In vitro and in vivo growth inhibition of human acute promyelocytic leukemia HL-60 cells by *Guatteria megalophylla* Diels (Annonaceae) leaf essential oil

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**ABSTRACT**

*Guatteria megalophylla* Diels (Annonaceae) is an 8–10 m tall tree that grows near streams and is widely spread throughout Colombian, Ecuadorian, Peruvian, Brazilian and Guianan Amazon rainforest. Herein, we investigated for the first time the chemical composition and in vitro and in vivo anti-leukemia potential of *G. megalophylla* leaf essential oil (EO) using human promyelocytic leukemia HL-60 cells as model. EO was obtained by a hydrodistillation clevenger-type apparatus and characterized qualitatively and quantitatively by GC–MS and GC–FID, respectively. In vitro cytotoxic potential of EO was evaluated in human cancer cell lines (HL-60, MCF-7, CAL27, HSC-3, HepG2 and HCT116) and in human non-cancer cell line (MRC-5) by Alamar blue method. Annexin V/propidium iodide staining, cell cycle distribution and reactive oxygen species (ROS) were assessed by flow cytometry for HL-60 cells treated with EO. In vivo efficacy of EO (50 and 100 mg/kg) was evaluated in C.B-17 SCID mice with HL-60 cell xenografts. Chemical composition analyses showed spathulenol, γ-muurolene, bicyclogermacrene, δ-elemene and β-elemene as main constituents of assayed sample. EO displayed in vitro cytotoxicity, including anti-leukemia effect with IC<sub>50</sub> value of 12.51 μg/mL for HL-60 cells. EO treatment caused augment of phosphatidylinerine externalization and DNA fragmentation without increasing of ROS in HL-60 cells. In vivo tumor mass inhibition rates of EO was 16.6–48.8 %. These data indicate anti-leukemia potential of *G. megalophylla* leaf EO.

**A R T I C L E   I N F O**

Keywords:
- *Guatteria megalophylla*
- Essential oil
- Spathulenol
- Anti-leukemia

**1. Introduction**

Cancer is a disease with high incidence and mortality worldwide. The new global cancer data indicate one-in-five men and one-in-six women in the world will develop cancer, leading to one-in-eight men and one-in-ten women deaths, respectively. In relation to leukemia, GLOBOCAN database estimated 437,033 new cases and 309,006 deaths worldwide in 2018 [1]. Therefore, researches on cancer biology, early diagnostic and new treatments are encouraged.

The genus *Guatteria* belongs to Annonaceae family and include about 307 species that are distributed through neotropical regions between Mexico and Brazil [2,3]. Anticancer potential of species from this genus have been reported for *Guatteria fricianna* (W. A. Rodrigues) Erkens & Maas [4–7], *Guatteria boliviensis* H.J.P. Winkl. [8], *Guatteria pogonopus* Mart. [9,10], *Guatteria calva* R.E. Fries [11], *Guatteria australis* A.St.-Hil. [12,13], *Guatteria ferruginea* A.St.-Hil. [13], *Guatteria latifolia* R.E.Fr. [13], *Guatteria sellowiana* Schltdl. [13], *Guatteria blepharophylla* Mart. [14–16] and *Guatteria hispida* (R.E. Fr.) Erkens & Maas [15,16] against different histological kind of cancer. 

*Guatteria megalophylla* Diels is an 8–10 m tall tree that grows near water streams and is widely distributed throughout the Amazon basin in Colombia, Ecuador, Peru, Brazil and Guiana. In Brazil, it can be found in states of Acre, Amazonas, Para and Rondônia [2,17]. Previsiously, alkaloids O,O-dimethylcurine, 12′-O-methylcurine and isochondodendrin were isolated from stems of *G. megalophylla* [18]. Additionally, in vitro antioxidant activity of ethanol extracts from *G.
megalophylla leaves and stems were assessed, but no antioxidant effect was observed at concentrations of 10 and 50 μg/mL. Despite of anticancer potential of plants belonging to genus Guatteria, G. megalophylla had never been studied in relation to its cytotoxic and anti-tumor properties. Herein, we investigated for the first time chemical composition and in vitro and in vivo anti-leukemia potential of G. megalophylla leaf essential oil (EO) using human promyelocytic leukemia HL-60 cells as model.

