National Park, sample B was collected at “Mata do Crasto” and sample C was collected near the campus of the Federal University of Sergipe.

(9.09–60.44%), bicyclgermacrene (7.00–14.63%), and (*E*)-caryophyllene (5.43–7.98%) were verified as major constituents in all samples of the essential oil.

In a second set of experiments, anticancer activity of the essential oil samples was evaluated in different bioassays. The effect of the essential oil samples was assessed on normal and tumor cells in culture. Genotoxic activity on human lymphocytes and lytic activity on mouse erythrocytes were also determined. In addition, *in vivo* antitumor activity was assessed in Sarcoma 180-bearing mice.

The cytotoxicity of all essential oil samples was evaluated for tumor cell lines from different histotypes using the MTT assay. Doxorubicin was used as a positive control. The cytotoxicity of the essential oil was similar for all samples. **Table 2** summarizes the IC₅₀ data for cytotoxic activity. IC₅₀ values ranging from 14.4 to 31.6 µg/mL were obtained in SF-295 and OVCAR-8 cell lines for sample A, from 17.9 to 27.1 µg/mL in SF-295 and OVCAR-8 cell lines for sample B, and from 18.5 to 27.6 µg/mL in OVCAR-8 and HCT-116 cell lines for sample C, respectively. Doxorubicin, used as a positive control, showed IC₅₀ values ranging from 0.02 to 1.36 µg/mL in HL-60 and OVCAR-8 cell lines, respectively.

The cytotoxicity of the essential oil was also evaluated with normal cells (PBMC). The results, presented in **Table 2**, show that these essential oil samples were also cytotoxic to normal cells. Since the inhibition of tumor cell lines can be related to membrane disruption, all essential oil samples were also tested for the ability to induce lysis of mouse erythrocytes; however, they were not hemolytic, even at the highest concentration tested (500 µg/mL, data not shown). Triton X-100, used as a positive control, showed potent hemolytic effects.

The genotoxicity of a representative sample of the essential oil (sample A) was evaluated in human lymphocytes in culture by

the alkaline comet assay at concentrations of 50, 100, and 200 µg/mL. Genotoxicity assay was performed with only one sample since the *in vitro* cytotoxic effects were similar among the samples. The essential oil did not show genotoxic activity at any experimental concentration tested (data not shown). Doxorubicin, used as a positive control, showed potent genotoxic activity.

To investigate whether the essential oil has *in vivo* antitumor activity, mice were subcutaneously transplanted with sarcoma 180 cells and treated by the intraperitoneal route once a day for 7 consecutive days with the essential oil. Since the *in vitro* cytotoxic effects were similar among the samples, we decided to perform the *in vivo* antitumor study with only one sample. Sample B was used due to the amount of samples available. The effects of the essential oil on mice transplanted with sarcoma 180 tumor are presented in **Table 3**. On day 8, the average tumor weight of the control mice was 1.97 ± 0.17 g. In the presence of the essential oil (50 and 100 mg/kg/day), the average tumor weights were 1.24 ± 0.09 and 1.13 ± 0.27 g, respectively. Tumor growth inhibition rates were 37.3–42.5%. The inhibition was significant at both doses in relation to the control group (*p* < 0.05). With the 25 mg/kg/day dose, 5-FU reduced tumor weight by 67.8% within the same period.

Systemic toxicological parameters were also examined in essential oil-treated mice. For these, body weight loss, organ weight alteration, and leukogram were determined. No statistically significant changes in the essential oil-treated mice were seen in any toxicological parameters analyzed (*p* > 0.05, data not shown). In contrast, 5-FU, used as a positive control, reduced the body weights and spleen organ weights and induced a decrease in total leukocytes (*p* < 0.05, data not shown).

Table 1 Chemical constituents of the essential oil from leaves of *Xylopi laevigata*.