2. Material and methods

2.1. Botanical material

G. megalophylla leaves were collected on September 19, 2018 at Adolfo Ducke Forest Reserve in Manaus, Amazonas, Brazil (coordinates 2° 56’ 0” S, 59° 58’ 35” W). Botanists of National Institute of Amazonian Research (INPA) identified this species, and a voucher specimen number #2807 was deposited at INPA’s Herbarium. The species (access) was registered in SISGEN with the record A70EDCD.

2.2. Chemical evaluation

2.2.1. Essential oil extraction

G. megalophylla leaves were oven dried with air circulation at 40 °C for 24 h and subjected to hydrodistillation for 4 h using a Clevenger type apparatus (Amitel, São Paulo, Brazil). EO was dried over anhydrous sodium sulphate and percentage content (w/w) was calculated based on plant material dry weight. Hydrodistillation extractions were performed in triplicate. EO was stored in freezer prior chemical and biological analyses.

2.2.2. GC-FID analysis

Gas chromatography coupled to flame ionization detection (GC-FID) analyses were carried out using a Shimadzu GC-2010 Plus (Shimadzu Corporation, Kyoto, Japan) fitted with a flame ionization detector and equipped with a self-injector, AOC-20i (Shimadzu Corporation). The separation of the oil constituents was achieved by employing an Rtx*-5 fused capillary column (30 m X0.25 mm X0.25 μm film thickness) coated with 5 %-phenyl-95 %-methylpolysiloxane) (30 m X0.25 mm X0.25 μm film thickness) was used as stationary phase. Helium (99.99 %) was the carrier gas at a flow rate of 1.0 mL/min. The column temperature program was 40 °C kept for 4 min, a heating ramp at a rate of 4 °C/min up to 240 °C, followed by a rate of 10 °C/min until 280 °C, and then280 °C kept for 2 min. The injector and detector temperatures were 250°C and 280°C, respectively. Samples (10 mg/mL in CH2Cl2) were injected with a 1:50 split ratio. Retention indexes were calculated according to Van den Dool and Kratz equation [20] in comparison with a standard solution of C6-C20 n-alkanes (Sigma-Aldrich Co., Saint Louis, MO, USA). 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.

2.2.3. GC–MS analysis

Gas chromatography coupled to mass spectrometry (GC–MS) analyses were also performed on a Trace gas chromatography ultra-system (Thermo-Scientific) coupled with an ISQ mass spectrometer equipped with a Tri Plus RSH auto-injector. An Rtx*-5MS fused capillary column (coated with 5 %-phenyl-95 %-methylpolysiloxane) (30 m X0.25 mm X0.25 μm film thickness) was used as stationary phase. MS data were taken at 70 eV with a scan interval of 0.5 s and mass spectra acquisition at the m/z 40 – 500 Da range. All injection and separation conditions were the same from GC-FID analysis. The identification of EO components was achieved based on similarity with data from Nist library [21].

Table 1

| Compounds       | RI1 | RI2 | Peak area % |
|-----------------|-----|-----|-------------|
| α-Pinene        | 930 | 932 | 0.90 ± 0.48 |
| β-Pinene        | 972 | 974 | 0.26 ± 0.14 |
| Limonene        | 1026| 1024| 0.15 ± 0.06 |
| Linalool        | 1099| 1095| 0.10 ± 0.03 |
| N.I.            | 1111|     | 0.17 ± 0.02 |
| δ-Elemene       | 1336| 1335| 5.15 ± 0.36 |
| α-β-Cubebene    | 1348| 1348| 0.37 ± 0.09 |
| α-Vlangeine     | 1369| 1373| 0.45 ± 0.13 |
| α-Copaene       | 1373| 1374| 1.37 ± 0.06 |
| β-Bourbonene    | 1382| 1387| 0.66 ± 0.11 |
| β-Elemene       | 1390| 1389| 7.48 ± 0.58 |
| Cyperene        | 1396| 1398| 0.41 ± 0.10 |
| α-Gursunene     | 1407| 1409| 0.42 ± 0.03 |
| E-Caryophyllene | 1416| 1417| 3.25 ± 0.11 |
| γ-Elemene       | 1431| 1434| 1.24 ± 0.07 |
| α-Guaiene       | 1436| 1437| 0.39 ± 0.03 |
| Aromadendrene   | 1441| 1439| 0.38 ± 0.01 |
| α-Humulene      | 1450| 1452| 1.37 ± 0.08 |
| N.I.            | 1475|     | 0.82 ± 0.04 |
| γ-Muurolene     | 1479| 1478| 13.43 ± 0.53 |
| Bicyclorigermacene | 1494| 1500| 10.47 ± 0.68 |
| α-Cadinene      | 1512| 1513| 0.84 ± 0.13 |
| δ-Cadinene      | 1522| 1522| 1.78 ± 0.04 |
| Hedycaryol      | 1547| 1546| 1.47 ± 0.26 |
| Germacrane B    | 1554| 1559| 2.71 ± 0.03 |
| N.I.            | 1564|     | 1.73 ± 0.25 |
| Spathulenol     | 1576| 1577| 27.76 ± 0.79 |
| Guaiol          | 1596| 1600| 3.86 ± 0.59 |
| N.I.            | 1627|     | 5.62 ± 0.80 |
| N.I.            | 1636|     | 1.60 ± 0.17 |
| N.I.            | 1656|     | 0.90 ± 0.09 |
| Bulnesol        | 1666| 1670| 0.40 ± 0.15 |
| N.I.            | 1672|     | 0.45 ± 0.06 |
| Cycloclorenone  | 1755| 1759| 0.73 ± 0.22 |

Monoterpenes

- 1,4-Disubstituted
- Sesquiterpenes

Table data are presented as mean ± S.D. of three analyses. RI (retention indices): a calculated on RTX*-5MS column according to Van Den Dool and Kratz [20], based on a homologous series of normal alkanes; b according to Adams [21]. N.I. = Not identified.

2.3. In vitro evaluation

2.3.1. Cells

HL-60 (human promyelocytic leukemia), MCF-7 (human breast adenocarcinoma), CAL27 (human oral squamous cell carcinoma), HSC-3 (human oral squamous cell carcinoma), HepG2 (human hepatocellular carcinoma), HCT116 (human colon carcinoma) and MRC-5 (human lung fibroblast) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and were cultured as recommended by ATCC animal cell culture guide. All cell lines were tested for mycoplasma using a mycoplasma stain kit (Sigma-Aldrich) to validate the use of cells free from contamination.

2.3.2. Cytotoxicity assay

For cytotoxicity assay, cell viability was quantified by Alamar blue method as previously described [22–24]. For all experiments, cells were plated in 96-well plates. EO was dissolved in dimethyl sulfoxide (DMSO, Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil) and added to each well and incubated for 72 h. Doxorubicin (doxorubicin hydrochloride, purity ≥ 95 %, Laboratory IMA S.A.I.C., Buenos Aires, Argentina) and 5-fluorouracil (purity ≥ 95 %, Sigma-Aldrich Co., Saint Louis, MO, USA) were used as positive controls. At the end of treatment, 20 μL of a stock solution (0.312 mg/mL) of resazurin (Sigma-Aldrich Co.) was added to each well. Absorbances at 570 nm and 600 nm were measured using a SpectraMax 190 Microplate Reader (Molecular
formed in duplicate, quantified obtained by nonlinear regression from three independent experiments per-
study. Cells were counted. Cell counting was performed using a light microscope with a hemocytometer
Trypan blue) cells were counted. Cell counting was performed using a light microscope with a hemocytometer

2.3.3. Trypan blue exclusion assay

Trypan blue exclusion assay was used to confirm the cytotoxic effect of EO, and the number of viable cells and non-viable (stained with trypan blue) cells were counted. Cell counting was performed using a light microscope with a hemocytometer filled with a homogenized cell suspension.