Compounds	RI ^a	RI ^b	Leaf oil %			
			Sample A	Sample B	Sample C	
1	α -Pinene	931	932	0.48 ± 0.14	0.81 ± 0.01	2.08 ± 0.06
2	Camphene	946	946	–	–	0.34 ± 0.01
3	β -Pinene	974	974	0.15 ± 0.05	–	2.16 ± 0.10
4	Limonene	1027	1024	1.41 ± 0.35	0.34 ± 0.01	2.19 ± 0.14
5	(Z)- β -Ocimene	1034	1032	0.11 ± 0.16	–	0.51 ± 0.01
6	δ -Elemene	1333	1335	0.72 ± 0.01	1.64 ± 0.10	4.50 ± 0.10
7	α -Cubebene	1345	1345	2.54 ± 0.00	0.48 ± 0.04	0.21 ± 0.01
8	α -Ylangene	1367	1373	1.00 ± 0.01	–	–
9	α -Copaene	1374	1374	5.98 ± 0.08	3.33 ± 0.06	3.56 ± 0.04
10	β -Bourbonene	1381	1387	0.42 ± 0.00	–	0.72 ± 0.00
11	β -Cubebene	1386	1387	0.95 ± 0.06	0.98 ± 0.04	0.79 ± 0.10
12	β -Elemene	1387	1389	–	0.48 ± 0.01	1.06 ± 0.07
13	(E)-Caryophyllene	1417	1417	5.43 ± 0.01	6.37 ± 0.15	7.98 ± 0.00
14	γ -Elemene	1427	1430	–	–	0.88 ± 0.01
15	β -Copaene	1427	1430	1.82 ± 0.01	0.61 ± 0.03	–
16	Aromadendrene	1436	1439	3.99 ± 0.02	0.43 ± 0.04	–
17	trans-Muurolo-3,5-diene	1447	1451	0.31 ± 0.04	–	–
18	α -Humulene	1453	1452	0.97 ± 0.11	0.61 ± 0.01	0.67 ± 0.02
19	Allo-Aromadendrene	1458	1458	0.33 ± 0.04	–	0.25 ± 0.01
20	cis-Cadina-1(6),4-diene	1460	1461	0.23 ± 0.00	–	–
21	trans-Cadina-1(6),4-diene	1469	1475	0.30 ± 0.02	–	–
22	γ -Muurolole	1474	1478	17.99 ± 0.25	2.41 ± 0.22	0.60 ± 0.01
23	Germacrene D	1480	1484	9.09 ± 0.64	60.44 ± 0.71	43.62 ± 0.49
24	δ -Selinene	1485	1492	0.42 ± 0.04	–	–
25	γ -Amorphene	1490	1495	3.92 ± 0.05	–	–
26	Bicyclogermacrene	1494	1500	7.00 ± 0.23	11.65 ± 0.49	14.63 ± 0.19
27	α -Muurolole	1496	1500	2.50 ± 0.03	0.33 ± 0.03	–
28	(Z)- α -Bisabolene	1498	1506	–	–	1.68 ± 0.01
29	δ -Amorphene	1503	1511	1.38 ± 0.02	–	–
30	β -Bisabolene	1505	1505	–	–	0.42 ± 0.01
31	γ -Cadinene	1511	1513	4.52 ± 0.04	1.16 ± 0.12	0.37 ± 0.01
32	δ -Cadinene	1516	1522	13.45 ± 0.42	3.38 ± 0.34	1.15 ± 0.01
33	trans-Calamenene	1520	1521	0.17 ± 0.23	–	–
34	trans-Cadina-1,4-diene	1530	1533	0.63 ± 0.06	–	–
35	α -Cadinene	1534	1537	1.06 ± 0.04	–	0.17 ± 0.00
36	α -Calacorene	1539	1544	0.63 ± 0.06	–	–
37	Germacrene B	1557	1559	3.22 ± 0.00	3.67 ± 0.04	7.31 ± 0.18
38	β -Calacorene	1560	1564	0.30 ± 0.01	–	–
39	Spathulenol	1575	1577	1.91 ± 0.12	0.67 ± 0.04	0.50 ± 0.01
40	Caryophyllene Oxide	1580	1582	0.37 ± 0.00	–	0.16 ± 0.00
41	α -Muurolol	1642	1644	1.04 ± 0.34	–	–
42	δ -Cadinol	1653	1649	0.85 ± 0.06	–	–
43	Pimaradiene	1915	1948	–	–	0.11 ± 0.01
44	Kaur-16-ene	1977	1979 ^c	–	–	0.11 ± 0.00
	Monoterpene identified			2.14	1.15	7.28
	Sesquiterpene identified			95.35	98.60	91.18
	Diterpene identified			–	–	0.22
	Total identified			97.49	99.75	98.68