2.3.4. Annexin-V-FITC/propidium iodide staining assay

FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) was used for apoptosis quantification, and the analysis was performed according to manufacturer's instructions. At least 10^4 events were recorded per sample using a flow cytometry with a BD LSRFortessa cytometer, BD FACSDiva Software (BD Biosciences) and FlowJo Software 10 (FlowJo Lcc; Ashland, OR, USA). Cellular debris were omitted from analysis.

2.3.5. Internucleosomal DNA fragmentation and cell cycle distribution

Internucleosomal DNA fragmentation and cell cycle distribution were assessed by quantification of DNA content [25]. Cells were harvested in a permeabilization solution containing 0.1 % triton X-100, 2 μg/mL propidium iodide (PI), 0.1 % sodium citrate and 100 μg/mL RNase (all from Sigma-Aldrich Co.) and incubated in dark for 15 min at room temperature. Cell fluorescence was determined by flow cytometry, as described above.

2.3.6. Intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were measured using 2′,7′-dichlorofluorescin diacetate (DCF-DA) (Sigma-Aldrich Co.) [26]. Cells were collected, washed with saline and suspended in tubes with saline containing 5 μM DCF-DA for 30 min. Then, the cells were washed with saline, and cell fluorescence was determined by flow cytometry, as described above.

2.4. In vivo evaluation

2.4.1. Animals

Forty-four C.B-17 severe combined immunodeficient (SCID) mice (males, six weeks old, 25–30 g) were obtained and maintained at Gonçalo Moniz Institute-FIOCRUZ animal facilities (Salvador, Bahia, Brazil). Animals were housed in cages with free access to food and water. All animals were subjected to a 12:12 h light-dark cycle (lights on at 6:00 a.m.). A local animal ethics committee approved the experimental protocol employed (number #06/2015).

2.4.2. Human leukemia xenograft model

HL-60 cells (1.5 × 10^7 cells per 500 μL) were implanted

![Fig. 1. Effect of Guatteria megaphylla leaf essential oil (EO) in HL-60 cells viability, as measured by trypan blue dye exclusion method, incubated for 24 h and 48 h with vehicle (0.1 % DMSO) used for diluting EO, and doxorubicin (DOX, 1 μg/mL) was used as positive control. Data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. * P < 0.05 compared with negative control by ANOVA, followed by Bonferroni's Multiple Comparison Test.](image-url)
subcutaneously into left front armpit of mice. When the tumors reached 100 to 200 mm³, animals were treated through intraperitoneal route (200 μL per animal) once a day for nine consecutive days. At beginning of the experiment, mice were randomly divided into four groups: group 1 animals received injections of vehicle (5 % DMSO solution) used for diluting EO (n = 11); group 2 animals received injections of doxorubicin (0.8 mg/kg, n = 11); group 3 animals received injections of EO at 50 mg/kg (n = 11); and group 4 animals received injections of EO at 100 mg/kg (n = 11). One day after the end of treatment, the animals were anesthetized, and peripheral blood samples were collected from the brachial artery. Animals were euthanized by anesthetic overdose, and tumors were excised and weighed. Inhibition ratio (%) was calculated by formula: inhibition ratio (%) = [(A–B)/A] x 100, where A is average tumor weight of negative control, and B is tumor weight of treated group.

2.4.3. Toxicological aspects

The mice were weighed at beginning and end of experiment to evaluation toxicological effects. Animals were observed for signs of abnormality throughout the study. A hematological analysis was performed using the Advia 60 hematology system (Bayer, Leverkusen, Germany). Livers, kidneys, lungs and hearts were removed, weighed and examined for signs of gross lesion formation, color change and/or hemorrhaging. After fixation in 4 % formaldehyde, histological analyses were performed for tumors and organs, under optical microscopy using hematoxylin-eosin and Periodic acid-Schiff (liver and kidney) staining.

2.5. Statistical analysis

Data were presented as mean ± S.E.M. or as IC₅₀ value with 95 % confidence intervals obtained by nonlinear regressions. Differences among experimental groups were compared through analysis of variance (ANOVA) followed by Bonferroni’s Multiple Comparison Test (P < 0.05). All statistical analyses were performed using GraphPad Prism (Intuitive Software for Science; San Diego, CA, USA).

Fig. 2. Effect of Guatteria megaphylla leaf essential oil (EO) on apoptosis induction in HL-60 cells, as determined by flow cytometry using annexin V-FITC/propidium iodide staining after 24 and 48 h incubation. (A) Representative flow cytometric dot plots. (B) Quantification of viable (annexin V-FITC/PI double negative cells), early apoptosis (annexin V-FITC positive, but PI negative cells), late apoptosis (annexin V-FITC/PI double positive cells) and necrosis cells (PI positive, but annexin V-FITC negative cells). Negative control (CTL) was treated with vehicle (0.1 % DMSO) used for diluting EO, and doxorubicin (DOX, 1 μg/mL) was used as positive control. Data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. Ten thousand events were evaluated per experiment, and cellular debris was omitted from analysis. * P < 0.05 compared with negative control by ANOVA, followed by Bonferroni’s Multiple Comparison Test.
3. Results and discussion

3.1. Chemical analysis of Guatteria megalophylla leaf essential oil

Hydrodistillation of G. megalophylla leaves resulted in a greenish-colored oil with a yield of 0.12 % ± 0.02 (w/w), based on weight of dried plant material. Twenty-seven compounds was identified according to GC-FID and GC-MS analyses (Table 1 and Figs. S1–11). The main compounds were the sesquiterpenes spathulenol (27.76 %), χymurolene (14.34 %), bicyclogermacrene (10.47 %), β-elemene (7.48 %) and δ-elemene (5.15 %).

The presence of spathulenol along with some of major compounds were also found in other EOs from other Guatteria species [9,27,28]. In fact, spathulenol is considered a plausible chemotaxonomic marker of EOs of Guatteria [29]. On the other hand, chemical constituents of EO from this genus presented significant variations, which could be explained by climatic conditions, geographical localizations, soil characteristics and fertilization level, seasons, among other factors, which can cause such deviations.

3.2. In vitro cytotoxicity of Guatteria megalophylla leaf essential oil

In vitro cytotoxic potential of G. megalophylla leaf EO was assessed against six human cancer cell lines (HL-60, MCF-7 CAL27, HSC-3, HepG2 and HCT116) and against one human non-cancer cell line (MRC-5) using Alamar blue method after 72 h incubation. Half maximal inhibitory concentration (IC$_{50}$) obtained are shown in Table 2. EO displayed IC$_{50}$ values of 12.51 μg/mL for HL-60 cells, 35.45 μg/mL for MCF-7 cells, 7.58 μg/mL for CAL27 cells, 14.90 μg/mL for HSC-3 cells, 21.62 μg/mL for HepG2 cells, 30.27 μg/mL for HCT116 cells and 29.80 μg/mL for MRC-5 cells. Doxorubicin, with IC$_{50}$ values ranging from 0.02 to 6.16 μg/mL for cancer cells HL-60 and MCF-7, respectively (IC$_{50}$ of 3.32 μg/mL for MRC-5 cells), and 5-fluourouracil, with IC$_{50}$ values ranging from 0.53 to 13.71 μg/mL for cancer cells HCT116 and HepG2, respectively (IC$_{50}$ of 5.96 μg/mL for MRC-5 cells), were used as positive controls.

In our cytotoxic screening program, we used a cut off limit where extracts/EOs with IC$_{50}$ values below 30 μg/mL are considered promising samples and are selected for our anticancer drug development program [15,30–32]. G. megalophylla leaf EO presented IC$_{50}$ values below 30 μg/mL for most of cell lines tested and was selected for further studies.