Data are presented as mean ± SD of three analyses. ^aRI (calc.): retention indices on DB-5MS column calculated according to Van Den Dool & Kratz [8]; ^bRI: retention indices according to Adams [9]; Sample A: collected at Itabaiana Mountain National Park; Sample B: collected at "Mata do Crasto"; Sample C: collected near the campus of the Federal University of Sergipe

Discussion

The present work investigated the phytochemical and antitumor activity of the essential oil from leaves of *X. laevigata* collected from wild plants growing at three different sites of the remaining Atlantic forest in Sergipe State (Brazilian Northeast). The chemical composition and anticancer activity of the essential oil from leaves of *X. laevigata* were studied here for the first time.

The essential oil from the leaves of *X. laevigata* was chemically characterized by the presence of the major constituents γ -muurolole, δ -cadinene, germacrene B, α -copaene, germacrene D, bicyclogermacrene, and (E)-caryophyllene. Among them, germacrene D, bicyclogermacrene, and (E)-caryophyllene could be considered chemotaxonomic markers of this species since they are found in a high concentration in all essential oil samples investigated. In addition, the minor constituents α -pinene, limonene, α - and β -cubebene, α -humulene, and spathulenol have been re-

Table 2 *In vitro* cytotoxic activity of the essential oil from leaves of *Xylopi*a *laevigata*.

Cell lines	Histotype	IC ₅₀ (µg/mL)			
		Sample A	Sample B	Sample C	Doxorubicin
OVCAR-8	Ovarian carcinoma	31.6	27.1	18.5	1.36
		26.6–37.6	23.1–31.8	15.3–23.0	0.98–1.89
SF-295	Glioblastoma	14.4	17.9	20.5	0.16
		10.2–20.3	13.5–23.8	18.8–22.5	0.13–0.23
HCT-116	Colon carcinoma	22.2	25.5	27.6	Nd
		11.7–42.1	11.7–55.6	19.3–39.6	
HL-60	Promyelocytic leukemia	17.5	25.0	20.6	0.02
		11.7–22.4	10.4–36.2	16.3–25.9	0.01–0.02
PBMC	Peripheral lymphoblast	63.7	35.3	56.9	0.97
		39.1–103.7	21.2–59.0	38.6–83.8	0.52–1.80

Data are presented as IC₅₀ values, and their 95% confidence interval obtained by nonlinear regression from two independent experiments performed in duplicate. Doxorubicin was used as a positive control. Sample A was collected at Itabaiana Mountain National Park, sample B was collected at "Mata do Crasto", and sample C was collected near the campus of the Federal University of Sergipe. Nd = not determined

Table 3 *In vivo* antitumor activity of the essential oil from leaves of *Xylopi*a *laevigata*.

Drug	Dose (mg/kg/day)	Tumor (g)	Inhibition (%)
5% DMSO	–	1.97 ± 0.14	
5-FU	25	0.63 ± 0.16*	67.8
Essential oil	50	1.24 ± 0.09*	37.3
	100	1.13 ± 0.27*	42.5

Mice were injected with sarcoma 180 (2.0×10^6 cells/animal, s. c.). Starting one day after tumor implantation, the animals were treated for seven consecutive days by intraperitoneal route. Data are presented as mean ± SEM for 8–12 animals. Negative control was treated with the vehicle used for diluting the test substance (5% DMSO). 5-Fluorouracil (5-FU) was used as a positive control. A representative sample of the essential oil (sample B) was used. Sample B was collected at "Mata do Crasto".

* $p < 0.05$ compared with the 5% DMSO group by ANOVA followed by Student-Newman-Keuls

ported in essential oils of several other species belonging to the genus *Xylopi*a, some of them, such as α -pinene, limonene, and spathulenol, have been found in high concentrations [20–22]. The presence of the major compounds along with the minor constituents identified α -pinene, limonene, and spathulenol, indicating that this species is a typical member of the Annonaceae family. Although some of these volatile compounds are also present in essential oils of other families, their occurrence in Annonaceae in high concentrations, mainly in the case of bicyclogermacrene, (*E*)-caryophyllene, and germacrene, is very common and has an important chemotaxonomic relevance [7, 20–22].

Although the essential oil samples have shown some difference in chemical composition, as the concentrations of germacrene D and γ -muurolene, they presented similar *in vitro* cytotoxic effect. Probably, the associations of the major and minor constituents are responsible for the cytotoxic activity of this essential oil. The differences in chemical composition can be related to soil and climate conditions, water stress, collection place, nutrition, and other abiotic factors.

All samples of the essential oil displayed cytotoxicity to all tumor cell lines tested, with IC₅₀ values ranging from 14.4 to 31.6 µg/mL. According to the preclinical anticancer drug-screening program used in this study, only those extracts/oils presenting IC₅₀ values below 30 µg/mL in tumor cell line assays are considered promis-

ing for anticancer drug development [23]. Therefore, the essential oil obtained from *X. laevigata* presented promising results. In addition, it inhibited tumor growth in mice in a dose-dependent manner. On the other hand, the essential oil was also cytotoxic to normal cells, presenting low selectivity, but it was not hemolytic or genotoxic.

The erythrocyte membrane is a dynamic structure that can dictate significant changes in its interaction with drugs [24]. The absence of lytic effects suggests that the cytotoxicity of the essential oil is not related to membrane disruption; probably a more specific cellular pathway is responsible for its action. On the other hand, genotoxic effects could be biologically relevant as an alternative strategy for killing tumor cells. The lack of a genotoxic effect suggests that the cytotoxicity of the essential oil is not related to DNA damage. Moreover, this effect needs to be extensively evaluated to assess the safety of novel drugs.

For the *Xylopi*a genus, as cited above, some studies have reported that plants belonging to this genus present cytotoxic activity. The ethanolic extract of *X. aethiopica* fruits was able to induce DNA damage, cell cycle arrest in the G₁ phase, and apoptotic cell death. These effects seem to be related to the diterpene ent-15-oxokaur-16-en-19-oic acid [1]. The methanol extract of *X. aethiopica* seeds was cytotoxic to some tumor cell lines [25]. Interestingly, the essential oil of *X. aethiopica* fruits also showed cytotoxic activity to epidermoid carcinoma cells (Hep-2) [26]. Extracts of the bark, wood, and stem of *X. aromatica* [2, 27] as well as numerous acetogenins isolated from this species [28, 29] showed cytotoxicity to several tumor cell lines. In *X. langsdorffiana*, the isolated compound xylodiol was able to inhibit cell proliferation probably by involvement of the induction of cell differentiation and apoptosis induction [3, 30]. Pita et al. [31] showed that trachylobane-360, a diterpene isolated from *X. langsdorffiana*, displays antitumor effects *in vitro* and *in vivo*, without substantial systemic toxicity. In addition, the diterpenoid kaurenoic acid, isolated from *X. sericea*, showed cytotoxic, genotoxic, and mutagenic effects in many experimental protocols because it induced DNA double-strand breaks and/or inhibition of topoisomerase I [4]. In this present work, the essential oil from leaves of *X. laevigata* showed significant *in vitro* and *in vivo* anticancer potential.

Some constituents of the essential oil from leaves of *X. laevigata* have been studied for their cytotoxic activity. (*E*)-caryophyllene and α -pinene were previously tested by our group and showed IC₅₀ values > 25 µg/mL [32]. In another study, (*E*)-caryophyllene

showed cytotoxic activity with an IC₅₀ value of ~20 µg/mL to renal cell adenocarcinoma (ACHN) and amelanotic melanoma (C32) [33]. Amiel et al. [34] reported that (*E*)-caryophyllene has cytotoxic activity to tumor cell lines but not to normal cells. Moreover, (*E*)-caryophyllene caused a potent induction of apoptosis, accompanied by DNA ladder and caspase-3 catalytic activity in tumor cell lines [34]. α -Humulene showed cytotoxic activity with an IC₅₀ value of 11.2 µg/mL to hormone-dependent prostate carcinoma cells (LNCaP) [35]. Areche et al. [36] showed that spathulenol was cytotoxic to gastric adenocarcinoma cells (AGS). Limonene inhibits the growth of human gastric cancer cells through a mechanism of inducing their apoptosis [37]. In addition, limonene showed anti-metastatic and anti-angiogenic effects in animal models [38]. Others constituents such as γ -muurolene, δ -cadinene, and α -copaene have not been previously tested against tumor cell lines.