Some previously works evaluated the cytotoxic action of Guatteria species leaves EOs with promising outcomes. Britto et al. [4] reported cytotoxic effect of G. friesiana leaf EO against human cancer cell lines HL-60, MDA-MB-435 (melanoma), HCT-8 (colon carcinoma) and SF-295 (glioblastoma) with IC$_{50}$ values below 10 μg/mL. This bioactivity reported was attributed to isomers α-, β- and χ- eudesmols. Fontes et al. [9] evaluated effect of G. poganopus leaf EO against human cancer cell lines OVCAR-8 (ovarian adenocarcinoma), NCI-H358 M (bronchoalveolar lung carcinoma) and PC-3 M (metastatic prostate carcinoma) and observed IC$_{50}$ values below 25 μg/mL, which were associated to its main (γ-patchoulene, (E)-caryophyllene and β-pinene) and minor constituents mixture. Similarly, G. blepharophylla and G. hisida leaves EOs showed cytotoxicity against cancer cell lines HCT-8, MDA-MB-435, SF-295, HepG2, K562 (human leukemia) and B16-F10 (mouse melanoma) and HL-60 with IC$_{50}$ values below 25 μg/mL. However, no potent cytotoxicity was found for major constituent of G. blepharophylla, caryophyllene oxide or main molecules of G. hisida (α-pinenene, β-pinene and (E)-caryophyllene) leaves EOs, indicating the associations of these constituents or minor constituents are responsible for their cytotoxicity [15,16]. Herein, G. megalophylla leaf EO presented spathulenol as its main constituent. Spathulenol have been previously reported as a cytotoxic agent against B16-F10 (IC$_{50}$ of 1.67 μg/mL), HepG2 (IC$_{50}$ of 11.19 μg/mL), K562 (IC$_{50}$ of 3.79 μg/mL) and HL-60 (IC$_{50}$ of 11.38 μg/mL) cells [33]. These data suggest that this compound is responsible, at least in part, for cytotoxic effect of G. megalophylla leaf EO.

To confirm the results obtained by Alamar blue method, we quantified the number of viable HL-60 cells by trypan blue exclusion method and annexin V-FITC/PI staining assay after 24 and 48 h incubation with EO at concentrations of 10, 20 and 40 μg/mL. EO significantly reduced the number of viable cells (Fig. 1). At concentrations of 10, 20 and 40 μg/mL, EO reduced the number of viable cells by 29.5, 40.5 and 59.2 % after 24 h, and 74.0, 76.0 and 89.4 % after 48 h, respectively. Doxorubicin, at 1 μg/mL, reduced the number of viable cells by 64.4 % after 24 h and 90.0 % after 48 h. In annexin V-FITC/PI staining assay, we measured the percentage of cells in viable (annexin V-FITC/PI double negative), early apoptotic (annexin V-FITC positive, but PI negative), late apoptotic (annexin V-FITC/PI double positive cells) and necrotic stages (PI positive, but annexin V-FITC negative cells). (Fig. 2). At concentrations of 10, 20 and 40 μg/mL, EO augments the percentage of early apoptotic cells by 36.4, 62.2 and 61.5 % after 24 h, and 46.9, 96.6 and 95.5 % after 48 h, respectively. Doxorubicin, at 1 μg/mL, increased the number of early apoptotic cells by 18.1 % after 24 h and

\[ \text{IC}_{50} \text{ values} \]
Fig. 4. Effect of Guatteria megalophylla leaf essential oil (EO) in the cell cycle distribution of HL-60 cells, as determined by flow cytometry using propidium iodide staining after 24 and 48 h incubation. (A) Representative flow cytometric histograms. (B) Quantification of sub-G1 (internucleosomal DNA fragmentation), G0/G1, S and G2/M percentage distribution. Negative control (CTL) was treated with vehicle (0.1 % DMSO) used for diluting EO, and doxorubicin (DOX, 1 μg/mL) was used as positive control. Data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. Ten thousand events were evaluated per experiment, and cellular debris was omitted from analysis. *P < 0.05 compared with negative control by ANOVA, followed by Bonferroni’s Multiple Comparison Test.