The systemic toxicological aspects were also subjected to investigation in the present study. Essential oil-treated animals did not show any significant change in body weight, macroscopy of the organs (kidney, liver, and spleen), or blood leukocyte counts. Unlike the essential oil, 5-FU, used as a positive control, reduced the body weights and spleen organ weights and induced severe leukopenia, which represents an important immunosuppression side effect [39].

In conclusion, the essential oil from leaves of *X. laevigata* is chemically characterized by the presence of γ -muurolene, δ -cadinene, germacrene B, α -copaene, germacrene D, bicylogermacrene, and (*E*)-caryophyllene as major constituents and possesses significant *in vitro* and *in vivo* anticancer potential. Therefore, further investigations to identify the molecule(s) responsible for its activity as well as elucidate the mechanism(s) of the antitumor effect exhibited are necessary.

Acknowledgements

This work was financially supported by Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and FAPITEC/SE (Fundação de Amparo à Pesquisa e à Inovação Tecnológica do Estado de Sergipe). The authors thank Richard Berger for editing the English manuscript.

Conflict of Interest

There is no conflict of interest among the authors.

Affiliations

- 1 Department of Physiology, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil
- 2 Department of Physiology and Pharmacology, Federal University of Ceará, School of Medicine, Fortaleza, Ceará, Brazil
- 3 Department of Chemistry, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil
- 4 Department of Biology, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil

References

- 1 Choumssi AT, Danel M, Chassaing S, Truchet I, Penlap VB, Pieme AC, Asonganyi T, Ducommun B, Valette A. Characterization of the antiproliferative activity of *Xylopiya aethiopicum*. Cell Div 2012; 7: 1–8
- 2 Taylor P, Arsenak M, Abad MJ, Fernández A, Milano B, Gonto R, Ruiz MC, Fraile S, Taylor S, Estrada O, Michelangeli F. Screening of Venezuelan medicinal plant extracts for cytostatic and cytotoxic activity against tu-