Fig. 5. Effect of Guatteria megalophylla leaf essential oil (EO) in reactive oxygen species (ROS) levels of HL-60 cells after 1 and 3 h incubation, as determined by flow cytometry using DCF-DA staining. Negative control (CTL) was treated with vehicle (0.1 % DMSO) used for diluting EO, and hydrogen peroxide (H2O2, 200 μM) was used as positive control. Data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. Ten thousand events were evaluated per experiment, and cellular debris was omitted from analysis. *P < 0.05 compared with negative control by ANOVA, followed by Bonferroni’s Multiple Comparison Test.
66.2 % after 48 h. In addition, EO induced cell shrinkage, as observed by reduction in forward light scatter (FSC), and nuclear condensation, as observed by increasing in side scatter (SCC), both morphological alterations characteristic of apoptotic cells (Fig. 3).

Next, we examined intracellular DNA content and ROS levels in EO-treated HL-60 cells. Quantification of intracellular DNA content allowed measurement of internucleosomal DNA fragmentation and the cell cycle distribution. In this assay, all DNA that was sub-diploid in size (sub-G1) was considered internucleosomal DNA fragmentation. EO-treated HL-60 cells presented an internucleosomal DNA fragmentation significantly increased after 48 h incubation \( (P < 0.05) \) (Fig. 4). At the concentrations of 10, 20 and 40 \( \mu \text{g/mL} \), the sample increased DNA fragmentation to 31.5, 54.7 and 58.7 %, against 11.6 % observed for negative control, respectively. Doxorubicin (1 \( \mu \text{g/mL} \)) increased DNA fragmentation up to 68.1 % at same incubation time. The cell cycle phases, G1, S and G2/M, were reduced proportionally. Furthermore, the effect of EO in ROS levels was investigated in HL-60 cells after 1 and 3 h incubation. However, EO did not induce a significant increase in ROS levels (Fig. 5).

Corroborating with these data, Ferraz et al. [16] observed cell morphology consistent with cell death by apoptosis, increased internucleosomal DNA fragmentations and activation of caspase-3 in HepG2 cells treated with G. blepharophylla and G. hispida leaf EOs.

### 3.3. In vivo anti-leukemia effect of Guatteria megalophylla leaf essential oil

In vivo anti-leukemia effect of G. megalophylla leaf EO was evaluated in C.B-17 SCID mice with HL-60 cell xenografts. When the tumors reached 100 to 200 mm\(^3\), animals were treated through intraperitoneal route once a day for nine consecutive days. EO significantly reduced tumor development at highest dose. One day after the end of treatment, the mean tumor weight of negative control group was 2.48 ± 0.40 g (Fig. 6A). In EO-treated groups, the mean tumor mass weights were 2.07 ± 0.19 g and 1.27 ± 0.24 g at lowest and highest doses, respectively. Tumor mass inhibition rates of EO was 16.6–48.8 % (Fig. 6B). Doxorubicin, at dose of 0.8 mg/kg, was used as positive control and reduced tumor weight by 49.4 %. Histology analysis of xenograft tumors showed a proliferation of malignant cells with abundant and granular cytoplasm, with two-or more distinct nucleoli, with characteristics similar to myeloid cells (Fig. 6C). In negative control and EO (50 mg/kg) groups, these malignant cells were often organized into small clusters of cells with scarce extracellular matrix. Instead, comparing with doxorubicin group, extensive necrosis areas were more pronounced in both EO (50 and...
Some toxicological aspects were also investigated in C.B-17 SCID mice with HL-60 cell xenografts treated with EO. After treatment, 100% survival (11/11) rates have been observed for all groups with exception for doxorubicin-treated mice that showed survival rate of ∼73% (8/11). No significant changes on body and organs (liver, kidney, lung and heart) weights were seen on EO-treated groups (P > 0.05) (Fig. 7). A decrease of body weight was found in doxorubicin-treated group (P < 0.05). In hematological analysis, all parameters remained unchanged after treatment with G. megalophylla leaf EO (P > 0.05) (Fig. 8).