- mor cell lines. Phytother Res advance online publication 2012; DOI: 10.1002/ptr.4752
- 3 Castello-Branco MV, Anazetti MC, Silva MS, Tavares JF, Diniz MF, Frungillo L, Haun M, Melo PS. Diterpenes from *Xylopiya langsdorffiana* inhibit cell growth and induce differentiation in human leukemia cells. Z Naturforsch C 2009; 64: 650–656
- 4 Cavalcanti BC, Ferreira JR, Moura DJ, Rosa RM, Furtado GV, Burbano RR, Silveira ER, Lima MA, Camara CA, Saffi J, Henriques JA, Rao VS, Costa-Lotufo LV, Moraes MO, Pessoa C. Structure-mutagenicity relationship of kaurenoic acid from *Xylopiya sericeae* (Annonaceae). Mutat Res 2010; 701: 153–163
- 5 Pontes AF, Barbosa MRV, Maas PJM. Flora Paraibana: Annonaceae Juss. Acta Bot Braz 2004; 18: 281–293
- 6 Silva DM, Costa EV, Nogueira PCL, Moraes VRS, Cavalcanti SCH, Salvador MJ, Ribeiro LHG, Gadelha FR, Barison A, Ferreira AG. Ent-kaurane diterpenoids and other constituents from the stem of *Xylopiya laevigata* (Annonaceae). Quim Nova 2012; 35: 1570–1576
- 7 Costa EV, Dutra LM, De Jesus HCR, Nogueira PCL, Moraes VRS, Salvador MJ, Cavalcanti SCH, Dos Santos RLC, Prata APN. Chemical composition and antioxidant, antimicrobial, and larvicidal activities of the essential oils of *Annona salzmannii* and *A. pickleii* (Annonaceae). Nat Prod Commun 2011; 6: 907–912
- 8 Van Den Dool H, Kratz PD. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr 1963; 11: 463–471
- 9 Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry. Illinois: Allured Publishing Corporation; 2007
- 10 Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55–63
- 11 Ahmed SA, Gogal RM, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes an alternative to [3H] thymidine incorporation assay. J Immunol Methods 1994; 170: 211–224
- 12 Jimenez PC, Fortier SC, Lotufo TMC, Pessoa C, Moraes MEA, Moraes MO, Costa-Lotufo LV. Biological activity in extracts of ascidians (Tunicata, Ascidiacea) from the northeastern Brazilian coast. J Exp Mar Biol Ecol 2003; 287: 93–101
- 13 Singh NP, McCoy MT, Tice RR, Schneider ELA. Single technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988; 175: 184–191
- 14 Hartmann A, Speit G. The contribution of cytotoxicity to DNA effects in the single cell gel test (comet assay). Toxicol Lett 1997; 90: 183–188
- 15 Speit G, Hartmann A. The comet assay (single-cell gel test). A sensitive genotoxicity test for the detection of DNA damage and repair. Methods Mol Biol 1999; 113: 203–212
- 16 Bezerra DP, Castro FO, Alves APNN, Pessoa C, Moraes MO, Silveira ER, Lima MAS, Elmiro FJM, Costa-Lotufo LV. *In vivo* growth-inhibition of sarcoma 180 by piplartine and piperine, two alkaloid amides from *Piper*. Braz J Med Biol Res 2006; 39: 801–807
- 17 Bezerra DP, Castro FO, Alves APNN, Pessoa C, Moraes MO, Silveira ER, Lima MAS, Elmiro FJM, Alencar NMN, Mesquita RO, Lima MW, Lotufo LVC. *In vitro* and *in vivo* antitumor effect of 5-FU combined with piplartine and piperine. J Appl Toxicol 2008; 28: 156–163
- 18 Britto ACS, Oliveira ACA, Henriques RM, Cardoso GMB, Bomfim DS, Carvalho AA, Moraes MO, Pessoa C, Pinheiro MLB, Costa EV, Bezerra DP. *In vitro* and *in vivo* antitumor effects of the essential oil from the leaves of *Gutteria friesiana*. Planta Med 2012; 78: 409–414
- 19 Bezerra DP, Marinho Filho JD, Alves AP, Pessoa C, de Moraes MO, Pessoa OD, Torres MC, Silveira ER, Viana FA, Costa-Lotufo LV. Antitumor activity of the essential oil from the leaves of *Croton regelianus* and its component ascaridole. Chem Biodivers 2009; 6: 1224–1231
- 20 Lago JHG, De Ávila Jr. P, Moreno PRH, Limberger RP, Apel MA, Henriques AT. Analysis, comparison and variation on the chemical composition from the leaf volatile oil of *Xylopiya aromatica* (Annonaceae). Biochem Syst Ecol 2003; 31: 669–672
- 21 Maia JGS, Andrade EHA, Da Silva ACM, Oliveira J, Carreira LMM, Araújo JS. Leaf volatile oils from four Brazilian *Xylopiya* species. Flavour Fragr J 2005; 20: 474–477
- 22 Tavares JF, Silva MVB, Queiroga KF, Martins RM, Silva TMS, Camara CA, Agra MF, Barbosa-Filho JM, Da Silva MS. Composition and molluscicidal properties of essential oils from leaves of *Xylopiya langsdorffiana* A. St. Hil. Et Tul. (Annonaceae). J Essent Oil Res 2007; 19: 282–284