In vivo antitumor effect of G. friesiana and G. pogonopus leaves EOs was previously investigated in murine model using Sarcoma 180-bearing mice [4,9]. The first presented tumor growth inhibition rates of 43.4–54.2% and 6.6–42.8%, when administrated by intraperitoneal (50 and 100 mg/kg) and oral (100 and 200 mg/kg) routes, respectively [4]. The second displayed tumor growth inhibition rate of 25.3–42.6%, when administrated by intraperitoneal route at 50 and 100 mg/kg, respectively [9]. Herein, G. megalophylla leaf EO inhibited leukemia cells development as observed in a xenograft model in mice. Tumor growth inhibition rate was 16.6–48.8%, when administrated by intraperitoneal route at 50 and 100 mg/kg, respectively. Moreover, although low selectively of EO against cancer cells versus non-cancer cells was found in vitro experiments, no significant side effect was observed in vivo model, suggesting safe anticancer potential of G. megalophylla leaf EO.

Morphological analyses of lungs, liver, kidneys and heart were compared with negative control by ANOVA, followed by Bonferroni’s Multiple Comparison Test.

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**Fig. 7.** Effect of Guatteria megalophylla leaf essential oil (EO) on body and relative organ weight from C.B-17 SCID mice with HL-60 cell xenografts. Negative control (CTL) was treated with vehicle (5% DMSO) used for diluting EO, and doxorubicin (DOX, 0.8 mg/kg) was used as positive control. When the tumors reached 100 to 200 mm³, animals were treated through intraperitoneal route once a day for nine consecutive days. Data are presented as mean ± S.E.M. of 8–11 animals. *P < 0.05 compared with negative control by ANOVA, followed by Bonferroni’s Multiple Comparison Test.

**Fig. 8.** Effect of Guatteria megalophylla leaf essential oil (EO) on hematological parameters of peripheral blood from C.B-17 SCID mice with HL-60 cell xenografts. Negative control (CTL) was treated with vehicle (5% DMSO) used for diluting EO, and doxorubicin (DOX, 0.8 mg/kg) was used as positive control. When the tumors reached 100 to 200 mm³, animals were treated through intraperitoneal route once a day for nine consecutive days. Data are presented as mean ± S.E.M. of 7 animals.
performed for all experimental groups. In liver, acinar architecture and centrilobular vein were preserved in all groups. Histopathological changes included congestion, hydropic degeneration, chronic inflammation in liver portal space and focal areas of coagulation necrosis, ranging from mild to moderate. It is important to note that these histopathological characteristics were more pronounced in EO (100 mg/kg) group and doxorubicin than in other groups (negative control and EO group - 50 mg/kg). In lungs, architecture of parenchyma was partially maintained in all groups. Histopathological changes ranged from mild to severe and, frequently, focal inflammation, edema, congestion, hemorrhage, and increased airspace were observed in all experimental groups. In kidneys, tissue architecture was maintained; however, some histopathological changes were observed in all experimental groups, such as moderate vascular congestion and thickening of basal membrane of renal glomerulus, ranging from mild to moderate, with decreased urinary space. Importantly, focal areas of coagulation necrosis were observed in some animals treated with EO (100 mg/kg). The hearts of animals did not show alterations in any group.

In conclusion, *G. megalophylla* leaf EO has anti-leukemia potential, in which the main constituents spathulenol, γ-muuroleone, bicyclo-germacrene, δ-elemenone and δ-elemene may play a central for the recorded activities.

Data availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

The study was done after agreement from the local ethics committee.

Funding statement

This work was financially supported by Brazilian agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) and Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB).

Declaration of Competing Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors are grateful to histotechnology and flow cytometry cores of FIOCRUZ-Bahia for performing histological techniques and collecting flow cytometric data, and Analytical Center of the Federal University of Amazonas (CA-UFAM) for GC-MS analyses.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109713.

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