- 23 Suffness M, Pezzuto JM. Assays related to cancer drug discovery. In: Hostettmann K, editor. *Methods in plant biochemistry: assays for bioactivity*. London: Academic Press; 1990: 71–133
- 24 Aki H, Yamamoto M. Drug binding to human erythrocytes in the process of ionic drug-induced hemolysis. *Flow microcalorimetric approaches*. *Biochem Pharmacol* 1991; 41: 133–138
- 25 Kuete V, Krusche B, Youns M, Voukeng I, Fankam AG, Tankeo S, Lacmata S, Efferth T. Cytotoxicity of some Cameroonian spices and selected medicinal plant extracts. *J Ethnopharmacol* 2011; 134: 803–812
- 26 Asekun OT, Adeniyi BA. Antimicrobial and cytotoxic activities of the fruit essential oil of *Xylopiya aethiopica* from Nigeria. *Fitoterapia* 2004; 75: 368–370
- 27 Suffredini IB, Paciencia ML, Varella AD, Younes RN. *In vitro* cytotoxic activity of Brazilian plant extracts against human lung, colon and CNS solid cancers and leukemia. *Fitoterapia* 2007; 78: 223–226
- 28 Colman-Saizarbitoria T, Gu ZM, Zhao GX, Zeng L, Kozlowski JF, McLaughlin JL. Venezenin: a new bioactive Annonaceous acetogenin from the bark of *Xylopiya aromatica*. *J Nat Prod* 1995; 58: 532–539
- 29 Colman-Saizarbitoria T, Zambrano J, Ferrigni NR, Gu ZM, Ng JH, Smith DL, McLaughlin JL. Bioactive annonaceous acetogenins from the bark of *Xylopiya aromatica*. *J Nat Prod* 1994; 57: 486–493
- 30 Castello-Branco MVS, Tavares JF, Silva MS, Filho JMB, Anazetti MC, Frungillo L, Haun M, Diniz MFFM, Melo PS. Xylodiol from *Xylopiya langsdorffiana* induces apoptosis in HL60 cells. *Braz J Pharmacogn* 2011; 21: 1035–1042
- 31 Pita JC, Xavier AL, de Sousa TK, Mangueira VM, Tavares JF, de Oliveira Júnior RJ, Veras RC, Pessoa Hde L, da Silva MS, Morelli S, Ávila Vde M, da Silva TG, Diniz Mde F, Castello-Branco MV. *In vitro* and *in vivo* antitumor effect of trachylobane-360, a diterpene from *Xylopiya langsdorffiana*. *Molecules* 2012; 17: 9573–9589
- 32 Ribeiro SS, Jesus AM, Anjos CS, Silva TB, Santos ADC, Jesus JR, Andrade MS, Sampaio TS, Gomes WF, Alves PB, Carvalho AA, Pessoa C, Moraes MO, Pinheiro MLB, Prata APN, Blank AF, Silva-Mann R, Moraes VRS, Costa EV, Nogueira PCL, Bezerra DP. Evaluation of the cytotoxic activity of some Brazilian medicinal plants. *Planta Med* 2012; 78: 1601–1606
- 33 Tundis R, Loizzo MR, Bonesi M, Menichini F, Dodaro D, Passalacqua NG, Statti G, Menichini F. *In vitro* cytotoxic effects of *Senecio stabianus* Lacaite (Asteraceae) on human cancer cell lines. *Nat Prod Res* 2009; 23: 1707–1718
- 34 Amiel E, Ofir R, Dudai N, Soloway E, Rabinsky T, Rachmilevitch S. β -Caryophyllene, a compound isolated from the biblical balm of gilead (*Commiphora gileadensis*), is a selective apoptosis inducer for tumor cell lines. *Evid Based Complement Alternat Med* 2012; 2012: 872394
- 35 Loizzo MR, Tundis R, Statti GA, Menichini F. Jacaranone: A cytotoxic constituent from *Senecio ambiguus* subsp. *ambiguus* (Biv.) DC. against renal adenocarcinoma ACHN and prostate carcinoma LNCaP cells. *Arch Pharm Res* 2007; 30: 701–707
- 36 Areche C, Schmeda-Hirschmann G, Theoduloz C, Rodríguez JA. Gastroprotective effect and cytotoxicity of abietane diterpenes from the Chilean Lamiaceae *Sphacele chamaedryoides* (Balbis) Briq. *J Pharm Pharmacol* 2009; 61: 1689–1697
- 37 Lu XG, Feng BA, Zhan LB, Yu ZH. D-limonene induces apoptosis of gastric cancer cells. *Zhonghua Zhong Liu Za Zhi* 2003; 25: 325–327
- 38 Lu XG, Zhan LB, Feng BA, Qu MY, Yu LH, Xie JH. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by d-limonene. *World J Gastroenterol* 2004; 10: 2140–2144
- 39 Takiguchi N, Saito N, Nunomura M, Kouda K, Oda K, Furuyama N, Nakajima N. Use of 5-FU plus hyperbaric oxygen for treating malignant tumors: evaluation of antitumor effect and measurement of 5-FU in individual organs. *Cancer Chemother Pharmacol* 2001; 47: 11–14